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

Aurore Touzart[1*], Etienne Lengliné[2*], Mehdi Latiri[1], Mohamed Belhocine[3], Charlotte Smith[1], Xavier Thomas[4], Salvatore Spicuglia[3], Denis Puthier[3], Françoise Pflumio[5], Thibaut Leguay[6], Carlos Graux[7], Yves Chalandon[8], Françoise Huguet[9], Stéphane Leprêtre[10], Norbert Ifrah[11], Hervé Dombret[2], Elizabeth Macintyre[1], Mathilde Hunault[12], Nicolas Boissel[2] and Vahid Asnafi[1]

[*] These authors contributed equally

**Affiliations:**

[1]Université Paris Descartes Sorbonne Cité, Institut Necker-Enfants Malades (INEM), Institut national de recherche médicale (INSERM) U1151, and Laboratory of Onco-Hematology, Assistance Publique-Hôpitaux de Paris (AP-HP), Hôpital Necker Enfants-Malades, Paris, [2]Université Paris Diderot, Institut Universitaire d'Hématologie, EA-3518, Assistance Publique-Hôpitaux de Paris, University Hospital Saint-Louis, Paris, [3]Aix-Marseille University, Inserm, TAGC, UMR1090, Marseille, France. Equipe Labélisée Ligue Contre le Cancer, Marseille, France, [4]Division of Hematology, Hospices Civils de Lyon, INSERM U1052–Centre National de la Recherche Scientifique UMR 5286, Centre Hospitalier Lyon Sud, Pierre Benite, France, [5]INSERM UMR967, CEA/DSV/iRCM, Laboratory of Hematopoietic Stem cells and Leukemic Cells, Equipe labellisée par la Ligue Nationale Contre le Cancer, Université Paris Diderot, Université Paris-Saclay, Commissariat à l'Energie Atomique et aux Energies Alternatives, 92265 Fontenay-aux-Roses, France, [6]Division of Hematology, Hôpital du Haut-Levêque, Pessac, France, [7]Department of Hematology, Mont-Godinne University Hospital, Yvoir, Belgium, [8] Department of Oncology, Hematology Division, University Hospital, Genève, Switzerland. Swiss Group for Clinical Cancer Research, Bern, Switzerland. [9] Centre Hospitalier Universitaire de Toulouse, Institut Universitaire du Cancer de Toulouse Oncopole, Toulouse, [10] Institut National de la Santé et de la Recherche Médicale (INSERM) U1245, Department of Hematology, Centre Henri-Becquerel and Normandie Univ UNIROUEN, Rouen, France, [11]PRES LUNAM, CHU Angers service des Maladies du Sang et INSERM U 892, [12]Division of Hematology, Centre Hospitalier Universitaire d'Angers, Angers, France; INSERM U892/Centre National de la Recherche Scientifique 6299, Angers, CRCINA, INSERM, Université de Nantes, Université d'Angers, Angers, France

**Running title:** *ASNS* promoter methylation predicts outcome in T-ALL

**Keywords :** T-ALL, Asparagine Synthetase, DNA methylation, pronostic biomarker

**Financial support :** The GRAALL was supported by grants P0200701 and P030425/AOM03081 from Le Programme Hospitalier de Recherche Clinique, Ministère de

l'Emploi et de la Solidarité, France and the Swiss Federal Government in Switzerland. This work was supported in the Necker hematology department by grants from the SFCE (Société Française des Cancers de l'Enfant), ITMO (Institut Thématique Multi-Organisme) épigénétique et cancer (PLBIO PLAN CANCER 2014-19-ASNAFI). EL was supported by a grant from the French "Académie Nationale de Médecine". AT was supported by a grant from the French program CARPEM (CAncer Research for PErsonalized Medicine) and the FRM (Fondation pour la Recherche Médicale).

**Corresponding author**:

-Vahid Asnafi, Hôpital Necker Enfants Malades, Laboratoire d'hématologie, 149 rue de Sèvres, 75015 Paris, France,

e-mail: vahid.asnafi@nck.aphp.fr. Phone : (+33) 144 49 49 33; Fax : (+33) 144 38 17 45

**Conflict of interest disclosure** : the authors declare no potential conflicts of interest

**Word count :** abstract : 245 ; text :4191,

**6 figures**

**49 references**

**Translational relevance:** Despite recent advances, "personalized" medicine does not currently benefit most patients individually and classical chemotherapies still remain the cornerstone of first line treatment. We report, using a leukemic 'proof-of-concept' model, the implication of epigenetic modulation in individual patient-related drug response. Methylation status may therefore allow individual adaptation of chemotherapy dose.

**Abstract**

**Purpose**: Biological explanations for discrepancies in patients related response to chemotherapy depending on the underlying oncogenic events is a promising research area. TLX1 or TLX3 deregulated T-ALLs (TLX1/3+) share an immature cortical phenotype and similar transcriptional signatures. However, their prognostic impacts differ and inconsistent clinical outcome has been reported for TLX3. We therefore hypothesized that the overlapping transcriptional profiles of TLX1+ and TLX3+ T-ALLs would allow identification of candidate genes which might determine their distinct clinical outcomes.

**Experimental design**: 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**: We show that TLX1+ patients expressed low levels of Asparagine Synthetase (*ASNS*) when compared to TLX3+ and TLX negative patients, due to epigenetic silencing of *ASNS* by both DNA methylation and a decrease of active histone marks. Promoter methylation of the *ASNS* gene correlated with L-asparaginase sensitivity in both T-ALL cell lines and Patient Derived Xenografts. Finally, *ASNS* promoter methylation was 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 and also in an independent series of 47 LL03 treated T lymphoblastic lymphomas (p=0.012).

**Conclusion**: We conclude that *ASNS* methylation status at diagnosis may allow individual adaptation of L-Asparaginase dose.

## 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. Genome wide expression [1-3] 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) proteins 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. Physiological expression of TLX1 and TLX3 is restricted to embryonic development [4,5], and no specific function of these genes has been reported in T-cell lymphopoiesis. We recently demonstrated that TLX1/3 proteins repress TCR$\alpha$ rearrangements [6], 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 prognosis [7-13], data regarding the prognostic impact of TLX3 are more conflicting [1,14,15]. The molecular explanation of this heterogeneity is unknown. We therefore hypothesized that the differences in transcriptional profiles of TLX1+ and TLX3+ T-ALLs would allow identification of candidate genes which might determine their 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.

