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ANALYSIS OF *NOTCH1* MUTATION STATUS IN PRECURSOR T-CELL LYMPHOBLASTIC LEUKEMIA OF CHILDHOOD: PROGNOSTIC VALUE AND CORRELATION WITH EARLY TREATMENT RESPONSE.

DISSERTATION

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SUMMARY

The presence of activating *NOTCH1* mutations in about 60% of pediatric T-ALL and the fact that different studies have shown a favorable effect on early treatment response and long term outcome, originated the question of the prognostic implication in patients treated on German Co-Operative Study Group for Childhood Acute Lymphoblastic Leukemia study (COALL-92-97-03) protocols. We retrospectively investigated the prognostic relevance of *NOTCH1* mutations for 133 pediatric patients enrolled in COALL-92-97-03 and analyzed the relationship with early treatment response [according to the incidence of high minimal residual disease (MRD) levels] and long term outcome.

Overall, 80 out of 133 (60.2%) patients were *NOTCH1*+. In 47/80 samples mutations were detected only in the HD domain (58.8%), in 11/80 in the PEST domain (13.8%) and in 19/80 in both domains (23.7%). Only one patient (1.25%) had a mutation in the TM domain. Noteworthy, are the two mutations (2.5%) found in the LNR repeat domain, a very rarely affected domain, which to our best knowledge, have not been previously described.

Statistical analysis of 127 patients failed to confirm a lower relapse rate (same incidence of relapse for both groups: 27% (12/50 *NOTCH*- versus 18/77 *NOTCH*+; p=0.95) and overall favorable effect of activating *NOTCH1* mutations. However, *NOTCH1* mutations were associated with low levels of MRD on day 29 (P=.009) but no correlation was found with levels of MRD on day 43.

In this study, 38 patients treated according the ALL IC-BFM protocol (Garrahan Hospital, Buenos Aires, Argentina) were included. When considering patients in the BFM-like cohort, mutations were associated with good prednisone response (P=.009); but no relationship with long-term prognosis.

Our data support the statement that the effect of NOTCH1 is generally treatment related and may depend on the intensity of the induction chemotherapy specifically. Therefore, they do not seem to improve our current risk-stratification strategies.

Of note, an in-frame mutation was found in the PEST domain (exon 34) that to date, has not been described, and does not follow the usual pattern of mutations in this domain, that normally lead to premature stop codons and a truncated PEST domain. The novel mutations found in exon 25, may contribute to a better understanding of the structure of the NOTCH1 negative regulatory region (NRR). Antibodies or small molecule inhibitors targeting the NRR of NOTCH1 might find future utility as targeted therapeutics in the management of T-ALL.

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ABBREVIATIONS

6-MP	6-Mercaptopurine
ABC	Absolute blast count
ALL	Acute lymphoblastic leukemia
ALL IC-BFM	International BFM (Berlin-Frankfurt-Münster) study group
ANK	Ankyrin domain
ARA-C	Cytarabin
ASP	Asparaginase
BM	Bone marrow
C-ALL	Common acute lymphoblastic leukemia
CR	Complete remission
CCR	Complete continuous remission
CD	Cluster of differentiation
CNS	Central nervous system
COALL	Cooperative study group for childhood acute lymphoblastic leukemia
CPM	Cyclophosphamide
CR	Complete remission
CSF	Cerebrospinal fluid
dATP	Deoxyadenosine triphosphate
DCOG	Dutch Childhood Oncology Group
DNA	Deoxyribonucleic acid
DNR	Daunorubicin
DOX	Doxorubicin (Adriamycin)
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EFS	Event-free survival
EGF	Epidermal growth factor
EORTC-CLG	European Organization for Research and Treatment of Cancer-Children Leukemia Group
et al.	et alii / alia
Ex	exon
FBXW7	F-box and WD-40 domain protein 7
G0/G1	Gap 0/Gap1 (Cell cycle)
GC	Glucocorticoid

GSI	γ-secretase inhibitor
HD	Heterodimerization domain
HD-C	C-terminal of heterodimerization domain
HD-N	N-terminal of heterodimerization domain
HLA	Human leukocyte antigen
HR	High-Risk group
ICN	NOTCH1 intracellular subunit
IT	Intrathecal
LBD	Ligand-binding domain
LNR	LIN-12/NOTCH repeats
MAML	Mastermind-like
MFD	Matched family donor
MRD	Minimal residual disease
mRNA	Messenger RNA
MTX	Methotrexate
MUD	Matched-unrelated-donor
NEC	N-terminal extracellular subunit
NRR	Negative regulatory region
NTM	C-terminal transmembrane subunit
N-terminus	Amino-terminus
OS	Overall survival
PB	Peripheral blood
PCR	Polymerase chain reaction
pCRT	Prophylactic cranial raiotherapy
PEG-ASP	Pegylated type of *E.coli* asparaginase
PEST	Proline-Glutamate-Serine-Threonine
PGR	Prednisone good response
PPR	Prednisone poor response
PVA	Prednisolone, vincristine and L-asparaginase
RAM	RBPJk-associated molecule
RIP	Intramembrane proteolysis
RNA	Ribonucleic acid
rpm	Revolutions per minute
RXT	Radiotherapy
S1	Furin-like protease cleavage site
S2	Metalloprotease cleavage site
S3	γ-secreatase cleavage site

SCT	Stem-cell transplantation
SE	Standard Error
SMN	Second malignancies
T-ALL	T-cell acute lymphoblastic leukemia
TAD	Transactivation domain
Taq	Thermophilus aquaticus
TCR	T-cell receptor
tCRT	Therapeutic cranial radiotherapy
TM	Transmembrane domain
TP	Time point
WBC	White blood cells
WT	Wild-type

1. INTRODUCTION

1.1 Acute lymphoblastic leukemia of childhood

Acute lymphoblastic leukemia (ALL) is the most common malignant disease in children. Great improvements have been made in the treatment of childhood leukemia; consequently the outcome has improved remarkably in the past 3 decades. With the actual treatment strategies for ALL, the five-year-event-free survival (EFS) rate is nearly 80% (Pui *et al*., 1998; Schrappe *et al*., 2000; Silverman *et al*., 2001). This success has been achieved with individualized treatment strategies, adapting the treatment's intensity according to each patient's risk of relapse.

This approach was developed after understanding that childhood ALL is a heterogeneous disease, which consists of several subtypes of leukemia with markedly different response to chemotherapy (Pui *et al*., 1998). When the intensity of the treatment is adapted to the relative risk of relapse of the patient, therapy is intensified in those patients with higher risk, and diminished in those with smaller risk, and therefore patients are provided with a higher cure probability. An aspect that has allowed the success of this approach is the precise assignation of patients in specific risk groups. The individual risk assessment of each patient is achieved through the identification of clinical and biological (genetic/molecular alterations) prognostic markers, that determine the pathogenesis of the individual subtypes of leukemia (Silverman *et al*., 2001).

Despite this progress, approximately 25% of the patients develop recurrent disease, and the prognosis of relapsed ALL remains poor (Herold *et al*., 2004). On the other hand, one third of the patients are probably over treated, when we consider the survival rates of 30% with less intensive therapies in the 1970s. Furthermore, surviving patients often experience significant toxicities related to the therapy. By the end of the 1980s, researchers started to take into account the acute and long-term toxicity of such treatments. Thus, the universal trend is to carry out better risk-adapted treatment strategies, which can improve the long-term outcome of high risk patients and diminish toxicity and other long term effects in those with standard risk.

Childhood leukemia's are usually subdivided according to their immunophenotype, and recently, according to their gene expression profiling (Yeoh *et al*., 2002). Precursor B-cell lymphoblastic leukemia account for 85% to 90% of all pediatric ALLs, and a number of clinically important genetic markers have been identified (e.g., t(1;19), t(4;11), t(9;22), t(12;21) and hyperploidy) (Pui *et al*., 2004). These chromosome alterations are important parameters in the risk stratification algorithms in the currently used treatment strategies. As an example, we can refer to the trial ALL-BFM 2000, that carried out a risk-adapted treatment strategy (standard, medium and high risk patients), using cytogenetic markers [t(9;22) and t(4;11)] or it's

molecular equivalents (*BCR-ABL* and *MLL-AF4*) and the *in vivo* response to treatment, among several parameters for risk-group definition. The response to treatment was cytomorphologically evaluated by the initial cytoreduction (number of peripheral blood blasts per micro liter on day 8) or according to the disease (minimal residual disease - MRD) kinetics (Cavé *et al*., 1994; Cavé *et al* 1998) that were assessed at 2 different time points (TPs), at days 33 (TP1) and week 12 of treatment, after induction and consolidation phase, respectively. In this study, Cario, Stanulla and associates reached the conclusion that drug resistance in leukemic cells is an intrinsic feature of lymphoblasts reflected in the gene expression pattern and that resistance to chemotherapy could be predicted before treatment (Cario *et al*., 1999).

It is well known that initial response to glucocorticoid therapy is a strong independent prognostic factor of outcome ALL (Dordelmann *et al*., 1999; Schrappe *et al*., 2000). Resistance to glucocorticoids *in vitro* is associated with an unfavorable prognosis and most patients who present ALL relapse show increased resistance to glucocorticoid therapy, identifying this biological feature as a major contributor to treatment failure (Kaspers *et al*., 2005). Yeoh *et al.* also suggest that, within some genetic subgroups, gene expression profiling enhance the accurate risk stratification of childhood ALL patients and can identify those patients that would eventually fail therapy (Yeoh *et al*., 2002).

T-cell lymphoblastic leukemias (T-ALLs) are characterized by infiltration of the bone marrow with inmature lymphoblasts expressing T-cell inmunophenotypic markers and account for 10% to 15% of all childhood ALLs. These patients frequently show a large tumor burden with hyperleukocytosis, large mediastinal masses, pleural effusions and have a higer risk of leptomeningeal (central nervous system-CNS) infiltration at the moment of diagnosis.

However, no solid prognostic genetic markers have been shown to be of clinical relevance for precursor T-ALL, although an overexpression of *HOX11, TAL1 or LYL1* has been reported to confer a favorable or an unfavorable prognosis in a small number of patients. Thus, risk-adapted treatment strategies depend on response to treatment parameters (Ferrando *et al*, 2002; Ferrando *et al*., 2004).

The presence of activating *NOTCH1* mutations in about 60% of pediatric T-ALL that result in constitutively active NOTCH1 signaling has opened the question of the prognostic significance in childhood T-ALL (Weng *et al*., 2004). Although an initial study associated these mutations with improved long term prognosis (Breit *et al*., 2006), other studies failed to demonstrate improved outcome in patients with *NOTCH1* mutations (Zhu *et al*., 2006), thus prognostic implications of *NOTCH1* mutations in T-ALL remains unclear.

1.2 The NOTCH1 signaling pathway

In order to correctly interpret the investigated mutations and there prognostic significance, it is important to understand NOTCH1 receptor's function and structure.

The NOTCH signaling pathway is an evolutionary conserved mechanism responsible for the direct transduction of extracellular signals into changes in gene expression in the nucleus of cell. The NOTCH family of receptors is composed of four different proteins, NOTCH1-4, that share a similar structure. NOTCH proteins define a unique class of highly conserved transmembrane receptors regulating cell growth, differentiation, and death in different tissues of multicellular organisms (Milner *et al*., 1999; Sanchez-Irizarry *et al*., 2004). Moreover, NOTCH is a general regulator of cell fate determination and interacts with the host factors that are of known significance in hematopoiesis.

The NOTCH1 receptor functions as a ligand-activated transcription factor (expressed on adjacent cells) that directly transduces extracellular signals in the cell surface into changes in the gene expression in the nucleus (Aster *et al*., 2008). The NOTCH1 receptors are class 1 transmembrane glycoproteins expressed at the cell surface as heterodimers consisting of a noncovalently associated N-terminal extracellular (NEC) fragment and a C-terminal transmembrane (NTM) subunit that is noncovalently joined to a intracellular (ICN) subunit. The NEC subunit consists of 36 iterated epidermal growth factor (EGF)-like repeats that include the region responsible for ligand binding with Delta-like and Jagged ligands [Delta/Serrate/LAG-2 (DSL) family] ligands, followed by three LIN-12/NOTCH repeats (LNR) (Aster *et al*., 2008).

A heterodimerization domain (HD) is responsible for stable subunit association. The HD consists of the C-terminal part of the NEC and the N-terminal part of the NTM subunits. The receptor is normally held in a resting metalloprotease-resistant conformation (auto-inhibited state) by a juxtamembrane negative regulatory region (NRR) that contains three cysteine-rich LIN-12-NOTCH repeats (LNRs domain) and the heterodimerization domain (HD) that flanks the S1 cleavage site (Kopan *et al*., 1996; Sanchez-Irizarry *et al*., 2004).

The NTM subunit contains a single-pass transmembrane domain (TM) followed by a RAM domain [RBP-Jκ (recombination-signal-sequence-binding protein for Jκ genes) associated molecule], seven ankyrin (ANK) repeats (exon 31, 32 and 33), a transactivation domain and several nuclear localization signals. The C-terminal part of the receptor contains a PEST [proline (P) glutamic acid (E) serine (S) and threonine (T) rich] sequence, which limits the intensity and duration of NOTCH activation.

Functionally, the RAM and ANK domains of the ICN participate in CSL binding, while the ANK domain is also essential for recruitment of activators and transctivation (Sanchez-Irizarry *et al*., 2004). The transactivation domain (TAD) serves to recruit coactivator molecules and the

PEST degron domain is believed to be essential for proteosome-dependent degradation of NOTCH1 (Weng *et al*., 2004).

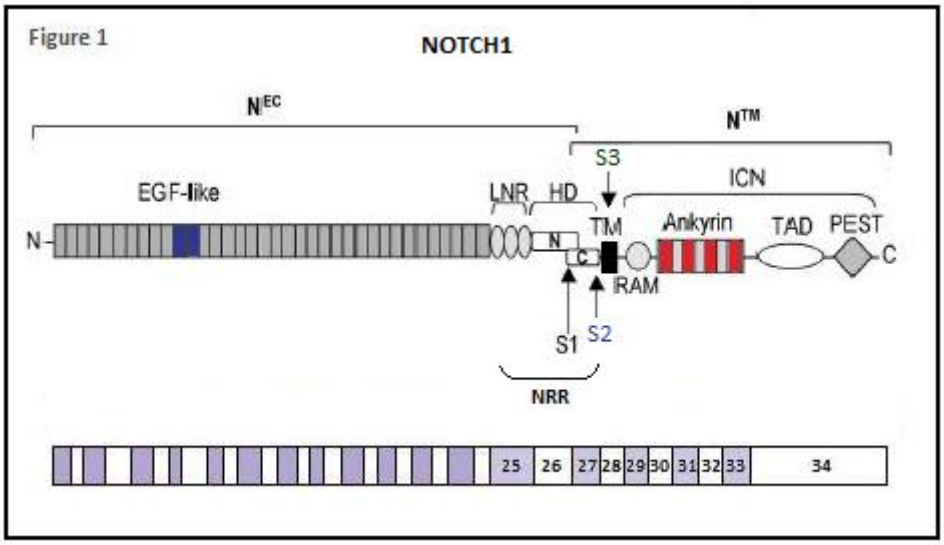

Figure 1. Domain organization of human NOTCH1. The NRR consists of the LNR and HD domains.

Binding of NOTCH ligand to EGF-like repeats 11 and 12 on an adjacent NOTCH receptor initiates a process called intramembrane proteolysis (RIP) that induces a conformational change in the NOTCH extracellular domain, resulting in the exposure of an S2 cleavage site within the extracellular domain. This S2 site is found in the C-terminal portion of the heterodimerization domain (exon 27) about 12-13 amino acids external to the transmembrane domain (Mumm *et al*., 2000). This leads to the proteolytic cleavage of the transmembrane-intracellular domain of the receptor by an ADAM metalloprotease. After this first cleavage, NOTCH receptors undergo regulated intramembraneous proteolysis, by the γ-secretase complex, at a conserved S3 cleavage site located within the transmembrane domain. Hence, the NOTCH intracellular domain (ICN) is released into the cytoplasm, which subsequently translocates into the nucleus, and complexes with CSL DNA binding proteins [C-promoter binding factors (CBF-1) Suppressor of Hairless, and LAG-] and the co activator, MAM (Master mind) to activate responsive genes (Leong *et al*., 2006). CSL is a DNA-binding transcription factor that orchestrates the transcriptional response to NOTCH receptor activation. Mastermind, a glutamine-rich non-DNA binding co-activator, recruits CycC:CDK8 (protein kinase) to phosphorylate the NOTCH ICN and coordinate activation with turnover (Fryer *et al*., 2004). Activation of transcription at CSL-binding sites also appears to depend on the recruitment of additional co-activators, such as CBP and p300 (Figure 2) (Aster *et al*., 2008).

Among the best characterized, NOTCH target genes are basic helix-loop-helix proteins that block cellular differentiation, such as mammalian Hairy/Enhancer of split (HES1, a transcriprional repressor). In the absence of ligand binding, heterodimeric NOTCH receptors are inactive. NOTCH target genes are actively repressed in the absence of signaling by the association of CSL proteins with histone deacetylases and CtBP, SMRT, or CIR co repressors. It is also suggested that the NOTCH enhancer complex may recruit transcription elongation factors (Fryer *et at*., 2004).

Actual models of NOTCH1 activation support that the binding of a ligand induces a conformational change in the LNR repeats which causes: (a) dissociation of the HD subunits, (b) exposure of normally protected metalloprotease cleavage site in the C-terminal of the HD domain, and (c) release of the ICN domain from the membrane via the proteolytic process previously described (Ferrando, 2009).

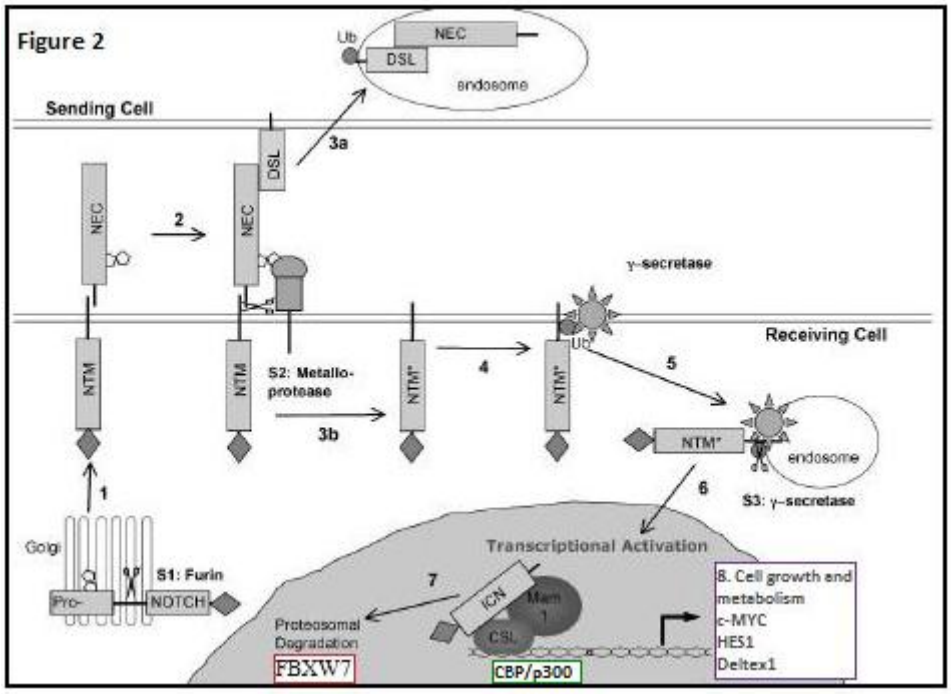

Figure 2. Pathophysiologic NOTCH1 signaling: Normal NOTCH1 is processed and glycosylated in the endoplasmic reticulum/golgi compartment to produce a surface heterodimer (1). Binding of ligand expressed on neighboring cells (2) results in removal of NEC (3) and cleavage of NTM at site S2 to create NTM* (3b), which is a substrate for monoubiquitination in at least some cell types (4). NTM* is endocytosed and cleaved at site S3 by γ-secretase (5). This permits nuclear translocation of ICN1and activation of target genes (6). ICN1 normally is short-lived, being terminated by the FBXW7/SCF mediated ubiquitination and subsequent proteosomal degradation of the ICN1, *via* mechanisms involving the C-terminal PEST domain. (8) The ICN associates with CSL and MAM1 to activate the expression of target genes which regulate cell growth and metabolism *(Adapted from Pear et al., 2004).*

The proper timing of NOTCH signaling is specified by events that control ICN levels in the nucleus. Several E3 ubiquitin ligases have been implicated in ICN turnover, including FBXW7 (F-box and WD repeat domain containing 7 isoform). FBXW7 is a potent tumor suppressor, which not only promotes PEST-dependent ICN degradation in the nucleus but also targets other proteins such as cyclin E and c-myc (Fryer *et al*., 2004). Thus, FBXW7 induces ICN inactivation via binding to a phosphothreonin centered degron (specific F-Box binding sites, spanning from L2511 to 2519) present in the PEST domain of NOTCH1 and targeting activated NOTCH1 to the proteosome (Figure 2) (Thompson *et al*., 2007). *FBXW7* mutations, found in 20% of T-ALL cases, abrogate the recognition of NOTCH1 by FBXW7 leading to the inhibition of the degradation of the activated form of NOTCH1. Thus *FBXW7* mutations are mechanistically related to *NOTCH1* PEST mutations as they result in increased ICN protein stability.

1.3 Abnormal NOTCH signaling is associated with human cancer

Aberrations in NOTCH signaling have been linked to several diseases in mammals. The first human homologue of NOTCH, *NOTCH1*, was identified through its involvement in chromosomal translocation (7;9) (q34; q34.3) found in human T-ALLs (Ellisen *et al*., 1991). This translocation juxtaposes a truncated *NOTCH1* gene (missing the EGF–like, LNR and HD domain) next to the *TCRB* locus, where fusion genes are generated and are essential for the commitment of pluripotent progenitors to the T-cell fate and for the subsequent assembly of pre-T-cell receptor complexes in immature thymocytes (Leong *et al*., 2006).

The alterations in the components of the NOTCH signaling pathway are also linked to a great variety of human diseases, specifically neoplasms. Amongst the most frequent alterations, we find the presence of point mutations, deletions, translocations and viral insertions. In humans, aberrant NOTCH1 expression has been identified as a causative factor in the development of T-cell acute lymphoblastic leukemia and lymphoma. In T-ALL, leukemic thymoblasts can be arrested at different intrathymic maturation stages. Immunophenotyping by using common cell surface markers helps to distinguish between the maturation stages. There are 4 main phenotypes: pro-T, pre-T, cortical and mature phenotype (Bene *et al*., 1995).

The expression of an activated form of NOTCH in developing T-cells biases the choice between the CD4 and CD8 lineages in such a way that CD8 cell development is favored over CD4 cell development. The activation of the NOTCH1 receptor produces the maturative arrest of T-cell blasts in the stage where the cells are both positive for CD4 and CD8 (Robey *et al*., 1996; Li *et al*., 2008). This event correlates to the development of T-ALL, because potentially, NOTCH1 owns a transforming activity that allows the survival of double positive cells, that otherwise would be destined to cell death (apoptosis). NOTCH signaling limits the number of cells that adopt a particular fate and leaves some progenitors uncommited but competent to adopt alternative fates, thus, normal differentiation is impaired and proliferation of immature cells continues.

These alterations cause the expression of the NEC and NTM subunits but exclude the ICN domain. Recently, Weng and associates demonstrated that over 50% of human T-ALL present activating mutations of the NOTCH1 receptor, making *NOTCH1* the most prominent oncogene specifically involved in the pathogenesis of this disease. The most common affected domains are the extracellular heterodimerization domain and the negative regulatory PEST sequence lying at the C-terminus of the NOTCH1 NTM. The mutations in the heterodimerization domain cause destabilization of the association between the NOTCH1 extracellular and transmembrane subunits, resulting in increased NOTCH1 rates of activated intracellular NOTCH1 production in the absence of ligand stimulation. In addition, when the PEST domain is affected, it prolongs the half-life of the intracellular NOTCH1-containing transcriptional activation complex. In

about 25% of T-ALL cases HD mutations are associated with PEST mutations so that these leukemias have a dual mechanism of NOTCH1 activation that combines ligand independent activation and prolonged ICN1 stability, causing synergistic activation of NOTCH1 signaling pathways (Sanchez Irizarry *et al*., 2004; Weng *et al*., 2004; Aster *et al* 2008). These alterations in NOTCH1 structure result in an aberrant up-regulation of the transcriptional pathways depending on NOTCH1 (Weng *et al*., 2004).

These mutations in the *NOTCH1* gene have not been identified in precursor B-cell ALLs; therefore these alterations are unique to T-ALLs (Breit *et al*., 2006).

1.4 Prognostic significance of activating *NOTCH* mutations

In 2006, Breit and associates demonstrated that the presence of the *NOTCH1* mutations in the context of the ALL-BFM treatment strategy significantly correlated with a good prednisone response and favorable minimal residual disease (MRD) kinetics, which is independent from sex, age, white blood cell count and T-cell immunophenotype at the time of diagnosis (Breit *et al*., 2006). It has been observed, that most of the T-ALL with cortical immunophenotype pattern present *NOTCH1* mutations, therefore linking the activated form of the *NOTCH1* and a predisposition to malignant transformation in an intermediate stage of the T-cell differentiation process. In previous reports from the study groups ALL-BFM, COALL and POG, an association between cortical immunophenotype T-cells and favorable clinical response has been reported (Breit *et al*., 2006), especially related with the evaluation of Minimal Residual Disease in these patients. Favorable MRD status was defined as the absence of leukemic cells in 10^4 cells.

As it was previously mentioned, the *NOTCH1* mutation were more common in patients with prednisone good responder subgroup than in the poor responder subgroup (p=.001). In a logistic regression analysis, Breit and associates observed that T-ALL patients with *NOTCH1* mutations were 3 times less likely to show a poor prednisone response (relative risk = 0.33, 95% CI = 0.17-0.65; p = .001) than patients with wild-type *NOTCH1* (Breit *et al*., 2006). Treatment response is further assessed by the measurement of MRD after 33 days and 78 days of treatment. At both time points, most of the T-ALLs with a favorable MRD status ($<10^{-4}$) were *NOTCH1* mutated, whereas in most patients with an unfavorable MRD response ($\geq 10^{-4}$) this mutation was not found. The mechanism of NOTCH1 activation does not appear to play a clinically relevant role.

