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Epigenetic silencing affects L-Asparaginase sensitivity and predicts outcome in T-ALL

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Running head: *ASNS* methylation in T-ALL

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Abstract

Purpose: T-Acute Lymphoblastic Leukemias are heterogeneous diseases that account for 20% of ALL in adults. TLX1 or TLX3 deregulated T-ALL (TLX1/3+) share an immature cortical phenotype and close transcriptional signatures. However, their prospective impacts differ and inconsistent impact has been reported for TLX3 in different trials.

Patients and Methods: we compared TLX1+ and TLX3+ adult T-ALL outcome in the successive French national LALA-94 and GRAALL-2003/2005 multicentric trials and analyzed transcriptomic data to identify differentially expressed genes. Epigenetic regulation of *ASNS* and *in vitro* L-asparaginase sensitivity were evaluated for T-ALL cell-lines and primary samples.

Results: Three-year event-free survival (EFS) of TLX3+ adult patients was significantly improved when treated in the, pediatric-inspired, asparaginase-based GRAALL-2003/05 compared to the previous LALA-94 trial (3y-EFS: 66%, 95%CI[39-84] vs. 0% respectively, $p=0.001$). This difference was not observed for TLX1+ patients (3y-EFS: 65%, 95%CI[46-79] vs. 46%, 95%CI[19-70] respectively, $p=0.15$). Transcriptome analysis revealed that TLX1+ blasts expressed lower levels of Asparagine Synthetase (*ASNS*) than TLX3+ blasts. Epigenetic silencing of *ASNS* in TLX1+ blasts was evidenced by an increase in DNA methylation and decrease of active histone marks. Promoter methylation of the *ASNS* gene correlated with asparaginase sensitivity in both T-ALL cell lines and patient-derived xenografts. Finally, *ASNS* promoter methylation was found to be an independent prognostic factor for both EFS (HR, 0.42 [95% CI, 0.24 to 0.71], $p=0.001$) and OS (HR, 0.40 [95% CI, 0.23 to 0.70], $p=0.02$) in 160 GRAALL2003/2005 T-ALL patients.

Conclusion: As asparaginase may be poorly tolerated in adults, *ASNS* promoter methylation status analysis may allow integration of individual variability in leukemic cell sensitivity and optimization of asparaginase therapy in T-ALL patients.

Introduction

T-cell Acute Lymphoblastic Leukemias (T-ALL) are aggressive and highly heterogeneous malignancies that predominate in the 10-39 year age group, where they account for 20% of ALL.¹ Genome wide expression²⁻⁴ assays led to the identification of several oncogenic T-ALL subgroups, namely the immature/Early Thymic Precursor (ETP) (Lyl1, MEF2C), late cortical (TAL1), and early cortical (TLX1/3 and NKX2.1) clusters (supplementary Figure S1). T-cell leukemia homeobox genes *TLX1* and *TLX3* belong to the NKL subclass of homeodomain (HD) proteins⁵ and contain a highly conserved HD known to be involved in DNA-protein and protein-protein interactions. Both of these genes are frequently deregulated in T-ALL (TLX1/3+ T-ALL), predominantly by involvement in chromosomal translocations which lead to constitutive expression.

During embryonic development, HOX proteins in general, and TLX in particular, repress transcriptional events.⁶⁻⁸ Physiological expression of TLX1 and TLX3 is restricted to embryonic development^{9,10}, and no specific function of these genes has been reported in T-cell lymphopoiesis. We recently demonstrated that TLX1/3 proteins repress TCR α rearrangements¹¹, leading to the early cortical maturation arrest observed in TLX1/3+ T-ALLs. Despite the highly related oncogenic functions and structural features of these proteins, TLX1/3+ T-ALLs are paradoxically heterogeneous in their clinical outcome. Although several adult and pediatric clinical trials have shown that T-ALL over-expressing TLX1 display a favorable prognosis¹²⁻¹⁸, data regarding the prognostic impact of TLX3 are more conflicting.^{2,19,20} The molecular explanation of this heterogeneity is unknown. We hypothesized that the very close gene expression pattern of TLX1+ and TLX3+ T-ALLs could represent a favorable setting to identify candidate genes which might determine distinct

clinical outcomes. We identified Asparagine Synthetase gene (*ASNS*) repression by hypermethylation in TLX1+ T-ALLs and demonstrated that *ASNS* methylation, but not expression, could represent a prognostic biomarker in T-ALLs treated within the GRAALL (Group for Research in Adult Acute Lymphoblastic Leukemia) trials.

Patients, materials and methods

Patients and treatments

Adult patients (15-60 years old), included in 3 successive French ALL cooperative group trials (LALA-94, GRAALL-2003 and GRAALL-2005) with T-ALL, defined according to the 2008 WHO classification, were analyzed. The LALA-94 protocol was a multicenter Phase III trial, which enrolled 239 T-ALL from June 1994 to January 2002.¹⁸ Of 97 patients with material available for oncogenic analysis, 25 demonstrated TLX1/3 overexpression. 97 LALA-94 patients were included in this study purely on the basis of material availability and were representative of the entire group (supplementary Table S1 and supplementary Figure S3). The GRAALL-2003 protocol was a multicenter Phase II trial, which enrolled 76 adults with T-ALL between November 2003 and November 2005 of whom 50 had sufficient diagnostic tumor material available.²¹ The multicenter randomized GRAALL-2005 Phase III trial was very similar to the GRAALL-2003 trial, with the addition of a randomized evaluation of an intensified sequence of hyperfractionated cyclophosphamide during induction and late intensification.²² Between May 2006 and May 2010, 337 adults with T-ALL were randomized in the GRAALL-2005, of which 185 had available diagnostic material. TLX1/3 over expression was found in 77 of the 235 GRAALL T-ALL samples (Figure S2). All samples contained >80% blasts. Phenotypic and oncogenetic characteristics were as described.^{12,14}

The LALA94, GRAALL-2003 and GRAALL-2005 protocols are briefly described in Supplementary Table S2. Informed consent was obtained from all patients at enrollment. All trials were conducted in accordance with the Declaration of Helsinki and approved by local and multicenter research ethical committees.

Microarray Gene Expression Profiling analyzes

The Affymetrix U133A microarray analyzes⁴ were performed on the quantile-normalized data using dChip1.3 (<http://biosun1.harvard.edu/complab/dchip/>) software. Locus Link symbols and ID (<http://www.ncbi.nih.gov/entrez/query.fcgi?db=gene>) were used when the information was available to label genes corresponding to the probe sets. The Affymetrix U133 plus 2.0 microarrays analyzes^{3,11} were performed with R version 3.1.1 using the “Affy” package from Bioconductor 3.1. The probe intensities were log2 transformed and normalized using RMA methodology. Identification of differentially expressed genes was performed by Significance Analysis of Microarrays, using 1000 permutations and a false discovery–rate threshold of 5%. The Custom-designed Agilent microarray covering all protein coding genes (33,128 mRNA probes, Human Sureprint G3 8x60k Agilent microarrays) analyzes²³ were performed with R v3.1.1. ([R Foundation for Statistical Computing, Vienna, Austria](#)). Expression data were normalized using the VSN-package (Bioconductor release 3.1) in R. Differential expression analysis was performed in R using Limma package. The genes were said to be differentially expressed if there was more than one Log2 fold change in expression between the two conditions with a p-value less than or equal to 0.05.

ChIP-seq and RNA-seq

Data of H3K4me3 ChIP-seq and RNA-seq in ALL-SIL and CCRF-CEM cell lines and H3K4me3 and H3K36me3 ChIP-seq in one TLX1+ and three TLX- patients were generated and processed within the framework of the Blueprint consortium and visualized using the Integrated Genome Browser (<http://bioviz.org/igb/>). [ChIP antibodies used were H3K4me3: catalog number C15410003-50 \(diagenode\) and H3K36me3: catalog number C15410192 \(diagenode\).](#)

Western Blot

Cells were washed in cold PBS and lysed by RIPA buffer supplemented with protease and phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor Cocktail, Thermo scientific). Proteins were separated by SDS-PAGE gels (MiniProtean, Bio-Rad, Les Ulis, France) and transferred to nitrocellulose membranes (Bio-Rad). Immunoblotting was performed with primary antibodies to ASNS²⁴ (kindly provided by D. Bouscary, Cochin hospital, Paris) and Actin (Abcam) followed by bovine anti-mouse-IgG-HRP (Santa Cruz Biotechnology, Dallas, TX) secondary antibodies. Chemiluminescence was detected by ChemiDoc XRS+ (Bio-Rad) using the West Dura SuperSignal kit (ThermoScientific).