## 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 [13]. 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 S2 and 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 [16]. 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 [17]. 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 [7,9]. *Trial registration:* GRAALL-2003 and GRAALL-2005 trials were registered at http://www.clinicaltrials.gov as #NCT00222027 and #NCT00327678, respectively. 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.

***TLX1 inactivation***

MISSION TRC shRNA Target Set vectors for TLX1 (TRCN0000014995) were purchased from Sigma Chemicals (St Louis, MO). Knockdown of the *TLX1* endogenous RNA transcripts was performed by transduction of the ALL-SIL cell line as described [6].

***Genetic inactivation of ASNS***

Two different SiRNA, SiRNA-1 and SiRNA-2 and a scrambled universal negative control RNA duplex (SR300319, Origene, Rockvile, MD), were used to perform transient genetic Knock-Down of *ASNS*. SiRNA sequences are specified in supplementary **Table S3**.

***Microarray Gene Expression Profiling analyzes***

The Affymetrix U133A microarray analyzes [3] 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 [2,6] 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) analyzes [18] 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 wereH3K4me3: catalog number C15410003-50 (diagenode) and H3K36me3: catalog number C15410192 (diagenode)

***Western Blot***

Immunoblotting was performed with primary antibodies to ASNS [19] (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.

***Quantification of ASNS by qRT-PCR***

qRT-PCR was performed using the Master SYBR-Green reagent kit. Primers sequences used were: ASNS-Forward: 5'-GAAATGAGAATTCCAAAGAATGG-3' and ASNS-Reverse: 5'-CTTTTGGTCGCCAGAGAATCT-3'. The change-in-threshold (-ΔΔCt) 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 previously [20] using 2 µg of sonicated genomic DNA. MeDIP samples were directly subjected to labeling and hybridization on a custom human promoter array (Agilent, Santa Clara, USA)[6], following the manufacturer's instructions. Finally, median-normalized log2 enrichment ratios (MeDIP/Input) were calculated for each probe using CoCAS software[21] 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).

Patients analyzed for global DNA methylation have clinico-biological characteristics similar to patients not included in this analysis. (**Table S5**).

***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. 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-variates: 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/m$^2$/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 S6**).

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, although a trend, 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 statistically significant 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. We therefore hypothesized that the overlapping transcriptional profiles of TLX1+ and TLX3+ T-ALLs would allow identification of candidate genes which might determine their distinct clinical outcomes.

***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 patients [3] to establish a list of differentially expressed transcripts in TLX1+ (n=11) as compared to TLX3+ (n=18) patients. This list (supplementary **Table S7**) contains 259 unique genes (p<0.05, fold change=2). Two other transcriptomic data sets were obtained by RNAi inactivation of TLX1 [18] and TLX3 [6] in the TLX overexpressing ALL-SIL (TLX1) and DND41 (TLX3) cell lines. This identified 929 and 1259 (supplementary **Table S7**) 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 on purified blast cells [2,22] (**Figure 2B-C**). These observations were confirmed, despite a larger overlap, using 86 unpurified samples from GRAALL treated T-ALL including 19 TLX1+ and 12 TLX3+ cases (**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 line [18], 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 hypermethylation leads to L-asparaginase sensitivity***

We then hypothesized that the favorable outcome of TLX1+ T-ALL could be associated with the low *ASNS* protein expression *via ASNS* promoter methylation. In order to test L-asparaginase *in vitro* sensitivity according to *ASNS* status, the TLX1+ ALL-SIL (high level ASNS promoter methylation) and the CCRF-CEM (Lowest level ASNS methylation level) 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 protein expression* 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, as expected, an inverse correlation between *ASNS* expression and L-asparaginase sensitivity. As L-Asparaginase efficacy correlates with exogenous asparagine depletion, this assumes that the leukemic cells are incapable of synthesizing asparagine. We then reasoned that if the T-ALL cells are perfectly capable of synthesizing asparagine (i.e. by ASNS protein expression due to hypomethylated *ASNS* promoter), they should be essentially insensitive to asparaginase therapy. TAL1+ cases harbor the lowest methylation levels (**Figure 3D**). This suggests that the TAL1+ subgroup of T-ALL should be L-Asparaginase insentitive or require the highest level of L-asparaginase, potentially not reached in the adult GRAALL protocol. In line with this, as shown in supplementary **Figure S5**, TAL1+ GRAALL treated cases benefitted less from GRAALL intensification than the TLX3+ cases. Moreover we evaluated *in-vitro* L-asparaginase sensitivity of 8 T-ALL cells from patient-derived primary T-ALL xenografts (PDX) in NSG mice. Importantly and as expected, PDX with hypomethylated *ASNS* promoters (UPNT-374 methylation ratio 0.08, M106 methylation ratio 0.33 and UPNT-420 methylation ratio 0.25) and high *TAL1* expression (Supplementary Figure S6) were strongly 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 an inverse correlation between *ASNS* promoter methylation (leading to decrease ASNS expression) and *in vitro* sensitivity to L-asparaginase.

***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 GRAALL03/05 trials. No significant correlation between *ASNS* expression and promoter methylation was observed (supplementary **Figure S7A**), probably due to high level *ASNS* expression by both normal peripheral blood lymphocytes and bone marrow normal residual cells (Supplementary **Figure S7B**). We used two strategies to purify blast cells (i) blast- and non-blast cells sorting and (ii) Primary Derived Xenograft (PDX), for *ASNS* quantification. As for T-ALL cell-lines (**Figure S7E**), we then observed a significant correlation confirming this hypothesis (supplementary **Figure S7C and S7D**). *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 S8**). 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 S8**). 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 S9**). 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 protocol [23] which used similar L-asparaginase schedules to the GRAALL trial (**Figure 5E**). 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 [24,25 26-28] 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 [16]. 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/$m^2$ cumulative dose in GRAALL induction, and 16 times more L-asparaginase in the whole GRAALL protocol (144,000 UI/ $m^2$) than in the LALA-94 (9,000 UI/ $m^2$) [13,16].