Furthermore, this study group demonstrated that the activation mutations of this gene determine an important group of patients with excellent long-term prognosis (median length of follow-up 3.35 years) with a significantly better relapse-free survival compared with those without mutations (90% vs. 71%, p=.004). The cumulative incidence of relapse was significantly higher in patients without mutations than in those with *NOTCH1* mutations (16% vs. 4% p=.02). In a multivariate analysis controlled for immunophenotype, the *NOTCH1* mutation status retained its significant effect on long-term event-free survival (EFS), which shows that the *NOTCH1* status is an independent prognostic factor on event-free survival in the context of ALL-BFM 2000 therapy (Breit *et al*., 2006).

In contrast, analysis of a series of 72 pediatric T-ALL patients, treated in the Dutch Childhood Oncology Group (DCOG) protocols ALL-7, ALL-8 or ALL-9, failed to demonstrate a different prognosis for patients whose leukemias harbored activating mutations in *NOTCH1*, suggesting differences in therapy may influence the effect of *NOTCH1* mutations on prognosis (van Grotel

et al., 2006). Furthermore, such opposite findings were also observed by Zhu YM *et al*, where *NOTCH1* mutations in 77 patients with T-ALL correlated with decreased survival time (Zhu *et al*., 2006).

In 1980 the COALL Study Group, part of the German Society of Pediatric Oncology and Hematology, was founded. The main objective of the first multicenter trial, COALL-80, was to reduce the treatment-related morbidity and mortality in induction without loss of efficacy.

The basic guidelines corresponded to BFM-76/79, of proved effectivity (Harms *et al*., 2000). However, asparaginase was omitted from the four-drug induction phase and intercalated between induction and CNS treatment. During 1982 and 1997, four trials for the treatment of childhood ALL were completed by the COALL Study Group. After informed consent 1191 patients with newly diagnosed precursor B-ALL or T-ALL were entered into the protocols from 24 children's hospitals all over Germany. The actual rates of pEFS of the COALL are near 80%. An interesting aspect of the COALL therapy protocols is the *in vitro* drug resistance analysis, developed since the 1990s. With these studies, the prognostic value of a drug resistance profile was confirmed, and the *in vitro* sensitivity of blasts to prednisolone, vincristine and L-asparaginase was identified as an independent prognostic marker, defining a numeric score for the sensitivity /resistance of blasts named PVA score. This score (PVA score) identifies patients at high risk of early treatment failures and may, therefore, be used to improve risk-group stratification of children with ALL. Thus, patients with a favorable resistance profile (Score 3+4) can receive a reduced therapy without loss of effectiveness, whilst those patients with an unfavorable resistance profile can benefit from a more intensive therapy (Janka-Schaub *et al*., 1999; Den Boer *et al*., 2003). In the COALL-97 study, patients are first stratified according to the high-risk and low-risk criteria, and are then once again stratified according to the PVA score.

1.5 Development of childhood ALL treatment in Argentina

In the city of Buenos Aires, malignancies are the first cause of death associated with diseases in children between 1 and 15 years old (De Sarasqueta *et al*.,1991; SAP-Unicef, 2005). In this age group we can observe 12,4 new cases every 100.000 children per year.

Since the year 2000, the Argentine Oncopediatric Hospital Register (Registro Oncopediátrico Hospitalario Argentino (ROHA)-Fundación Kaleidos) collected data from patients under 15 years old with new diagnosis of cancer. This office was developed following the international alignment (OMS/IARC) as sole model (Moreno *et al*., 2007) and its objective is the coordination and centralization of all the information for the statistical analysis of the data in a local, provincial and national level. The actual estimated local coverage of ROHA of the registered cases, in relationship with the expected cases in all the country is of 92%. In the last 9 years, since the opening of this register, the number of new cases recorded by the ROHA has been stable, both in Argentina and most of the provinces. Between 2000-2008 period, 11.445 children under 15 years old with oncologic diseases were registered, representing approximately 1270 new cases per year. The Argentine frequency of oncologic diseases coincides with the information internationally published, with leukemia being the most frequent oncologic disease, followed by the central nervous system tumors and lymphomas.

Comparing the relative frequencies found in the ROHA with international publications from the German Society of Pediatric Oncology and Hematology Registry, we can see in the following table that there is a great similarity in the distribution of pathologies, which allows us to assume that information of the ROHA is of consistent quality.

Table 1: Comparison of relative frequencies ROHA 2000-2005 German register 1980-2004

ICCC	Description	ROHA Register %	German Register %
I	Leukemia	36.7	34.2
II	Lymphomas and Reticuloendotelial neo.	12.7	12.3
III	CNS and misc intracranial & intraspinal neo.	18.9	20
IV	Neuroblastoma & other peripheral nervous cell Tum.	5.6	8
V	Retinoblastoma	3.2	2.3
VI	Renal Tumors	5.0	6.2
VII	Hepatic Tumors	1.5	1
VIII	Malignant Bone Tumors	4.9	4.9
IX	Soft tissue and other extraosseous sarcomas	6.2	6.6
X	Germ cell & trophoblastic tumors & neo. of gonads	3.0	3.3
XI	Carcinomas and Other malignant epithelial neo.	1.6	1.1
XII	Various and non-specific Tumors	0.7	0.1

ICCC: International Classification of Childhood Cancer-1996)

From what is previously reported, we can state that the data published by ROHA are comparable to international publications.

According to the analysis carried out by ROHA, of the 7127 children registered since the year 2000 to 2005, 86% of them were attended in 30 public institutions and the rest in private institutions. Approximately 50% of the cases are treated in 5 hospitals. Due to the complexity of the treatment, 40% of the patients have to, at some point, migrate to hospitals where high complexity treatment is offered.

The Juan P. Garrahan Children's Public Hospital (GH) receives between 300 and 350 new oncologic patients per year, representing approximately one third of all new cases in Argentina (Felice *et al*., 2007). Around 90 to 100 patients have a diagnosis of acute leukemia, and of these, 75% approximately correspond to ALL. Since the opening of Garrahan Public Hospital in August 1987, till November 2002, 989 consecutive patients were enrolled with diagnostic of ALL, without previous treatment, and were included in three therapy protocols (7-LLA 87, 1-LLA 90 and 1-LLA 96) (Sackmann-Muriel *et al*., 1996; Sackmann-Muriel *et al*., 1999; Felice *et al*., 2011). The diagnosis was based according to morphology, cytochemistry, immunophenotype and cytogenetic criteria of the European Group for Diagnosis of Leukemia. In all three protocols, early treatment response was considered as evaluation parameter. This early treatment response was the response to a pre-phase with 7 days of prednisone as only drug, together with one dose of intrathecal chemotherapy during the same week. In all three studies, bad response to prednisone, together with the presence of cytogenetic markers of bad prognosis, as t (9;22), or not achieving complete remission (CR) after induction phase, defined patients in the high-risk group.

The three treatment strategies were elaborated taking into account the basic guidelines used by the German group BFM protocols (Schrappe *et al*., 2000), with local adjustments according to the drug and diagnostic studies availability in Argentina. Local experience in this field was also taken into account.

The analysis of the induction phase response, the description of the events presented by the patients in all three studies, the pEFS (pSLE Probabilidad de Sobrevida Libre de Eventos) in each protocol in general and of the risk groups in particular, and the patients that remained in CR, are described in Table 2 (Felice *et al*., 2007).

In agreement with international literature, the main adverse event observed in the three studies was relapse or recurrent disease, most commonly in bone marrow, and in a smaller proportion, death during induction phase and complete remission (CR).

The analysis of pEFS of the protocols 7-LLA 87, 1-LLA 90 y 1-LLA 96, updated in December 2006, shows values of 61%, 63% y 72%, respectively. The difference between these results are statistically significant (p=.0237).

The decrease observed in the death rates during the induction phase as well as during CR, indicates an indirect measurement of the improvements achieved in the clinical support offered to patients over the years. This decrease in the number of deaths related to the treatment, together with the lower percentage of relapses, has allowed a gradual and significant increase in the pEFS (Felice *et al*., 2007).

Table 2: Induction phase response, events and evolution.

	7-LLA 87	1-LLA 90	1-LLA	P Value
Evaluable Patients	92	374	430	
RESPONSE TO INDUCTION THERAPY				
-Complete Remission (CR) (%)	88 (95,6)	353 (94,4)	417 (96,9)	
-Death during Induction (%)	2 (2,1)	11 (2,9)	8 (1,8)	
-Non response (%)	4 (4,3)	10 (2,7)	5 (1,3)	
EVENTS				
-Relapse	27	100	90	
-Death in CR (%)	6 (6)	17 (4,8)	17 (4,3)	
-Second Malignancy	2	2	5	
P(EFS)				
-Total pEFS (SE)	61 (51)	63 (%)	72 (6)	0.0237
-Standard-Risk Group	72	73	85	
-Intermediate-Risk Group	-	69	71	
-High-Risk Group	56	37	42	
Median in months of follow-up time (range)	116 (230-240)	102 (203-131)	65 (130-48)	
Continue in CCR	53	234	305	

CR: Complete Remission; **CCR:** Complete and continuous Remission; **pEFS:** Event-free survival probability; **SE:** Standard Error.

When we analyze the situation of leukemia in Argentina in relationship with the diagnostic stage, we observe that the Garrahan Hospital can carry out all the necessary determinations for a clear definition of the ALL subtypes, that is, cytochemic, immunophenotype and cytogenetic studies. In recent years, molecular biology studies have been incorporated.

An interesting event to highlight is the incorporation, in 1994 of the immunophenotyping with flow cytometry techniques. Since the 1990s, Garrahan Hospital has also incorporated cytogenetic studies. At the moment, the percentage that can be submitted to evaluation is similar to international standard values. Since 2002, molecular biology studies are carried out systematically in all the patients (Alonso *et al*., 2006) allowing the researchers not only to

complete the diagnostic stage, but also count on a very necessary tool for the determination of MRD kinetics, which is considered, by most international groups, as one of the markers with the highest sensitivity and high correlation with the different prognostic groups.

1.6 International research groups in a globalized world

A collaborative research project of this magnitude is of enormous value for Garrahan Hospital in Argentina. Although Garrahan Hospital has achieved a gradual and significant increase of the pEFS and a decrease in deaths during induction phase and CR (Moreno *et al*., 2007), the results have not yet reached the rates published by international centers of reference.

Another factor to take into account is the technical development of the Argentine institution in comparison with centers in developed countries. As was previously mentioned, important advances in the development of molecular biology studies have been achieved, allowing Garrahan Hospital (GH) to carry out minimal residual disease kinetics (MRD). Nonetheless, the experience in Argentina is not so broad when compared to other centers, specifically German centers, which possess invaluable experience and a long history. The world's tendency reflects the need to work together with other hospitals around the globe. In this way, experiences are shared and the number of patients that can be analyzed is increased. GH can profit from the technological advances and knowledge of reference centers, allowing this institution to offer a better attention to its patients.

The diagnostic stage of this disease is essential for the posterior classification of the patients in the different risk groups, and determines the treatment they will receive. Throughout the years, cytochemistry, immunophenotype, cytogenetic and molecular biology studies have been incorporated. Important prognostic markers and risk factors have been identified, allowing a more adequate attention of the patients with leukemia in Argentina. Nevertheless, the universal tendency is the development of new molecular biology studies, to improve the identification of the different risk groups, genetic characterization of the disease and a better adjustment of the treatment.

Even though near 70% of the patients with T-cell ALL have a good prognosis with the current intensive cytotoxic therapies, new therapies are required for patients who do not respond to the treatment or that are resistant to it. On the other hand, progress in the molecular classification of ALL, through use of DNA microarrays coupled with methods to assess the functional significance of newly discovered genes, will most certainly lead to the identification of targets for specific treatments. The mentioned publications identify the NOTCH1 pathway as a relevant molecular target for new anticancer drugs, through the development of specific inhibitors. A clear example is imatinib mesylate for the treatment of BCR-ABL positive chronic myeloid leukemia. This agent inhibits the BCR-ABL fusion protein and other constitutively active tyrosine kinases and induces transient remissions of BCR-ABL positive ALL and partial response in other cancers (Pui *et al*., 2004).

In relation to the NOTCH1, even though the samples that will be analyzed in this project correspond to patients treated in two centers (Garrahan Hospital and University Medical

Center Hamburg-Eppendorf), which apply different treatments; both therapy protocols have their origin in the BFM study strategy. Furthermore, the analysis of the *NOTCH1* gene mutations will be done in bone marrow samples taken at the moment of the initial diagnosis, which means that they will be free of all treatment.

As previously described, both groups of patients are treated with different chemotherapy protocols, therefore, early treatment response will be evaluated separately for both groups of patients. For the patients of Garrahan Hospital, early treatment response will be evaluated according to the response to prednisone. MRD studies are being incorporated nowadays in the new schedules of treatment. The patients treated according to the COALL study, will be evaluated according to the MRD kinetics at days 29-33 and with the evaluation of the PVA score, previously mentioned. *NOTCH1* mutations will be analyzed as a prognostic factor independent from sex, age, white blood cell count and T-cell immunophenotype at the time of diagnosis.

The establishment of a new relationship between the University Medical Center Hamburg-Eppendorf (UKE) and Garrahan Hospital will allow a fluent, satisfying exchange of knowledge for both entities. In this way, the Argentine hospital will benefit from the long and vast experience of the German center, applying the technological and scientifically advances, within GH possibilities, incorporating Argentine patients in the data analysis of one of the most important reference centers worldwide.

The posterior implementation of molecular biology techniques for the identification of mutations in the *NOTCH1* gene, in the molecular biology lab in Garrahan Hospital are of invaluable importance for the future development of both hospitals, allowing GH to reach international standards comparable to the best reference centers in the world.

Further refinements in the molecular classification of ALL, together with the identification of genetic features that affect the efficacy and toxicity of antileukemic therapy, will afford unique opportunities to devise treatment plans for individual patients and thus to reach the elusive goal of cure of all patients, regardless of their initial characteristics upon diagnosis.

Research in this field is crucial, since the prognosis of relapsed ALL patients remains poor, and therapy can be tailor-made, so that each patient receives precisely the right treatment, thus avoiding unnecessary toxicity. In this study we investigate the potential association between NOTCH1 and prognosis, in an attempt to better understand the relationship with disease progression and treatment response.

2. MATERIALS AND METHODS

2.1 Patients and cell samples

A cohort of 171 children with newly diagnosed T-cell lymphoblastic leukemias were analyzed, that include 38 patients treated according the ALL IC-BFM protocol (Garrahan Hospital, Buenos Aires, Argentina) and 133 German patients treated according COALL protocol (German Co-Operative Study Group for Childhood Lymphoblastic Leukemia). Informed consent was provided at the time of diagnosis according to the Declaration of Helsinski. Mononuclear cells (MNCs) were isolated from bone marrow (BM) samples and stored at - 80°C until DNA extraction. DNA was isolated using the Qiagen QiAmp DNA mini kit (Qiagen-USA, Valencia, CA) per the manufacturers' directions.

MRD monitoring was based on quantitative detection of leukemic clone-specific T-cell-receptor gene rearrangements as previously described (Cavé *et al*., 1994; Cavé *et al*., 1998).

Surface markers analysis was performed on mononuclear cells from bone marrow aspirates. Immunophenotyping of ALL was carried out as previously described (Bene *et al*., 1995) and the criteria for sub classification of T-ALL were adopted according the guidelines proposed by the European Group for immunological Characterization of Leukemias (Bene *et al*., 1995).

2.2 *NOTCH1* mutations screening

Mutation screening was performed by direct sequencing of PCR-amplified DNA fragments. For all 171 patients, *NOTCH1* exons 25 to 34 were screened for mutations. These exons include the negative regulatory regions covering the 3 lin12/NOTCH repeats (LNR) and the heterodimerization domain (HD), the juxtamembrane (JM), the RBP-JKappa-associated module (RAM), the ankyrin-repeat (ANK), the transactivation (TAD), and the proline, glutamic acid, serine, threonine rich (PEST) domains. PCR reactions were performed using 50ng of genomic DNA (10ng/µl), 10µM of upstream primer and 10µM of downstream primer, Nuclease-Free Water 22 µl and 25µl of GoTaq ® Green Master Mix, 2X, that contains 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3mM $MgCl_2$ (Promega Corporation, Madison, WI, USA) in a total volume of 50 µl. After initial denaturation at 95°C for 5', PCR was performed at 40-50 cycles of 95°C for 40", 65°C for 30" and 72°C for 1'. 22 primer pairs were developed to amplify exons 25 till 34 (Table 1, Appendix A). PCR products were visualized on an ethidium bromide-stained 1% agarose gel (1x TAE-Tris-acetate EDTA buffer® (Millipore, Bedford, MA, 01730, USA) and purified with Millipore Ultrafree®-DA filter device (Millipore, Bedford, MA, 01730, USA), and subsequently sequenced with the BigDYE Terminator v3.1

Cycle sequencing KIT (Applied Biosystems) according to the manufactures' protocol on an ABI PRISM 3130 DNA Analyzer (Applied Biosystems).

Forward and reverse sequences were aligned using SeqMan and frameshifts were analyzed using EditSeq (DNASTAR Inc). All sequences were compared with the reference sequences for genomic DNA. GenBank accession numbers for *NOTCH* genomic sequence is NM_ 017617.3. Presence of single-nucleotide polymorphisms was checked consulting the National Center for Biotechnology information (NCBI) browser (http://www.ncbi.nlm.nih.gov/SNP). Prediction of structural effects of nucleotide substitutions, deletions or insertions on the structure of the *NOTCH1* protein was achieved using dedicated prediction software (EditSeq, Lasergene). All these DNA sequence analysis programs are part of the DNASTAR Lasergene 7.1 software package (DNASTAR, Inc., Madison, WI).

Mutations were designated according to the "Nomenclature for the description of sequence variants" issued by the Human Genome Variation Society (HGVS), URL: http://www.hgvs.org/mutnomen/.

PCR products from exons 26, 27, 28 and 34 amplified from DNA obtained from 14 patients were TA-cloned into the plasmid vector pCR®4-TOPO® and chemically competent *E.coli.* (Invitrogen Corporation, Camarillo, CA, USA) and between 12 and 48 clones from each amplification product were sequenced. Sequences were analyzed for the presence of mutations using EditSeq (DNASTAR Inc). The procedure used for cloning is described in Appendix A.

2.3 Treatment Protocol

As previously mentioned, in treatment protocol ALL IC-BFM 2002 (Garrahan Hospital) early *in vivo* response to a 7-day prednisone treatment prophase and a single dose of intrathecal methotrexate on day 1 served to assess the effect of early treatment. Early prednisone response was defined as the number of peripheral blood blasts per micro liter on day 8). According to treatment response, patients were classified into good responders (<1000 blasts/µl at day 8) or poor responders (≥1000 blasts/µl at day 8).

For patients treated according to the COALL protocol, treatment response was further evaluated by determination of MRD kinetics that were analyzed at two different time points (TPs), at day 29 (TP1) and 43 (TP2) of treatment. Favorable MRD status was defined as the absence of leukemic cells in 10^4 cells.

Complete remission (CR) was defined as <5% blasts in a regenerating bone marrow, the absence of leukemic blasts in the peripheral blood and cerebrospinal fluid, and no evidence of localized disease. *Null response* was defined as the failure to achieve CR after induction phase, and children who died before achieving CR were defined as death during induction cases. *Relapse* was defined as occurrence of lymphoblasts or localized infiltrates at any site.

Central nervous system (CNS) involvement was established by the presence of at least 5 nucleated cells per micro liter in cerebrospinal fluid (CSF) with blasts detected by cytospinning and confirmed by immunophenotyping studies.

2.4 Statistical Analyses

Event-free survival (EFS) was measured from the time from diagnosis to the date of last follow-up in complete remission or to the first event. *Events* were resistance to therapy (non-response), relapse, secondary neoplasm or death due to complications. *Failure to achieve remission* (early death or resistant leukemia) was considered as event at time zero. *Survival* was defined as the time of diagnosis to death from any cause or last follow-up.

Proportional differences between groups were analyzed by chi-squared (X^2) or Fisher's exact tests. The association between *NOTCH1* mutations and prednisone response or MRD was examined by use of unconditional logistic regression analysis to calculate relative risks (RR) and their 95% confidence intervals (CI). For these analyses, MRD loads smaller than 10^3 was defined as negative. The Kaplan-Meier method was used to estimate survival rates. Differences in prognosis between groups were evaluated using the 2-sided log-rank test. Gray's test was used to analyze differences in the cumulative incidence of relapse between patients with *NOTCH1* mutations and those without. Relative risks were estimated according to the Cox proportional-hazards model. Data were considered significant when the P value was ≤ 0.05 (two sided).

Estimated probability of 5-years EFS (pEFS) and estimated cumulative incidence of relapse for the Cooperative study group for childhood acute lymphoblastic leukemia (COALL) cohort were initially done by Martin Zimmerman, Hannover. Estimated probability of 5-years EFS (pEFS) for the Argentine patients treated according to the International BFM (Berlin-Frankfurt-Münster) study group (ALL IC-BFM) protocol were performed using SPSS 17.0 software (SPSS INC., Chicago, IL, USA).

3. RESULTS

3.1 *NOTCH1* mutations in pediatric T-ALL

Primary bone marrow samples were obtained from 133 children and adolescents with T-ALL (median age of 8.0 years; range: 1.0 - 17.0 years), who were enrolled in the German Co-Operative Study Group for Childhood Lymphoblastic Leukemia Study (COALL-92-97-03) between the year 1992 and 2007. Heterozygous *NOTCH1* mutations were identified in 80 of 133 T-ALL samples (80/133; 60.2%). In 47/80 samples mutations were detected only in the HD domain (58.8%), in 11/80 in the PEST domain (13.8%) and in 19/80 in both domains (23.7%) (Figure 3).

Two patients (2/80; 2.5%) presented mutations in the LNR domain (exon 25); one of them was simultaneously affected by a mutation in the PEST domain (patient 8). Only one patient (1.25%) had a mutation in the TM domain. This is in line with previous reports on childhood T-ALL, (Weng *et al*., 2004; Breit *et al*., 2006) and confirms a slightly lower incidence in childhood T-ALL compared with adult T-ALL (Mansour *et al*., 2007).

When analyzing the ALL IC-BFM cohort (median age of 11 years; range: 1.0 - 16.0 years), we identified heterozygous *NOTCH1* mutations in 24 of the 38 T-ALL samples (24/38; 63.2%). In 17/24 samples mutations were detected only in the HD domain (70.8%), in 1/24 in the PEST domain (4.2%) and in 4/24 in both domains (16.7%). Interestingly, one patient (4.2%) (patient 6) was found to have two mutations, one in exon 26 and in exon 31, the ankyrin domain, a domain that is very rarely affected. Only one patient (4.2%) had a mutation in the TM domain. Furthermore, 1 sample presented the c.4077C>T point mutation in exon 25, a silent mutation (since AAT and AAC both encode Asparagine; p.N1359N). From the 17 mutations found in the HD domain, 11 (64, 7%) introduced Proline residues in the protein which are known to alter α-helical structures, essential for the secondary structure of proteins, causing disruptions of folded structures and therefore reducing the stability of the heterodimer. This high frequency of mutations that introduce Pro-residues in the HD domain was also observed in the COALL cohort; 27 cases were observed, representing 57.4 % of all the HD mutations. This is in accordance with other publications (Malecki *et al*., 2006; Breit *et al*., 2006).

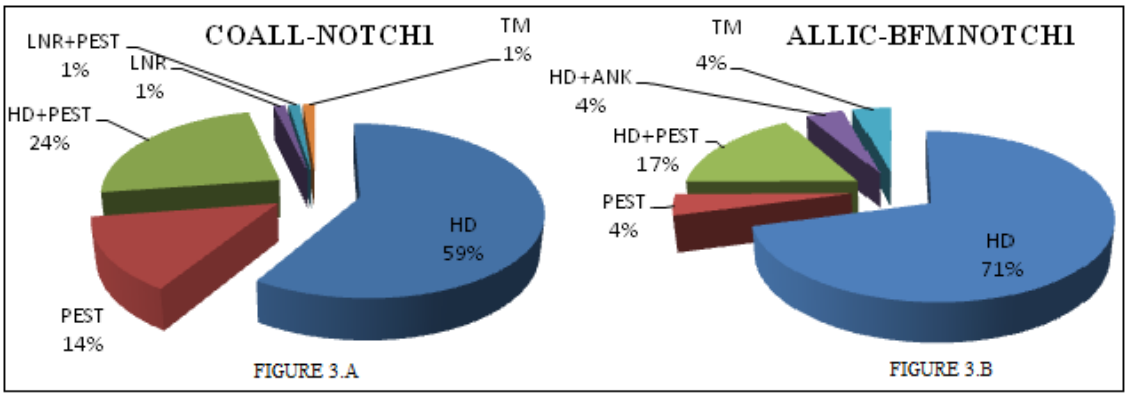

Figure 3. Location of *NOTCH1* mutations in pediatric T-ALL. The distribution of *NOTCH1* mutation types in the ALL IC-BFM (T-ALL n=38) and COALL (T-ALL n=133) cohort. Heterodimerization domain (HD) juxtamembrane or transmembrane domain (TM), PEST domain, lin12/NOTCH repeats (LNR), Ankyrin-repeat domain (ANK).

3.2 Basic clinical and biological characteristics of T-ALL according to *NOTCH1* status

The main characteristics of the patients are described in Table 3 for the COALL cohort and Table 4 for the ALL IC-BFM cohorts. The clinical and biological features of patients with T-ALL were analyzed according to the presence or absence of *NOTCH1* mutations. Both study groups were analyzed separately. In the COALL cohort, 6 patients were left out of the analysis of event-free survival and relapse free survival; 4 of these didn't complete the treatment, one had received previous treatment and one patient was treated according the AML protocol since it was affected by biphenotypic leukemia. The 6 patients are evenly distributed in the *NOTCH*+ and *NOTCH*- groups.