Quantification of ASNS by qRT-PCR

RNA was extracted using an RNeasyMicroKit (Qiagen, Venlo, The Netherlands) and retrotranscribed using Superscript III reverse transcriptase (ThermoScientific). qRT-PCR was performed using the Master SYBR-Green reagent kit. Primers sequences used were: ASNS-Forward: 5'-GAAATGAGAATTCCAAGAATGG-3' and ASNS-Reverse: 5'-CTTTTGGTCGCCAGAGAATCT-3'. The change-in-threshold ($-\Delta\Delta C_t$) method was used to quantify transcript levels following PCR amplification. ASNS levels were calculated relative to the reference genes *ABL1* or *GAPDH*.

DNA methylation study

Global DNA methylation was assessed by Methylated DNA Immunoprecipitation (MeDIP) assay in a series of 24 T-ALLs, including 6 TLX1 positive cases and 3 human thymii. Briefly, methylated DNA was immunoprecipitated as described previously²⁵ using 2 μ g of sonicated genomic DNA. MeDIP samples were directly subjected to labeling and hybridization on a

custom human promoter array (Agilent, Santa Clara, USA)¹¹, following the manufacturer's instructions. Finally, median-normalized log₂ enrichment ratios (MeDIP/Input) were calculated for each probe using CoCAS software²⁶ and visualized using IGB tool (<http://bioviz.org/igb>). Direct ASNS methylation levels were analyzed by MS-MLPA (Methylation Sensitive-Multiple Ligation-dependent Probe Assay) with custom probes (supplementary **Table S4**) and, SALSA® MLPA® P200 Reference-1 probemix and EK1 reagent kits from MRC-Holland (Amsterdam, The Netherlands), according to manufacturer's recommendations. Data were analysed with the Coffalyser software (MRC-Holland).

Statistical analysis

Comparison of continuous and categorical variables between subgroups was performed by Mann-Whitney test and Fisher's exact test, respectively. Censored data (i.e. Overall Survival (OS), Event-Free Survival (EFS) and Disease-Free Survival (DFS)) were analysed using Cox models. OS and EFS were calculated from the date of prephase initiation. **Kaplan-Meier method was used to estimate the probability of survival.**²⁷ Events accounting for EFS were induction failure, first haematological relapse, and death from any cause in first complete remission (CR). DFS were calculated from the date of CR achievement. Events accounting for DFS were first haematological relapse, and death from any cause in first CR. In some analyses, outcome variables were censored at allogeneic hematopoietic stem cell transplant (HSCT) time. Univariate and multivariate analysis assessing the impact of categorical and continuous variables were performed with a Cox model. Proportional-hazards assumption was checked before conducting multivariate analyses. Interaction between trial (LALA-94 vs. GRAALL-2003/2005) effect and TLX (TLX1+ vs. TLX3+) status was tested by introducing an interaction term in the multivariable model **comprising three co-variables: Trial, TLX1/3 status**

and the interaction term (Trial x TLX1/3). Statistical analyses were performed with STATA software (STATA 12.0 Corporation, College Station, TX). All tests were two-sided with a significance level of 0.05.

Results

TLX3+ Adult T-ALL outcome is improved by an intensive, pediatric-inspired, chemotherapy regimen

In order to compare the therapeutic response of TLX1+ and TLX3+ adult T-ALLs, we first analyzed their outcome within the successive French LALA-94 and GRAALL-2003/2005 multicenter trials. The main difference in chemotherapy was a global increase in dose-intensity in the GRAALL trials with, notably, addition of an intensive asparaginase sequence during induction therapy (6000U/m²/day see supplementary **Table S2** for details) which was repeated during delayed intensification, 12 weeks after complete remission. TLX over-expression was demonstrated in 25/97 (26%) and 77/235 (33%) LALA94 and GRAALL03/05 T-ALLs, respectively, including 65 (64%) TLX1+ and 37 (36%) TLX3+ cases (Supplementary **Figure S2**).

As previously reported, TLX3+ patients were slightly younger (28 years [15-55] vs. 35 years [17-57], p=0.03) and had higher initial white blood cell counts (WBC) (59 G/L, range [2-308] vs. 23 G/L, range [4-294], p=0.03) and less frequent cortical immunophenotypes compared to TLX1+ T-ALL cases. *NOTCH1*, *FBXW7*, *RAS*, *PTEN* and *NUP214-ABL* alterations were found at similar incidences in both groups (supplementary **Table S5**).

Complete remission (CR) was achieved in 65/65 TLX1+ patients (100%) and 34/37 TLX3+ patients (92%, p=0.05). For TLX3+ patients, the CR rate was 80% (8/10) in the LALA-94 trial vs. 96% (26/27) in the GRAALL trials (p=0.17). Since eligibility for HSCT differed among trials,

survival analyses were censored at the time of HSCT. As shown in **Figure 1**, 3-year EFS was 0% vs. 66% (95%CI[39-84]) for TLX3+ patients treated on the LALA-94 and GRAALL trials, respectively ($p=0.001$). In TLX1+ patients, this difference was not significant (3-year EFS: 46%, 95%CI[19-70] vs. 65%, 95%CI[46-79] in LALA-94 versus GRAALL trials, $p=0.16$). Similarly, 3-year OS was 11% (95%CI[2-72]) vs. 82% (95%CI[66-100]) for TLX3+ patients treated on the LALA-94 and GRAALL trials respectively ($p<0.001$). This difference was not observed in TLX1+ patients, with a 3-year OS of 60% (95%CI[39-95]) vs. 80% (95%CI[68-95]) for LALA-94 and GRAALL patients respectively ($p=0.15$). Moreover, there was a significant interaction, in a multivariable model between the trial and the TLX1 and TLX3 groups for EFS ($p=0.05$) and a trend for OS ($p=0.07$), suggesting that TLX3+, but not TLX1+, patients preferentially benefited from the more intensified GRAALL protocols.

Low Asparagine Synthetase transcript expression in TLX1+ T-ALL

In an attempt to identify the molecular mechanism underlying the disparity in response to treatment between TLX1+ and TLX3+ T-ALLs, differentially expressed genes in three data sets were compared. We first took advantage of public transcriptomic data of T-ALL patients⁴ to establish a list of differentially expressed transcripts in TLX1+ ($n=11$) as compared to TLX3+ ($n=18$) patients. This list (supplementary **Table S6**) contains 259 unique genes ($p<0.05$, fold change=2). Two other transcriptomic data sets were obtained by RNAi inactivation of TLX1²³ and TLX3¹¹ in the TLX overexpressing ALL-SIL (TLX1) and DND41 (TLX3) cell lines. This identified 929 and 1259 (supplementary **Table S6**) differentially expressed genes ($p<0.05$, fold change=2) in ALL-SIL and DND41, respectively, after TLX inactivation. Comparison of these three lists of differentially expressed genes identified only 4 overlapping genes (**Figure 2A**). Among those, the *ASNS* gene (Asparagine Synthetase) was

the only one which was less expressed in TLX1+ compared to TLX3+ patients. *ASNS* was, moreover, upregulated after TLX1 siRNA inactivation, as confirmed in an independent ALL-SIL cell line (**Figure 3E and 3F**). Conversely, *ASNS* was downregulated after TLX3 shRNA inactivation. Of note, *ASNS* was the third most differentially expressed gene between TLX1 and TLX3 T-ALL ($p=0.0025$) and demonstrated significantly lower expression in TLX1+ compared to TLX3+ and TLX negative patients in independent published gene expression profiling and RNA-seq analyses^{3,28} (**Figure 2B-C**). We confirmed this result in 86 GRAALL treated T-ALL including 19 TLX1+ and 12 TLX3+ T-ALLs (**Figure 2D**).