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 [29,30]. Primary ALL cells and many ALL cell lines exhibit a particularly low level of ASNS expression [31], 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). L-asparaginase is responsible for hepatic toxicity,

thrombosis, hypo-albuminemia and denutrition. There is a consequent need for tailored L-asparaginase use, based on leukemia biology and patient-related conditions.

It is noteworthy that L-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 L-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 [1,8,10-15,32-37] 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. Of course, it is likely that the protocols included in this analysis might have many other differences and further investigations are required to address a definitive conclusion.

The mechanisms of L-asparaginase resistance remain unclear and data from studies on the importance of ASNS are conflicting. In leukemic cell lines [38,39], 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* [40] and

that overexpression of exogenous ASNS protein could result in an L-asparaginase-resistant phenotype in the absence of drug selection [40]. Concerning primary ALL, data remain unclear and the role of ASNS expression with regard to L-asparaginase resistance may vary among genetic subtypes and, as suggested by others, may have little or no relevance to drug sensitivity in vivo [38,41-45]. 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 reported [46] 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. Advantage in methylation could also be explained by the lower disruption of input of contaminating cells at the genome level (two low methylation copies per contaminating normal cell) than at the mRNA level (multiple mRNA copies per contaminating normal cell).

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 [38,42-45,47]. One study did, however, report higher *ASNS* expression in T-ALL as compared to BCP-ALL [48]. 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 sarcomas or gliomas. *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 L-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 L-asparaginase total dose, potentially with a reduced incidence of side-effects.

**Aknowledgments:**

We thank all French, Swiss and Belgian participants, clinicians, biologists and clinical research assistants, in the LALA-94 and GRAALL 2003-2005 trials, for collecting and providing data and samples. We cordially thank Marie-Christine Bene, André Delannoy and Jonathan Bond for constructive editing. We thank the IBiSA "Transcriptomics and Genomics Marseille-Luminy (TGML)" platform for microarray hybridization and high-throughput sequencing.

**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.

**References**

1. Ferrando AA, Neuberg DS, Staunton J, et al. Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer Cell*. 2002;1(1):75-87.

2. Homminga I, Pieters R, Langerak AW, et al. Integrated transcript and genome analyses reveal NKX2-1 and MEF2C as potential oncogenes in T cell acute lymphoblastic leukemia. *Cancer Cell*. 2011;19(4):484-497.

3. Soulier J, Clappier E, Cayuela JM, et al. HOXA genes are included in genetic and biologic networks defining human acute T-cell leukemia (T-ALL). *Blood*. 2005;106(1):274-286.

4. Roberts CW, Shutter JR, Korsmeyer SJ. Hox11 controls the genesis of the spleen. *Nature*. 1994;368(6473):747-749.

5. Shirasawa S, Arata A, Onimaru H, et al. Rnx deficiency results in congenital central hypoventilation. *Nat Genet*. 2000;24(3):287-290.

6. Dadi S, Le Noir S, Payet-Bornet D, et al. TLX homeodomain oncogenes mediate T cell maturation arrest in T-ALL via interaction with ETS1 and suppression of TCRalpha gene expression. *Cancer Cell*. 2012;21(4):563-576.

7. Asnafi V, Buzyn A, Le Noir S, et al. NOTCH1/FBXW7 mutation identifies a large subgroup with favorable outcome in adult T-cell acute lymphoblastic leukemia (T-ALL): a Group for Research on Adult Acute Lymphoblastic Leukemia (GRAALL) study. *Blood*. 2009;113(17):3918-3924.

8. Baak U, Gokbuget N, Orawa H, et al. Thymic adult T-cell acute lymphoblastic leukemia stratified in standard- and high-risk group by aberrant HOX11L2 expression: experience of the German multicenter ALL study group. *Leukemia*. 2008;22(6):1154-1160.

9. Bergeron J, Clappier E, Radford I, et al. Prognostic and oncogenic relevance of TLX1/HOX11 expression level in T-ALLs. *Blood*. 2007;110(7):2324-2330.

10. Ferrando AA, Neuberg DS, Dodge RK, et al. Prognostic importance of TLX1 (HOX11) oncogene expression in adults with T-cell acute lymphoblastic leukaemia. *Lancet*. 2004;363(9408):535-536.

11. Kees UR, Heerema NA, Kumar R, et al. Expression of HOX11 in childhood T-lineage acute lymphoblastic leukaemia can occur in the absence of cytogenetic aberration at 10q24: a study from the Children's Cancer Group (CCG). *Leukemia*. 2003;17(5):887-893.

12. Schneider NR, Carroll AJ, Shuster JJ, et al. New recurring cytogenetic abnormalities and association of blast cell karyotypes with prognosis in childhood T-cell acute lymphoblastic leukemia: a pediatric oncology group report of 343 cases. *Blood*. 2000;96(7):2543-2549.

13. Thomas X, Boiron JM, Huguet F, et al. Outcome of treatment in adults with acute lymphoblastic leukemia: analysis of the LALA-94 trial. *J Clin Oncol*. 2004;22(20):4075-4086.

14. Attarbaschi A, Pisecker M, Inthal A, et al. Prognostic relevance of TLX3 (HOX11L2) expression in childhood T-cell acute lymphoblastic leukaemia treated with Berlin-Frankfurt-Munster (BFM) protocols containing early and late re-intensification elements. *Br J Haematol*. 2010;148(2):293-300.

15. Ballerini P, Blaise A, Busson-Le Coniat M, et al. HOX11L2 expression defines a clinical subtype of pediatric T-ALL associated with poor prognosis. *Blood*. 2002;100(3):991-997.

16. Huguet F, Leguay T, Raffoux E, et al. Pediatric-inspired therapy in adults with Philadelphia chromosome-negative acute lymphoblastic leukemia: the GRAALL-2003 study. *J Clin Oncol*. 2009;27(6):911-918.

17. Maury S, Chevret S, Thomas X, et al. Rituximab in B-Lineage Adult Acute Lymphoblastic Leukemia. *N Engl J Med*. 2016;375(11):1044-1053.