For the 127 patients analyzed (50 *NOTCH* WT [39.4%] versus 77 *NOTCH* mutated [60.6%]) from the German cohort, there was no association between gender, age, CNS involvement, mediastinal involvement or white blood cell count at diagnosis and the presence of *NOTCH1* mutations (Table 3).

When considering the 38 patients treated according to the ALL IC-BFM 2002 protocol, we did not observe a relationship between *NOTCH1* mutations with gender, age or WBC counts at diagnosis (Table 4). In contrast, there was a clear association between *NOTCH1* mutations and the common cortical immunophenotype (19/24, 79.2%; P value 0.022). This is accordance with previous publications (Breit *et al*., 2006) and confirms the theory that T-cell progenitors are specifically sensitive to transformation by NOTCH1 (Aster *et al*., 2008). This association with cortical immunophenotype was not observed in the COALL cohort. This could be explained by the fact that T-ALLs in the German group were classified in 5 different groups, therefore reducing its statistical significance.

Table 3: Patients treated according to the COALL protocol: Clinical and immunologic characteristics according to either presence or absence of *NOTCH1* mutations.

Variable/total (%)	Total population studied 127 (100%)	*NOTCH1* mutation **Negative** n (%) 50 (100%)	*NOTCH1* mutation **Positive** n (%) 77 (100%)	**P** [b]
Gender				.24
Male	89 (71.1)	38 (76.0)	51 (66.2)	
Female	38 (29.9)	12 (24.0)	26 (33.8)	
Age at diagnosis (years)				.79
<10	73 (57.5)	28 (56.0)	45 (58.4)	
≥10	54 (42.5)	22 (44.0)	32 (41.6)	
Median (years)	8.0	9.0	8.0	
Presenting on diagnosis WBC ($10^3/\mu l$) [a]				.39
<10	23 (18.1)	11 (22.0)	12 (15.6)	
10-<50	43 (33.9)	17 (34.0)	26 (33.7)	
50-<100	24 (18.9)	6 (12.0)	18 (23.4)	
≥100	37 (29.1)	16 (32.0)	21 (27.3)	
Median ($10^3/\mu l$)	45.9	44.15	57.2	
Range ($10^3/\mu l$)	1.0-784	1.3-621	1.0-784	
T-cell immunophenotype#				.36
Early-T (Pro/Pre)	20 (16.8)	10 (20.0)	10 (12.9)	
T-ALL (not further specified)	27 (21.2)	12 (24.0)	15 (19.5)	
Cortical	63 (49.6)	20 (40.0)	43 (55.9)	
Mature	17 (13.4)	8 (16.0)	9 (11.7)	
CNS involvement*				.88
No	115 [94.3]	46 [93.9]	69 [94.5]	
Yes	7 [5.7]	3 [6.1]	4 [5.5]	
Missing	5 (3.9)	1 (2.0)	4 (5.2)	
Mediastinal involvement*				.49
No	70 [57.4]	24 [53.3]	46 (59.7)	
Yes	52 [42.6]	21 [46.7]	31 (40.3)	
Missing	5 (3.9)	5 (10.0)	0	

[a] WBC: White blood cell count

[b] P X ² Tests

° Percentages between [] were calculated taking into account only documented cases.

Remark: For these criteria, X^2 value was calculated over a total of 120 patients.

* Remark: For these criteria, X^2 value was calculated over a total of 122 patients.

Table 4: Patients treated according to the ALL IC-BFM protocol: Clinical and immunologic characteristics according to either presence or absence of *NOTCH1* mutations

Variable/total (%)	Total population studied 38 (100%)	*NOTCH1* mutation Negative n (%) 14 (100%)	*NOTCH1* mutation Positive n (%) 24 (100%)	P [b]
Gender				.85
Male	32 (84.2)	12 (85.7)	20 (83.3)	
Female	6 (15.8)	2 (14.3)	4 (16.7)	
Age at diagnosis (years) *			*	.89
<10	13 [37.1]	5 (35.7)	8 [38.1]	
≥10	22 [62.9]	9 (64.3)	13 [61.9]	
Median (years)	11	11	11	
Presenting on diagnosis WBC (10^3/µl) [a]			*	.48
<10	1 [2.9]	1 (7.1)	0	
10-<50	10 [28.6]	5 (35.8)	5 [23.8]	
50-<100	4 [11.4]	1 (7.1)	3 [14.3]	
≥100	20 [57.1]	7 (50.0)	13 [61.9]	
Median (10^3/µl)	128	107	159	
Range (10^3/µl)	2.6-630.0	2.6-630.0	20.0-532.0	
T-cell immunophenotype				**.022**
Early-T (Pro/Pre)	4 (10.5)	2 (14.3)	2 (8.3)	
Cortical	24 (63.2)	5 (35.7)	19 (79.2)	
Mature	10 (26.3)	7 (50.0)	3 (13.5)	
CNS involvement			*	.81
No	32 [91.4]	13 (92.9)	19 [90.5]	
Yes	3 [8.6]	1 (7.1)	2 [9.5]	
Missing	3 (7.9)	0	3 (12.5)	
Mediastinal involvement			*	.32
No	21 [60.0]	7 (50.0)	7 [33.3]	
Yes	14 [40.0]	7 (50.0)	14 [66.7]	
Missing	3 (7.9)	0	3 (13.5)	

[a] WBC: White blood cell count

[b] P X ² Tests (only significant P-values are indicated in bold).

* Remark: For these criteria, positive *NOTCH1* mutation was calculated over a total of 21 patients.

° Percentages between [] were calculated taking into account only documented cases.

#T-Cell immunophenotype: Immature (cyCD3+, Cd7+, CD1-) Cortical (CD1a+), Mature (CD1a-, sCD3+).

3.3 Prognostic significance between *NOTCH1* mutations and treatment response

From the 127 patients enrolled in the cooperative study group for childhood acute lymphoblastic leukemia studies (COALL-92-97-03) COALL protocol, three patients did not reach CR (non response). With a median follow-up of 88 months, there was a total of 39 events [19 (38%) events in the *NOTCH*- group and 20 (25.97%) in the *NOTCH*+ group]. Within these events, 23 consisted in relapses, 6 patients presented an early death (2 died in CR) and 7 presented second malignancies. A total of 31 patients died during follow-up. There was loss of follow-up in two patients (one died at the age of 21 from leukemia relapse, no information was available from the other patient); 94 remained alive in complete continuous remission (CCR) (Table 5).

The effect of *NOTCH1* mutations on early treatment response and long-term outcome was also analyzed (Figure 4).

Early treatment response was also analyzed in relation to the *NOTCH1* status. For 96 patients, the MRD level at day 29 was known. It is important to remember, that as T-ALL often present a delayed MRD kinetic, the cut-off values of MRD are different for T-ALL and B-precursor ALL (Escherich *et al*., 2010). As reminder, COALL protocol defines at both time points (weeks 5 and 12 after start of therapy) a favorable MRD status ($< 10^{-3}$) by the absence of detectable leukemic cells in 10^3 cells, whereas an unfavorable MRD status ($\geq 10^{-3}$) is defined by the presence of at least one leukemic cell in 10^3 cells. Of interest, *NOTCH1* mutations were more common in MRD at day 29 $< 10^{-3}$ subgroup than in the unfavorable MRD at day 29 subgroup, and the two-tailed P-value is considered to be statistically significant (P=.0095) (Table 5). Although when compared to other protocols the cut-off value for MRD on day 29 tolerates a higher burden of leukemic cells, we can say that *NOTCH1* mutations represent, in relation to this parameter, a good prognostic factor.

When considering COALL patients, the outcome of *NOTCH*+ patients was similar to that of *NOTCH*- patients (Figure 4): the 8-year EFS rates were 71% and 59% (Log Rank p=.12), respectively and the relapse rate at 10 years were 20% for both groups [p(Gray)=0.99].

Table 5: COALL: Treatment results according to *NOTCH1* mutation status

		***NOTCH1* mutation**		
	Total Population studied 127 (100%)	**Negative** n (%) 50 (100%)	**Positive** n (%) 77 (100%)	**P ***
Events	39 (30.7)	**19** (38)	**20** (25.97)	
Relapse	23 (18.2)	**9** (18)	**14** (18)	(Gray).99
* BM only	15 (11.8)	6 (12.0)	9 (11.7)	
* CNS only	3 (2.4)	0	3 (3.9)	
* BM and CNS	3 (2.4)	2 (4.0)	1 (1.3)	
* Other	2 (1.6)	1 (2.0)	1 (1.3)	
Non-response	3 (2.4)	**3**	**0**	
Early death	6 (4.7)	**4**	**2**	
Second Malignancies	7 (5.5)	**3**	**4**	
MRD on day 29				**.0095**
Data not available	31 (24.4)	10 (20)	21 (27.2)	
Favorable $< 10^3$	51 (40.2)	15 (30.0)	36 (46.6)	
Unfavorable $\geq 10^3$	45 (35.4)	25 (50.0)	20 (26.9)	
MRD on day 43				
Data not available	80 (63.0)	30 (60)	50 (64.9)	
Favorable $< 10^3$	28 (22.0)	10 (20)	18 (23.4)	.2497
Unfavorable $\geq 10^3$	19 (15.0)	10 (20)	9 (11.7)	
CCR	94 (74.0)	35 (70.0)	59 (76.6)	
pEFS (SE)		**0.59 (.07)**	**0.71 (.06)**	

CCR: continuous complete remission

* P X^2 Test

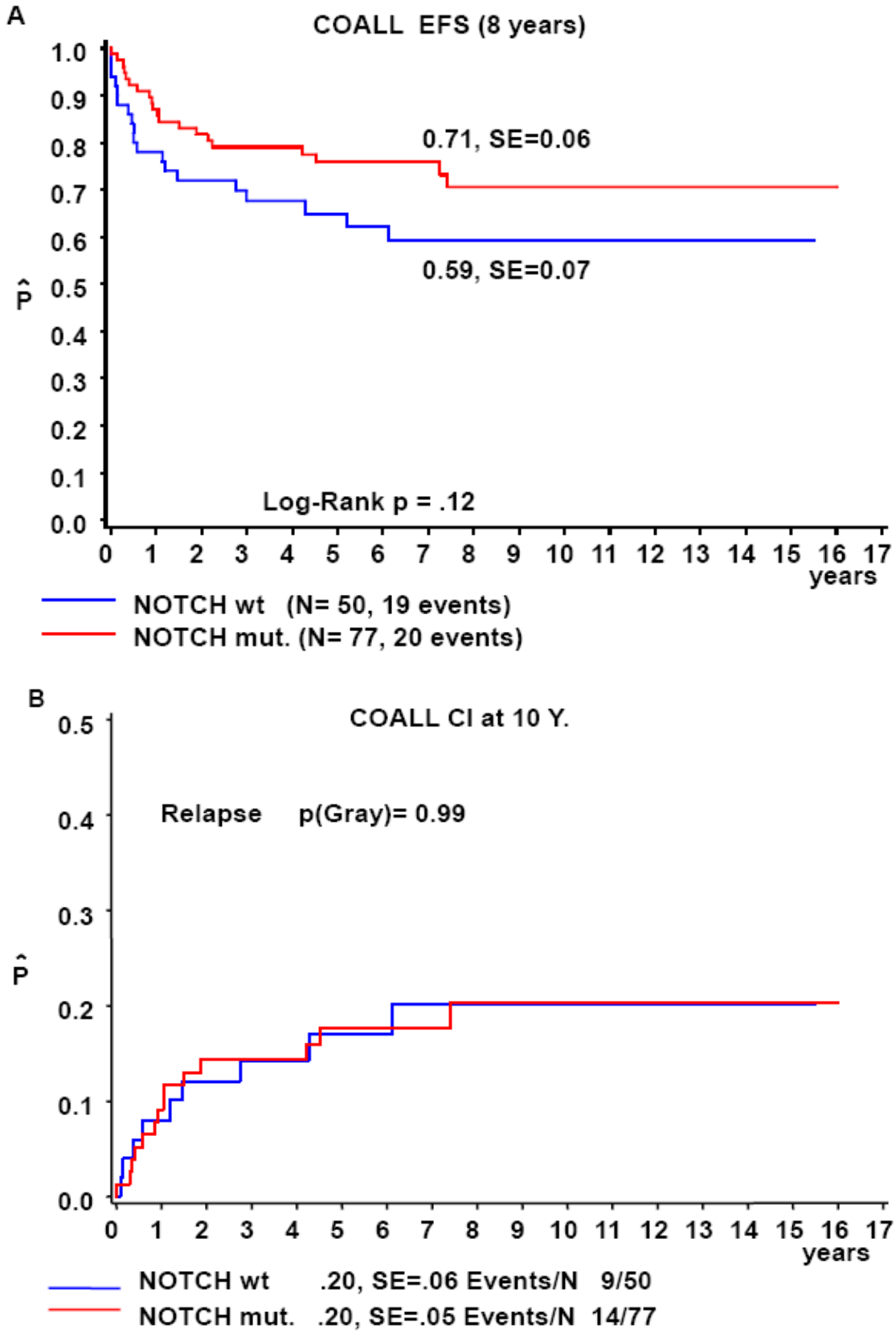

Figure 4. COALL study group: Effect of *NOTCH1* mutations on event-free survival in T-ALL. One-hundred and twenty seven children with precursor T-ALL were classified according to the presence or absence of *NOTCH1* mutations. (A) Kaplan-Meier estimate of event-free survival (EFS) at 8 years. (B) Cumulative incidence of relapse.

The median follow-up of the 38 patients enrolled in the International BFM (Berlin-Frankfurt-Münster) study group (ALL IC-BFM) was 40.3 months. Events in this cohort are summarized in Table 6. There was a total of 6 events [2 (14.3%) in the *NOTCH*- group and 4 (16.7%) in the *NOTCH*+ group]. Within the *NOTCH*- group, one patient died before starting treatment and the other died in complete remission. Among *NOTCH*+ patients, one patient suffered early death, one died in CR, one had a relapse and another patient developed a second malignancy (Ewing sarcoma). Thirty three remained alive in complete continuous remission (CCR) (Table 6).

Of the patients treated by the ALL IC-BFM protocol, the *in vivo* prednisone response was known for 36 individuals. *NOTCH* mutations were associated with prednisone good response (PGR) since 20 of the 27 patients with PGR were affected by one or more *NOTCH1* activating mutations. In contrast, only 3 of the 9 patients that presented a prednisone poor response presented mutations in *NOTCH1* gene, therefore the two-tailed P-value is considered to be statistically significant (P=0.028) (Table 6).

The effect of *NOTCH1* mutations on long-term prognosis was also analyzed (Figure 4). When considering ALL IC-BFM patients, the outcome of patients of *NOTCH*-mutated patients was similar to that of *NOTCH*- patients (Figure 5): the 5-year EFS rates were 65% and 86% (Log Rank p=.81), respectively.

Cumulative incidence of relapse was not analyzed for the Argentine cohort, since only 1 patient relapsed in the *NOTCH* mutated group. In order to avoid false conclusions this parameter was excluded from the analysis.

Table 6: ALL IC-BFM: Treatment results according to *NOTCH1* mutation status

		***NOTCH1* mutation**		
	Total population studied 38 (100%)	**Negative n (%)** 14 (100%)	**Positive n (%)** 24 (100%)	**P [a]**
Prednisone response [#]				**.028**
Good	27 (71.0)	7 [53.8]	20 [87.0]	
Poor	9 (23.7)	6 [46.7]	3 [13.0]	
Unknown	2 (5.3)	1 (7.1)	1 (4.2)	
Complete remission	37 (97.4)	13 (92.9)	24 (100)	
Non-response	-	-	-	
Events	6 (15.8)	**2** (14.3)	**4** (16.7)	
Death during induction	2 (5.3)	1 (7.1)	1 (4.2)	
Death in complete remission	2 (5.3)	1 (7.1)	1 (4.2)	
Relapse	1 (2.6)	-	1 (4.2)	
Second malignancies	1 (2.6)	-	1 (4.2)	
CCR	33 (86.8)	12 (85.7)	21 (87.5)	
pEFS (SE)		**0.86 (.094)**	**0.65 (.195)**	

CCR: continuous complete remission

[a] P X^2 Test

[#] Remark: For this criterion, X^2 was calculated over a total of 36 patients. Prednisone good response: < 100 leukemic blood blasts/µl on treatment day 8; poor: ≥100/µl.

° Percentages between [] were calculated taking into account only documented cases.

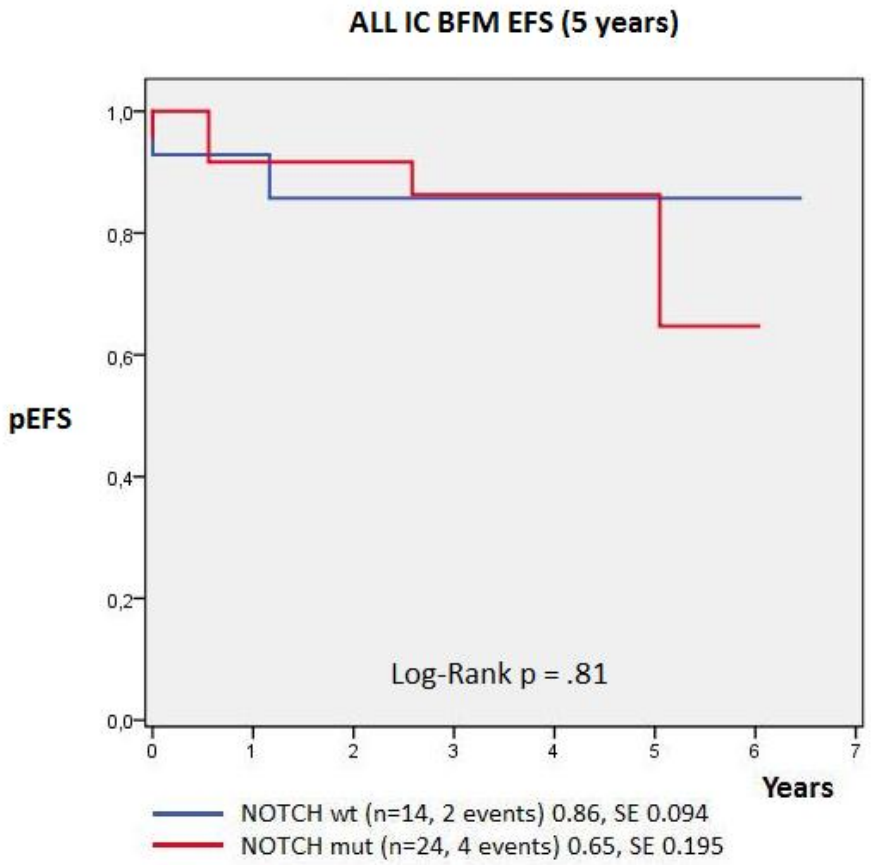

Figure 5. ALL IC-BFM study group: Effect of *NOTCH1* mutations on event-free survival in T-ALL. Thirty eight children with precursor T-ALL were classified according to the presence or absence of *NOTCH1* mutations. Kaplan-Meier estimate of event-free survival (EFS) at 5 years.

3.4 Prognostic relevance according to the affected NOTCH1 domains

Although up to date all mutations found in T-ALL affecting the *NOTCH1* gene are activating mutations, it is suggested that mutations in different gene domains may have a different effect on the activation of downstream target genes. Zuurbier and coworkers describe two different subgroups; those affecting the HD or PEST domain are considered as weak *NOTCH1* activating mutations. Strong activating mutations are those that affect the transmembrane domain (exon 28) or combinations of *NOTCH* HD and PEST mutations (Zuurbier *et al*., 2010).

Within the increasingly large number of distinct HD mutations and C-terminal deletions, variation in potency is likely. Whether this will translate into differences in clinical behavior or transforming activity remains unknown.

Regarding the COALL patients, when we analyze the clinical and immunologic characteristics according to the localization of *NOTCH1* mutations in 77 children with T-ALL, there seems to be no correlation with any of the clinical and immunologic characteristics (Table 7).

However, when analyzing early treatment response, we do observe a tendency to better outcome in patients with mutations in the HD domain only (exons 26 or 27) when compared to mutations in other domains.

When measuring treatment response by measurement of MRD at day 29, data was available for 34 patients. Most of the T-ALLs with a favorable MRD status ($<10^{-3}$) were *NOTCH1* mutated (24 out of the 34 patients), whereas most with an unfavorable MRD response ($\geq 10^{-3}$) presented no mutations. This observation was stronger when comparing this subgroup of patients with the wild-type group, where only 15 of the 40 patients had a favorable MRD response at day 29 (P=.004).

When we further analyze this same subgroup of mutation, even though the MRD status at day 43 was only known for 13 of the 45 patients, we still observe the same correlation, where 11 of the 13 presented a favorable MRD status ($< 10^{-3}$).

The correlation between HD mutations and a good early treatment response can also be observed in the ALL IC-BFM patients. The response to prednisone treatment was known for 16 of the 17 patients presenting mutations in the HD domain. In this case, most of the patients with HD mutations had a good prednisone response [15 out of 16 patients (93.8%)]. This tendency was also present when comparing this subgroup of patients with the *NOTCH*-non-mutated cases, where only 7 out of 13 patients presented a good prednisone response (P=.013). No other associations were observed with any of the clinical and immunologic characteristics (Table 8).

Table 7: COALL cohort: Clinical and immunologic characteristics according to the localization of *NOTCH1* mutations in 77 children with T-ALL

	***NOTCH1* mutation**				
	HD	**PEST**	**PEST + HD**	**Others**	**P [b]**
Variable/total (%)	**45 (100)**	**10 (100)**	**19 (100)**	**3 (100)**	
Gender					.629
Male	29 (64.4)	7 (70.0)	12 (63.2)	3 (100.0)	
Female	16 (35.6)	3 (30.0)	7 (36.8)	-	
Age at diagnosis (years)					.491
<10	29 (64.4)	4 (40.0)	10 (52.4)	2 (66.7)	
≥10	16 (35.6)	6 (60.0)	9 (47.4)	1 (33.3)	
Presenting on diagnosis WBC (10^3/µl)					.914
<10	7 (15.6)	2 (20.0)	3 (15.8)	-	
10-<50	17 (37.8)	2 (20.0)	5 (26.3)	2 (66.7)	
50->100	10 (22.2)	3 (30.0)	5 (26.3)	-	
≥100	11 (24.4)	3 (30.0)	6 (31.6)	1 (33.3)	
T-cell immunophenotype #					.390
Early- T (Pro/Pre)	6 [13.7]	2 (20.0)	1 [6.25]	-	
T-ALL (not further specified)	12 [28.6]	2 (20.0)	1 [6.25]	-	
Cortical	19 [45.2]	6 (60.0)	11 [68.7]	3 (100.0)	
Mature	5 [11.9]	-	3 [18.8]	-	
CNS involvement *					.073
No	41 (91.1)	10 (100.0)	16 (84.2)	2 (66.7)	
Yes	1 (2.2)	-	2 (10.5)	1 (33.3)	
Missing	3 (6.7)	-	1 (5.3)	-	
Mediastinal involvement					.674
No	26 (57.8)	6 (60.0)	13 (68.4)	1 (33.3)	
Yes	19 (42.2)	4 (40.0)	6 (31.6)	2 (66.7)	
MRD on day 29					.271
Data not available	11 (24.4)	6 (60.0)	4 (21.1)	-	
Favorable < 10^{-3}	24 (53.3)	1 (25.0)	8 (42.1)	2 (66.7)	
Unfavorable ≥10^{-3}	10 (22.2)	3 (75.0)	7 (36.8)	1 (33.3)	
MRD on day 43					**.043**
Data not available	32 (71.1)	8 (80.0)	9 (47.4)	1 (33.3)	
Favorable < 10^{-3}	11 (24.4)	-	5 (26.3)	2 (66.7)	
Unfavorable ≥ 10^{-3}	2 (4.4)	2 (20.0)	5 (26.3)	-	

[b] P X^2 Test

Remark: For these criteria, X^2 value was calculated over a total of 71 patients.

* Remark: For these criteria, X^2 value was calculated over a total of 73 patients.

° Percentages between [] were calculated taking into account only documented cases.

We also addressed the issue of whether specific *NOTCH1* mutation may also have a prognostic significance in long-term outcome: that is, if mutations in the HD or PEST domain only or both domains have a different outcome when compared between each other and the wild-type group. Events and the EFS rates are summarized in Figure 6. Patients affected with mutations in the HD domain are associated with a significant better outcome, with p (log-rank)-values of p=0.012 and p=0.0041, compared to patients without *NOTCH1* mutations (wild-type) or patients with PEST (exon 34) *NOTCH1* activating mutations. Interestingly, when both domains are affected (exon 34 + 26/27), the pEFS is similar to the wild-type group.