Promoter hypermethylation leads to Asparagine synthetase silencing in TLX1+ T-ALLs

To explore the mechanism underlying *ASNS* low level transcription in TLX1+ T-ALL, we performed global methylation analyses by MeDIP-array in 24 primary T-ALLs, including 6 TLX1+ cases. *ASNS* promoter methylation was significantly higher in TLX1+ cases compared to other T-ALL cases (**Figure 3A and 3B**). To validate this observation, 153 adult T-ALLs were analyzed by an in-house custom MS-MLPA assay which clearly demonstrated significantly higher *ASNS* promoter methylation in TLX1+ cases ($n=33$) as compared to TLX1- ($n=120$), including 25 TAL1+ and, importantly, 22 TLX3+ cases (**Figure 3C-D**). Interestingly, we observed a decrease of *ASNS* promoter methylation in the ALL-SIL cell line transduced with shRNA-TLX1 (fold change=-0.14 relative to shRNA-mock) (**Figure 3E-F**) suggesting a role of TLX1 in the methylation status of *ASNS* promoter methylation. An increase of *ASNS* expression and concomitant decrease of *ASNS* promoter methylation was also observed following a 5 day exposure of the ALL-SIL cell line to the 5-azacytidine hypomethylating agent, supporting the relationship between *ASNS* expression and promoter methylation (**Figure 3F**). To provide additional support for the epigenetic silencing of *ASNS* in TLX1+

blasts, we analyzed active histone marks in several T-ALL cell lines and primary blasts. The TLX- CCRF-CEM cell line, which displays high *ASNS* expression and low level *ASNS* promoter methylation (**Figure 4A-B**), demonstrated significant enrichment of H3K4-me3-active marks within the *ASNS* promoter region, as compared to the TLX1+ ALL-SIL cell line (**Figure S4A**). ChIP-seq analysis performed on primary T-ALL cells (one TLX1+ and three TLX-) also showed a decrease of H3K4me3 and H3K36-me3-active marks within the promoter and the gene body of *ASNS*, respectively, in the TLX1+ case as compared to the 3 TLX- cases (**Figure S4B**). In an attempt to identify the mechanism of *ASNS* repression by TLX1, we analyzed TLX1 ChIP-seq data in the ALL-SIL cell line²³, which failed to demonstrate direct TLX1 binding on *ASNS* (data not shown).

Altogether, these results suggest epigenetic silencing of *ASNS* in TLX1+ cases by both DNA methylation and a decrease of active histones marks.

ASNS methylation levels correlate with L-asparaginase sensitivity

We then hypothesized that the favorable outcome of TLX1+ T-ALL could be associated with the low *ASNS* expression *via* *ASNS* promoter methylation. In order to test L-asparaginase *in vitro* sensitivity according to *ASNS* status, the TLX1+ ALL-SIL and TLX- CCRF-CEM T-ALL cell lines were exposed to increasing doses of native *E. coli* L-asparaginase (Kidrolase, EusaPharma) (0-25 UI/mL) for 48h, followed by a cell viability assay. As expected, ALL-SIL was more sensitive to L-asparaginase as compared to CCRF-CEM (**Figure 4A-D**). Then two different *ASNS* siRNA, SiRNA-1 and SiRNA-2, were used to determine the relationship between *ASNS* levels and L-asparaginase sensitivity in the CCRF-CEM cell line. SiRNA-1 was more efficient compared to SiRNA-2, yielding a decrease of *ASNS* expression of 63% and 37% respectively (**Figure 4E-F**). Decreased *ASNS* expression led to a proportional increase in

sensitivity to L-asparaginase (**Figure 4G**), with LC50 values at 72h of 0.48, 0.23, 0.017 UI/mL for CCRF-CEM siRNA-mock, CCRF-CEM siRNA-2 and CCRF-CEM siRNA-1 respectively compared to less than 0.001 UI/mL for ALL-SIL, suggesting an inverse correlation between *ASNS* expression and L-Asparaginase sensitivity in these T-ALL cell lines. We then evaluated *in-vitro* L-asparaginase sensitivity of 9 T-ALL cells from patient-derived primary T-ALL xenografts (PDX) in NSG mice. Apart from one (M105 with an *ASNS* promoter methylation ratio at 0.38), PDX with hypomethylated *ASNS* promoters (UPNT-374 methylation ratio 0.08, M106 methylation ratio 0.33 and UPNT-420 methylation ratio 0.25) were less sensitive to L-asparaginase than those with hypermethylated *ASNS* promoters (UPNT-670 methylation ratio 0.86, UPNT-565 methylation ratio 1, M152 methylation ratio 1.04 and M149 methylation ratio 1.04) (**Figure 4H**).

These data strongly support a correlation between *ASNS* promoter methylation and *in vitro* sensitivity to L-asparaginase in both T-ALL cell lines and PDX.

ASNS Methylation, but not expression, is an outcome predictor in adult T-ALL

ASNS expression and promoter methylation by MS-MLPA were then evaluated for 86 and 160 T-ALL patients respectively from the GRAALLO3/05 trials. No significant correlation between *ASNS* expression and promoter methylation was observed (supplementary **Figure S5A**), probably due to high level *ASNS* expression by both normal peripheral blood lymphocytes and bone marrow normal residual cells (Supplementary **Figure S5B**). We used two strategies to purify blast cells (i) blast- and non-blast cells sorting and (ii) Primary Derived Xenograft (PDX), for *ASNS* quantification and confirmed this hypothesis (supplementary **Figure S5C**). *ASNS* expression levels had no prognostic impact on EFS nor OS (**Figure 5A-B**). In striking contrast, *ASNS* promoter methylation levels strongly correlated

with outcome. When considering the methylation ratio as a continuous variable, a higher ratio was significantly associated with better EFS (HR 0.33, 95%CI[0.17-0.64], $p=0.001$) and OS (HR 0.25, 95%CI[0.12-0.50], $p<0.001$). Patients in the lower methylation tertile T1 had a significantly lower EFS (3-year EFS: 36%, 95%CI[23-49]) and OS (3-year OS: 43%, 95%CI[30-56]) than patients in the T2 and T3 tertiles, who displayed comparable outcome (3-year EFS: 67%, 95%CI[57-75], $p<0.001$; 3-year OS: 75%, 95%CI[66-83], $p<0.001$) (**Figure 5C-D**). Similar results were observed after censoring outcome at the time of allogenic stem cell transplantation (data not shown). Of note the T1 tertile subgroup was significantly associated with a low rate of NOTCH1 pathway mutated status and unfavorable classifier (**Table 1**). It was also less likely to achieve CR, but did not demonstrate other early resistance to treatment as assessed by prednisone response, bone marrow blast clearance or MRD after induction (**Table 1**). After adjustment for age, WBC, CNS involvement, early therapeutic responses and the NOTCH1/FBXW7/RAS/PTEN molecular classifier, *ASNS* promoter methylation status was confirmed to be an independent prognostic indicator for both EFS (HR, 0.42 [95% CI, 0.24 to 0.71]; $p=0.001$) and OS (HR, 0.40 [95% CI, 0.23 to 0.70]; $p=0.02$) (**Table 2**). We also demonstrated a significant prognostic impact of *ASNS* promoter methylation in an independent series of 47 adult T Lymphoblastic Lymphomas (T-LBL) included in the French LL03 protocol²⁹ which used similar L-asparaginase schedules to the GRAALL trial (**Figure 5E**). Interestingly, the LALA94-treated T2 subgroup, with intermediate methylated *ASNS* status, demonstrated higher rates of relapse as compared to T2 GRAALL- and LL03-treated cases, which probably benefited from the 6-fold higher doses of L-Asparaginase (**Figure 5F**). Overall these data suggest that the methylation status of the *ASNS* promoter could represent a biomarker of L-asparaginase response and outcome of adult T-ALL/T-LBL.

Discussion

Despite recent insights into the molecular and cellular mechanisms responsible for T-ALL onset and progression, survival rates remain around 50% in adults, justifying the search for novel therapeutic options or more adapted/personalized regimens. Extensive clinical data support the benefit of more intensive L-asparaginase treatment in pediatric ALL^{30,31 32-34} and the GRAALL has reported a significant improvement in the outcome of adults with BCR-ABL-negative ALL using a pediatric-inspired intensified treatment protocol.²¹ The LALA-94 and GRAALL trials mostly differed in the use of L-asparaginase, with no L-asparaginase in LALA-94 induction vs. 48,000 UI/m² cumulative dose in GRAALL induction, and 16 times more asparaginase in the whole GRAALL protocol (144,000 UI/ m²) than in the LALA-94 (9,000 UI/ m²).^{18,21}

The present study focused on the TLX+ population of T-ALL and reports a clear benefit of an intensified pediatric-inspired regimen in TLX3+ patients, while TLX1+ patients display a comparable favorable outcome whatever the therapeutic approach. Taking advantage of the closely related transcriptional profiles of TLX1 and TLX3 T-ALLs, we demonstrate that *ASNS* silencing in TLX1+ cases is a plausible mechanistic explication for these discrepancies. The *ASNS* gene, which encodes for asparagine synthetase, is an aminotransferase enzyme that catalyzes the biosynthesis of the amino acid asparagine (Asn) from aspartic acid (Asp). Most tissues contain sufficient asparagine synthetase activity to maintain satisfactory asparagine levels and upregulate the enzymatic activity in response to asparagine depletion.^{35,36} Primary ALL cells and many ALL cell lines exhibit a particularly low level of *ASNS* expression^{37,38}, and as such, are usually sensitive to asparagine depletion. This relatively low *ASNS* expression in lymphoblasts is the basis for the use of L-asparaginase, leading to the depletion of plasma

asparagine, during chemotherapy in ALL and is now an essential component of most pediatric therapeutic protocols.