18. Durinck K, Van Loocke W, Van der Meulen J, et al. Characterization of the genome-wide TLX1 binding profile in T-cell acute lymphoblastic leukemia. *Leukemia*. 2015;29(12):2317-2327.

19. Hutson RG, Kitoh T, Moraga Amador DA, Cosic S, Schuster SM, Kilberg MS. Amino acid control of asparagine synthetase: relation to asparaginase resistance in human leukemia cells. *Am J Physiol*. 1997;272(5 Pt 1):C1691-1699.

20. Jia J, Pekowska A, Jaeger S, Benoukraf T, Ferrier P, Spicuglia S. Assessing the efficiency and significance of Methylated DNA Immunoprecipitation (MeDIP) assays in using in vitro methylated genomic DNA. *BMC Res Notes*. 2010;3:240.

21. Benoukraf T, Cauchy P, Fenouil R, et al. CoCAS: a ChIP-on-chip analysis suite. *Bioinformatics*. 2009;25(7):954-955.

22. Atak ZK, Gianfelici V, Hulselmans G, et al. Comprehensive analysis of transcriptome variation uncovers known and novel driver events in T-cell acute lymphoblastic leukemia. *PLoS Genet*. 2013;9(12):e1003997.

23. Lepretre S, Touzart A, Vermeulin T, et al. Pediatric-Like Acute Lymphoblastic Leukemia Therapy in Adults With Lymphoblastic Lymphoma: The GRAALL-LYSA LL03 Study. *J Clin Oncol*. 2016;34(6):572-580.

24. Amylon MD, Shuster J, Pullen J, et al. Intensive high-dose asparaginase consolidation improves survival for pediatric patients with T cell acute lymphoblastic leukemia and advanced stage lymphoblastic lymphoma: a Pediatric Oncology Group study. *Leukemia*. 1999;13(3):335-342.

25. Sallan SE, Hitchcock-Bryan S, Gelber R, Cassady JR, Frei E, 3rd, Nathan DG. Influence of intensive asparaginase in the treatment of childhood non-T-cell acute lymphoblastic leukemia. *Cancer Res*. 1983;43(11):5601-5607.

26. Moghrabi A, Levy DE, Asselin B, et al. Results of the Dana-Farber Cancer Institute ALL Consortium Protocol 95-01 for children with acute lymphoblastic leukemia. *Blood*. 2007;109(3):896-904.

27. Pession A, Valsecchi MG, Masera G, et al. Long-term results of a randomized trial on extended use of high dose L-asparaginase for standard risk childhood acute lymphoblastic leukemia. *J Clin Oncol*. 2005;23(28):7161-7167.

28. Silverman LB, Gelber RD, Dalton VK, et al. Improved outcome for children with acute lymphoblastic leukemia: results of Dana-Farber Consortium Protocol 91-01. *Blood*. 2001;97(5):1211-1218.

29. Chen H, Pan YX, Dudenhausen EE, Kilberg MS. Amino acid deprivation induces the transcription rate of the human asparagine synthetase gene through a timed program of

expression and promoter binding of nutrient-responsive basic region/leucine zipper transcription factors as well as localized histone acetylation. *J Biol Chem*. 2004;279(49):50829-50839.

30. Kilberg MS, Pan YX, Chen H, Leung-Pineda V. Nutritional control of gene expression: how mammalian cells respond to amino acid limitation. *Annu Rev Nutr*. 2005;25:59-85.

31. Miller HK, Salser JS, Balis ME. Amino acid levels following L-asparagine amidohydrolase (EC.3.5.1.1) therapy. *Cancer Res*. 1969;29(1):183-187.

32. Cave H, Suciu S, Preudhomme C, et al. Clinical significance of HOX11L2 expression linked to t(5;14)(q35;q32), of HOX11 expression, and of SIL-TAL fusion in childhood T-cell malignancies: results of EORTC studies 58881 and 58951. *Blood*. 2004;103(2):442-450.

33. Gottardo NG, Jacoby PA, Sather HN, Reaman GH, Baker DL, Kees UR. Significance of HOX11L2/TLX3 expression in children with T-cell acute lymphoblastic leukemia treated on Children's Cancer Group protocols. *Leukemia*. 2005;19(9):1705-1708.

34. Mauvieux L, Leymarie V, Helias C, et al. High incidence of Hox11L2 expression in children with T-ALL. *Leukemia*. 2002;16(12):2417-2422.

35. van Grotel M, Meijerink JP, Beverloo HB, et al. The outcome of molecular-cytogenetic subgroups in pediatric T-cell acute lymphoblastic leukemia: a retrospective study of patients treated according to DCOG or COALL protocols. *Haematologica*. 2006;91(9):1212-1221.

36. van Grotel M, Meijerink JP, van Wering ER, et al. Prognostic significance of molecular-cytogenetic abnormalities in pediatric T-ALL is not explained by immunophenotypic differences. *Leukemia*. 2008;22(1):124-131.

37. Beldjord K, Chevret S, Asnafi V, et al. Oncogenetics and minimal residual disease are independent outcome predictors in adult patients with acute lymphoblastic leukemia. *Blood*. 2014;123(24):3739-3749.

38. Fine BM, Kaspers GJ, Ho M, Loonen AH, Boxer LM. A genome-wide view of the in vitro response to l-asparaginase in acute lymphoblastic leukemia. *Cancer Res*. 2005;65(1):291-299.

39. Scherf U, Ross DT, Waltham M, et al. A gene expression database for the molecular pharmacology of cancer. *Nat Genet*. 2000;24(3):236-244.

40. Aslanian AM, Fletcher BS, Kilberg MS. Asparagine synthetase expression alone is sufficient to induce l-asparaginase resistance in MOLT-4 human leukaemia cells. *Biochem J*. 2001;357(Pt 1):321-328.

41. Haskell CM, Canellos GP. l-asparaginase resistance in human leukemia--asparagine synthetase. *Biochem Pharmacol*. 1969;18(10):2578-2580.

42. Hermanova I, Zaliova M, Trka J, Starkova J. Low expression of asparagine synthetase in lymphoid blasts precludes its role in sensitivity to L-asparaginase. *Exp Hematol*. 2012;40(8):657-665.

43. Holleman A, Cheok MH, den Boer ML, et al. Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. *N Engl J Med*. 2004;351(6):533-542.