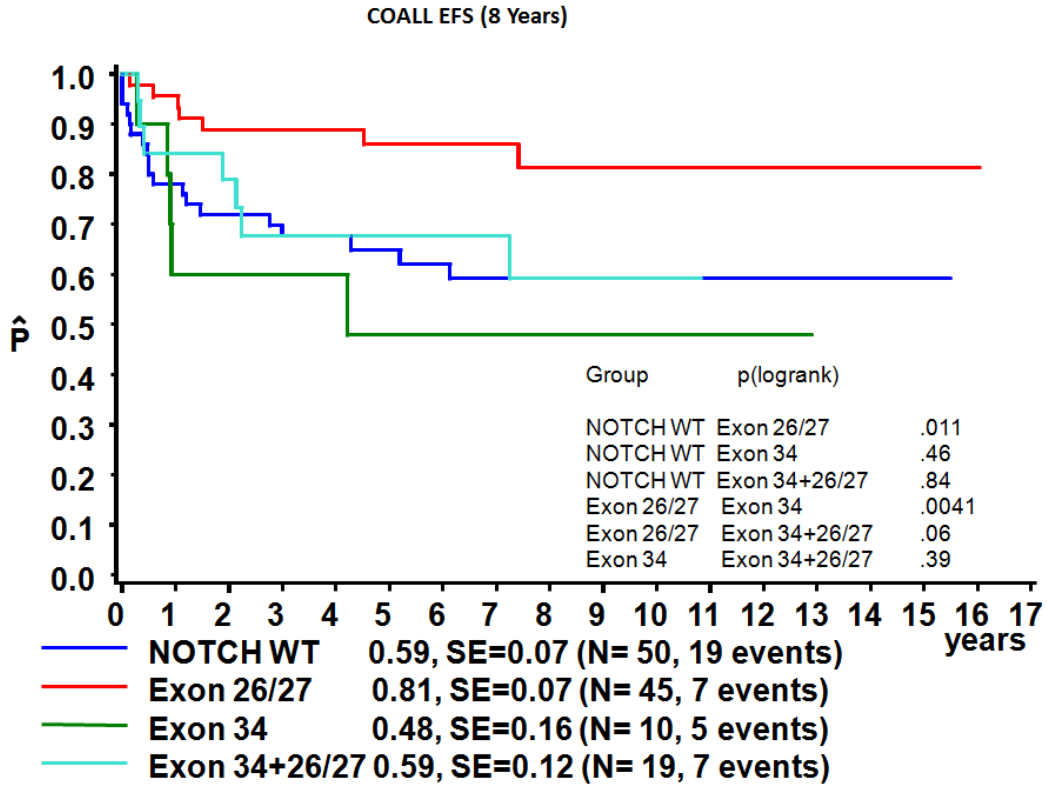

Figure 6. COALL study group: Effect of *NOTCH1* mutations on event-free survival in T-ALL. One-hundred and twenty seven children with precursor T-ALL were classified according to the presence or absence of *NOTCH1* mutations in specific exons. Kaplan-Meier estimate of event-free survival (EFS) at 8 years.

Table 8: ALL IC-BFM cohort: Clinical and immunologic characteristics according to the localization of *NOTCH1* mutations in 24 children with T-ALL

	***NOTCH1* mutation**				
	HD	**PEST**	**PEST + HD**	**Others**	**P [b]**
Variable/total (%)	**17 (100)**	**1 (100)**	**4 (100)**	**2 (100)**	
Gender					.824
Male	13 (76.5)	1 (100.0)	3 (25.0)	2 (100.0)	
Female	4 (23.5)	-	1 (25.0)	-	
Age at diagnosis (years) *					.799
<10	5 [35.7]	-	2 (50.0)	1 (50.0)	
≥10	9 [64.3]	1 (100.0)	2 (50.0)	1 (50.0)	
Presenting on diagnosis WBC (10^3/µl) *					
<10	-	-	-	-	
10-<50	5 [35.7]	-	-	-	
50->100	2 [14.3]	-	1 (25.0)	-	
≥100	7 [50.0]	1 (100.0)	3 (75.0)	2 (100.0)	
T-cell immunophenotype					.577
Early-T (Pro/Pre)	3 (17.6)	-	-	-	
Cortical	12 (70.6)	1 (100.0)	4 (100.0)	1 (50.0)	
Mature	2 (11.8)	-	-	1 (50.0)	
CNS involvement *					.776
No	12 [85.7]	1 (100.0)	4 (100.0)	2 (100.0)	
Yes	2 [14.3]	-	-	-	
Mediastinal involvement #					.4290
No	4 [28.6]	1 (100.0)	1 (25.0)	-	
Yes	10 [71.4]	-	3 (75.0)	1 [100.0]	
Prednisone response					**.016**
Good	15 (88.2)	-	4 (100.0)	1 (50.0)	
Poor	1 (5.9)	1 (100.0)	-	1 (50.0)	
Unknown	1 (5.9)	-	-		

[b] P X^2 Test

* Remark: For these criteria, positive *NOTCH1* mutation was calculated over a total of 21 patients.

Remark: For these criteria, positive *NOTCH1* mutation was calculated over a total of 20 patients.

° Percentages between [] were calculated taking into account only documented cases.

3.5 Different molecular mechanisms are involved in activating *NOTCH1* mutations

In three cases of the COALL cohort (cases 14, 93 and 132), when exon 34 was cloned between 2 and 3 different mutant clones were identified. This was surprising since in the initial PCR only one mutant clone was suspected. The exact mutations descriptions can be found in the supplementary data, Appendix C.

This can be explained by the fact that some mutations are observed at low levels despite a high blast count. This would suggest that they were acquired as secondary events in a subclone. Although it would be interesting to analyze if one clone was selected over the other one, patients 14 and 132 did not present any relapse. Patient 93 developed two years later a second neoplasm disease, a Langerhans cell Histiocytosis. Relapse material is currently being analyzed.

Another interesting mutation is the one found in exon 27 of patient 9 of the Argentine cohort. In the initial PCR we could observe a frameshift involving more than 50 base pairs. By cloning we observed an in frame duplication of a total of 69 nucleotides, 40 of them were found in the 3'of exon 27 and 29 nucleotides of intronic adjacent sequence (IVS27+29DUP69bp), leading to a duplication of the exon's donor splice site. An explanation of the potential consequence in the function and structure of NOTCH1 receptor will be offered in the discussion.

Case 70 from the COALL cohort is also particularly interesting. This patients presented an in-frame 27 nucleotide insertion (c.7414_7415insATCGAGGTAAGTATATACCGTGGTCTG) in exon 34, corresponding to a 9 amino acid insertion (p.L2472insIEVSIYRGL) in the PEST domain (Figure 7). This was verified when the initial sequence was clonated and also checked using the sequence analysis program DNASTAR Lasergene 7.1. To our best knowlegde, in-frame mutations in the PEST domain, that do not lead to premature codons have not been previously described. Once again, a thorough analysis is found in the discussion.

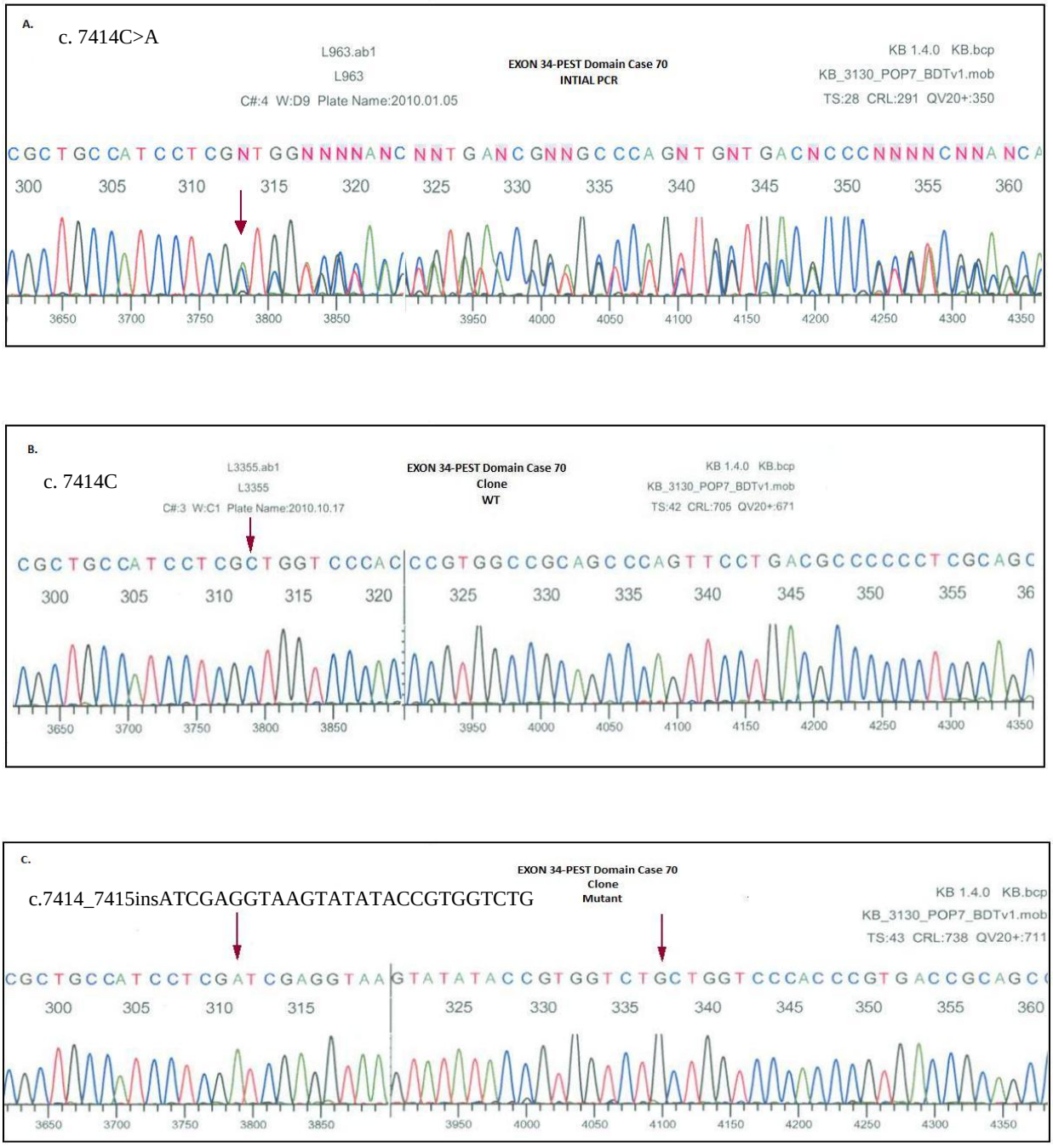

Figure 7. Exon 34 (PEST Domain) case 70 from COALL cohort. (A) The initial PCR showed a frameshift, starting at c.7414 (arrow). After cloning two clones were identified (B) being the wild-type and (C) being the mutant clone. As observed, there is an insertion of 27 basepairs (showed between arrows), which is in frame since the normal wild-type sequence continues after the insertion and didn't lead to a premature STOP codon.

Other interesting mutations are individually commented in the discussion.

4. DISCUSSION

Since the 1970s, the clinical outcome and five-year survival rates of patients with T-ALL in childhood and adolescents have improved considerably. Although the probability of cure has dramatically improved, the children who relapse face a dismal prognosis. It is suggested that an unrecognised biological heterogeniety could contribute to drug resistance, and therefore explain the 20% of children with T-ALL that die due to this disease.

One of the key factors of this true success of modern clinical oncology is the proper risk stratification of patients, allowing a risk-adapated and individualized therapies. Currently, the individual risk assessment of patients is achieved through the identification of clinical and biological (genetic/molecular alterations) prognostic markers that determine the pathogenesis of the individual subtypes of leukemia (Silverman *et al*., 2001).

Over the last decade, new genetic abnormalities have been described in T-ALL (Ferrando *et al*., 2004; van Grotel *et al*., 2006; van Grotel *et al*., 2008), therefore it has become possible to examine the chronology in which these molecular events are acquired. This information could help to understand the mechanisms by which oncogenic pathways interact, characterize the leukemic stem cell responsible for relapse and, eventually develop new therapeutic strategies that target rationally, specifically and effectively on molecular pathway.

4.1 Prognostic significance of T-ALL according to *NOTCH1* mutation status

The prognostic significance of *NOTCH1* mutations in T-ALL remains controversial. Although two studies in children T-ALL (n=53 and n=70, respectively) showed there was no association with prognosis (Zhu *et al*., 2006; van Grotel *et al*., 2008), a recent study of 157 pediatric T-cell-ALL treated according to the ALL-Berlin-Frankfurt-Münster strategy established an association between *NOTCH1* mutations and an excellent prognosis, and reported to be an independent good prognostic factor for EFS and overall survival (Breit *et al*., 2006). The same group confirmed this association in a second extended analysis in a larger group of 301 patients study claiming a favorable effect of activating *NOTCH1* receptor mutations on long-term outcome in T-ALL patients treated on the ALL-BFM 2000 protocol (Kox *et al*., 2010).

The results are also discrepant in adult T-ALL studies. Analysis of 141 patients treated in the Lymphoblastic Acute Leukemia in Adults (LALA)-94 (n=87) and the GRAALL-2003 (n=54) protocols showed positive prognosis for patients with *NOTCH1* mutations (Asnafi *et al*., 2009). These observations were not confirmed by Mansour and coworkers, since the analysis of a serie of 88 patients treated in the MRC UKALLXII/ECOG E2993 protocol failed to demonstrate an improved outcome for patients affected by *NOTCH1* mutations (Mansour *et al*.,2009).

In this work, a total of 171 pediatric T-ALL were screened for *NOTCH1* mutations, of which, 133 patients were enrolled in the German COALL protocol, and 38 Argentine patients were treated according to the ALL IC-BFM protocol. Heterozygous *NOTCH1* mutations were identified in 60.2% of the German patients and in 63.2% of the Argentine patients. The frequency of mutations is in accordance with other publications (Breit *et al*., 2006; Zuurbier *et al*., 2010; Kox *et al*., 2010; Clappier *et al*., 2010).

In relation to early treatment response, we observed an association with good prednisone response in the BFM-like cohort (P=.009); but no relationship with long-term prognosis. In contrast, previous studies that include patients treated with BFM-based strategy have observed an improved long-term outcome (Breit *et al*., 2006; Kox *et al*., 2010). One reason that could explain this difference is the small number of patients screened (n=38) or the shorter time of follow-up when compared to the COALL cohort (BFM patients: median follow-up 40.3 months versus 88 months COALL patients). However, it is well known that relapses usually occur during early phases in precursor T-ALL.

When considering the COALL cohort, *NOTCH1* mutations could also represent a good prognostic factor when considering early treatment response, since most T-ALLs with favorable MRD status at day 29 ($<10^3$) were mutated (P=.009), therefore allowing patients with mutated *NOTCH1* to be stratified in a reduced High-risk group. This improved initial treatment response is not observed when considering long-term outcome, since the event-free survival rate and cumulative incidence of relapse didn't differ between *NOTCH+* patients and *NOTCH-* patients (Figure 4).

From what has been previously described, it is currently not possible to state whether *NOTCH1* mutated patients represent a group with improved outcome, or in fact NOTCH effects are treatment dependent.

4.2 Main differences between COALL and ALL IC-BFM protocols

The previous mentioned studies suggest that *NOTCH1* mutations prognostic value in T-ALL could be associated or influenced by differences in therapy. Although it is only a hypothesis, we will here analyze the main differences between both protocols, that is the ALL IC-BFM 2002 protocol (n=38) and the German Co-Operative Study Group for Childhood Lymphoblastic Leukemia Study (COALL-92-97-03) (n=127). In both protocols patients with T-ALL are considered as patients of high-risk. The induction phase aims to induce complete morphologic remission in 4 to 6 weeks. Both protocols contain an induction therapy that includes at least three systemic drugs [i.e., prednisone (60 mg/m^2/day for 28 days), vincristine and 4 doses of an anthracyclin]. In addition, ALL IC-BFM protocol includes *E. coli* asparaginase (L-asparaginase) as fourth drug, therefore applies a more intensive induction in comparison to COALL protocol.

Although native *E. coli* asparaginase is the preparation of first choice; allergic reactions are common and are usually responsible for the discontinuation of asparaginase. Therefore both protocols contemplate the use of pegylated type of *E. coli* asparaginase (PEG-asparaginase) or Erwinia chrysanthemi-ASP (*Erwinase* ®). For more details refer to Appendix B.

One of the main differences observed between these two protocols is the parameter analyzed for *early treatment response* in the induction phase. The early response to therapy is the most important stratification principle for most protocols. The ALL IC-BFM 2002 trial assesses the prednisone response by the absolute blast count (ABC) in the peripheral blood (PB) on day 8 after 7 days of prednisone pre-phase and one dose of IT MTX on day 1. The day of the first dose of prednisone is day 1. Since the COALL protocol starts with a pre-phase that consists of one dose of anthracyclin (24 hours, daunorrubicin or adriamycin in equivalent doses) the initial treatment response is evaluated later. Therefore, prednisone response is not evaluated as early treatment response parameter in the COALL study group. This protocol also considers the day of the first dose of prednisone as day 1. In ALL IC-BFM 2002 trial bone marrow status are evaluated on day 15 and 33 of induction therapy by observation of bone marrow morphology with light microscopy.

The measurement of minimal residual disease (MRD), as a prognostic factor for risk of relapse, was introduced in COALL 97 trial at weeks 5 and 12. The assessment of MRD was performed separately in T-ALL and precursor B, since T-ALL often present a delayed MRD kinetics. Although nowadays MRD is performed in Garrahan hospital (ALL IC-BFM cohort), to the time the bone marrow samples were collected, this molecular biology assay was not available.

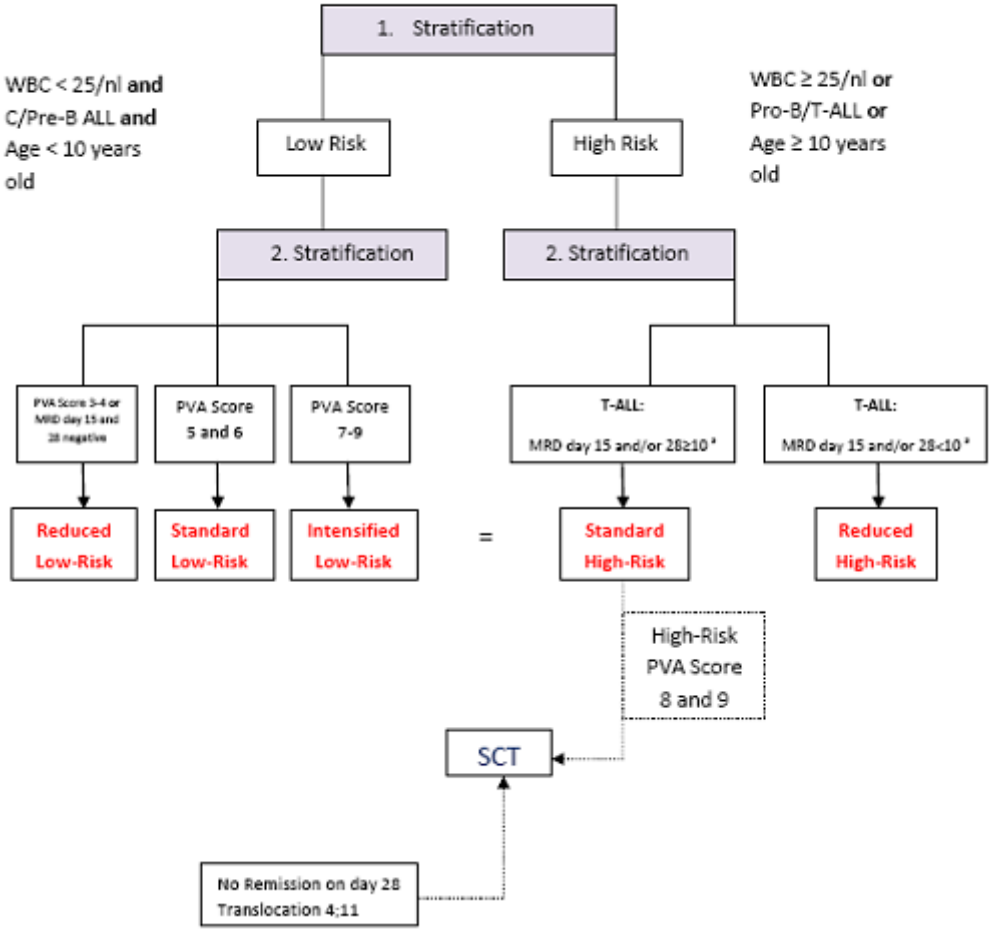

Figure 8. Risk Stratification: COALL 07-03 Protocol

Another difference in the initial stratification is the consideration of the PVA score in the COALL protocol (Figure 8). As it has been previously described, the PVA score is the result of the *in-vitro* resistance test (MTT-Test) from the leukemic cells to prednisone, vincristin and L-asparaginase, defining numeric scores for the sensitivity/resistance of blasts. A score of 3 shows the highest sensitivity of cells to these pharmaceutical drugs. A score of 9 respresents a strong resistance to these 3 chemotherapies, and therefore are associated with poor response recognizing a subtype of very high-risk patients. A recent publication showed that the PVA score's prognostic effect is inferior in comparison to MRD measurement at defined time points. By multivariate analysis, low MRD at the end of induction (week 5) predicted a greater probability of disease-free survival independently of the PVA score (Escherich *et al*., 2010). Escherich and coworkers suggest that PVA score's inferior prognostic power is due to the fact that it predicts only early rather than late response, and therefore looses its prognostic effect over time. However, the COALL protocol still considers the PVA score as a prognostic factor for patients in the HR group, since they frecuently present early relapse.

Different randomized studies have demonstrated that the substitution of prednisone by dexamethasone decreases the risk of bone marrow and CNS relapse (Mitchell *et al*., 2005). The benefit of dexamethasone may be due to a higher free plasma level and a better CNS penetration.

Patients treated according to the ALL IC-BFM 2002 protocol receive at least 4 weeks of dexamethasone and gradual tapering of the drug. Patients treated according to the COALL protocol also receive dexamethasone during the re-induction phase. Although they all receive 10 mg/m^2/day, patients assigned to the "Reduced high-risk group" receive only 2 weeks of dexamethasone and 2 doses instead of four doses of doxorubicin during the re-induction phase, without changing the total duration of therapy (Appendix B). These patients had a less favorable outcome compared with previous studies (pEFS 0.75 vs. 0.82) (Graubner *et al*., 2007). It is important to remember that the contribuition of specific parts of treatment depends on the TOTAL therapy administered to a patient.

There are some differences worth to mention when we consider the total dose of oral antimetabolite. During the induction phase, patients treated according to the BFM-ALL IC protocol receive oral antimetabolites during a longer period of time (Protocol I and Protocol mM: 84 days) but a lower dose when compared with CO ALL protocol, that only receive antimetabolites during 49 days. Therefore the cumulative dose during the induction phase is practically the same, that is 3220 mg/m^2 in the case of the BFM protocol versus the 3500 mg/m^2 of oral antimetabolite in the COALL protocol. During reinduction there is a considerable difference, since COALL includes only 14 days of 100 mg/m^2 6-Thioguanin daily (total dose 1400 mg/m^2) while BFM protocol includes 28 days of oral antimetabolite with a total cumulative dose of 2500 mg/m^2.

Like most ALL protocols, both COALL and ALL IC-BFM protocols are completed by a prolonged maintance therapy for a total treatment duration of 2 years. In both cases, all patients receive uniform oral antimetabolite therapy with daily 6-Mercaptopurine (6-MP 50mg/m^2/day) and weekly oral Methotrexate (MTX 20 mg/m^2/day). Continuous adaptations of the doses of MTX and 6-MP based on peripherial blood counts are necessary to reduce the risk of relapse and reduce the risk of infections (Relling *et al*., 1999). Both protocols include in the maintance therapy intrathecal MTX. In the case of the COALL cohort, all patients that do not qualify for radiotherapy receive 3, 6 and 9 months after finishing the reinduction 1 dose of MTX i.th. In the case of T-ALL, these are the patients that presented at initial diagnosis with < 50.000/nl leucocytes.

Since the introduction of central nervous system (CNS) therapy in the mid 60's and early 70's, there has been a dramatic decrease in the rate of CNS relapses from about 70% to less than 5%, thus improving the overall prognosis of children with acute lymphoblastic leukemia (ALL) (Pieters *et al*., 2010). CNS-targeted therapy addresses occult or manifest disease within the

CNS, a "natural reservoir" favored by malignant lymphoblasts. Intrathecal therapy is present in all protocols.

The reduction of CNS irradiation has been an important change within the last decade. The gradual reduction in CNS irradiation has led to a decrease in the incidence of brain tumors as second malignancy. After several publications, it has been concluded that radiotherapy can be replaced by intrathecal doses of chemotherapy and intravenous MTX reduces systemic relapses. The ALL IC-BFM protocol includes 4 bi-weekly cycles with high dose of MTX (5 g/m^2) and preventive radiotherapy for all T-ALL patients or therapeutic in the case of CNS envolvement at the moment of diagnosis. In the COALL protocol, this intravenous high-dose MTX is replaced with a medium-dose MTX (1 g/m^2, 4 doses every 2 weeks), high-doses of cytarabin (ARA-C), and several repetitve high doses of ASP that lead to an extended depletion of asparaginase in CSF. This study group suggests that this drug regimen could compensate the reduction of MTX (Escherich *et al*., 2010). Cycles of high- dose cytarabin are also included in the HR arm of the ALL IC-BFM protocol.

In the BFM protocol, prophylactic CNS therapy (CNS Status 1) includes 12 Gy prophylactic cranial radiotherapy (pCRT) and high dose of MTX (5 g/m^2/day); addressing adequatly CNS reservoir and avoid relapses. In the case of CNS Status 2, both protocols include 2 additional intrathecal MTX (IT MTX) doses. Patients with positive CNS at diagnosis are treated with therapeutic cranial radiotherapy (tCRT); that is for patients aged $\geq 1 < 2$ years with 12 Gy and patients aged $\geq$ 2 years with 18 Gy. The COALL group also applies the same doses of radiotherapy for patients with primary CNS affectation. In contrast, BFM groups considers not only additional intrathekal (IT) MTX doses, but also triple IT (prednisone, Methotrexate and cytarabine). This is not the case in the COALL treatment protocol, which only includes IT MTX doses. A Children's Cancer Group study suggested that intratecal triple therapy prevented CNS relapse but did not improve overall survival (OS) since fewer bone marrow relapses were observed when intrathecal MTX was applied as single agent (Matloub *et al*., 2006).

Both protocols have a similar approach when considering overt testicular leukemia at diagnosis. Up to 25 % of males with ALL have subclinical leukemic infiltration of their gonads. However, only 2 % present with evident disease, the majority being infants or adolescents with T-ALL, high WBC count, and usually a mediastinal mass. With actual therapies, the occurrence of testicular relapse is rare, even when hematological relapses occur as the most frequent adverse event. Historically it accounted for 10% of all relapses, but this rate has been decreased with therapy directed schedules. BFM experience has demonstrated that in the mayority of cases testicular radiotherapy can be avoided. A testicular tumor at diagnosis has no influence on the initial risk classification. Nevertheless, if the testicular size has not normalized after the induction phase, the local status should be exhaustively examined. In case of doubt, sonography and eventually biopsy are necessary. A persistent leukemia of both testes may ultimately need

local RXT of 18 Gy at the end of the intensive phase. When only one testicle is affected, COALL protocol considers the alternative of a orchiectomy, since through radiotherapy the not affected testicle may also suffer irradiation.