Asparaginase has been recently added to adult ALL trials as a pediatric inspired approach. Despite the benefits of this strategy, these dose intense regimens are far more toxic in adults and cumulative incidence of non-relapse mortality is as high as 40% in patients over 55 years (Huguet *et al*, ASH 2016). Asparaginase is responsible for hepatic toxicity, thrombosis, hypo-albuminemia and denutrition. There is a consequent need for tailored asparaginase use, based on leukemia biology and patient-related conditions.

It is noteworthy that asparaginase is the drug with the most heterogeneous usage within different ALL cooperative groups. We hypothesized that prognosis could be related to differences in L-asparaginase dosage in therapeutic protocols and to differential sensitivity between TLX1+ and TLX3+ patients based on their ASNS activity. Although it is unknown if the total cumulative amount of asparaginase is more relevant than the overall time spent with effective depletion, this data is difficult to assess and is not commonly reported. We therefore analyzed published outcomes of TLX patients as a function of their cumulative L-asparaginase treatment. After excluding duplicates and articles that did not specify the chemotherapy regimen used, we found 14 publications^{2,13,15-20,39-44} that report on the survival of TLX3 and TLX1 patients. TLX1 overexpression is constantly associated with favorable EFS and OS in all reported trials (Figure 6) and seems to be independent of L-asparaginase cumulative dose. Conversely, the outcome of TLX3+ patients is highly heterogeneous between different trials, but demonstrates an approximately “linear” correlation between L-asparaginase cumulative dose and outcome, suggesting that this could account for the variable prognostic impact of TLX3+ (and potentially other TLX1 negative) T-ALLs.

The mechanisms of L-asparaginase resistance remain unclear and data from studies on the importance of ASNS are conflicting. In leukemic cell lines^{45,46}, correlations between *ASNS* transcriptional expression and L-asparaginase sensitivity seem reliable. It has been shown that drug-selected L-asparaginase-resistant cell lines exhibit a high expression of *ASNS*⁴⁷ and that overexpression of exogenous *ASNS* protein could result in an L-asparaginase-resistant phenotype in the absence of drug selection⁴⁷. Concerning primary ALL, data remain unclear and the role of *ASNS* expression with regard to asparaginase resistance may vary among genetic subtypes and, as suggested by others, may have little or no relevance to drug sensitivity *in vivo*^{45,48-52}. Moreover, most studies correlated *ASNS* expression and L-asparaginase sensitivity at a transcriptional level but the relationship between *ASNS* mRNA and intracellular protein concentrations has not been established. It has been reported⁵³ that the correlation between *ASNS* protein levels and IC50 was much better than that between the IC50 and *ASNS* mRNA content, suggesting that transcriptional expression might not be the best marker of *ASNS* activity. In addition, we found that normal blood and bone marrow cells express high level of *ASNS*. As such, mRNA content could be accurately correlated to *ASNS* activity in ALL cell lines but not in primary ALLs, because of potential contamination with residual normal blood or bone marrow cells. **The fact that *ASNS* methylation is not dependent on exponential PCR amplification, unlike transcript quantification, probably contributes to its greater predictive value and a more reliable and accurate way to assess *ASNS* activity.**

No study has specifically focused on the role of *ASNS* in T-ALL; the majority of studies were conducted in B-cell precursor (BCP)-ALL.^{45,49-52,54} One study did, however, report higher *ASNS* expression in T-ALL as compared to BCP-ALL.⁵⁵ Therefore, it would be interesting to study whether *ASNS* methylation could also be a biomarker of L-asparaginase response in BCP-ALL.

Amino acid metabolism is essential for cancer cell proliferation in general and not only for ALL. L-asparaginase is also used in chemotherapy protocols to treat NK/T cells lymphomas and other aggressive malignancies such as soft-tissues sarcomas⁵⁶ or gliomas.⁵⁷ *In vivo*, genetic silencing of ASNS in mouse sarcoma cells combined with depletion of plasma asparagine by L-asparaginase inhibited tumor growth. It could therefore be advantageous to combine asparagine depletion and ASNS inhibition for tumors which exhibit high ASNS activity. Despite recent advances in “personalized” medicine, targeted therapies do not currently benefit most ALL patients and classical chemotherapies still remain the cornerstone of first line treatment. Although asparaginase management optimization mainly relies on monitoring of immune inactivation of the drug and adaptation of effective asparagine depletion, we report for the first time on a leukemic cell biological explanation for discrepancies in patient related outcomes to this major ALL drug. **If these observations are confirmed in larger scale and independent T-ALL trials, ASNS methylation status at diagnosis may allow individual adaptation of asparaginase total dose, potentially with a reduced incidence of side-effects.**

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Figure legends

Figure 1. Impact of *TLX1* and *TLX3* deregulation and treatment era on adult T-ALL patient outcome. Kaplan-Meier graphs for (A) EFS and (B) OS censored at HSCT are shown according to TLX status (*TLX1+* vs. *TLX3+*) and trial (LALA-94 vs. GRAALL-2003/2005). A significant impact of treatment is shown within the *TLX3+* subgroup.

Figure 2. The *ASNS* gene is expressed at lower levels in *TLX1+* T-ALL. (A) Venn diagram comparing three transcriptomic signatures, (i) genes differentially expressed between *TLX1+*

and *TLX3*+ T-ALL⁴ (green circle), (ii) genes differentially expressed after *TLX1* siRNA inactivation in the *TLX1* overexpressing ALL-SIL cell line²³ (red circle) and (iii) genes differentially expressed after *TLX3* shRNA inactivation in the *TLX3* overexpressing DND41 cell line¹¹ (blue circle). Four genes overlap, including the *ASNS* gene which is the only one to be expressed at lower levels in *TLX1*+ patients (D), down-regulated after *TLX3* inactivation (D) and up-regulated after *TLX1* inactivation (U). (B) public gene expression profiling³ and (C) RNA-seq data²⁸ showing significantly reduced *ASNS* expression in *TLX1*+ patients compared to *TLX3*+ and *TLX*- patients. (D) *ASNS* expression normalized to *GAPDH* by RTQ-PCR in an adult T-ALL series included in the GRAALL 2003/20005 trials including 26 *TLX1*+, 10 *TLX3*+ and 55 *TLX*- cases. *TLX1*+ patients expressed significantly less *ASNS* than other patients.

Figure 3. Hypermethylation of the *ASNS* promoter in *TLX1*+ cases. (A) MeDIP-array data focused on *ASNS* promoter in 3 thymii, 6 *TLX1*+ cases (in red) and 18 *TLX1*- cases (in blue) including two *TLX3*+ cases (T-ALL #233 and T-ALL #454). (B) Diagram of MeDIP-array data focused on CpG in the dashed box. (C) Examples of MS-MLPA profiles; left, a *TLX1*+ case; right, a *TLX*- case. Signals for three reference probes and four probes located on the *ASNS* promoter (*ASNS*-1, *ASNS*-2, *ASNS*-4, and *ASNS*-6) are shown. Upper part, profiles of control MLPA analysis; bottom part, profiles with *Hha*I restriction endonuclease showing *ASNS* promoter methylation ratios. (D) *ASNS* promoter methylation by MS-MLPA in GRAALL patients according to oncogenotype (*TLX1*+, *TLX3*+, *TAL1*+ and others). (E) Western blots for expression of *TLX1* and the actin control (bottom). (F) Fold Change (FC) of *ASNS* expression and promoter methylation in the ALL-SIL cell line transduced with shRNA-*TLX1* relative to shRNA-mock and in the ALL-SIL cell line exposed to 5-azacytidine during 5 days (1 μ M and 2.5 μ M) relative to cells treated with vehicle.

Figure 4. L-Asparaginase sensitivity according to *ASNS* expression and methylation ratios.