44. Stams WA, den Boer ML, Beverloo HB, et al. Sensitivity to L-asparaginase is not associated with expression levels of asparagine synthetase in t(12;21)+ pediatric ALL. *Blood*. 2003;101(7):2743-2747.

45. Stams WA, den Boer ML, Holleman A, et al. Asparagine synthetase expression is linked with L-asparaginase resistance in TEL-AML1-negative but not TEL-AML1-positive pediatric acute lymphoblastic leukemia. *Blood*. 2005;105(11):4223-4225.

46. Su N, Pan YX, Zhou M, Harvey RC, Hunger SP, Kilberg MS. Correlation between asparaginase sensitivity and asparagine synthetase protein content, but not mRNA, in acute lymphoblastic leukemia cell lines. *Pediatr Blood Cancer*. 2008;50(2):274-279.

47. Stams WA, den Boer ML, Beverloo HB, van Wering ER, Pieters R. Upregulation of asparagine synthetase and cell cycle arrest in t(12;21)-positive ALL. *Leukemia*. 2005;19(2):318-319; author reply 319-321.

48. Iwamoto S, Mihara K, Downing JR, Pui CH, Campana D. Mesenchymal cells regulate the response of acute lymphoblastic leukemia cells to asparaginase. *J Clin Invest*. 2007;117(4):1049-1057.

49. Trinquand A, Tanguy-Schmidt A, Ben Abdelali R, et al. Toward a NOTCH1/FBXW7/RAS/PTEN-based oncogenetic risk classification of adult T-cell acute lymphoblastic leukemia: a Group for Research in Adult Acute Lymphoblastic Leukemia study. *J Clin Oncol*. 2013;31(34):4333-4342.

**Figures legends**

**Figure 1. Impact of *TLX1* and *TLX3* deregulation and treatment era on adult T-ALL patient outcome.** Kaplan-Meier graphs for (A-B) EFS and (C-D) OS censored at HSCT in LALA-94 and GRAALL-2003/2005 respectively are shown according to oncogenetic status (TLX1+ *vs.* TLX3+ *vs.* others). 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-ALL [3] (green circle), (ii) genes differentially expressed after *TLX1* siRNA inactivation in the *TLX1* overexpressing ALL-SIL cell line [18] (red circle) and (iii) genes differentially expressed after T*LX3* shRNA inactivation in the *TLX3* overexpressing DND41 cell line [6] (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 profiling[2] and (C) RNA-seq data[22] 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 HhaI 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 (1µM and 2.5µM) 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$^{TM}$) 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$^{TM}$) exposure. (H) *In vitro* sensitivity experiments of primary T-ALL xenografted in NSG mice upon

L-asparaginase (Kidrolase$^{TM}$) treatment. Eight 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 GRAALL03-05 trial. (C) and (D) Kaplan-Meyer graphs according to *ASNS* methylation tertiles for EFS and OS respectively for patients included in GRAALL03-05 trial. (E) EFS according to methylation tertiles for patients included in the LL03 Lymphoblastic lymphoma trial.