In ALL IC-BFM 2002, allogenic hematopoietic stem-cell transplantation (HSCT) from an HLA-identical family donor (MFD= matched family donor), when available, is a therapeutic option for a subgroup of high-risk patients. The addition of MFD SCT has improved the outcome of patients with prednisone poor response (PPR) and T-ALL. COALL protocol also considers the PVA score as an independent prognostic factor. In high-risk patients with a score of 8 and 9, the availabilty of HLA-identical family donor should be assessed as soon as patients achieve their first remission. Non- responders or late responders in remission are also eligible for transplantation. For this subgroup of patients, allogenic transplatation from unrelated donors [matched-unrelated-donor (MUD) transplantation] is also taken into consideration.

The COALL study group has recently described a significantly higher frecuency of second malignancies (SMN) in T-ALL when compared with B-precursor ALL. Other studies have also observed a higher incidence of SMN in T-ALL patients (Löning *et al*., 2000).

Finally, the comparison between the COALL protocol and the ALL IC-BFM protocol suggests that the NOTCH effect is in general treatment dependent and may depend specifically on the intensity of the induction therapy and central nervous system-directed therapy. In fact, the inability of this study to establish a statistically significant association between relapse or event free survival rate and *NOTCH* mutations in the German and Argentine cohort could be to various levels of overall signaling resulting from different activating potencies for *NOTCH1* mutations and/or the fact that the T-ALL samples were from patients treated with different protocols which would suggest that aberrant NOTCH1 signaling may impact sensitivities of leukemic blasts to various cytostatic drugs at different extents.

4.3 Prognosis of *NOTCH* mutations in different European Protocols

The question about the prognostic significance of NOTCH1 activation in childhood T-ALL has been recently revisited. Three different European working groups observed that *NOTCH1* activating mutations define a particular group of T-ALL, characterized by a more favorable early treatment response. However, whether patients with mutations in this receptor represent an improved general outcome is still unclear.

Clappier and coworkers analyzed a series of 134 patients treated according to the Children Leukemia Group of the European Organization for Research and Treatment of Cancer (EORTC-CLG) 58881 and 589951. Of note, trials conducted by the EORTC-CLG are based on BFM-derived protocols. The authors describe an association with rapid initial therapeutic response, decreased levels of MRD disease, but no statistical association with long term overall survival (Clappier *et al*., 2010). This is in accordance with the results observed in this work. However, they observed that *NOTCH1* mutated patients and high MRD levels had a worse prognosis when compared to *NOTCH1* negative patients. This does not apply to the COALL cohort in our present study, in which *NOTCH1*+ patients with high MRD levels had a 5-years EFS of 45% (SE=0.10) versus 47% (SE=0.13) for the wild-type group.

Kox and coworkers published an extended series of 301 patients treated in the ALL-BFM 2000 study (Kox *et al*., 2010). They confirmed the initial observations published by Breit and colleagues (Breit *et al*., 2006) that activating mutations in *NOTCH1* were not only associated with prednisone good response, and improved response to initial induction therapy (low MRD levels), but also with a favorable long-term outcome. In contrast, similar to the observations of Clappier and coworkers, Kox *et al*. also reported that *NOTCH1*+ patients but initial poor prednisone response or high MRD levels after induction do not have an improved overall long-term outcome.

The third study included 146 T-ALL cases: 72 were treated on DCOG (Dutch Childhood Oncology Group) protocols ALL-7/8 and ALL-9 and 74 cases were treated in the COALL97 clinical trial (Zuurbier *et al*., 2010), respectively. Although an association between *NOTCH1* mutations and initial prednisone response was reported, the prednisone response was known only in a very limited number of patients (n=23). Interestingly, in this publication a significantly poorer outcome for patients having a strong *NOTCH1* activating mutation (considered as JM mutations or HD mutations in combinations with PEST mutations) when compared to weak *NOTCH1* mutations (considered as *NOTCH1* HD or PEST mutations) were reported. In contrast, in the report of Breit and coworkers, the mechanism of *NOTCH1* mutations did not appear to play a clinically relevant role (Breit *et al*., 2006).

4.4 Mechanisms of Aberrant NOTCH1 Activation in T-ALL

As previously mentioned, *NOTCH1* mutations in human T-ALL cluster in 2 general regions of the protein. One cluster lies at the C-terminal end of the receptor, and consists of nonsense or frameshift mutations that result in the deletion of a **PEST** domain (exon 34) which regulates ICN1 degradation (Chiang *et al*., 2006). Apparently these mutations increase NOTCH1 activity by stabilizing ICN1, therefore aberrantly prolonging NOTCH1 activation.

Although these mutations are scattered throughout the 3-end of exon 34, most of these deletions eliminate at least residues 2524 to 2556, suggesting that this minimal region contains at least one important motif that negatively regulates NOTCH1 signal strength (Weng *et al*., 2004). Morevover, Chiang and coworkers indicate that the sequence WSSSSP, which spans residues 2520 to 2525 of human NOTCH1 receptor, exerts a functionally important restraint on NOTCH1 signal strength (Chiang *et al*., 2006).

In relationship with the FBXW7 degron, Thompson and co-workers suggest three categories for the mutations found in the PEST domain (Thompson *et al*., 2007). Most of the mutations insert a translational termination codon. In our study such termination codons started as early as amino acid 2348 [position numbers are based upon *NOTCH1* sequence (NM_017617.3)], and thus delete almost the entire PEST domain. The second category they described includes mutations that introduce termination codons immediately upstream of L2510 residue (e.g. samples 35, 39 and 42 from the German cohort). We were able to confirm what was previously stated, since the most common nucleotide deletion found was the mutation p.P2514R (c.7540_7541delCT) predicted to result in a stop codon in residue 2518, therefore removing the ^{2520}WSSSSP^{2525} sequence. This mutation is an example of Thompson and coworkers third category which specifically targets the FBXW7 degron.

In patients 9 and 10 of the Argentine cohort, and patients 8 and 29 of the German cohort, previously published common deletions that include the sequence ^{2481}FLTPPSQ^{2487} were identified (Aster *et al*., 2008).

As previously mentioned, recent studies have elucidated the amino acid degron sequence required for the NOTCH1-FBXW7 interaction, This degron, found in the NOTCH1 PEST domain, is centered at a conserved threonine T2511, and spans residues ^{2509}FLTPSE^{2516} (Thompson *et al*., 2007; O´Neil *et al*., 2007). The functional consequences of the missense mutation p.V2536I found in this study have not been analyzed, but it could be postulated that it also stabilizes the ICN and therefore prevents PEST-dependent degradation by affecting the region where the NOTCH1-FBXW7 interaction takes place.

Another missense mutation p.G2152R (c.6454 G>C in exon 34) in the NOTCH1 TAD region was found in case 12 of the COALL cohort. We suggest that this point mutation could affect the binding of the NOTCH1 intracellular domain with downstream effectors, since the TAD

region serves to recruit co activator molecules. Furthermore, previous studies have shown that the TAD domain is essential for T-cell transformation by NOTCH1. Aster and coworkers based their conclusion on the finding that four different constructs encoding polypeptides that lack the entire TAD domain or part of it uniformly failed to induce leukemic transformation (Aster *et al*., 2000).

The two mutations identified in patient 74 (COALL cohort) deserve particular mention. This patient presented an inframe deletion/insertion mutation in exon 26 (p.P1582 delEQLRinsRAEQ) and a novel missense mutation, p.V2536I (c.7606G>A, exon 34), in the PEST domain. As it was previously mentioned, point mutations in this domain are rare. Although p.V2536I has not been described as a conserved residue, its position seems to be important for the region that negatively regulates NOTCH1 signaling. Fryer and coworkers reported that point mutations affecting conserved Serine residues within the PEST motif (S2480, S2483, S2505) prevent hyperphosphorylation by the CycC:CDK8 protein kinase and stabilize the ICN in vivo. They noted that the NOTCH ICN contains multiple conserved cyclin-dependent kinase phosphorylation sites within the TAD and PEST domains. Under normal conditions, expression of CycC:CDK8 promotes hyperphosphorylation of the ICN and facilitates PEST-dependent degradation of the ICN by the FBXW7 ubiquitin ligase (Fryer *et al* 2004).

As mentioned earlier patient 70 from the COALL German cohort had an <u>in-frame</u> 9 amino acid insertion in the PEST domain (p.L2472insIEVSIYRGL, c.7414_7415 insATCGAGGTAAGTATATACCGTGGTCTG) (Figure 7). To confirm this unusual finding and to increase the sensitivity of the analysis, the PCR product was cloned and sequenced. Whereas most PEST mutations introduce frameshifts or stop codons upstream S2513 (a highly conserved serine residue, implicated in NOTCH degradation by FBXW7) (Fryer *et al*., 2004), in-frame mutations have not been reported and may provide a potentially novel mechanism of NOTCH1 activation. The functional significance of this mutation has not been analyzed, but it is a very remarkable case since it is not in accordance with the theory that truncated PEST domain avoids the degradation of the ICN. We hypothesize that an abnormal "long" PEST domain could also interfere with the degradation of NOTCH1 ICN. Another hypothesis could be that, although the original ^{2520}WSSSSP^{2525} sequence is kept intact; the tertiary structure of the domain could be affected in such a way that could also decrease the ICN1 phosphorylation and consequently increase the ICN1 stability.

As previously mentioned, three cases of the COALL cohort (cases 14, 93 and 132) presented different mutant subclones. This pattern of *NOTCH1* mutations suggests a stepwise acquisition of additional genetic hits, with dose-dependent effect of the NOTCH1 pathway activation on T-cell leukemogenesis and a strong selection pressure for acquired mutations that activate the pathway. In fact, when mutations are present at both HD and PEST sites, it has been shown that

they are always located in *cis* on the *NOTCH1* allele and reporter assays have shown that they induce a synergistic increase in transcriptional activity (Weng *et al*., 2004). Moreover, the presence of different mutant clones could be explained by the fact that there is a positive selective pressure for ever-increasing levels of NOTCH1 activation (Aster *et al*., 2008).

Patient 93 developed Langerhans Cell Histiocytosis (LCH) as a second neoplasm two years later. Initially, this patient presented at the age of 4;2 years with a white blood cell count of 108,000 cells/µl but without central nervous system or mediastinal involvement. Unfortunately, despite treatment the patient died at the age of 11;5 years due to progressive LCH. Molecular analysis revealed an amino acid deletion/insertion (p.E1583 delQRLinsPPEELD) in the NOTCH1 HD domain and three different clones with mutations in exon 34. Characteristically, all three mutations introduced a premature stop codon that results in the deletion of C-terminal negative regulatory PEST region (S2467D fsX12, R2431 fsX4, A2479Q fsX8).

LCH is a rare, clinically heterogeneous neoplasm of immature dendritic cells (its manifestations range from isolated bone lesions to systemic disease with involvement of two or more visceral organs) that is most frequent in children (Jaffe *et al*., 2001). Development of LCH following T-ALL is extremely rare. Few brief reports have stated a relationship between LCH and systemic Juvenile Xantogranuloma (JXG) following T-ALL (Rodig *et al*., 2007; Perez-Becker *et al*., 2010). It is suggested that LCH/JXG are clonally related to T-ALL and present persistent expression of constitutively active NOTCH1. Perez-Becker *et al.* reported the case of a 5-year-old female that developed an aggressive JXG only 5 months after the diagnosis of T-cell acute lymphoblastic leukemia. They reported identical bi-allelic T-cell receptor-γ (TCR-γ) rearrangement in both neoplasms. Rodig and coworkers describe the case of a 3;7 year old patient who eighteen months after initial diagnosis developed an aggressive Langerhans cell Histiocytosis. Both neoplasms harbored not only the same (TCR-γ) rearrangement, but also identical, synergistic activating *NOTCH1* mutations, affecting exons 27 and 34. Moreover, the analysis of 24 cases of LCH and Rosai-Dorfman disease in patients without prior history of T-ALL revealed no mutations. These reports highlight the unique nature of the mutations found in this work, emphasizing its exceptional character.

The second "hotspot" of mutations affects the **HD** domain of the negative regulatory region (NRR) and the region at the boundary between the extracellular and transmembrane of the protein (exons 26 and 27). This group includes the most common *NOTCH1* mutations found in human T-ALL (Leong *et al*., 2006; Zhu *et al*., 2006). There are 2 different classes of mutations in this group, both that cause ligand-independent NOTCH1 signaling (Malecki *et al*., 2006). **Class I** mutations, the most common mutations affecting the NOTCH1 receptor, are single amino acid substitutions or short insertions or deletions that cause increased sensitivity of NOTCH1 heterodimers to subunit dissociation (S2 cleavage) under native or mildly denaturating conditions, leading to decreased NRR stability (Gordon *et al*., 2007). These

mutations reside in the hydrophobic core of the HD domain and could act by partially or completely unfolding the domain, destroying the pocket that masks the S2 site and therefore preventing the LNR from protecting this cleavage site (Gordon *et al*., 2007).

In this study we found several patients that presented more than one heterozygous class I mutation in the same exon (Figure 9). Although we expected that these point mutations would be found in different alleles, since it is suggested that *NOTCH1* mutations occur in a stepwise aquisition pattern, we found at least 3 patients that presented point mutations on the same allele. By cloning we found wild-type alleles, alleles that presented only one of the point mutations and alleles that presented both point mutations. Since they all affect the hydrophobic core, we hypothesize that with the acquisition of more mutations, the association between the LNR repeats and HD domain is increasingly affected and would therefore increase the tendency to dissociate in absence of NOTCH1 receptor ligands.

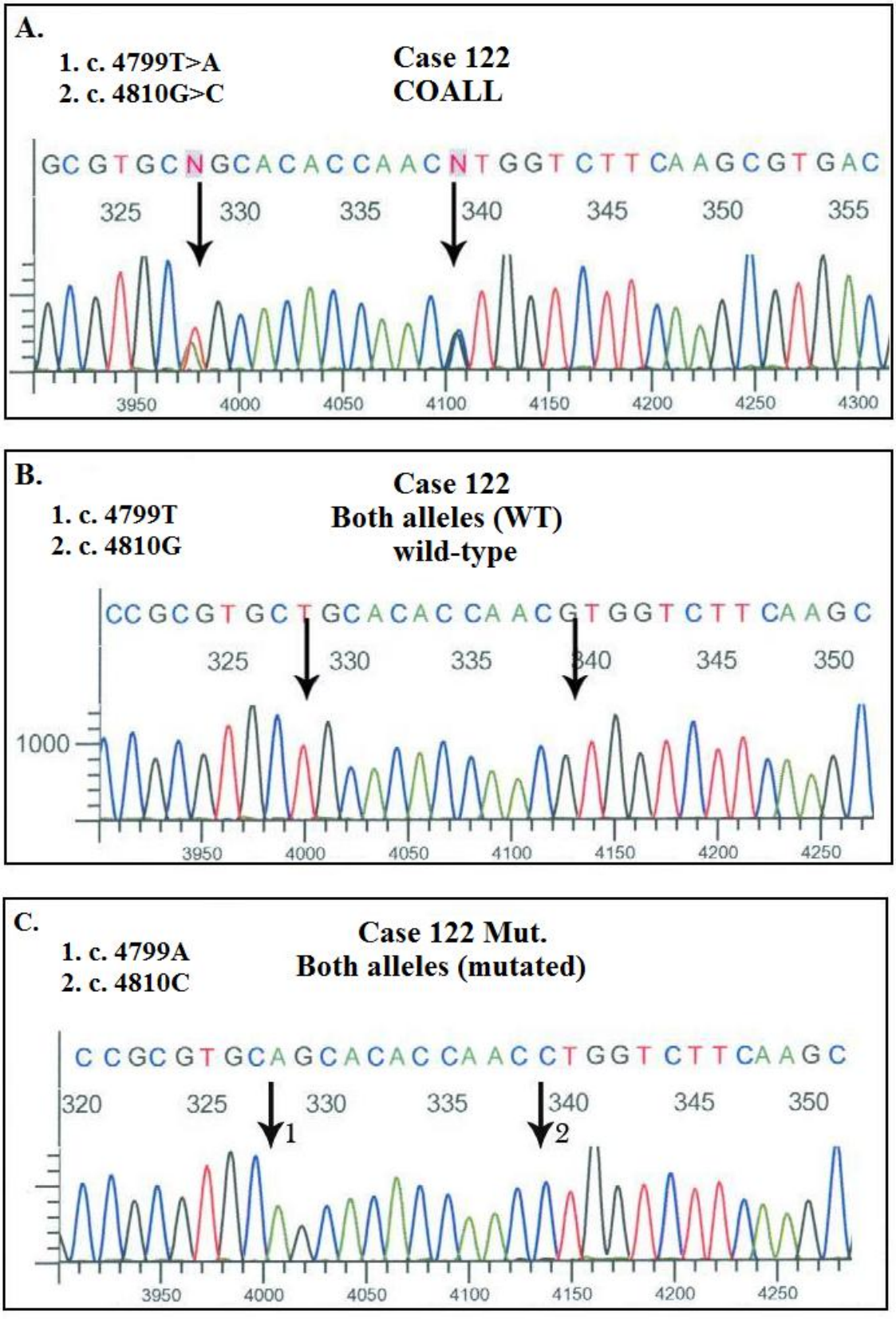

Figure 9. Patient 122 from the COALL cohort showed two heterozygous missense mutations in exon 26 (HD domain). (A) initial PCR showing two heterozygous point mutations (c.[4799T>A; c.4810G>C]). (B) shows the wild-type clone. (C) both missense mutations affect the same alelle.

Class II mutations consist of insertions of at least 12-15 residues near the C-terminal end of the heterodimerization domain creating a second S2 cleavage site. This kind of mutation produce ligand-independent S2 cleavage and strong increases in signaling without any apparent destabilization of the receptor; therefore they would displace the NRR leaving the S2 site unprotected (Malecki *et al*., 2006).

A clear example of class II mutations is the inframe duplication in patient 9 of the Argentine cohort. This duplication of a total of 69 nucleotides, 40 of them in the 3' region of exon 27 and 29 bp in the intronic adjacent sequence (IVS27+29dup69), results in a duplication of the exon's splice site. When comparing the mutated sequence with the wild-type sequence [using Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Berkeley Drosophila Genome Project internet link (http://www.fruitfly.org/)], both splice sites (the one found in the original sequence and the one created by the duplication) showed exactly the same score (0.99), with a confidence value of 0.96. Displacement of a splice site, leading to inclusion or exclusion of more RNA could be expected to result in longer exons, which would also include 29 nucleotides of intron 27. Since both splice variants have a score of 0.99, alternative splicing would probably result in both, the WT sequence and the longer mutated sequence. Although we did not experimentally show the longer translation product, we nevertheless suggest that this *de novo* 2nd cleavage site would be left unprotected by the NNR and could subsequently lead to ligand-independent S2 activation (Figure 10).

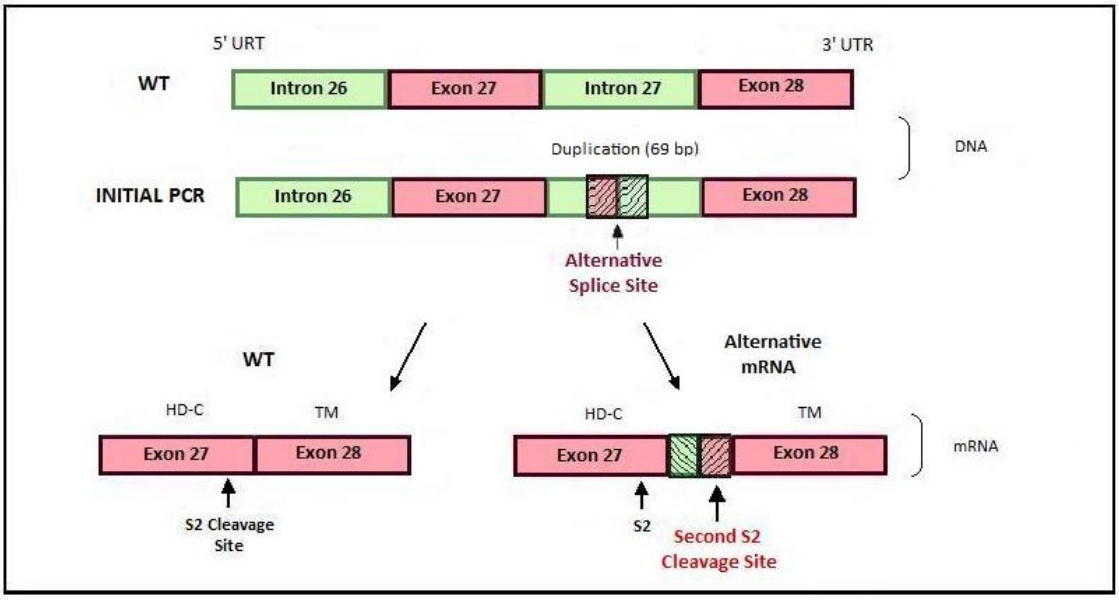

Figure 10. In-frame duplication (striped) of a total of 69 nucleotides, 40 of them were found in the 3'of exon 27 and 29 nucleotide from intronic adjacent sequence leading to a duplication of the exon's donor splice site. Alternative mRNA products could be created that include 29 bp from intron 27. A second cleavage site for the ADAM-type protease (S2) could lead to ligand-independent activation since the second S2 is not protected by the NRR.

In the present series of T-ALL patients, HD-N and HD-C mutations were mutually exclusive, consistent with the fact that these two regions are components of a single functional domain.

Sulis and colleagues identified a fourth family of *NOTCH1* activating mutations in T-ALL that show elevated ICN1 but none of the previous mentioned mutations in exons 26, 27 or 34. These mutations affect the extracellular juxtamembrane domain. They consist of internal tandem duplications in the 3´end of intron 27 and/or the proximal region of exon 28. These **juxtamembrane extension mutants (JEMs)** distance the entire HD-LNR complex from the membrane, and presumably allow ligand-independent proteolytic processing of S2 (Sulis *et al*., 2008). It is presumed, that the activity of JEMs depends on the length of the inserted sequence, i.e., the increased distance from the membrane rather than the sequence of the insertion *per se*. Notably, JME mutations result in very high levels of NOTCH1 signaling and may represent a unique mechanism of NOTCH1 activation.

In this study we observed only two mutations that affected the **TM** domain (exon 28) but none of these fulfills the previous mentioned characteristics. This domain is rarely affected therefore these mutations should be shortly discussed. One of them is seen in patient 8 from the Argentine cohort and consists of an in-frame duplication and insertion of a total of 9 amino acids (p.P1728dupPAGAAALHVR+insL). The second mutation found in exon 28 is observed in case 78 of the COALL cohort and consists of an in-frame insertion of 33 nucleotides (p.A1741insEARQLHFMYVA). Theoretically, these insertions also increase the length of the TM domain, affect the HD-LNR complex and therefore ligand to ligand-independent activation.

The H1544P substitution is a recently identified novel point mutation found in a T-ALL located in the third LNR (Mansour *et al*., 2007; Gordon *et al*., 2009), a position distinct and distant from the typical NRR mutations found in the heterodimerization domain (Figure 11). This novel mutation has been shown to trigger ligand-independent increases in NOTCH1 signaling, despite inducing only mild stabilization of the LNR-HD complex, suggesting that it may activate NOTCH1 cleavage by selectively releasing the inhibitory effect of the LNR repeats on the ADAM cleavage site located in the C-terminal HD domain (Gordon *et al*., 2009). Moreover, several studies suggest that the NRR domain could be the "activation switch" of the receptor (Sanchez-Irizarry *et al*., 2004; Gordon *et al*., 2008).

It is noteworthy to mention, that although the H1544P mutation was not found in any patient, we did find, in two cases from the German cohort, mutations in the **LNR** domain (exon 25). Patient 98 presented the insertion/deletion p.N1468ILIL (c.4403_4404delinsTTCTCATTTTA) that, to our knowledge, has not been previously described. The other interesting case is patient 8, that presented the p.G1433R point mutation. This patient also presented a mutation in the PEST domain, the frameshift p.V2443D fsX38 (c.7328_7329 ins ATCGACTCGCC) which, as previously described, stabilize the nuclear ICN containing transcriptional activation complex. It remains to be determined whether these mutations have a dual mechanism of NOTCH1

activation that combines ligand independent activation and prolonged ICN1 stability. We suggest that the mutations found in exon 25 also cause a mild stabilization of the LNR-HD complex, since the conformational changes are necessary for subunit dissociation and exposure of the cleavage site.

As it was previously mentioned, the NRR domain is constituted by the HD and the LNR domain. Previous studies have suggested that HD mutants are weak transcriptional activators (Thompson *et al*., 2007; Zuurbier *et al*., 2010). We hypothesize that mutations affecting both LNR and PEST domains simultaneously could represent a mechanism of signal amplification for LNR mutations. Up to date, it has not been determined whether mutations in LNR also act as "weak" transcriptional activators.

Although the functional significance of mutations in exon 25 has not been analyzed, these mutations are of great interest, since the NOTCH1 NRR could provide a mechanism based target therapy (Malecki *et al*., 2006).

The structure of the NOTCH1 NRR could also serve to identify compounds that directly stabilize the resting metalloprotease-resistant conformation of the HD domain or that mimic the LNR domain in masking the S2 site and therefore confer resistance to cleavage at the S2 site (Gordon *et al*., 2009).