(A) *ASNS* expression normalized to *ABL1* evaluated by RTQ-PCR in the ALL-SIL and CCRF-CEM cell lines and in normal thymus. (B) *ASNS* promoter methylation ratio by MS-MLPA in ALL-SIL and CCRF-CEM cell lines and in normal thymus. (C) Western-Blot for *ASNS* or actin expression in ALL-SIL and CCRF-CEM cell lines. (D) Sensitivity experiments of ALL-SIL and CCRF-CEM T-ALL cell lines upon L-asparaginase (Kidrolase™) exposure. Results are expressed as the relative number of surviving cells, as compared to an internal control (NaCl). (E) Decrease of *ASNS* expression after *ASNS* siRNA inactivation in the CCRF-CEM cell line. Two independent siRNA were used, siRNA-1 and siRNA-2, with a decrease of *ASNS* expression of 63% and 37% respectively compared to siRNA control (siRNA-mock). (F) Western-Blot for *ASNS* or actin expression in CCRF-CEM cell line after *ASNS* siRNA inactivation; *ASNS*/actin protein ratios are indicated below the blot. (G) Sensitivity experiments of ALL-SIL cell line and CCRF-CEM cell line after *ASNS* siRNA inactivation upon L-asparaginase (Kidrolase™) exposure. (H) *In vitro* sensitivity experiments of primary T-ALL xenografted in NSG mice upon L-asparaginase (Kidrolase™) treatment. Nine primary T-ALL were exposed during 72h and apoptosis was analyzed by FACS after annexin V/ propidium iodide staining. *ASNS* promoter methylation was analyzed by MS-MLPA and ratios are noted in brackets. T-ALL with higher methylation ratios (in red) appear more sensitive to L-asparaginase than T-ALL with lower methylation ratios (in blue).

Figure 5. Outcome of patients according to *ASNS* expression and methylation ratio. (A) and (B) Kaplan-Meyer graphs according to *ASNS* expression tertiles for EFS and OS respectively for patients included in GRAALLO3-05 trial. (C) and (D) Kaplan-Meyer graphs according to

ASNS methylation tertiles for EFS and OS respectively for patients included in GRAALL03-05 trial. EFS according to methylation tertiles for patients included in (E) the LL03 Lymphoblastic lymphoma trial and (F) the LALA-94 trial. Outcome comparison (p) between subgroups is shown.

Figure 6: Reported 2 to 5 year event free survival in published series of adult and pediatric TLX1+/3+ patients according to the cumulative amount of asparaginase. Mean Asparaginase dose was calculated in case of paper reporting about a mix of patients included in various trials. Each circle represents a published series. Circle sizes are proportional to the number of TLX+ subjects reported. The numbers next to the circles correspond to the references reporting the respective trials.

Tables

	Total	T1	T2+T3	p-value† T1 vs T2+T3
n (%)	160	54	106	
TCR subsets analysed				
Immature (IM0. Imd. Img)	38/140 (27)	12/46 (26)	26/94 (28)	1
αβ lineage	89/140 (64)	31/46 (67)	58/94 (62)	0.57
γδ lineage	13/140 (9)	3/46 (7)	10/94 (11)	0.55
ETP immunophenotype	29/139 (21)	9/47 (19)	20/92 (22)	0.83
oncogenetic category				
<i>NOTCH1/FBXW7</i> ^{mutated}	110/160 (69)	25/54 (46)	85/106 (80)	<0.0001***
unfavorable classifier*	69/158 (44)	37/53 (70)	32/105 (30)	<0.0001***
<i>TLX1</i> +	33/153 (22)	1/52 (2)	32/101 (32)	<0.0001***
<i>TLX1</i> -	120/153 (78)	51/52 (98)	69/101 (68)	<0.0001***
Clinical subsets analyzed				
Age median, years	30.6	28.2	31.2	0.32
Age>35	57/160 (36)	20/54 (37)	37/106 (35)	0.86
Sex ratio M/F	2.4	4.4	1.86	0.04*
White blood cell count median, x10 ⁹ /L	34.2	73	28.6	0.02*
Central nervous system involvement	23/157 (15)	10/51 (20)	13/106 (12)	0.2
Corticosenitivity	88/160 (55)	31/54 (57)	57/106 (54)	0.7
Chemosenitivity	91/157 (58)	33/51 (65)	58/106 (55)	0.3
Complete Remission	149/160 (93)	45/54 (83)	104/106 (98)	0.001**
MRD ≥ 10 ⁻⁴	29/88 (33)	5/21 (24)	24/67 (36)	0.4

Table 1. Clinico-biological characteristics of T1 (lower ASNS methylation tertile) and T2+T3 (higher methylation tertiles) adult T-ALL. TCR: T-cell receptor; ETP: early thymic precursor. *

The unfavorable classifier includes NOTCH1, FBXW7, RAS and PTEN ⁵⁸. † (Mann-Whitney test) or Fisher exact test were used where appropriate. Statistically significant differences are highlighted in bold.

Event-free Survival	Univariate			Multivariate		
	HR	95%CI	p	HR	95%CI	p
Age*	1.03	[1.00 ; 1.05]	0.023	1.04	[1.02 ; 1.07]	0.001
LogWBC*	1.55	[1.05 ; 2.28]	0.028	1.58	[0.99 ; 2.51]	0.05
CNS	1.91	[1.10 ; 3.33]	0.022	1.90	[1.04 ; 3.47]	0.04
Prednisone response	0.52	[0.32 ; 0.82]	0.005	0.71	[0.39 ; 1.30]	0.27
D8 BM response	0.60	[0.38 ; 0.96]	0.033	0.67	[0.40 ; 1.14]	0.14
Unfavorable classifier	3.56	[2.17 ; 5.84]	<0.001	2.39	[1.36 ; 4.20]	0.002
ASNS promoter methylation	0.41	[0.26 ; 0.66]	<0.001	0.42	[0.24 ; 0.71]	0.001

Overall Survival	Univariate			Multivariate		
	HR	95%CI	p	HR	95%CI	p
Age*	1.03	[1.01 ; 1.05]	0.01	1.05	[1.02 ; 1.08]	<0.001
LogWBC*	1.71	[1.11 ; 2.62]	0.02	1.85	[1.11 ; 3.09]	0.02
CNS	2.29	[1.28 ; 4.11]	0.01	2.46	[1.34 ; 4.51]	0.004
Prednisone response	0.62	[0.37 ; 1.02]	0.06	0.85	[0.47 ; 1.56]	0.61
D8 BM response	0.68	[0.41 ; 1.13]	0.14	-	-	-
Unfavorable classifier	3.43	[2.00 ; 5.87]	<0.001	2.38	[1.33 ; 4.27]	0.004
ASNS promoter methylation	0.38	[0.23 ; 0.62]	<0.001	0.40	[0.23 ; 0.70]	0.02

* continuous variables

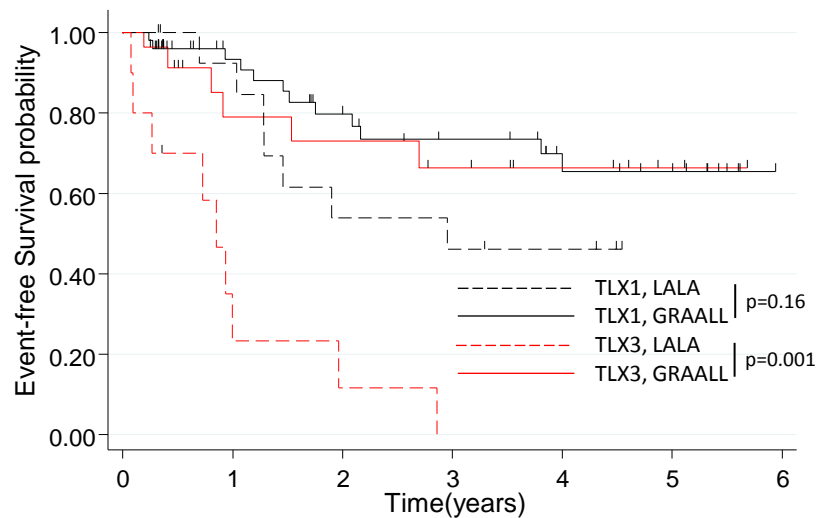
Table 2. Univariate and multivariate analysis for EFS and OS

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Authorship contributions: AT, ML, MB and SS performed diagnostic and experimental analyses. AT, EL, NB and VA analyzed clinical data and wrote the manuscript. All authors contributed to supervision of clinical research and data collection. VA designed and oversaw conceptual development of the project.