**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.

Figure 1

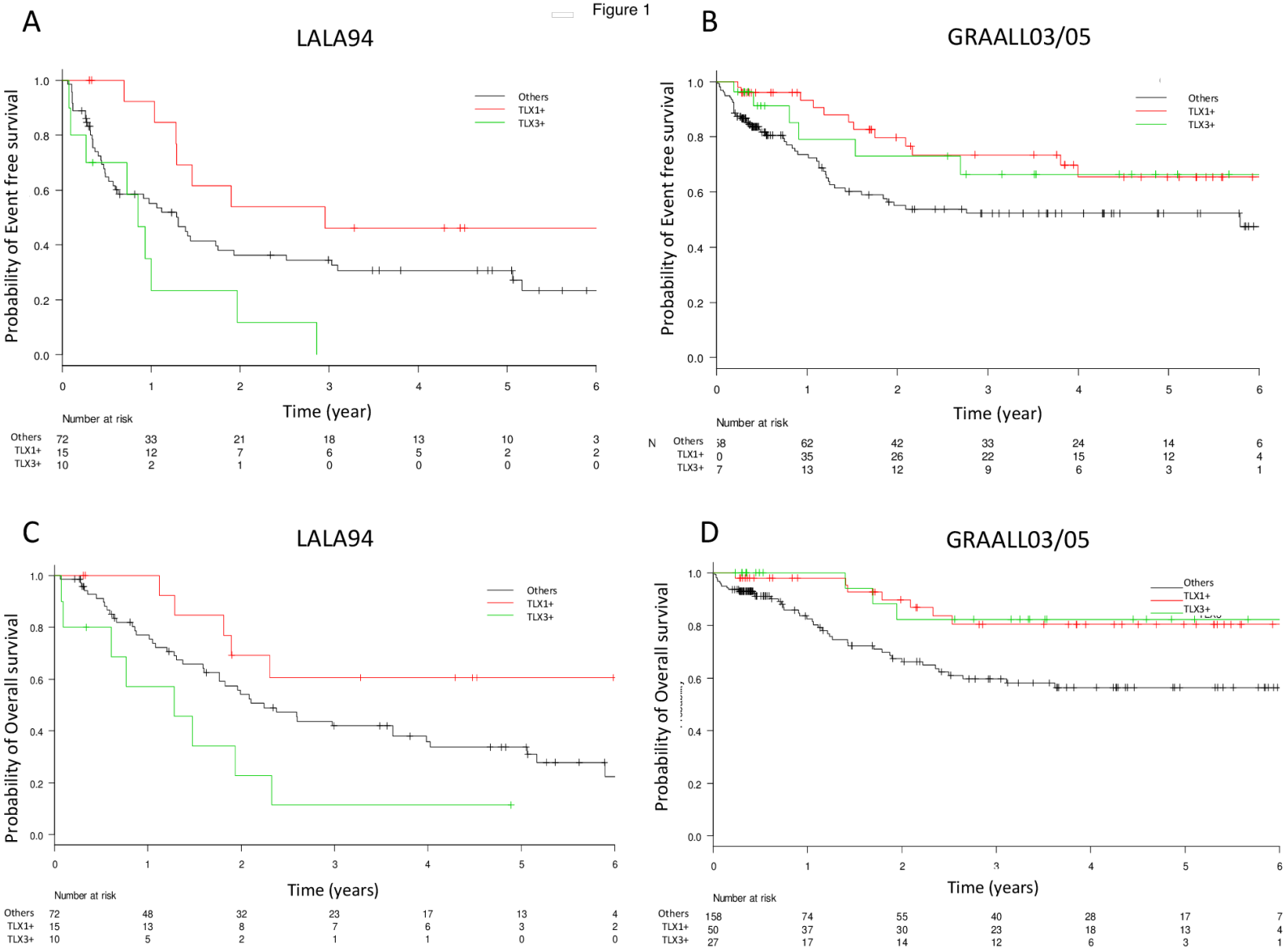


Figure 2

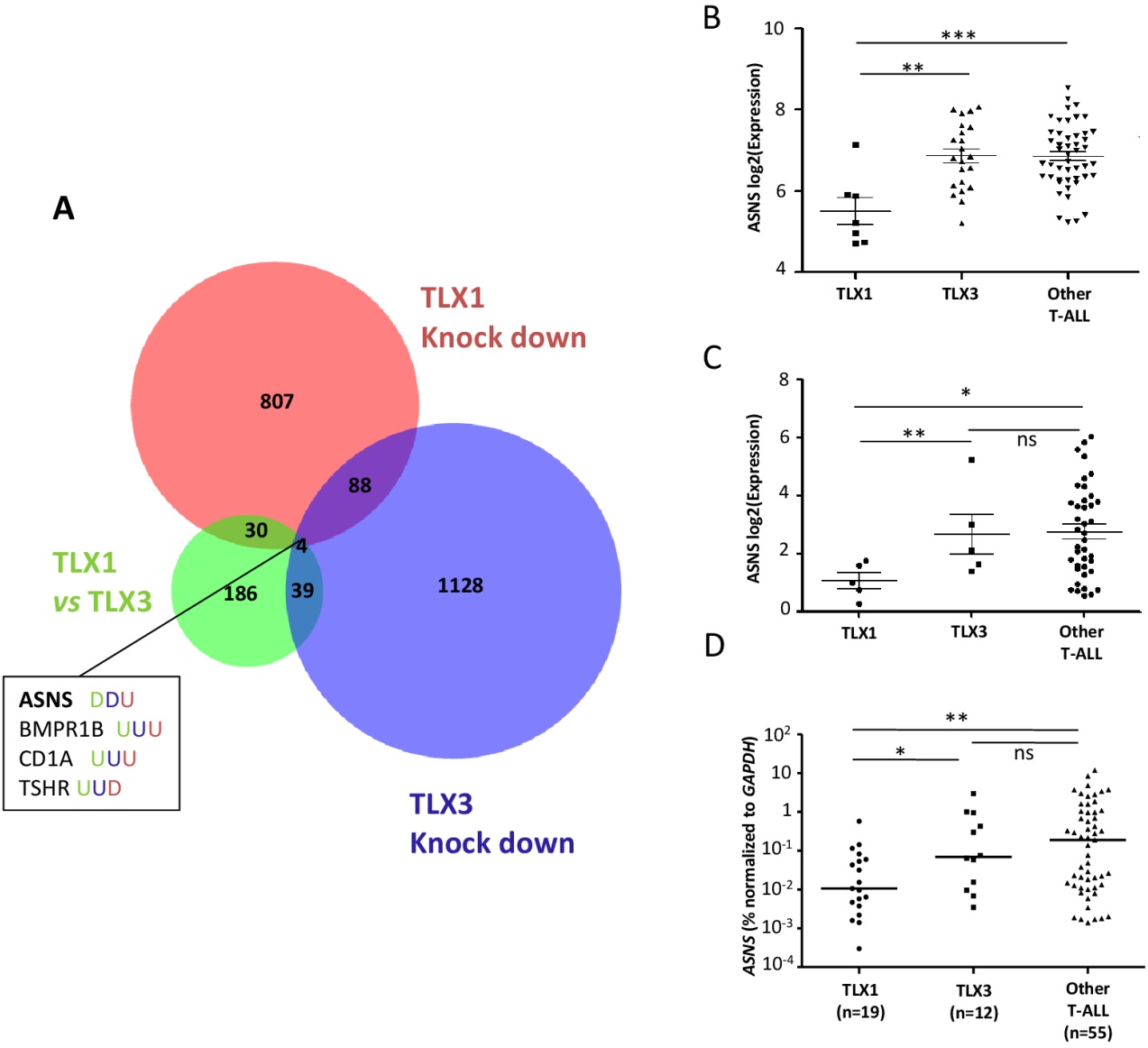


Figure 3

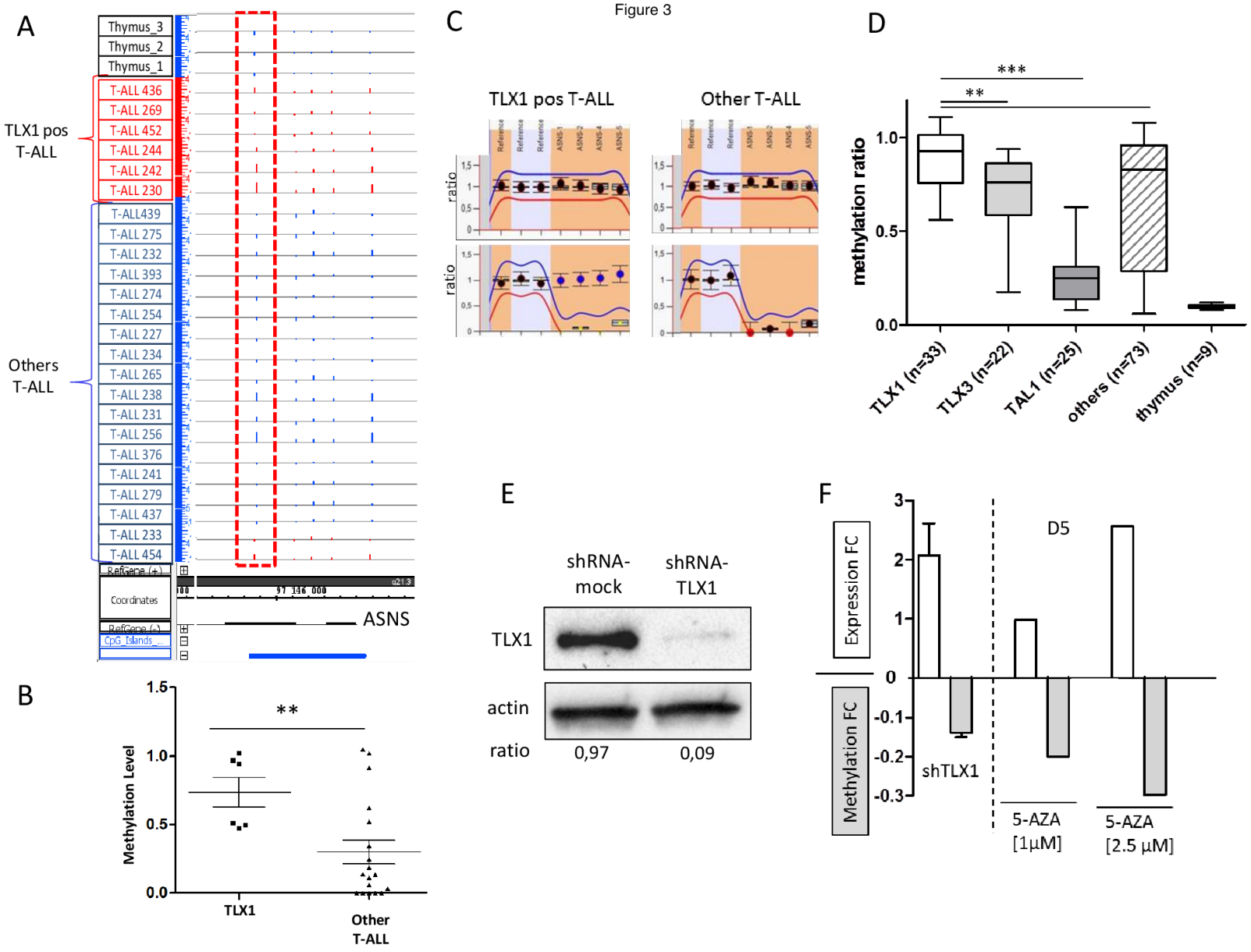