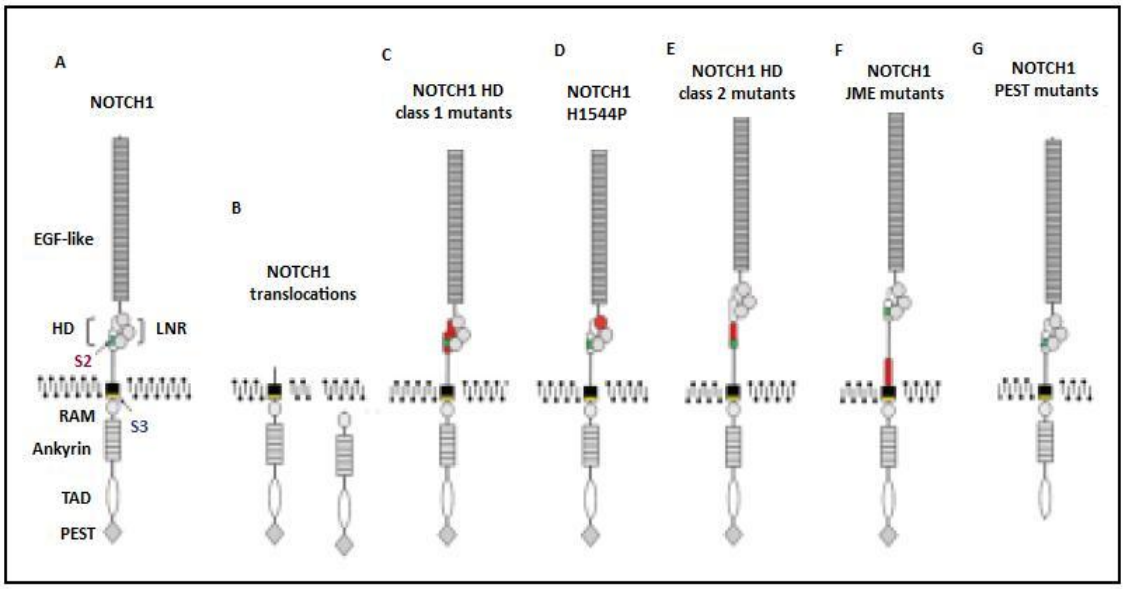

Figure 11. Oncogenic forms of NOTCH1 in T-ALL. Aberrant activation of NOTCH signaling can be triggered by mutations in the *NOTCH1* gene. (a) Structure of the wild-type NOTCH1 receptor. Functional domains of NOTCH1 are annotated. (b) Translocations of *NOTCH1* to the *TCR* loci induce the expression of truncated forms of NOTCH1. (c) *NOTCH1 HD class1* mutations destabilize the structure of the HD-LNR repeats responsible for maintaining the receptor in resting configuration. (d) The *NOTCH1 H1544P* mutation impairs the protection of the S2 cleavage site by the HD-LNR repeat complex. (e) *NOTCH1 HD class2* mutations displace the S2 metalloprotease cleavage site outside the HD-LNR complex. (f) *NOTCH1 JME* alleles increase the separation of the HD-LNR repeat complex from the membrane. (g) *NOTCH1 PEST* mutations delete the C-terminal part of the receptor and impairing the degradation of activated NOTCH1 in the nucleus. EGF-like indicates EGF-like repeats; HD, heterodimerization domain; LNR, LNR repeats; RAM, RAM domain; Ankyrin, ankyrin repeats; TAD, transactivation domain; PEST, PEST domain; S2, metalloprotease cleavage site (green); S3, γ-secretase cleavage site (yellow). Sequences altered by the different *NOTCH1* mutations are highlighted in red. (*Adapted from Ferrando AA, 2009)*.

The mutations identified in patient 6 of the ALL IC-BFM cohort also deserve particular mention. The *NOTCH1* gene harbored mutations in two domains. The HD domain was affected by two mutations, the point mutation p.R1608H (c.4623G>C) and the deletion/insertion p.I1616S (c.4848_4852insTT). The **ankyrin** domain (exon 31) was affected by the point mutation p.R1946H (c.5837 G>A). This domain is only rarely affected; it interacts with downstream transcriptional factors and is believed to be the most conserved region of the ICN, being essential for the NOTCH receptor function (Aster *et al*., 20009). To our knowledge, this last mutation has not been reported before.

The functional consequences of mutations in the ANK domain could be clarified by a thorough understanding of the mechanism of nuclear-complex assembly and the posterior induction of target-gene transcription.

Although the ICN is thought to carry a transcription activation domain (TAD), incorporation of MAML into the ICN:CBF1 complex is essential for transcription initiation on chromatin templates in vitro (Figure 11) (Fryer *et al*., 2002). MAML1 interacts with the ICN-CBF1 complex, stabilizes its binding to DNA and provides an activation domain necessary for NOTCH regulated transcription in vitro (Fryer *et al*., 2002).

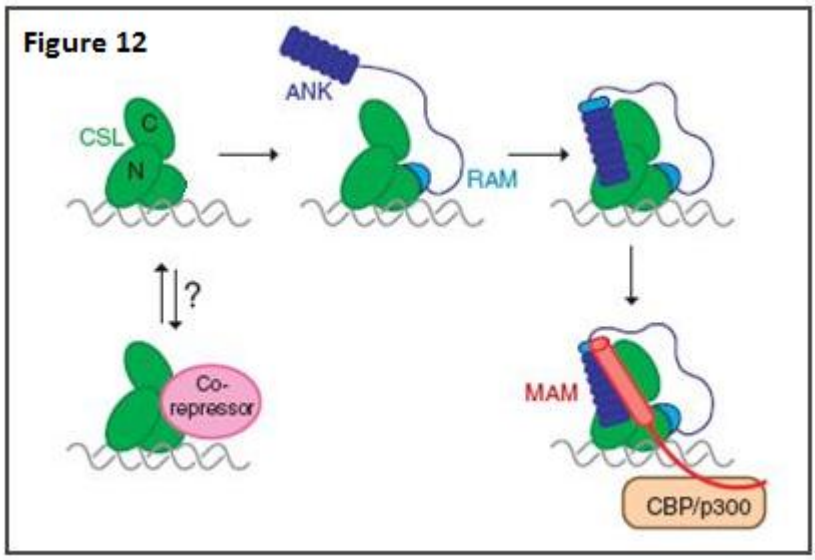

Figure 12. Model for the assembly of NOTCH ternary complexes. A high-affinity interaction between the N-terminal RAM peptide of NOTCH and the CSL is likely to be the first event in the assembly of NOTCH transcriptional activation complexes. This step allows the lower-affinity ANK domain to bind at its docking site, resulting in ordering of the ankyrin-like N-cap and first repeat of the ANK domain. The interface between the ANK domain and the (N) and (C) domains of CSL create a composite surface for the binding of MAM, which recruits p300 or CBP *(Adapted from Gordon et al., 2008).*

Previous studies have shown that the ANK and RAM regions of the NOTCH-ICN mediate binding to CBF1, whereas the central region contains the transactivation (TAD) and nuclear localization (NLS) domains (Fryer *et al*., 2002; Fryer *et al*., 2004).

ICN likely binds initially on CSL through a high-affinity binding site in the RAM domain, which stabilizes binding of the ankyrin repeat domain (ANK) to CSL through a second lower-affinity site. When ICN is expressed at high levels, the RAM domain is dispensable for the generation of T-ALL in mice (Aster *et al*., 2000). In contrast, the ANK domain is absolutely required for all known NOTCH functions, including leukemogenesis. The ankyrin domain generally serves as site of protein-protein interaction and is crucial for association of ICN1 with downstream molecules (Aster *et al*., 2000).

Fryer and coworkers conclude that the primary role of the ICN is to tether MAML to CBF1 through the ANK repeats. Moreover, Gordon and coworkers suggest that the NOTCH ICN, at least in its unmodified state, does not contain an independent activation domain sufficient to initiate transcription on chromatin (Gordon *et al*., 2008).

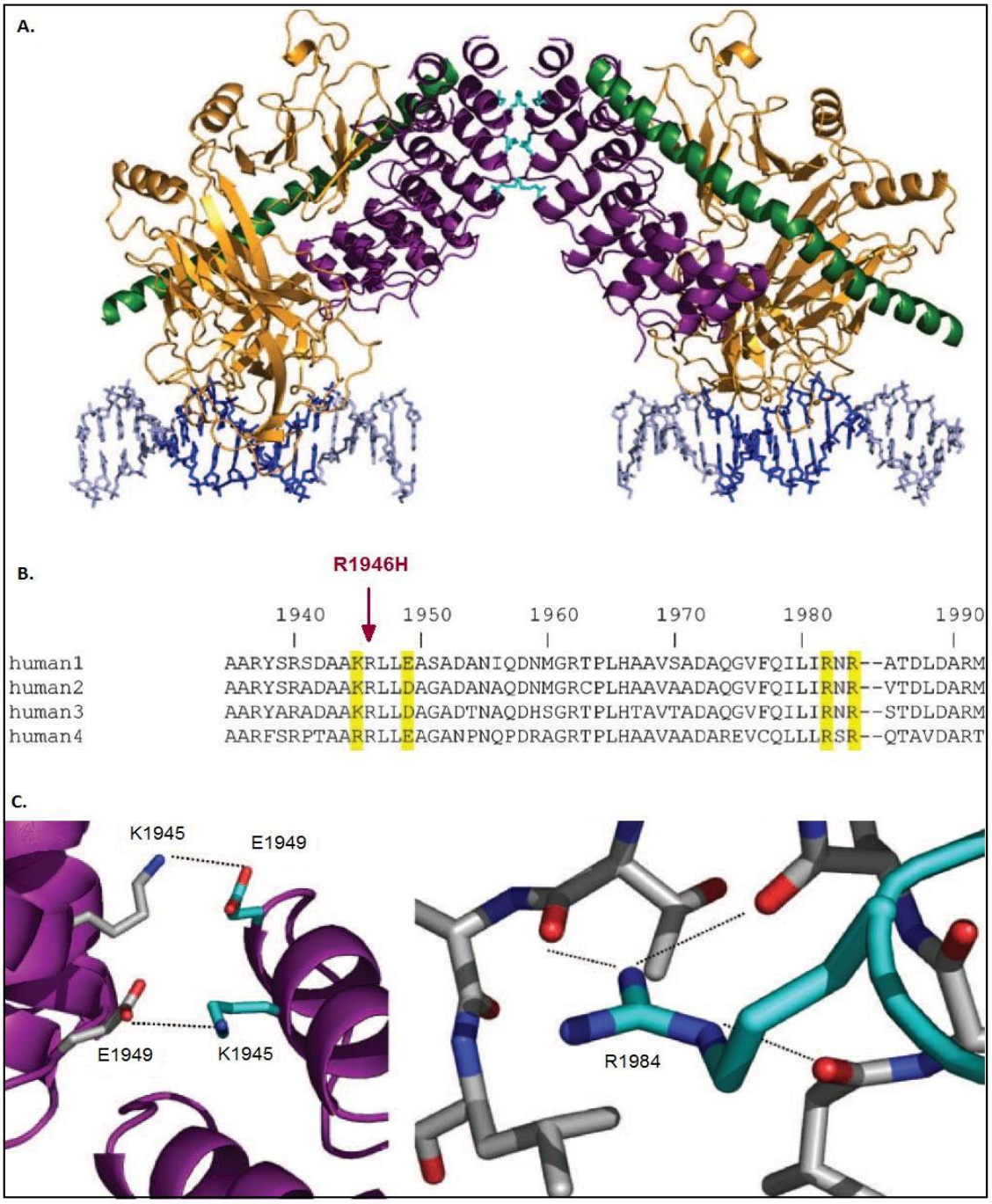

Figure13. ANK-ANK interactions between conserved residues in the crystal lattice. (A) Structure of two copies of the MAML1/ANK/CSL/DNA complex. The protein subunits are rendered as ribbons (ANK, purple; CSL, gold; and MAML-1, green), and the DNA is rendered as blue sticks. Residues of ANK engaged in lattice contacts are shown as cyan sticks. (*B*) Sequence alignment of the ANK which participates in the dimer interface. Key residues that participate in NOTCH1 lattice contacts are highlighted in yellow. [position numbers are based upon *NOTCH1* sequence (NM_017617.3)] (*C*) Specific contacts observed between two symmetry related ANK molecules (one in gray and the other in cyan) in the crystal structure. (*Left*) Salt bridges in ankyrin repeat two between K1945 of one ANK subunit and E1949 of the other. (*Right*) Interactions in ankyrin repeat between R1985 of one ANK subunit and three backbone carbonyl groups of the other. *(Adapted from Nam et al., 2007).*

Experiments show that the binding of MAML1 drives dimer formation on DNA. Mutations of the key residue R1984, which participates in the ANK/ANK contacts in crystals, not only abolish dimerization on DNA in solution (Nam *et al*., 2007) but abrogate the activation from receptor genes that contain paired sites (such as Hes1 promoter) (Figure13) .

It has not been determined whether the mutation R1946H found in this work is pathogenically significant, but it could provide useful information to further understand the NOTCH pathway, since early experiments suggest that mutations such as R1984A prevent induction of T-ALL by ICN1 in mice (W.S. Pear, unpublished data).

Although the R1946H could be thought of as a "loss of function" mutation, this patient also presented a well known point mutation in the heterodimerization domain, therefore it is difficult to say which "effect" predominated. On the basis of immunohistochemical staining intensity (or through Western Blot using specific commercial antibodies), the level of ICN1 in the patient's tumor cells could be evaluated in order to analyze which mutation plays a stronger role.

4.5 Therapeutic implications for T-cell acute lymphoblastic leukemia - NOTCH1: as a molecular target?

4.5.1 γ-Secretase Inhibitors (GSIs)

In the last years, NOTCH1 signaling has been identified to playing a central role in human T-ALL pathogenesis. Although general outcome in pediatric T-ALL has improved, the most frequent cause of treatment failure in leukemia is relapse of the disease, generally associated with acquired chemotherapy resistance, and this adverse event still represents a challenge. The Berlin-Frankfurt-Münster Group 87 in 2005 published their results from a group of children with relapsed ALL, observing an EFS of near 30% for all subtypes combined. T-cell ALL were observed to have a particular grim prognosis, especially when initial slow response to chemotherapy was present; with overall survival approximately 15% (Einsiedel *et al*., 2005). Therefore, development of new drugs and drug combinations has become a priority in this field.
As it has been previously mentioned, activation of *NOTCH1* mutant alleles depend on the S3, the γ-secretase-mediated cleavage of the receptor. Since the presenilin γ-secretase complex plays an important role in the generation of pathogenic amyloid deposits in the brain of patients with Alzheimer's disease, it has gained much attention as a therapeutic target for this disease (Kimberley *et al*., 2003). The first proof of principle of the potential of GSI in the context of T-ALL was provided by the observation that five out of thirty tested T-ALL cell lines went into G0/G1 cell cycle arrest after 4 to 8 days of γ-secretase inhibition (Weng *et al*., 2004). Inhibition of NOTCH1 signaling with GSIs in T-ALL results in a rapid clearance of the ICN1 and transcriptional down-regulation of NOTCH1 target genes, for example MYC and DELTEX1 (Weng *et al*., 2004; Palomero *et al*., 2006).
Following these results, an open-label, non-randomized phase I clinical trial was started to test the activity of the MK0752 GSI in patients with relapsed T-ALL. Unfortunately, the results of this study were discouraging, showing very limited antitumor activity and severe gastrointestinal toxicity, presumably resulting from inhibition of NOTCH signaling in the gut (Deangelo *et al*., 2006). Furthermore, only a fraction of the T-ALL cell lines harboring mutations in NOTCH1 respond with altered proliferation, cell cycle arrest and increase in apoptosis, suggesting that primary resistance to GSI therapy may be present in a significant fraction of T-ALLs (Palomero *et al*., 2007).
Despite these disappointing results, De Keersmaecker and coworkers have shown that the use of γ-secretase inhibitor for 5-7 days reversibly inhibited cell proliferation, caused cell cycle block in sensitive T-cell acute lymphoblastic leukemia cell lines and caused differentiation of some T-ALL cell lines. Although these results suggest that GSIs may sensitize T-ALL cells to

chemotherapy, the fact that GSI only induce apoptosis after a long period of treatment (14 days or longer) and that the effects of γ-secretase inhibition are reversible, suggest that the use of a GSI as a single agent for the treatment of T-ALL could be limited (De Keersmaecker *et al*., 2008).

The intestinal epithelium seems to be very sensitive to systemic inhibition of NOTCH signaling, and GSI treatment is associated with dose-limiting gastrointestinal toxicity, resulting from the accumulation of mucus-secreting goblet cells in the gut (Deangelo *et al*., 2006). This problem is probably accentuated by the fact that GSIs developed for the treatment of Alzheimer's disease are formulated as oral drugs.

Intriguingly, recent data have shown that glucocorticoid (GC) can avoid the development of goblet cell metaplasia in mice treated with a GSI. These results suggest that glucocorticoid might antagonize the effects of NOTCH1 inhibition in the intestinal epithelium and protect from GSI-induced gut toxicity (Real and Ferrando, 2009). Treatment with GC could not only reduce toxicity but also restore glucocorticoid sensitivity in patients with glucocorticoid-resistant T-ALL (Real and Ferrando, 2009). Real and coworkers show that inhibition of NOTCH1 signaling in glucocorticoid-resistant T-ALL restored GC receptor auto-up regulation and induced apoptotic cell death. These results could have a significant impact on future T-ALL treatment, since *in vitro* resistance to glucocorticoid is associated with an unfavorable prognosis (Hongo *et al*., 1997). The majority of patients with T-ALL relapse show an increased resistance to glucocorticoid treatment, identifying glucocorticoid resistance as a major contributor to therapy failure (Yilmaz *et al*., 2006). NOTCH1 signaling plays a critical role in promoting cell growth, proliferation and survival in immature T-cells, which is in a way opposed to glucocorticoid-induced cell death (Aster *et al*., 2008). However, short-term or periodic treatment with γ-secretase inhibitors may produce an acceptable level of toxicity in T-ALL patients, for whom such inhibitors represent a rational, targeted molecular therapy. Clinical trials using these drugs in patients with refractory T-ALL are still in planning stage, but hopefully we will soon know whether their promise as new chemotherapeutic agents will be met. Second generation γ-secretase inhibitors, which apparently exhibit decreased toxicity, are currently being evaluated in clinical trial adult malignancies.

The resistance of cancer cells to chemotherapy and targeted molecular drugs has been well established over the last years (Mellor and Callaghan, 2008). The general mechanisms of drug resistance is by decreasing the effective intracellular concentration of the drug. This can be due to either decreased drug uptake, increased drug export or increased drug metabolism. When considering molecular targeted drugs, mutations are the most common cause of drug resistance. These mutations usually block the interaction of the drug with its specific molecular target. Unfortunately, the presence of activating mutations in *NOTCH1* or constitutive expression of

ICN levels does not seem to effectively predict response of T-ALL cell lines to GSIs (Rao *et al*., 2009).

4.5.2 Understanding the mechanisms of GSI resistance

Mutations in other downstream genes affected by the NOTCH1 signaling pathway could also be responsible for resistance to γ-secretase inhibitors. As previously mentioned, studies have shown that 50% of human T-cell lines bearing HD mutations are unaffected by exposure to NOTCH inhibitors (Weng *et al*., 2004). It has been suggested that when there is a loss of FBXW7, MYC concentrations are maintained above a critical threshold, even in the absence of NOTCH1 signals; therefore facilitating tumor cells a way of escape.

FBXW7 mutants, due to missense mutations or homozygous *FBXW7* deletions, are unable to bind to NOTCH intracellular domain (NIC) and define the phosphodegron region of NIC domain necessary for FBXW7 binding (Thompson *et al*., 2007; O'Neil *et al*., 2007). O'Neil and coworkers identified seven T-ALL cell lines that showed activated NOTCH signaling, but did not display decreased MYC expression upon GSI treatment. Interestingly, all of the cell lines in which MYC expression is not decreased upon GSI treatment, were resistant to the drug, therefore suggesting that lack of MYC down-regulation may contribute to GSI resistance. These results reinforce MYC gene importance for T-ALL cell growth.

Since mutation in the NOTCH PEST domain and loss of FBXW7-mediated regulation both lead to accumulation of the NIC domain due to altered degradation and prolonged NIC half-life, these mutations are thought to be mutually exclusive. On the other hand, O'Neil and coworkers also suggest that there is no selective pressure for *FBXW7* mutations in cells with stabilized NOTCH1.

These results could indicate that mutations in *FBXW7* in T-ALL could represent an alternative mechanism of NOTCH deregulation. Furthermore, it is suggested that *FBXW7* mutations may contribute to T-ALL pathogenesis and GSI resistance by leading to the stabilization not only of MYC, but of the NOTCH1 intracellular domain as well (O'Neil *et al*., 2007).

The association between *FBXW7* mutation and resistance to GSIs has implications for clinical evaluation of agents in patients whose leukemia show deregulation of the NOTCH1 signaling. Molecular analysis of *FBXW7* gene together with other key components of the NOTCH1 pathway may contribute to the identification of patients that are likely to respond to GSI therapy.

Another mechanism that could explain the resistance of T-ALL cells to GSI is the over expression of c-MYC, since NOTCH directly induces c-MYC transcription (Weng *et al*., 2006).

4.5.3 Alternative therapeutic strategies: NOTCH1 interactions with PI3-AKT pathway

Despite the growing interest for γ-secretase inhibitors, the clinical application of these small molecule inhibitors has not been successful, most probably due to our incomplete understanding of the effectors pathways controlled by NOTCH1.

A significant fraction of T-ALLs present a primary resistance to GSI therapy (Weng *et al*., 2004; Thompson *et al*., 2007). Palomero and coworkers support the hypothesis that activation of an alternative oncogenic pathway may bypass NOTCH1 signaling in GSI-resistant T-ALL cells and therefore make these tumors insensitive to NOTCH1 inhibition. They report that loss of the tumor suppressor gene PTEN (phosphatase and tension homolog**)** is associated with resistance to GSI in cell lines with *NOTCH1* mutations (Palomero *et al*., 2007).

PTEN is a tumor suppressor gene that encodes a lipid phosphatase, which inhibits the PI3K-AKT signaling pathway. Notably, PTEN functions as a critical negative regulator of the PI3K-AKT pathway and mutational loss of PTEN is associated with constitutively active AKT signaling which promotes increased cell growth, cell cycle progression and cell survival (Sulis and Parsons, 2003). Palomero and coworkers also demonstrated a mechanistic link between NOTCH1 and the PI3K-AKT pathway in normal T-cell development and T-ALL (Figure 14). NOTCH1 negatively regulates the expression of PTEN via up-regulation of HES1 (a transcriptional repressor), up-regulation of MYC and facilitates the activation of the PI3K-AKT pathway in thymocyte progenitors and T-ALL cells (Palomero *et al*., 2007).

PTEN mutations are frequent in solid tumors and loss of PTEN has been shown to promote self renewal of leukemic stem cells (Yilmaz *et al*., 2006). *PTEN* mutations and loss of PTEN protein expression are relatively frequent in T-ALL cell lines, also occur in a subset of human T-cell leukemias and lymphomas at diagnosis, and can be found also as a secondary event during disease progression, since the analysis of paired diagnostic and relapsed tumor samples demonstrate patients with loss of PTEN at relapse. Interestingly, PTEN loss is present in 17% of primary T-ALL samples. They hypothesize that primary resistance to GSI therapy could be due to aberrant activation of the PI3K-AKT signaling pathway and could be present in a significant fraction of T-ALL patients at diagnosis (Palomero *et al*., 2008).

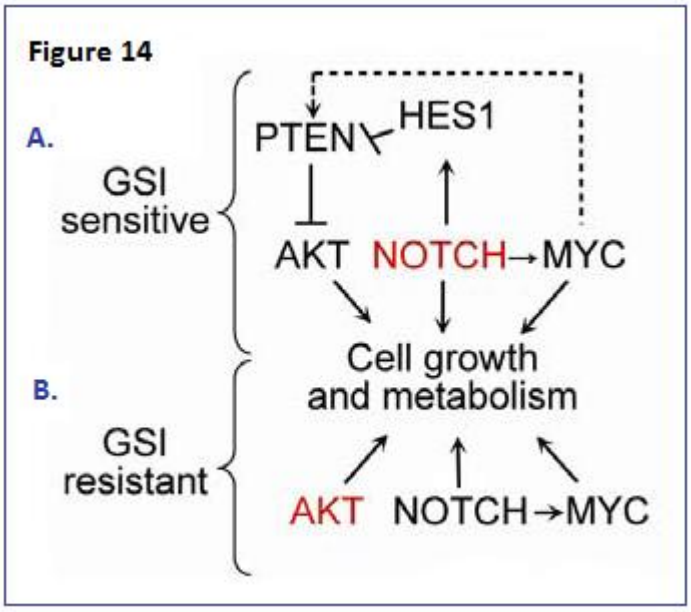

Figure 14. PTEN loss interrupts the pathway controlling oncogenic cell growth in T-ALL downstream of NOTCH1. A. NOTCH1 controls leukemic cell growth through different mechanisms, which include activation of target genes and up-regulation of *MYC*. Moreover, NOTCH1 via HES1 (a transcriptional repressor), negatively controls the expression of PTEN **B.** Mutational loss of *PTEN* induces constitutive activation of AKT and disrupts NOTCH1 signaling from the PI3K-AKT pathway. Therefore, T-ALLs with mutational loss of PTEN cells become insensitive to inhibition of NOTCH1 signaling with GSIs (Adapted from Palomero *et al*., 2007)

The complex network observed here is a clear example of the role of integrative transcriptional regulatory systems, which together adjust the kinetics and intensity of potential oncogenic pathways that control cell growth.

Targeting the PI3K-AKT pathway, with AKT inhibitors, could be a specific novel mechanism against tumors with PTEN loss and could represent an alternative therapeutic strategy for GSI-resistant T-ALLs. Moreover, if the results from T-ALL cell lines are confirmed in primary T-ALL lymphoblasts, analysis of PTEN expression might identify patients with poor response to GSI treatment and could thus serve as a biomarker for tumor progression in T-ALL (Palomero *et al*., 2007).

4.5.4 Utility of the NOTCH1 NRR in a search for compounds to inhibit NOTCH1 signaling in T-ALL

A recent report by Li and coworkers shows an antibody based approach for the inhibition of NOTCH3. They used anti-NOTCH3 inhibitory antibodies that bind to the HD-LNR repeat complex and block the processing and activation of NOTCH3 receptor. The inhibitory antibodies were highly potent, selective for NOTCH3, and block signaling from either Jagged or Delta-like ligands. Interestingly, the antibodies bind to overlapping epitopes within the juxtamembrane NRR that protects NOTCH3 from proteolysis and activation in its resting auto-inhibited conformation (Li *et al*., 2008).