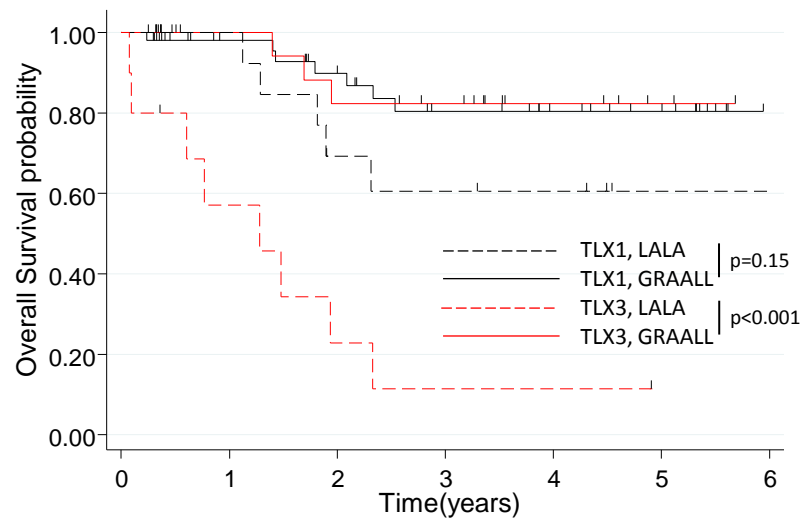
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Number at risk

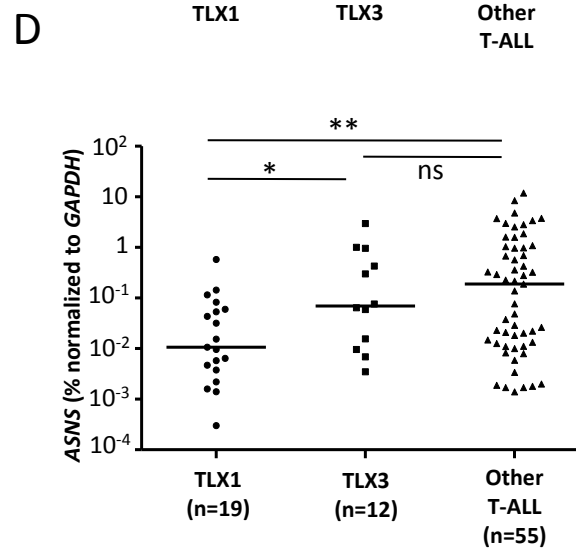
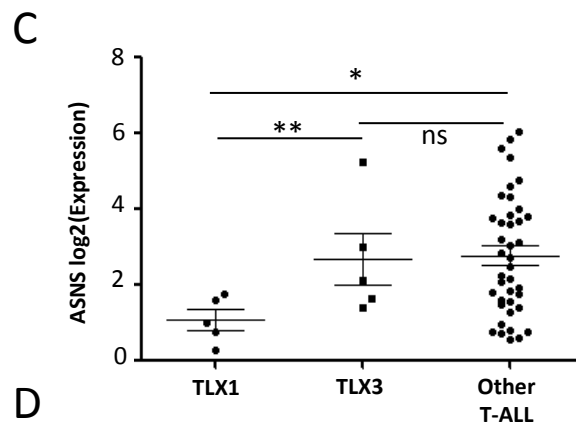
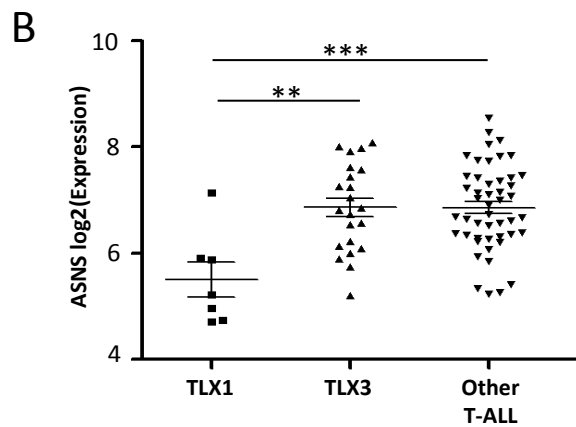
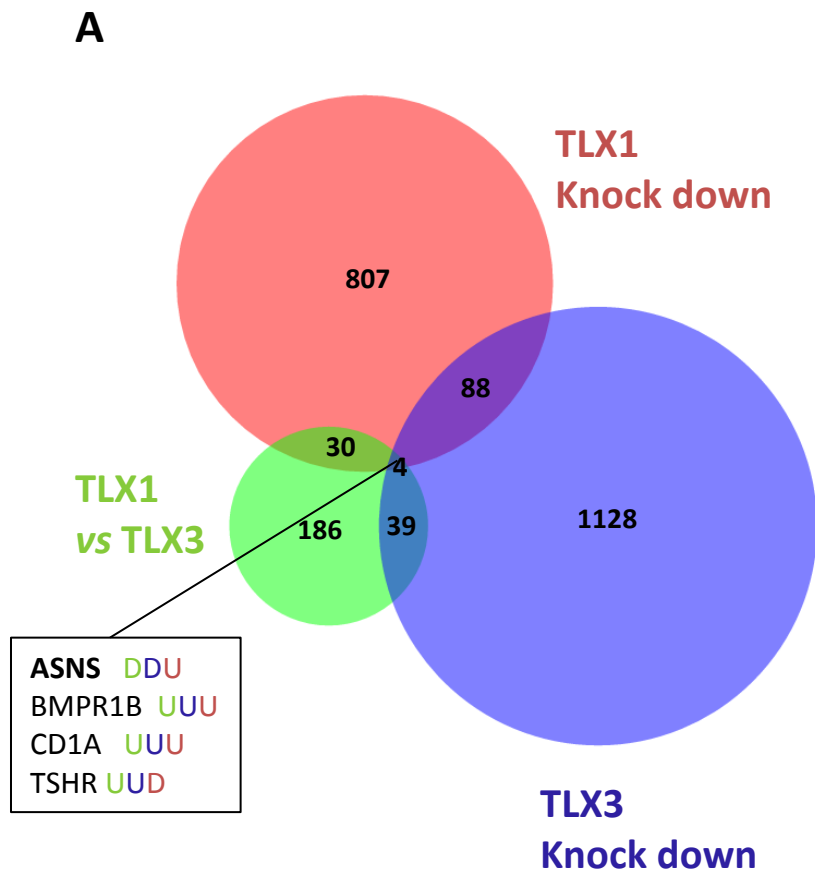
GRAALL, TLX1+	50	35	26	22	15	12	4
GRAALL, TLX3+	27	13	12	9	6	3	1
LALA, TLX1+	15	12	7	6	5	2	2
LALA, TLX3+	10	2	1	0	0	0	0