Figure 4

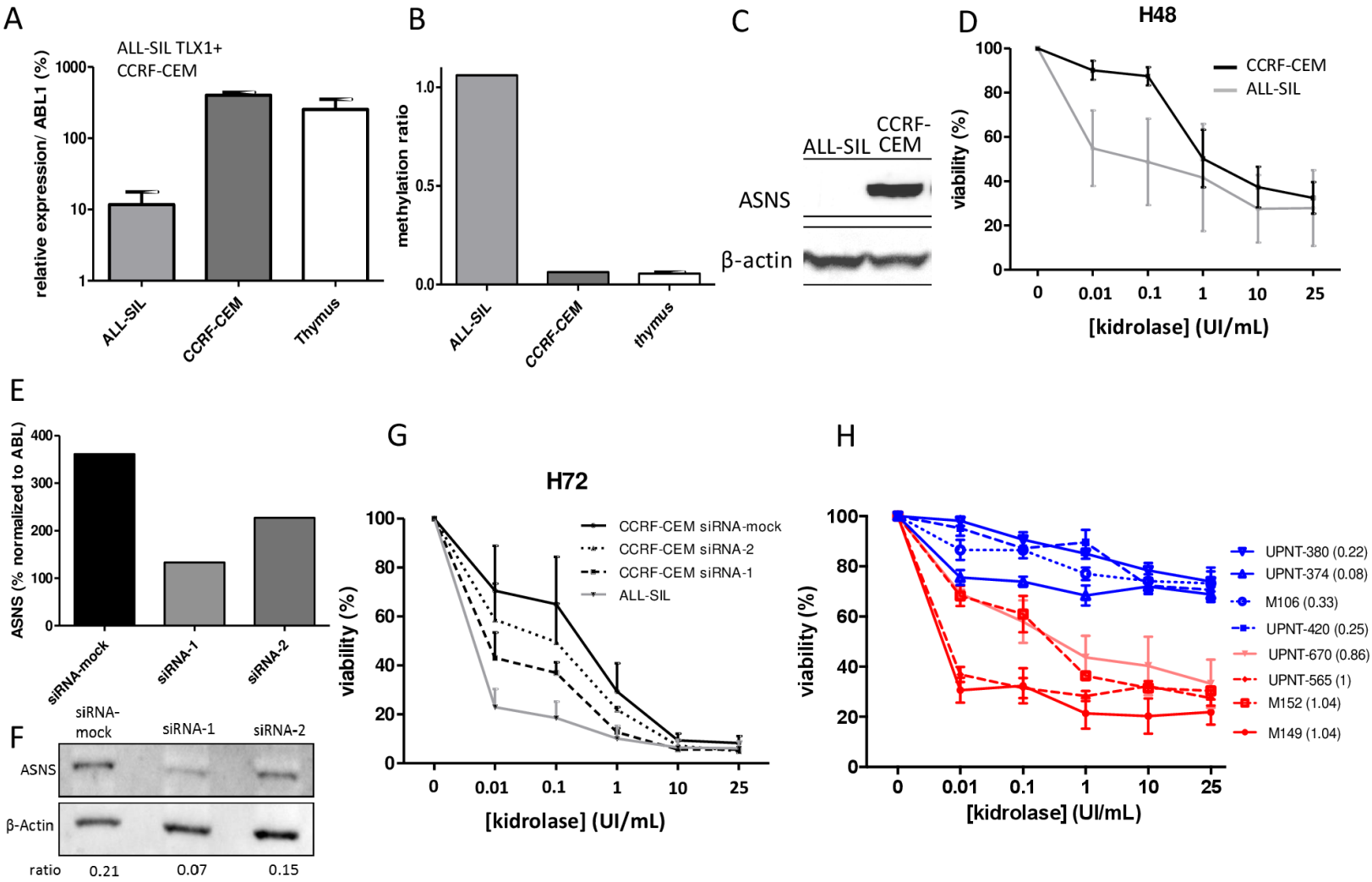


Figure 5

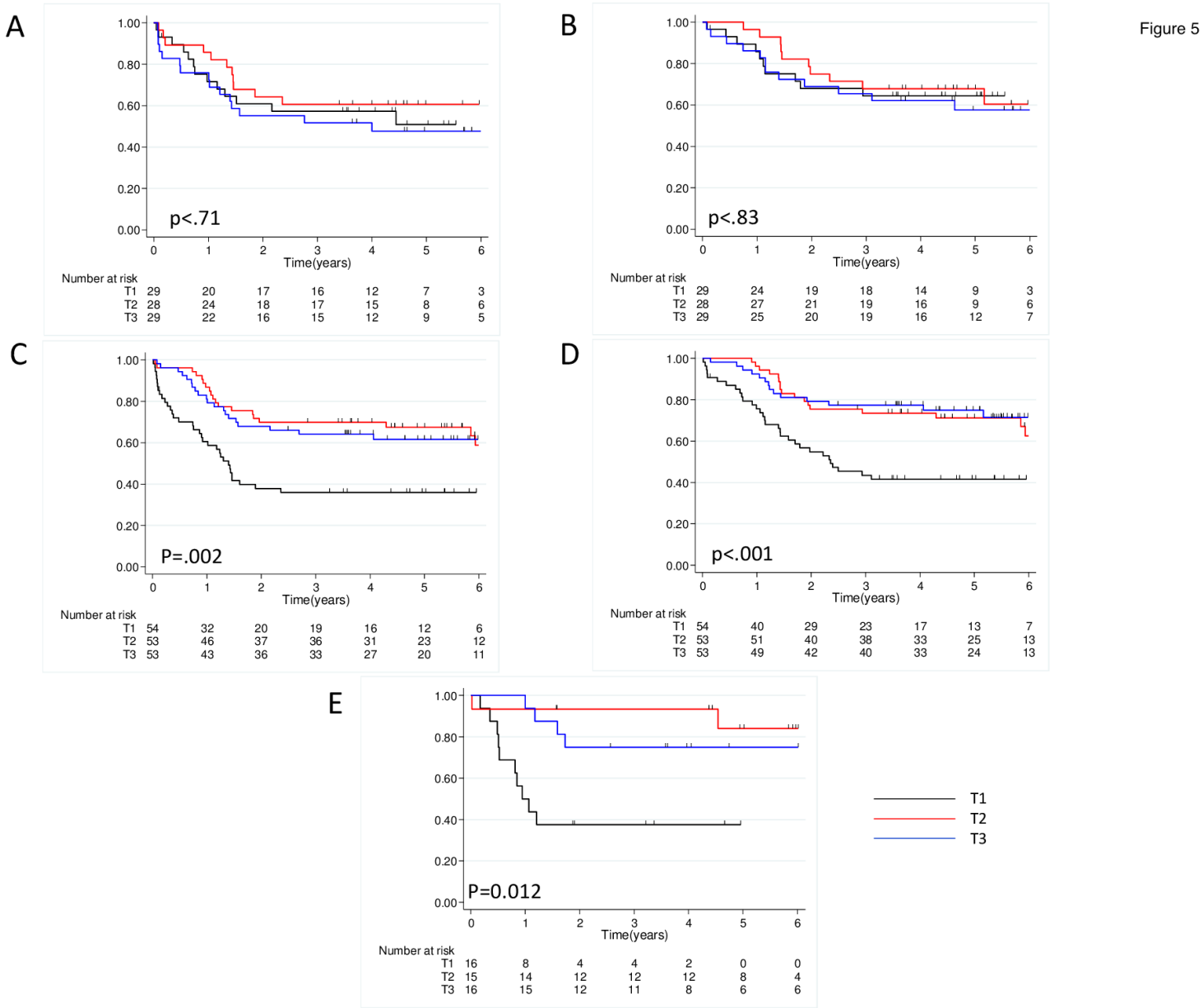
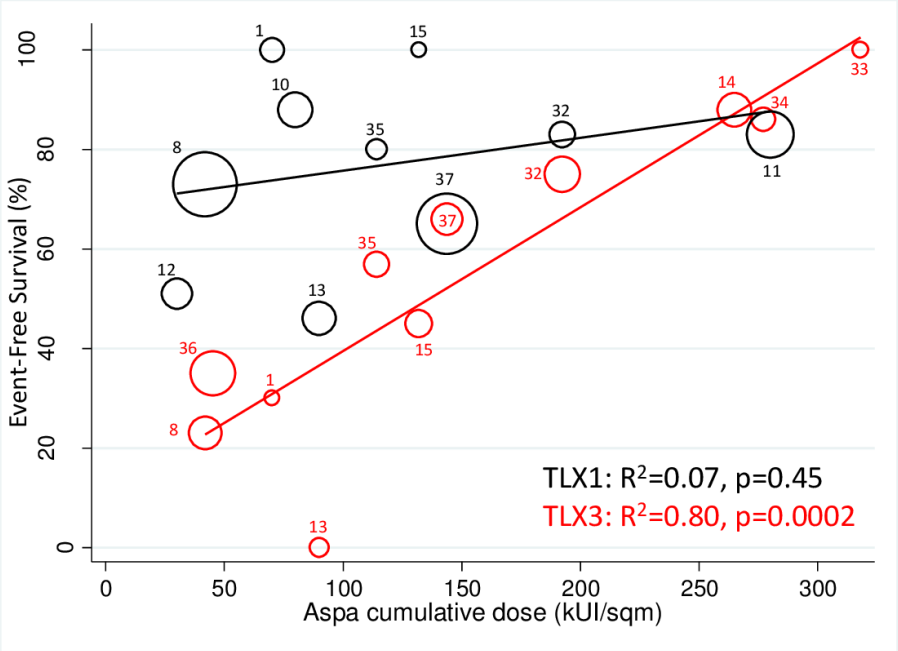


Figure 6



AACR American Association for Cancer Research

Clinical Cancer Research

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

Aurore Touzart, Etienne Lengline, Mehdi Latiri, et al.

*Clin Cancer Res* Published OnlineFirst January 18, 2019.