Similar antibodies or inhibitors targeting NOTCH1 could find utility for targeted therapy in the management of T-ALL. Aste-Amézaga and coworkers characterized two classes of selective inhibitory NOTCH1 monoclonal antibodies. One class is directed against the EGF-repeat region of the receptor, the ligand-binding domain (LBD) and is therefore ligand-competitive. The second class of antibodies is allosteric, and is directed against the NRR region, that, as previously mentioned, is the activation switch of the receptor (Figure 15). These antibodies allosterically inhibit ligand-induced conformation changes in the NRR (Aste-Amézaga *et al*., 2010).

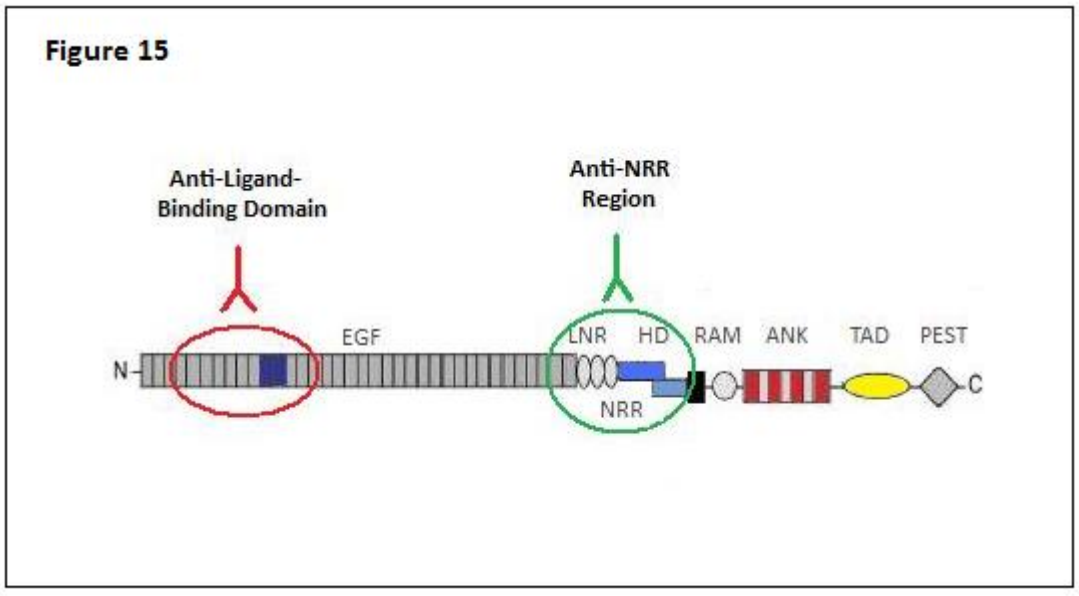

Figure 15. Schematic representation of antibodies or inhibitors targeting the NOTCH1 receptor. The NRR consists of the LNR and HD domains. *(Adapted from Ashworth TD. 2010).*

They described some difference in their activity. As predictable, the activity of the first class of antibodies is strongly dependent on the activating ligand, and, unfortunately has little effect on mutated NOTCH1 receptors.

On the other hand, the antibodies directed against the NRR region are more potent, but incomplete antagonist of NOTCH1 signaling and are able to inhibit ligand-independent signaling in NOTCH1 receptors containing "class 1" mutations, the most common type of mutations found in T-ALL. The antibodies alter the conformational state of the mutated receptor back toward the auto inhibited conformation, without causing total unfolding. This could, theoretically prevent ligand-independent activation of mutants affecting the HD domain, that destabilize the NRR (Gordon *et al*., 2009). Unfortunately they don't seem to affect NOTCH1 receptors bearing other types of mutations. Although, none of the antibodies showed significant anti-proliferative activity, therefore limiting their use as single therapeutic agent; they could eventually be used in combination with other agents.

These reports reinforce the concept that the NRR region is the key NOTCH-specific structural domain to understand how the activation of these receptors is normally regulated. With the description of novel mutations in the LNR domain, this study helps further characterization of the NRR, and could provide interesting information to further focus efforts on the development of potent, selective and rational antibodies or small-molecular-weight molecules, that target the NOTCH signaling pathway.

These novel compounds could contribute to a rational combination strategy, using different therapeutic agents simultaneously, which hopefully might increase the chances of response to T-ALL treatment.

5. CONCLUSION

In this work, a total of 171 pediatric T-ALL were screened for *NOTCH1* mutations, of which, 133 patients were enrolled in the German COALL protocol, and 38 Argentine patients treated according to the ALL IC-BFM protocol. Heterozygous *NOTCH1* mutations were identified in 60.2% of the German patients and in 63.2% of the Argentine patients. The frequency of mutations analyzed in this work coincides with other publications (Breit *et al*., 2006; Zuurbier *et al*., 2010; Kox *et al*., 2010; Clappier *et al*., 2010). In accordance to previous studies, the mutations concentrate in two domains, that is the HD domain and the PEST domain. Interestingly, in this study we also identified mutations in rarely affected domains, as the LNR region, the ANK domain and TAD domain (Zhu *et al*., 2006; Asnafi *et al*., 2009). These noteworthy mutations could offer interesting insight into the NOTCH1 signaling pathway and help to understand the impact of *NOTCH1* activating mutations.

It is well known that mutations that affect the PEST domain encode premature stop codons, leading to truncated forms of NOTCH1 receptor and, due to impaired proteosomal degradation, increase the levels of the active intracellular domain (Weng *et al*., 2004). Of interest, we here describe the case of an in-frame PEST mutation, that doesn't lead to the previously described consequence.

Although *NOTCH1* mutations are associated with early treatment good response, the prognostic impact of NOTCH1 in T-ALL is still unclear and could be influenced by differences in therapy.

This work also suggests that multiple factors should be contemplated when attempting to identify molecular-based prognostic factor for T-ALL in children. Moreover, a better characterization of the NOTCH1 signaling status could be the key element to introduce rational molecular targeted therapies to the standard chemotherapy protocols, combining agents that target not only the NOTCH pathway but also the PI3-AKT pathway.

In conclusion, despite the outcome of children with ALL has considerably improved, there is a long road to cover and a lot of work to do. This requires perseverance, patience, ongoing research and the strengthening of new working relationships. The incorporation of molecular biology studies into the stratification of patients and subsequent individualized treatment strategies will contribute to improved survival in high-risk patients and decreased toxicity and late effects in standard-risk patients with a better quality of life.

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Mutations were designated according to the "Nomenclature for the description of sequence variants" issued by the Human Genome Variation Society (HGVS), URL: http://www.hgvs.org/mutnomen/.

7. APPENDIX

Appendix A

A.1 DNA Extraction

DNA was isolated using the Qiagen QIAmp DNA mini kit (Qiagen-USA, Valencia, CA). Qiagen columns specifically bind DNA while contaminating RNA and proteins are removed. Recovery of DNA from the columns is highly efficient, and PCR inhibitors are effectively reduced.

Principles

Lysis with QIAGEN Protease or proteinase K

QIAamp DNA Blood Mini Kits contain QIAGEN Protease. Research has shown that QIAGEN Protease is the optimal enzyme for use with the lysis buffer provided in the QIAamp DNA Blood Mini Kit. QIAGEN Protease is completely free of DNase and RNase activity.

Purification on QIAamp Mini spin columns

The QIAamp DNA purification procedure comprises 4 steps and was carried out using QIAamp Mini spin columns in a standard microcentrifuge.

Adsorption to the QIAamp membrane

The lysate buffering conditions were adjusted to allow optimal binding of the DNA to the QIAamp membrane before the sample was loaded onto the QIAamp Mini spin column. DNA was absorbed onto the QIAamp silica membrane during a brief centrifugation step. Salt and pH conditions in the lysate ensure that protein and other contaminants, which can inhibit PCR, are not retained on the QIAamp membrane.

Removal of residual contaminants

DNA bound to the QIAamp membrane was washed in 2 centrifugation steps. The use of 2 different wash buffers, Buffer AW1 and Buffer AW2, significantly improves the purity of the eluted DNA. Wash conditions ensure complete removal of any residual contaminants without affecting DNA binding.

Elution of pure nucleic acids

Purified DNA was eluted from the QIAamp Mini spin column in a concentrated form in Buffer AE. The eluted genomic DNA was up to 50 kb in length (predominantly 20–30 kb) and was suitable for direct use in PCR.

Preparation of buffy coat

Buffy coat is a leukocyte-enriched fraction of whole blood. Buffy-coat fraction was prepared from whole blood by centrifuging whole blood at 2500 x g for 10 minutes at room temperature. After centrifugation, 3 different fractions are distinguishable: the upper clear layer is plasma; the intermediate layer is buffy coat, containing concentrated leukocytes; and the bottom layer contains concentrated erythrocytes.

Procedure

1. 20 µl QIAGEN Protease (or proteinase K) were pipeted into the bottom of a 1.5 ml microcentrifuge tube.
2. 200 µl buffy coat sample was added to the microcentrifuge tube.
3. 200 µl Buffer AL were added to the sample. It was then mixed by pulse-vortexing for 15 s.
4. The mixture was incubated at 56°C for 10 min.
5. The 1.5 ml microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid.
6. 200 µl ethanol (96–100%) were added to the sample, and mixed again by pulse-vortexing for 15 s. After mixing, the 1.5 ml microcentrifuge tube was once again briefly centrifuged to remove drops from the inside of the lid.
7. Step 6 mixture was carefully applied, without wetting the rim, into the QIAamp Mini spin column (in a 2 ml collection tube). The columns were then centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube (provided), and the tube containing the filtrate was discarded.
8. After carefully opening the QIAamp Mini spin column, 500 µl Buffer AW1 was added. The columns were then centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube and the collection tube containing the filtrate was discarded.
9. After carefully opening the QIAamp Mini spin column, 500 µl Buffer AW2 was added and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min.
10. The QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded. After carefully opening the QIAamp Mini spin column, 200 µl Buffer AE was added After incubation at room temperature (15-25°C) for 1 min, the columns were once again centrifuged at 6000 x g (8000 rpm) for 1 min.

Determination of DNA length

The length of genomic DNA can be determined through an agarose gel. The DNA should be concentrated by alcohol precipitation and reconstituted by gentle agitation in approximately 30 µl TE buffer, pH 8.0, for at least 30 minutes at 60°C.

A.2 Producing PCR products

PCR reactions were performed using GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA). The procedure was previously described.

A.3 Cloning

Cloning was fulfilled using the TOPO TA Cloning ® Kit for Sequencing (Invitrogen Corporation, Camarillo, CA, USA). This Kit allows an efficient strategy for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector (pCR®4-TOPO®) for sequencing. After PCR amplification, the PCR products were gel-purified (NucleoSEQ®-Macherey-Nagel Inc, Bethlehem, PA, USA). After purification, addition of a 3' A-overhangs post-amplification was performed. *Taq* polymerase buffer, dATP, and 0.5 unit of *Taq* polymerase were added. The mixture was then incubated for 20-30 minutes at 72 °C and was used for TOPO® cloning reaction.

Method and Materials

One Shot®Mach1-T1 Chemically Competent *E.coli* cells were used for cloning.

PCR product 3 µl, salt solution (1 µl), water (1 µl) and TOPO® vector (1 µl) were mixed gently and were incubated 5 minutes at room temperature (22-23°C). The mixture was then used to transform the pCR®4-TOPO® construct into the competent *E.coli*.

1. 3,5 µl of the TOPO® Cloning reaction was added into a vial of chemically competent E.coli and gently mixed.
2. The mixture was incubated on ice for 30 minutes.
3. Cells were heat-shocked for 30 seconds at 42°C. They were then immediately transferred to ice and incubated on ice for 2 minutes.
4. 250 µl of room temperature S.O.C medium was added.
5. The tubes were the shaken horizontally (200 rpm) at 37°C for 1 hour.
6. 120 µl from each transformation was spread on a prewarmed selective plate (kanamycin was used as the selective agent).
7. Plates were incubated at 37ºC overnight.
8. 12 to 24 colonies were picked for analysis.

PCR was used to directly analyze positive transformants. For each sample, 25µl GoTaq® Green Master Mix and 25µl Nuclease-free water were into a 0.5 ml microcentrifuge tube aliquoted. 1µl of M13 primer forward and 1µl M13 reverse primer were used. The selected colony was added in each tube respectively. After initial denaturation at 95°C for 5', PCR was performed at 40 cycles of 95°C for 40", 48°C for 30" and 72°C for 1'. The products were visualized by agarose gel electrophoresis and sequencing reactions were performed as previously described.

A.4 Primer sequences

Table A 1: Primer sequences

NOTCH1-Primer sequences		
	Foward	**Reverse**
Ex 25	CCTCCCAGGTTAGAGGAGAGCGGTGGC	GCCTTAAGAACTGCATGCTGGCCTCCG
Ex 26	CGGAGGCCAGCATGCAGTTCTAAG	GTGGGGAGAGTACTGCTTGCCATG
Ex 27	CTCACCATGTCCTGACTGTGGCGTC	GCTCACACCCGTGGGTAGCAACTG
Ex 28	GACCTTATTTTGGAGGGCGGGATGG	GATGCTCGGCCAGGTCCCACCTC
Ex 29	CCAGGGCCCACCTCCCACGCCAGG	GTCCAGGTCAGGCAGAACCACGGG
Ex 30	GGACCTGGAGACCAAGAAGTTCCGGG	GTATCAACTGTACCCCAGCCTCGGG
Ex 31	CCACACCGTGGCCCCTTGAGCTTGG	GTCCCGGACTCAGCTGTGCTCGG
Ex 32	CCGGAAGACAGTGGGGCTTCCCGT	CCAGTGCAGGGCGGACTTGCCTGC
Ex 33	GGCACGGCTGCTCTGTCGTGGG	GGTCAGGCCCTTGTGTCCCTGCGCC
Ex 34	CTGCTGCTTCCTCTGGTGATGGAACC seq1 GACTCCCTGGAGTCACCCCATGGCTAC seq2 CAGATGATGAGCTACCAGGGCCTGCCC	CATGTGTTTTAAAAAGGCTCCTCTGGTCG

Appendix B

Table B 1: Treatment Schedule, Phases and Doses of ALLIC-BFM 2002 Protocol

Time frame	Treatment
Protocol I (A +B) (8w)	Prednisone: 60 mg/m^2/day (28 days) Vincristine: 1.5 mg/m^2 every w (maximum, 2mg), days 8, 15, 22, 29 Daunorubicin: 30 mg/m^2, every w, days 8, 15, 22, 29 Asparaginase 10,000 U/m^2, days 15, 17, 19, 22, 24, 26, 29, 31 Cyclophosphamide: 1 g/m^2, days 36 and 64 Cytarabine: 75 mg/m^2, days38-41, 43-36, 50-53, 57-60 6-MP: 60 mg/m^2, days 26-64 Methotrexate IT (intrathecal), days 1, 12, (18 and 27 if CNS-2 or CNS-3 or traumatic LP), 33, 45, 59
Protocol M (8w)	Methotrexate: 5 mg/m^2 in 4 doses every 2 weeks. With leucovorin rescue 6-MP: 25 mg/m^2, 8 w Methotrexate IT, second day of every cycle (4 doses)
Protocol II (6w)	Dexamethasone: 10 mg/m^2, 3 w Vincristine: 1.5 mg/m^2 every w (maximum, 2mg), days 8, 15, 22, 29 Doxorubicin: 30 mg/m^2, every w, days 8, 15, 22, 29 Asparaginase 10,000 U/m^2, days 8, 11, 15, 18 Cyclophosphamide: 1 g/m^2, days 36 Cytarabine: 75 mg/m^2, days 38-41, 45-48 6-TG: 60 mg/m^2, days 36-49 Methotrexate IT,(day 1 and 18 if CNS positive) days 38 and 45
Protocol III (4w)	Dexamethasone: 10 mg/m^2, 2 w Vincristine: 1.5 mg/m^2 every w (maximum, 2mg), days 1, 8 Doxorubicin: 30 mg/m^2, every w, days 1, 8 Asparaginase 10,000 U/m^2, days 1, 4, 8, 11 Cyclophosphamide: 1 g/m^2, days 15 Cytarabine: 75 mg/m^2, days 17-20, 24-27 6-TG: 60 mg/m^2, days 15-28

	Methotrexate IT, (day 1if CNS-3 status), 17 and 24
HR1 block (6 days, every 3 w)	Dexamethasone: 20 mg/m^2, 5 days Vincristine: 1.5 mg/m^2 days 1 and 6 Methotrexate: 5 mg/m^2, day 1. With leucovorin rescue Cyclophosphamide: 200 mg/m^2, every 12hs, days 2-4 (5 doses) Cytarabine: 2 g/m^2, 3 hs infusion, 2 doses, day 5 Asparaginase 25,000 U/m^2, day 6 TIT, during MTX infusion
HR2 block (6 days, every 3 w)	Dexamethasone: 20 mg/m^2, 5 days Vindesine: 3 mg/m^2 days 1 and 6 Daunorubicin: 30 mg/m^2 24hs infusion, day 5 Methotrexate: 5 mg/m^2, day 1. With leucovorin rescue Iphosphamide: 800 mg/m^2, every 12hs, days 2-4 (5 doses) Asparaginase 25,000 U/m^2, day 6 TIT, during MTX infusion (in CNS-positive patients also day 5 during DNR infusion)
HR3 block (6 days, every 3 w)	Dexamethasone: 20 mg/m^2, 5 days Cytarabine: 2 g/m^2, 3 hs infusion, days 1 and 2 (4 doses) Etoposide: 100 mg/m^2, every 12 hs, days 3-5 (5 doses) Asparaginase 25,000 U/m^2, day 6 TIT: day 6
Radiotherapy	Preventive: 12 Gy Therapeutic 18 Gy
Maintenance Standard Pulses	 6-MP: 50 mg/m^2, daily, until 2 years from diagnosis Methotrexate: 20 mg/m^2, weekly, until 2 years from diagnosis Vincristine 1,5 mg/m^2 (maximum: 2 mg) day 1 and 8, every 10 w (6 pulses) Dexamethasone: 6 mg/m^2, days 1-7 of pulse (6 pulses)

TIT: triple intrathecal (MTX/ARA-C/PRED)

W: week

6-MP: 6-Mercaptopurine

6-TG: 6-Thioguanine

Table B 2: Treatment in COALL-07-03 trials for high-risk patients

Time frame	Treatment
Pre-Phase (1w)	Daunorubicin (36 mg/m^2 as 24-hours infusion) or Adriamicin (30 mg/m^2 as 24-hours infusion) Methotrexate IT (intrathecal), days -7
Induction (4w)	Vincristine: 1.5 mg/m^2 every w (maximum, 2mg), days 1, 8, 15 and 22 Daunorubicin 36 mg/m^2 (24-hours infusion) days 1, 8 and 15 Prednisone: 60 mg/m^2/day (28 days) * If CNS positive Methotrexate IT day 1 and 18
Intensive phase (14w)	Cyclophosphamide: 900 mg/m^2, days 29 and 43 Medium dose Methotrexate: 1 mg/m^2, day 30, 44, 57 and 71. With leucovorin rescue L- Asparaginase 45,000 U/m^2, day 32 and 46. (When allergic reactions are present substitution with PEG-Asparaginase 2,500 U/m^2) PEG-Asparaginase 2,500 U/m^2 on day 87 and 108 Teniposid (VM-26) 165 mg/m^2 on day 59 and 73 Cytarabine: 300 mg/m^2, days 59 and 73 High dose Cytarabine (HIDAC): 4 x 3 g/m^2 over 3 days every 12 hours on days 85/86 and 106/107 6-MP:100 mg/m^2, days 43-49 and 57-67 6-TG:100 mg/m^2, days 71-77 Methotrexate IT days 30, 44, 57 and 71
CNS Phase -No initial affectation and < 50.000/nl WBC at diagnosis - No initial affectation and > 50.000/nl WBC at diagnosis -Initial CNS affectation	3 Methotrexate IT doses and 6-MP 50 mg/m^2 during 4 weeks No Radiotherapy Preventive Radiotherapy: 12 Gy Therapeutic Radiotherapy: * 1-2 years: 12 Gy * > 2 years: 18 Gy
Re-induction (REDUCED High-Risk)	Dexamethasone: 10 mg/m^2 days 1-7 and 22-28 Vincristine: 1.5 mg/m^2 every w (maximum, 2mg), days 1, 8, 22 and 29

	Doxorubicin: 30 mg/m^2, days 8 and 22 PEG-Asparaginase 2,500 U/m^2 on day 9 and 30 Cyclophosphamide: 900 mg/m^2, days 36 and 50 Cytarabine (ARA-C) 90 mg/m² i.v. or 75 mg/m² Cytarabin i.m. days 37 -40 and 51-54 6-TG:100 mg/m^2, days 36-42 and 50-56 Methotrexate IT days 1, 22 and 36
Re-induction (STANDARD High-Risk)	Dexamethasone: 10 mg/m^2 days 1-14 and 22-35. Vincristine: 1.5 mg/m^2 every w (maximum, 2mg), days 1, 8, 22 and 29 Doxorubicin: 30 mg/m^2, every w, days 8, 15, 22, 29 PEG-Asparaginase 2,500 U/m^2 on day 9 and 30 Cyclophosphamide: 900 mg/m^2, days 36 and 50 Cytarabine (ARA-C) 90 mg/m² IV or 75 mg/m² Cytarabin IM days 37 -40 and 51-54 6-TG:100 mg/m^2, days 36-42 and 50-56 Methotrexate IT days 1, 22 and 36
Maintenance	6-MP: 50 mg/m^2, daily, until 2 years from diagnosis Methotrexate: 20 mg/m^2, weekly, until 2 years from diagnosis **Patients that didn't receive radiotherapy must receive 3 doses of Methotrexate IT (3, 6 and 9 months after ending the reinduction therapy)**

Methotrexate intrathecal (IT): ≥ 1 < 2 years: 8 mg IT.; ≥ 2 < 3 years: 10 mg IT, ≥ 3 years: 12 mg IT

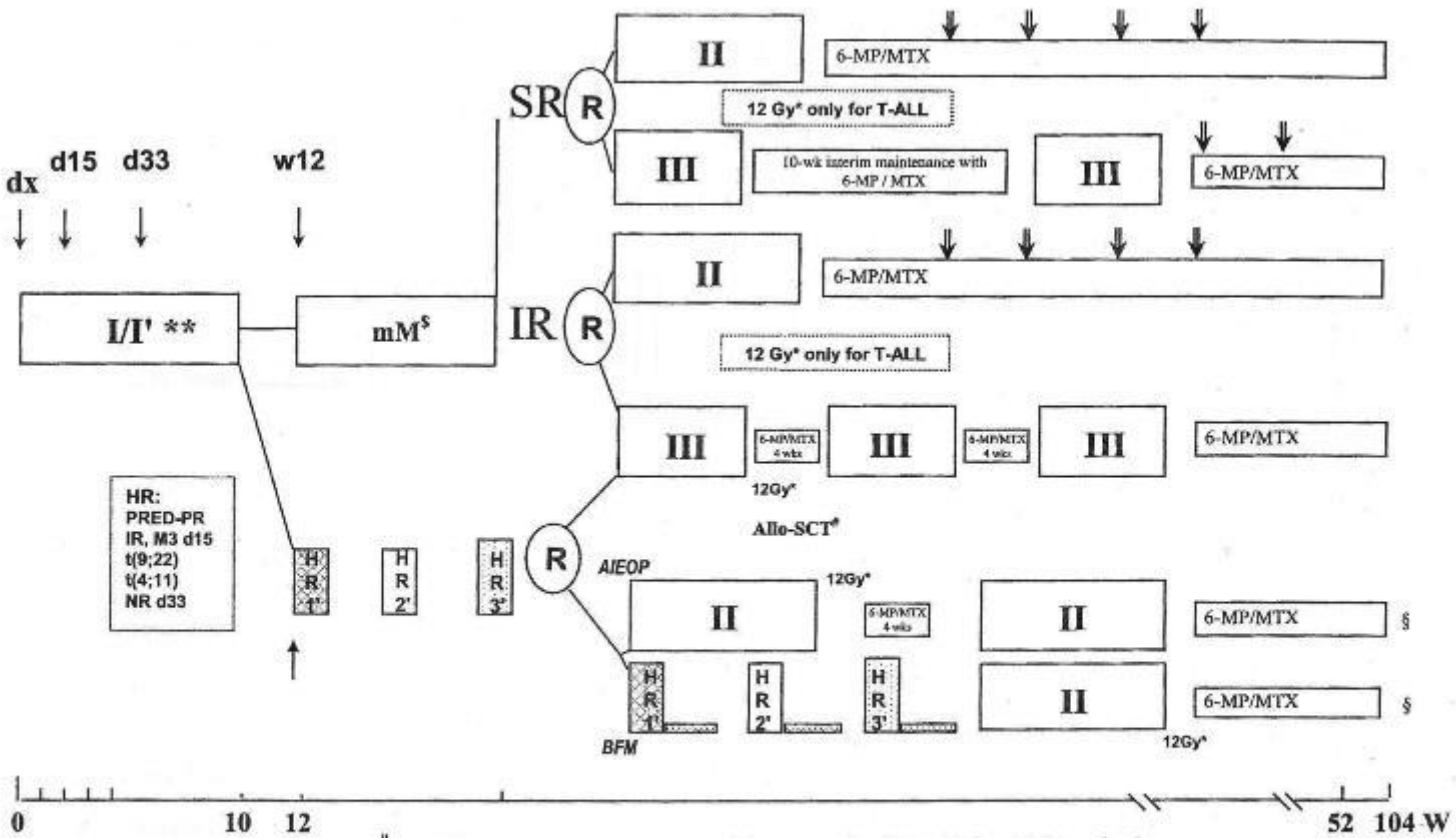
HIDAC: High dose Cytarabine

VM-26: Teniposid

6-TG: 6-Thioguanine

ARA-C: Cytarabine

ALL IC-BFM 2002



** Prot. I' (DNR 30mg/m^2 x2 **only for SR patients with BCP-ALL)**

$ **BCP-ALL: MTX 2g/m^2/24h x4; T-ALL: MTX 5g/m^2/24h x4**

* CNS status 1/2: pCRT = 12 Gy q 1.5 Gy per fraction
CNS status 3: tCRT: 12/18 Gy q 1.5 per fraction – dosage by age at treatment delivery
Infants < 1 yr of age: neither pCRT nor tCRT
See text for definition of CNS status, indications, timing & technique of CRT