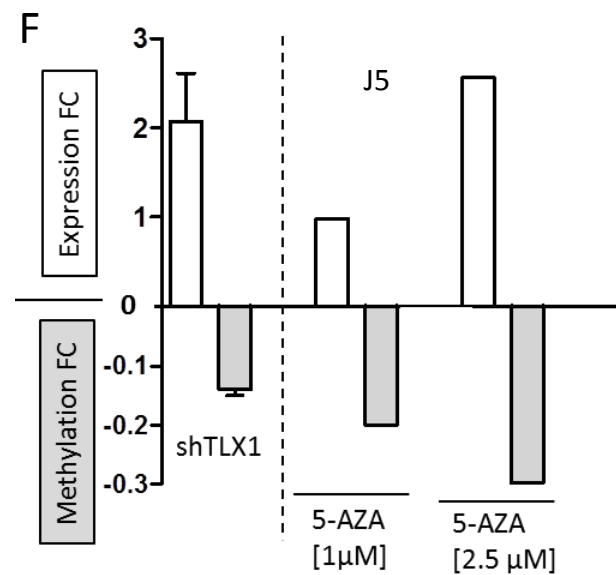
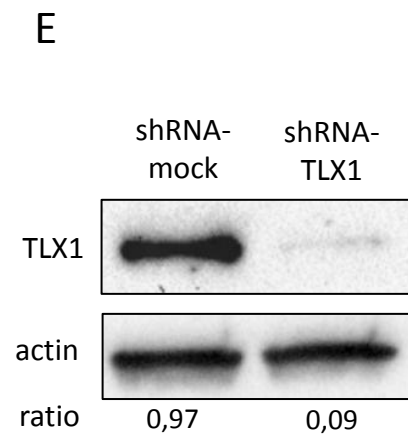
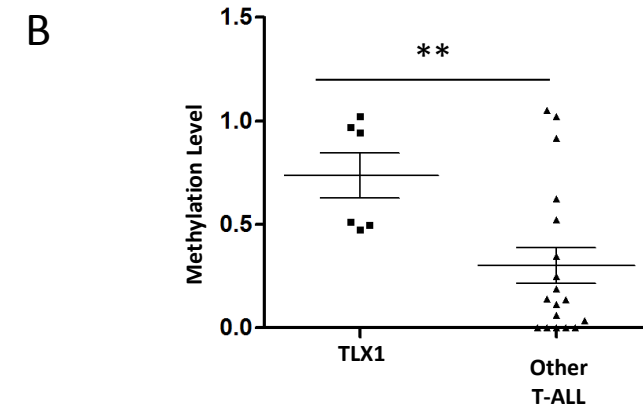
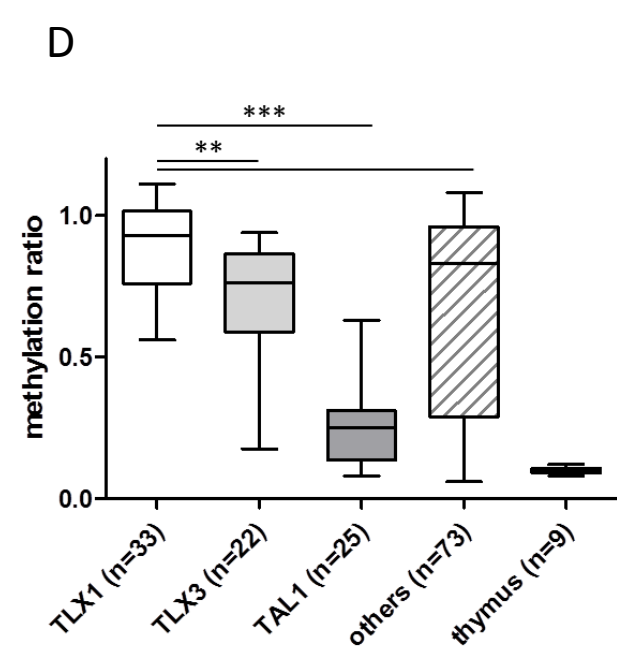
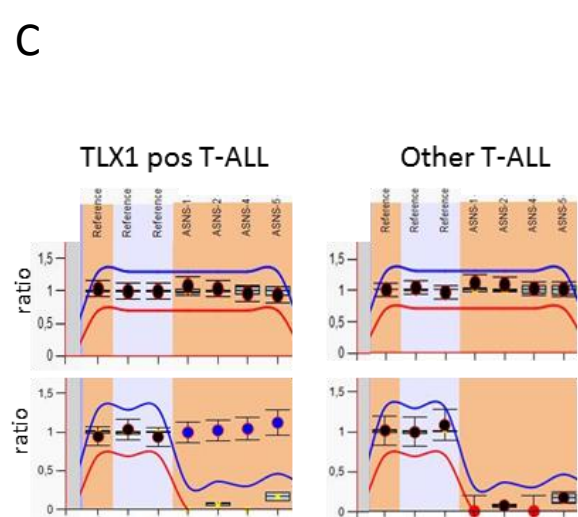
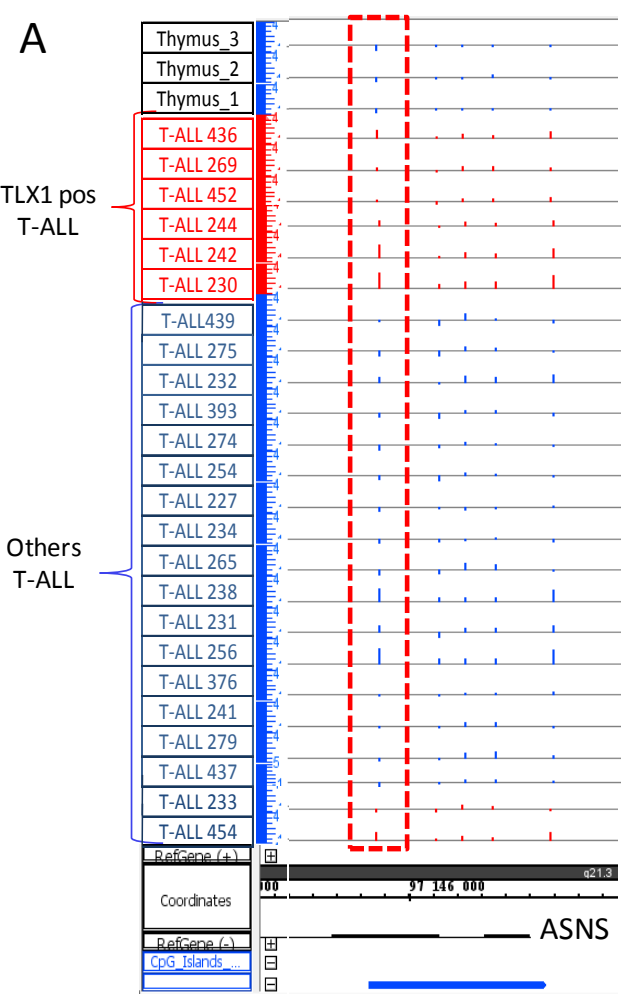
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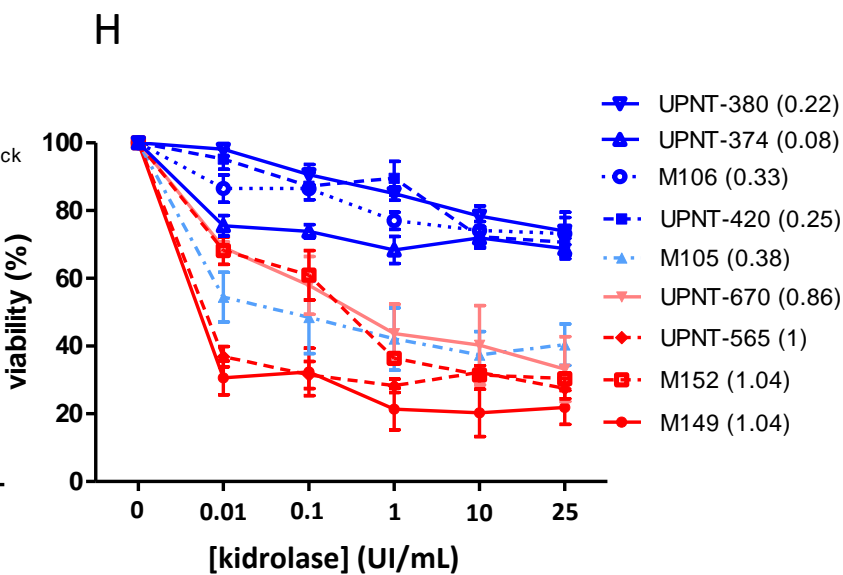
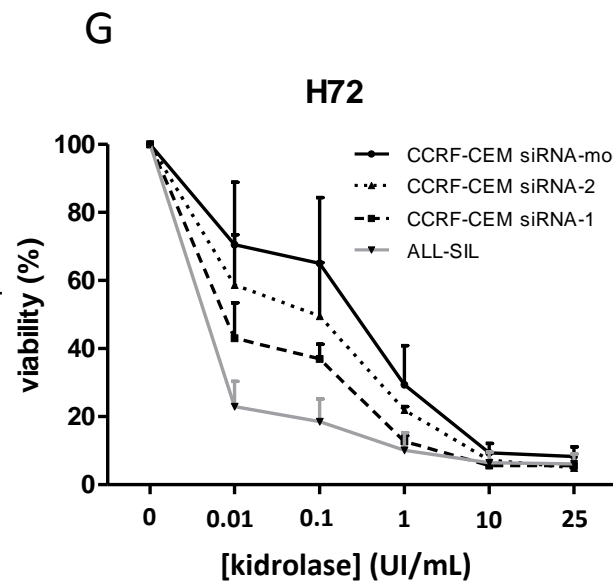
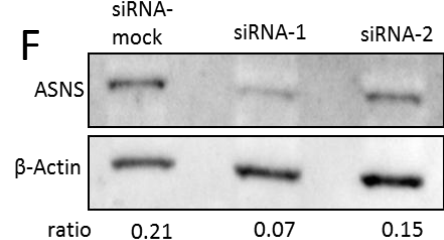
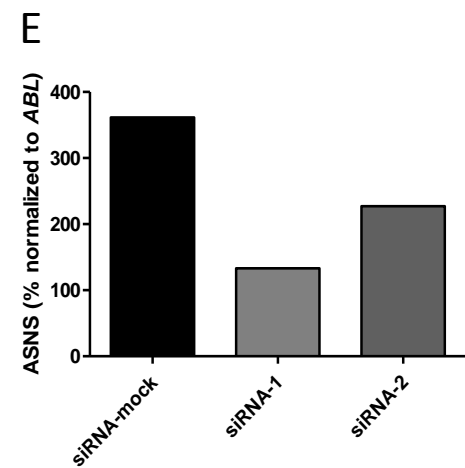
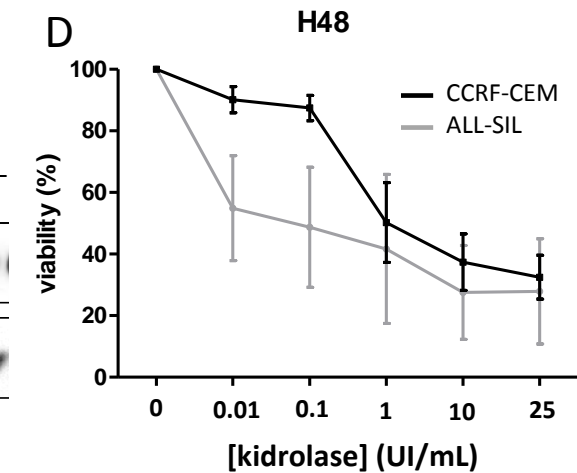
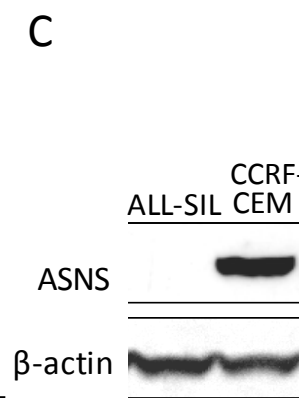
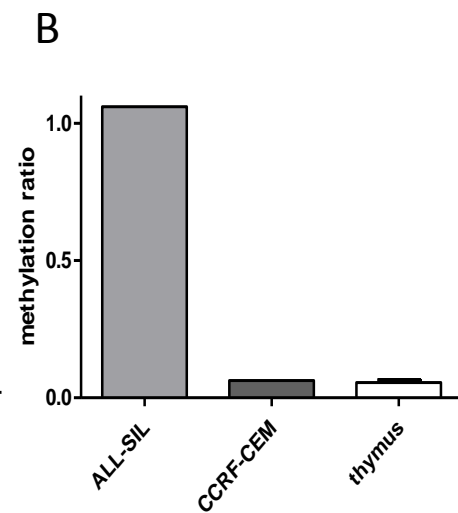
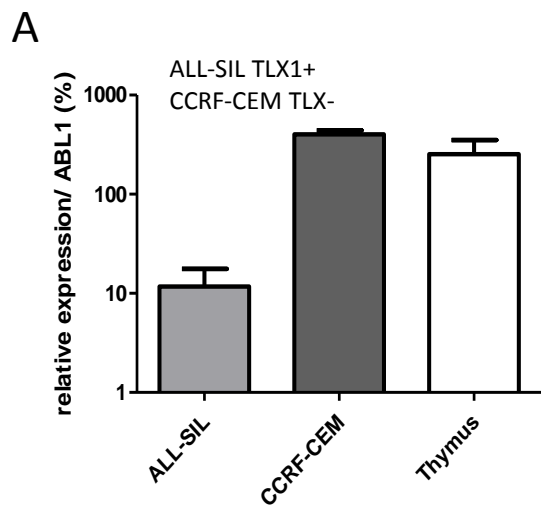


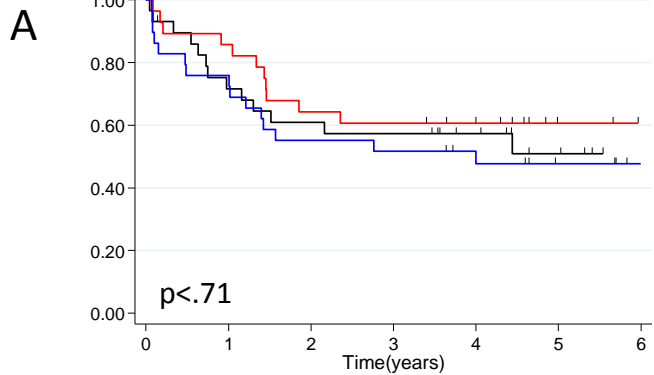
Number at risk

GRAALL, TLX1+	50	37	30	23	18	13	4
GRAALL, TLX3+	27	17	14	12	6	3	1
LALA, TLX1+	15	13	8	7	6	3	2
LALA, TLX3+	10	5	2	1	1	0	0



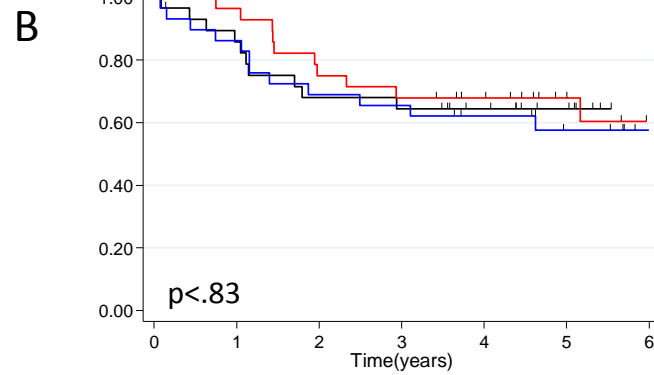






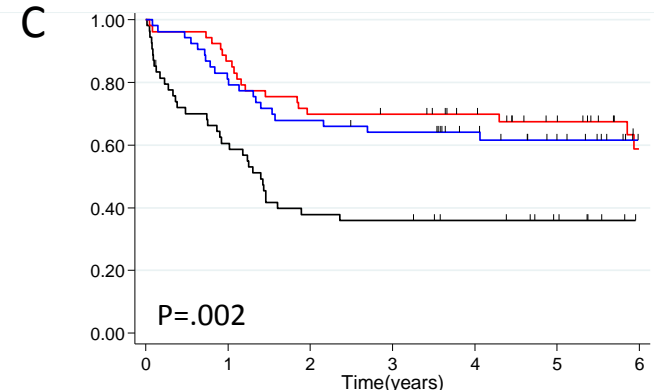
Number at risk

T1	29	20	17	16	12	7	3
T2	28	24	18	17	15	8	6
T3	29	22	16	15	12	9	5



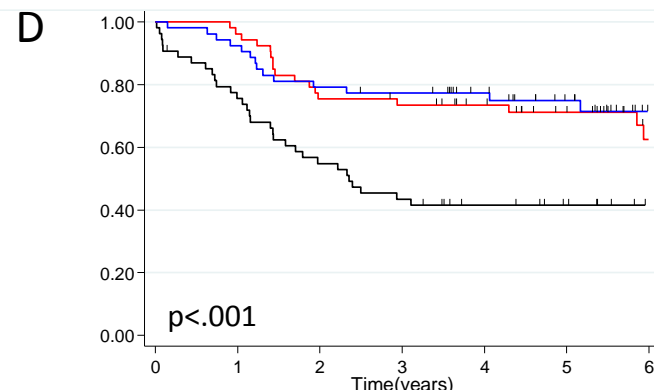
Number at risk

T1	29	24	19	18	14	9	3
T2	28	27	21	19	16	9	6
T3	29	25	20	19	16	12	7



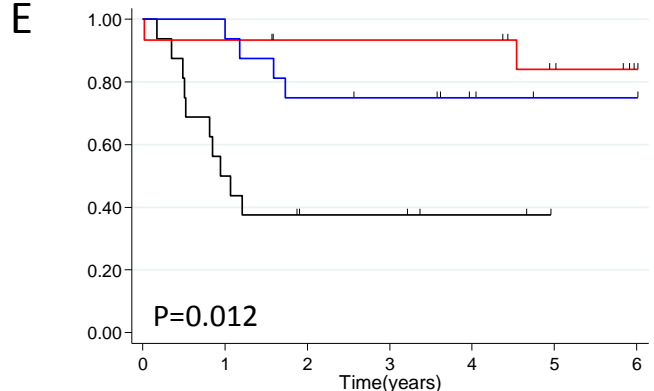
Number at risk

T1	54	32	20	19	16	12	6
T2	53	46	37	36	31	23	12
T3	53	43	36	33	27	20	11



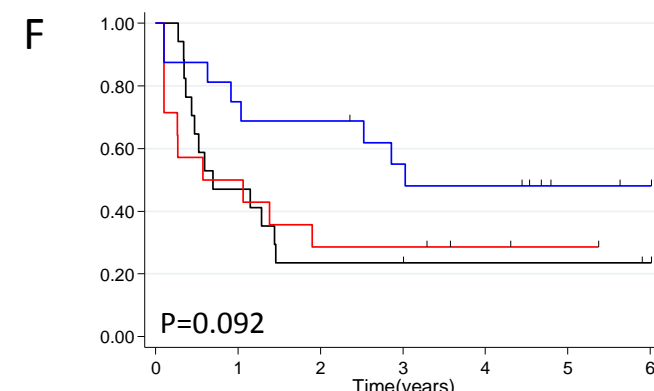
Number at risk

T1	54	40	29	23	17	13	7
T2	53	51	40	38	33	25	13
T3	53	49	42	40	33	24	13



Number at risk

T1	16	8	4	4	2	0	0
T2	15	14	12	12	12	8	4
T3	16	15	12	11	8	6	6



Number at risk

T1	17	8	4	3	3	3	2
T2	14	7	4	4	2	1	0
T3	16	12	11	8	7	3	2

— T1
— T2
— T3