\# Selected indications for allogeneic SCT in all strata of HR (See text)

§ No randomization of AIEOP *vs.* BFM but choice by group according to previous experience with one of the two high-risk strategies in trial 95

Appendix b

ALL IC-BFM 2002: Protocol I

Start Phase 1

Weight = _____ kg Height = _____ cm

BSA = _____ m^2

PRED *p.o.* 60 mg/m^2/d = _____ mg

VCR *i.v.* 1.5 mg/m^2/d = _____ mg
(maximum: 2.0 mg/SD)

DNR *p.i.* (1h) 30 mg/m^2/d = _____ mg

L-ASP *p.i.* (1h) 5,000 U/m^2/d = _____ U
(E.coli- MEDAC/KYOWA)

Start Phase 2

Weight = _____ kg Height = _____ cm

BSA = _____ m^2

CPM *p.i.* (1h) 1,000 mg/m^2/d = _____ mg
(+ MESNA: 400 mg/m^2 i.v. x3 at 0, 4, 8h)

ARA-C *i.v.* 75 mg/m^2/d = _____ mg

6-MP *p.o.* (28 d) 60 mg/m^2/d = _____ mg

MTX *I.T.* = _____ mg

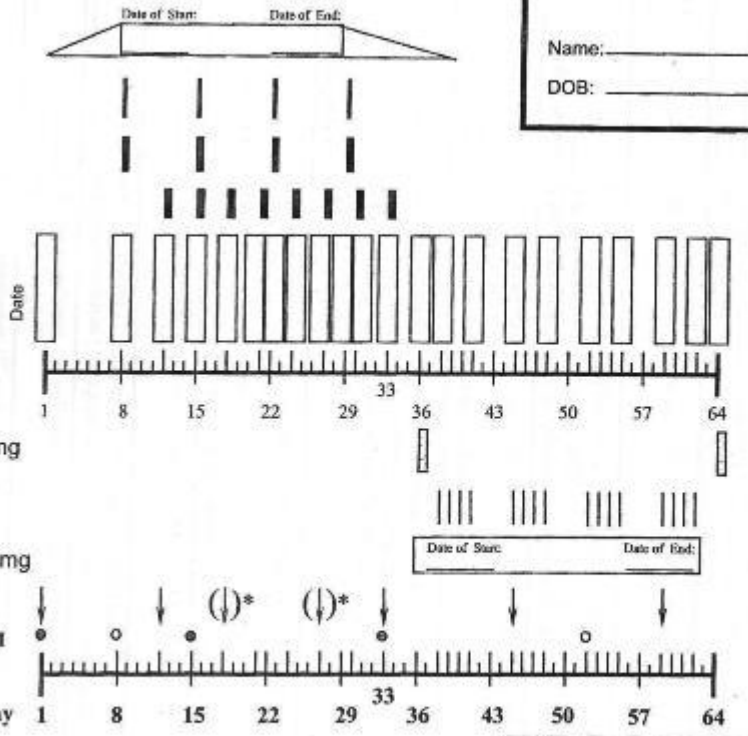
Dose age-adapted:	<1	1	2	≥3Y
MTX *IT* (mg)	6	8	10	12

* if CNS-2 or CNS-3 status, or traumatic LP:
additional MTX *IT* on d 18/27

Center:

Name: _____

DOB: _____



Dose modification? Cytostatic agents added or omitted? YES – NO
Description of modification(s) & reason(s):

_____

Fill in additional **toxicity forms** for Phase 1 & 2 !

_____
Signature

Send copy on completion to national study coordinator

Day 33: Remission?

☐ yes

☐ no ≥ 5% blasts in BM
Blasts in CSF
Mediastinal tumor
≥ 30% of initial size

ALL IC-BFM 2002 : Protocol II

Therapy arm: ☐ SR -1 ☐ IR-1 ☐ HR-2A ☐ HR-2B
HR-2A ☐ 1. ☐ 2.

Center:

Name: _____

DOB: _____

Start Phase 1

Weight = _____ kg Height = _____ cm

BSA = _____ m^2

DEXA *p.o./i.v.* 10 mg/m^2/d = ___ mg

VCR *i.v.* 1.5 mg/m^2/d = ___ mg
(maximum: 2.0 mg/SD)

DOX *p.i.* (1h) 30 mg/m^2/d = ___ mg

L-ASP *p.i.* (1h) 10,000 U/m^2/d = ___ U
(E.coli- MEDAC/KYOWA)

Start Phase 2

Weight = _____ kg Height = _____ cm

BSA = _____ m^2

CPM *p.i.* (1h) 1,000 mg/m^2/d = ___ mg
(+MESNA: 400 mg/m^2 i.v. x3 at 0, 4, 8h)

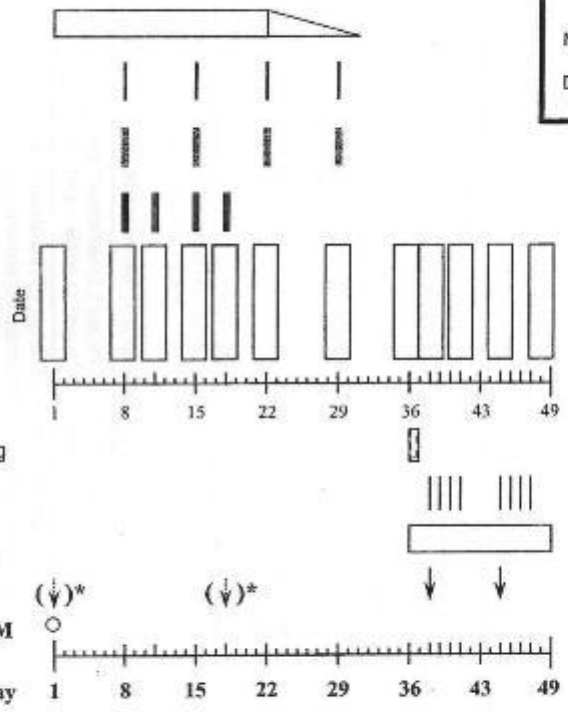
ARA-C *i.v.* 75 mg/m^2/d = ___ mg

6-TG *p.o.* (14 d) 60 mg/m^2/d = ___ mg

MTX *i.T.* = ___ mg

Dose age-adapted: <1 1 2 ≥3Y
MTX *IT* (mg) 6 8 10 12

* If CNS-positive: additional MTX *IT* on day 1 & 18



Start of 6- MP/MTX: ___ ___ ___

Cranial radiotherapy:

☐ No

☐ Yes, from ___ till ___

Total dose: ___ Gy

Fractions (n): ___

Dose modification? Cytostatic agents added or omitted? YES – NO

Description of modification(s) and reason(s):

_____

Fill in additional **toxicity forms** for Phase 1 & 2 !

_____
Signature

ALL IC-BFM 2002: Protocol M

Weight = _____ kg
Height = _____ cm
BSA = _____ m^2

Risk group: ☐ SR T-ALL ☐ IR T-ALL

Center:
Name: _____
DOB: _____

6-MP *p.o.* **(56 d)** 25 $mg/m^2/d$ = ___ mg/d
(in evening, on empty stomach, w/o milk)

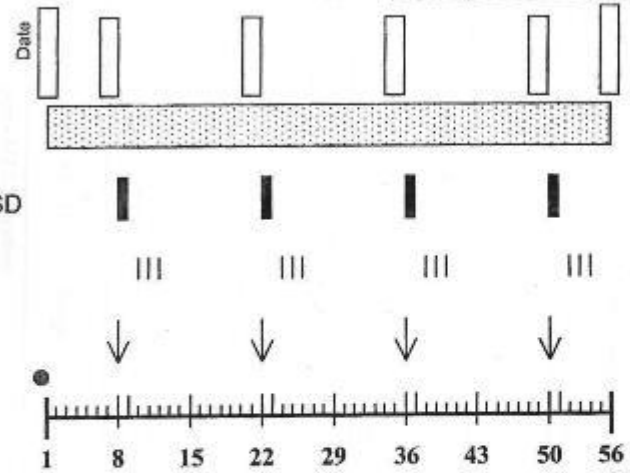
MTX *p.i.* **(24h)** **5,000 mg/m^2** = ____ mg/SD
(10% in 0.5 h, 90% in 23.5 h)

LCV-Rescue 15 mg/m^2 *i.v.* at h: 42, 48, 54

MTX *I.T.* (1h after start of MTX inf.) = ___ mg

Dose age-adapted:

<1y: 6 mg; 1y: 8 mg; 2y: 10 mg; >=3y: 12 mg



Dose modification?
Cytostatic agents added or omitted?
YES – NO
Description of modification(s)/reason(s):

_____
Signature

Fill in additional **toxicity form** for each HD MTX I

Send copy on completion to national study coordinator

1.MTX		2.MTX		3.MTX		4.MTX	
MTX (µmol/l)	CF-Rescue (mg)	MTX (µmol/l)	CF-Rescue (mg)	MTX (µmol/l)	CF-Rescue (mg)	MTX (µmol/l)	CF-Rescue (mg)
24 h		24 h		24 h		24 h	
36 h		36 h		36 h		36 h	
42 h		42 h		42 h		42 h	
48 h		48 h		48 h		48 h	
54 h		54 h		54 h		54 h	
Extended Rescue		Extended Rescue		Extended Rescue		Extended Rescue	
☐ Yes ☐ No		☐ Yes ☐ No		☐ Yes ☐ No		☐ Yes ☐ No	
until hour ☐		until hour ☐		until hour ☐		until hour ☐	

Appendix C

Table C. 1: *NOTCH1* mutations in T-ALL Argentine patients

Case no.	Domain	Classes	Type	Amino Acids involved
1	HD-C	PM	c.5033T>C	p.L1678P
4	HD-N	DEL/INS	c.4740_4742 delins CAGGCTAACTTCCAC	p.M1580IinsRLTST
	PEST	DEL	c.7375delC	p.Q2459R fsX17
6	HD-N	PM*+DEL/INS	c.4623G>C + 4848_4852insTT	p.R1608H + I1616S
	ANK	PM	**c. 5837 G>A**	**p.R1946H**
8	TM	DUP+INS	c.5186_5216dup36 + 5217_5218insCT	p.P1728dupPAGAAALHVR+insL
9	HD-C	INS	**IVS27+29DUP69bp**	
	PEST	INS	c.7327_7328insCCCCCCCC	p.V2443P fsX37
10	PEST	DEL/INS	c.7392_7401delinsACGTTCACCATACCAAGGCCC	p.L2464R fsX16
11	HD-C	PM	c.5026 G>T	p.V1676D
	PEST	DEL/INS	c.7393_7400delinsAGAAGGA	p.P2465R fsX12
13	HD-N	INS	c.4818_4819insTAGGATCGTCTCCTCGCGATCCTT	p.F1606insRIVSSRSF
14	HD-N	PM	c.4799T>C	p.L1600P
	PEST	DEL/INS	c.7308_7312insGAAG	p.G2436R fsX39
15	HD-N	PM (2)*	c.[4603T>C; 4754T>C]	p.[Y1535H; L1585P]
16	HD-N	PM	c.4778T>C	p.L1593P
17	HD-C	DUP+INS	c.5097_5148dup + 5149_5150insTCG	p.V1699dupAAFLGALASLGSLNIPY+1716insR
20	HD-N	PM	c.4721T>C	p.L1574P
21	HD-C	PM	c.5027 T>A	p.V1676D
23	HD-N	PM	c.4721T>C	p.L1574P
24	HD-N	INS	c.4754_4755insAGC	p.L1585insQ
25	HD-C	PM	c.5033 T>C	p.L1678P
26	HD-C	PM	c.5033 T>C	p.L1678P
28	HD-N	PM	c.4778T>C	p.L1593P
29	HD-C	PM	c.5033 T>C	p.L1678P

Case no.	Domain	Classes	Type	Amino Acids involved
34	HD-N	PM	c.4787T>A	p.L1596H
35	HD-N	PM	c.4754T>C	p.L1585P
37	HD-N	PM	c.4793G>C	p.R1598P
38	HD-N	INS	c.4776_4777 ins GGG	p.F1592 ins LG

Note: Based on accession number: NM_017617.3

Abbreviations: PM, point mutation; DEL, deletion; INS, insertion; DUP, duplication.

* **Remark**: Case number 6 had 2 PM in Exon 26 that were found in different alleles.
Case number 15 had also 2 PM in Exon 26, but these were found in the same allele.

Table C.2: NOTCH1 mutations in T-ALL german patients

Case no.	Domain	Classes	Type	Amino Acids involved	Remarks
1	HD-N	DEL	c.4729_4731delGTG	p.V1577L	
3	HD-N	PM	c.4799T>A	p.L1600Q	
7	HD-C	PM	c.5087C>A	p.A1696D	
8	LNR	PM	**c.4297G>C**	**p.G1433R**	Ex25
	PEST	INS	c.7328_7329 ins ATCGACTCGCC	p.V2443D fsX38	
10	HD-N*	PM (2)*	c.[4793G>C + 4799T>C]	p.[R1598P + L1600P]	in ≠ Clones (Trans)
12	TAD	PM	c.6454 G>C	p.G2152R	PM
13	HD-N	PM	c.4754T>G	p.L1585P	
14	HD-N	DEL/INS	c.4816_4816delinsCTTCGAACGG	p.F1606insLRTV	
	PEST	DEL	c.7423_723delC	p.P2475 fsX1	
		DEL/INS	c.7388_7401delinsA	p.A2463D fsX9	
15	HD-C	PM	c.5033T>C	p.L1678P	
17	HD-C	PM	c.5033T>C	p.L1678P	
18	HD-N	PM	c.4793G>C	p.R1598P	
	PEST	DEL	c.7541_7542delCT	p.P2514R fsX4	
19	HD-N	DUP	c.4815_4817dup3	p.V1605dupV	
20	HD-N	PM	c.4790G>T	p.S1597I	
	PEST	INS	c.7486_7487insCTCT	p.N2496L fsX2	
21	HD-C	PM	c.5126T>C	p.L1709P	
22	HD-N	DEL/INS	c.4838_4847delinsCCTGGCC	p.Q1613insPWP	
	PEST	PM	c.7216 C>T	p.Q2401Stop	
25	HD-N	DEL	c.4729_4734delGTGGTG	p.V1577L	
27	HD-N	DEL/INS	c.4816_4817delinsCCCCTCAGCCC	p.F1606insPLSP	
28	HD-C	PM	c.5027T>A	p.V1676D	
29	PEST	PM	c.7475C>A	p.S2491Stop	
32	HD-N	PM	c.4721T>C	p.L1574P	
33	HD-N	PM	c.4721T>C	p.L1574P	
35	PEST	INS	c.7445_7446insCGCACTTTCGACAGCCCAGTCC	p.L2482P fsX9	
36	HD-N	PM	c.4775T>C	p.F1592S	
39	HD-N	PM	c.4733T>A	p.V1578E	

Case no.	Domain	Classes	Type	Amino Acids involved	Remarks
39	PEST	DEL/INS	c.7397_7397delinsGGG	p.T2466R fsX12	
41	HD-N	DEL	c.4735_4737delCTG	p.1579delL	
42	HD-C	PM	c.5101G>C	p.A1701P	
	PEST	PM	c.7318 C/T	p.Q2440Stop	
43	HD-N	PM	c.4721T>C	p.L1574P	
46	HD-N	DUP	c.4689_4713dup27	p.E1563dupEHVPERLAA	
47	HD-N	INS	c.4748_4749insCTACCCCGG	p.E1583insATP	
48	PEST	INS	c.7330_7331 ins TG	p.Q2444C fsX34	
49	HD-N	PM	c.4847T>A	p.I1616N	
50	HD-N	PM	c.4754T>C	p.L1585P	
51	HD-N	PM	c.4799T>C	p.L1600P	
53	HD-N	DUP	c.4732_4734dup3	p.V1578dupV	
55	PEST	DEL	c.7005_7021del	p.L2335P fsX13	
57	HD-N	DEL	c.4815_4922del108	p.F1605del36	
58	HD-N*	PM (2)*	c.[4598A>G;4778T>C]	p.[D1533G;L1593P]	in = Clone
59	HD-N*	PM (2)*	c.[4778T>C + 4790G>T]	p.[L1593P + S1597I]	in ≠ Clones (Trans)
61	HD-N	INS	c.4818_4819insGGG	p.F1606insLG	
62	PEST	INS	c.7171_7172insTGAA	p.Q2391 fsX1	
63	HD-C	PM	c.5039T>A	p.I1680L	
	PEST	PM	c.7172C>T	p.Q2391Stop	
64	PEST	DEL	c.7541_7542delCT	p.P2514R fsX4	
65	HD-N	DEL/INS	c.4771_4775insAA	p.H1591N	
66	PEST	DEL	c.7541_7542delCT	p.P2514R fsX4	
67	HD-N	PM	c.4799T>C	p.L1600P	
68	HD-N	PM	c.4754T>G	p.L1585R	
70	HD-N	PM	c.4821G>T	p.K1607N	
	PEST	INS	7414_7415insATCGAGGTAAGTATATACCGTGGTCT	p.L2472insIEVSIYRGL	In Frame!!!
74	HD-N	DEL/INS	c.4745_4757delinsGGGCGGAGCAGCTGCGT	p.P1582RAEQ	
	PEST	PM*	c.7606G>A	p.V2536I	PM
76	HD-N	PM	c.4799T>C	p.L1600P	
78	TM	INS	c.5222_5223ins33	p.A1741insEARQLHFMYVA	
83	HD-N	PM	c.4754T>C	p.L1585P	

Case no.	Domain	Classes	Type	Amino Acids involved	Remarks
84	HD-C	PM	c.5126T>C	p.L1709P	
87	HD-C	PM	c.5126T>C	p.L1709P	
	PEST	INS	c.7401_7402insCC	p.S2467R fsX10	
88	HD-N	DEL/INS	c.4793_4794delinsAGAAA	p.R1598QK	
89	HD-N	PM	c.4754T>A	p.L1585Q	
93	HD-N	DEL/INS	c.4747_4757delinsCCGGAGCAGCTGGA	p.E1583insPPEELD	
	PEST*	DEL/INS	c. 7399_7401 ins GACCTCCT	p.S2467D fsX12	LCH
		INS	c.7293_7294 ins CAACC	p.R2431 fsX4	
		DUP/INS	c.74357445dup + insTCCTTC	p.A2479Q fsX8	
94	HD-C	PM	c.5044A>G	p.N1682D	
	PEST	PM	c.7375C>T	p.Q2459Stop	
96	HD-N	PM	c.4721T>C	p.L1574P	
97	HD-C	PM	c.5033T>A	p.L1678Q	
98	LNR	DEL/INS	c.4403_4404delinsTTCTCATTTTA	p.N1468ILIL	Ex25
99	HD-C	PM	c.5033T>C	p.L1678P	
	PEST	INS	c.7308_7309 ins CGTCTAACGG	p.G2436V fsX2	
100	HD-N	DEL	c.4732_4734del	p.1578delV	
102	HD-N	PM	c.4754T>C	p.L1585P	
	PEST	DEL/INS	c.7170_7182delinsTGGCCAAACCGGTTAA	p.L2390F fsX6	
104	HD-N	PM	c.4754T>C	p.L1585P	
105	HD-N	PM	c.4730T>A	p.V1577E	
	PEST	DEL	c.7540_7541delCT	p.P2514R fsX4	
106	PEST	DEL/INS	c.7327_7327delinsAGGTAGTCCC	p.V2443R fsX2	
108	HD-C	PM	c.5033T>C	p.L1678P	
111	PEST	INS	c.7302_7303insTCCCTCCT	p.L2434P fsX4	
113	HD-N	INS	c.4777_4778insACCCACTCGGCC	L1593insTHSA	
	PEST	INS/DUP	c.7432_7433insTTTA_7353_7432dup79	p.A2478F fsX27	
115	HD-N	DEL/INS	c.4850_4860delinsCTTACCCAAACCGACAT	p.F1617SYPNRH	
117	HD-N	DEL	c.4732_4734delGTG	p.1578delV	
	PEST	INS	c.7414_7415insGCTTG	p.L2472A fsX7	
118	HD-N	PM	c.4775T>C	p.F1592S	
119	HD-N	PM	c.4793G>C	p.R1598P	

Case no.	Domain	Classes	Type	Amino Acids involved
119	PEST	DEL/INS	c.7169_7169delinsCCCCTCCTTAGAAGCCCC	p.L2390S fsX4
122	HD-N	PM*	c.[4799T>A; 4810G>C]	p.[L1600Q; V1604L]
124	HD-N	PM	c.4799T>C	p.L1600P
125	PEST	DEL	c.7525_7535delTTCCTCACCCC	p.F2509V fsX3
126	HD-C	DEL/INS	c.5029_5030delinsGATTC	p.Y1677DS
127	HD-C	PM	c.5033T>C	p.L1678P
128	HD-N	DEL/INS	c.4851_4857delinsACTTCCGGAGGCGGGGAGT	p.F1617LLPEAGS
132	HD-N	DEL/INS	c.4806_4806delinsGGGA	p.T1602G
	PEST*	INS	c. 7318_7319insACCCCCTAG	p.Q2440T fsx3
		DEL/INS	c.7327_7327delinsCCT	p.V2443P fsX34

Remarks

in = Clone

Note: Based on accession number: NM_017617.3

Abbreviations: PM, point mutation; DEL, deletion;INS, insertion; DUP, duplication.

***Remark:** Cases 10 and 59 had 2 PM in exon 26 that were found in TRANS.

Case 58 and 122 had also 2 PM in exon 26, but these were found in the same clone.

Case 93 presented mutations in Ex26 and Ex34. In fact, 3 different mutated clones were identified in Exon34.

8. ACKNOWLEDGEMENTS

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Finally I would like to thank my family. Without your constant and loving support this project would have been much more difficult. Thank you.

9. CURRICULUM VITAE

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Schooling

February 1985 - December 1991	Primary Saint Andrew's Scots School
February 1992 - December 1997	Secondary Saint Andrew's Scots School Final average 9.25 (over 10) • Diplomas: International Exams: - International General Certificate of Secondary Education (IGCSE – Cambridge) - International Baccalaureate (Geneva-Switzerland) IB Bilingual Diploma (37 marks over 45) • Awards: Academic excellence: 1993, 1995, 1996, 1997

University Studies

October 1997 - March 1998	• Pre-university course of the "Universidad de Buenos Aires" (UBA) for the Faculty of Medicine
April 1998 - March 2004	• Degree in medicine of the "Universidad de Buenos Aires" (UBA), Faculty of Medicine - Plan B (Special programme for high average students) - Hospital de Clínicas San Martín, Buenos Aires Final average of the career: 9 (over 10). Diploma of Honors Matriculate number: MN 114.086
June/July 2005	• Training in Breast Feeding Instruction, "Hospital Materno-infantil Ramón Sarda", Buenos Aires
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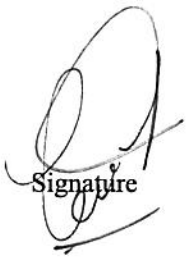
May 31, 2008	• University degree in Clinical Pediatrics of the "Universidad de Buenos Aires" (UBA) and "Sociedad Argentina de Pediatria" (SAP).
July 30, 2008	• Validation of Doctor's degree by the Science and Development Ministry of Spain. Credential number 2007/H04319.
August 2009 - May 2011	• Experimental part and data evaluation of my doctoral thesis on the prognostic value of NOTCH1 mutations in T-ALL in the Department of Pediatric Hematology and Oncology at the Faculty of Medicine – University Medical Center Hamburg-Eppendorf (UKE) with Prof. Dr. Schneppenheim. - August 2009-September 2010 financed by DAAD Scholarschip. - September 2010-May 2011 financed by Günther-Landbeck Scholarship.

Working Experience

May 2011-August 2011	• Employed by the University Medical Center Hamburg-Eppendorf (UKE) as a pediatrician in the Children´s Oncological Ward (Kinder 1).
August 2009-May 2011	• Worked in Pediatric Oncology Hematology at the Faculty of Medicine – UKE (Kinder Ward 1), with full authorization of the State Examination Office for Health Professionals (Landesprüfungsamt für Heilberufe, Hamburg, Germany).
March 2008-April 2009	• Out-patients shifts (replacement) Güemes Hospital, Buenos Aires, Argentina.
Sep. 2007-Feb. 2008	• Hospitation for scientific training in the Department of Pediatric Hematology and Oncology at the Faculty of Medicine – University Medical Center Hamburg-

	Eppendorf (UKE) with Prof. Dr G. Janka-Schaub (Günther-Landbeck Scholarship).
April 2007-July 2007	• Residency optional rotation in Pediatric Oncology Hematology in Charité-Universitätsmedizin Berlin (Campus Virchow-Klinikum, Ward 30) with Prof. Dr. Henze.
June 2004 - March 2007	• Residency in Clinical Pediatrics in the "Hospital de Pediatría Prof. Dr. Juan P. Garrahan", Buenos Airess, obtaining the title of National Specialist in Clinical Pediatrics.
Congress	• Assisted the XX Argentine National Congress of Hematology, Mar del Plata, Buenos Aires, October 2011.

Buenos Aires, Argentina, 16. March 2012


Signature

10. AFFIDAVIT

I hereby confirm that my thesis entitled "Analysis of *NOTCH1* mutation status in precursor T-cell lymphoblastic leukemia of childhood: prognostic value and correlation with early treatment response" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and tools applied are listed and specified on the thesis. They have been identified after the issue (issue and year of publication), volume and page of the used work. Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical no in similar form.

Buenos Aires, Argentina, 16. March 2012

Signature