# Adult B-cell acute lymphoblastic leukemia cells display decreased PTEN activity and constitutive hyperactivation of PI3K/Akt pathway despite high PTEN protein levels

A. Margarida Gomes,[1] Maria V. D. Soares,[1] Patrícia Ribeiro,[2] Joana Caldas,[2] Vanda Póvoa,[1] Leila R. Martins,[1] Alice Melão,[1] Ana Serra-Caetano,[1] Aida B. de Sousa,[2] João F. Lacerda,[1,3]* and João T. Barata[1]*

[1]Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa; [2]Hospital dos Capuchos, Lisboa; and [3]Hospital de Santa Maria, Lisboa, Portugal

*JFL and JTB contributed equally to this work.

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**SUPPLEMENTARY DATA**

**(Methods, Supplementary Figures 1-4)**

# Methods

**Primary samples and B-ALL cell lines.** Bone marrow samples from adult (n=21) or adolescent (n=2) B-ALL patients were collected in accordance with the Declaration of Helsinki, with informed consent after Ethical Committee approval from Hospital Santo António dos Capuchos (Lisbon, Portugal) and Hospital Santa Maria (Lisbon, Portugal). Patient characteristics are summarized in Table 1. Normal bone marrow samples were obtained from healthy individuals donating bone marrow for patients at Hospital Santa Maria. Samples were enriched by density centrifugation over Ficoll-Paque (GE Healthcare) and washed twice in culture medium (RPMI-1640 supplemented with 10% FBS, 2mM L-glutamine and penicillin/streptomycin). Human B-ALL cell lines, SUP-B15 (Ph+) and Nalm6 (Ph-), were maintained in RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine and penicillin/streptomycin and cultured at 37ºC in 5% $CO_2$.

**Intracellular phospho-specific flow cytometry.** To determine the phosphorylation status of PI3K/Akt and JAK/STAT pathways, cells were washed twice with PBS, pelleted by centrifugation, and fixed with Cytofix buffer (BD Biosciences) for 10min at 37ºC. Cells were then pelleted and permeabilized in ice-cold PERM buffer III (BD Biosciences) for 30min on ice. The cells were washed twice in staining buffer (BD Bioscience), and stained with the following antibodies (all from BD Biosciences): CD79a-APC; Akt-Alexa Fluor 488, PTEN-PE, pAkt S473-Alexa Fluor 488, pAkt T308-PE, and pSTAT5 Y694-Alexa Fluor 488. Following incubation for 30 min at room temperature, cells were washed in staining buffer and analyzed on a FACSAria or LSRFortessa (BD Biosciences). At least 100,000 events were collected for all samples.

Data were collected using DIVA software (BD Bioscience) and analyzed with FlowJo software (Tree Star). For each sample, live lymphocytes were gated based on their forward scatter (FSC) versus side scatter (SSC) profile. Single cells were then selected on a plot of forward scatter area (FSC-A) vs forward scatter width (FSC-W) to exclude signaling data from doublets. Lymphoblastic population was identified based on cCD79a staining and analysis of individual phospho-proteins was then performed in this cell population. Normalized basal phospho-protein levels were calculated as the ratio between the Mean Fluorescence Intensity (MFI) of cells stained with a phospho-specific antibody with MFI of control cells stained only with CD79a.

**Endogenous PTEN *in vitro* lipid phosphatase assay.** PTEN phosphatase activity was measured *in vitro*. Briefly, immunoprecipitations were carried out with an anti-PTEN antibody (Santa Cruz Biotechnology) overnight and a secondary agarose-conjugate antibody for 3 hours at 4ºC. Immunoprecipitated protein was washed, resuspended in enzyme reaction buffer (50mM Tris, pH 8; 50mM NaCl; 10mM DTT and 10mM $MgCl_2$), and incubated with 10µM PIP3 (Echelon) for 30 min at 37ºC, after which phosphatase reaction was stopped with 100µL malachite green reagent (Echelon). Free phosphatase levels were measured in an ELISA plate reader at 630nm. Absorbance was converted into pmol phosphate using a phosphate standard curve.

**Endogenous CK2 *in vitro* kinase assay**. CK2 activity was measured using the Casein Kinase 2 Assay Kit (Millipore), according to the manufacturer's instructions. Briefly, protein lysates were incubated for 10 minutes at 30°C in a reaction mixture containing a CK2-specific peptide, $[\gamma\text{-}^{32}P]ATP$ (PerkinElmer) and a PKA inhibitor cocktail. The radioactivity incorporated into the substrate was determined by scintillation counting. The kinase activity was calculated by subtracting the background for each sample (without substrate).

**Immunoblotting.** Cells were lysed in 50mM Tris-HCl pH 8.0, 150mM NaCl, 5mM EDTA, 1% (v/v) NP-40, 1mM Na3VO4, 10mM NaF, 10mM NaPyroph, 1mM 4-(2-aminoethyl) benzenesulfonyl (AEBSF), 10μg/ml leupeptin, 10μg/ml aprotinin, 1μg/ml Pepstatin, resolved by 10% SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with the following antibodies: p-PTEN (S380), PTEN (Cell Signaling Techonology), actin, CK2α, and CK2α′ (Santa Cruz Biotechonology).

**Treatment with CX-4945.** In order to assess the effect of CK2 inhibition in PTEN expression, leukemia cells were cultured in 24-well plates as $2 \times 10^6$ cells/ml at 37ºC with 5% CO2 in control medium, or in the presence of 10 or 20μM of CX-4945 (Adooq.com Bioscience). After 24h cells were harvested and fixed immediately with Cytofix buffer (BD Biosciences) for 10 min at 37ºC. The cells were then pelleted, resuspended in ice-cold PERM buffer III (BD Biosciences), and incubated for 30 min on ice. Cells were washed twice with staining buffer (BD Bioscience), resuspended at $1\times10^7$cells/mL, and stained with CD79a-APC, PTEN-PE, or pAkt T308-PE for 30min at room temperature. The cells were washed with staining buffer and analyzed in LSRFortessa (BD Bioscience).

**Analysis of cell viability and apoptosis.** Cells were cultured in 24-well plates as 2 x $10^6$ cells/ml at 37ºC with 5% CO2 in control medium or with the indicated concentrations of LY294002 (Calbiochem) and CX-4945. After 48 hours, cells were harvested and viability was determined by double-staining with APC or FITC-conjugated Annexin V (R&D systems) and propidium iodide (PI) (Sigma). Briefly, cells were washed with PBS and resuspended in 100µL of binding buffer with annexin V and PI. After 15 min of incubation at room temperature in the dark, 100µL of binding buffer were added and the samples were analyzed by flow cytometry. Viability index was calculated as the ratio of experimental to control conditions.

**Statistical analysis.** GraphPad Prism version 5.00 for windows (GraphPad Software) was used for statistical analyses. Differences between populations were calculated using unpaired two-tailed Students´s t test, Mann-Whitney test, or One-way ANOVA, as appropriate. Correlations were analyzed using the Pearson's correlation coefficient. *P* values lower than 0.05 were considered significant.
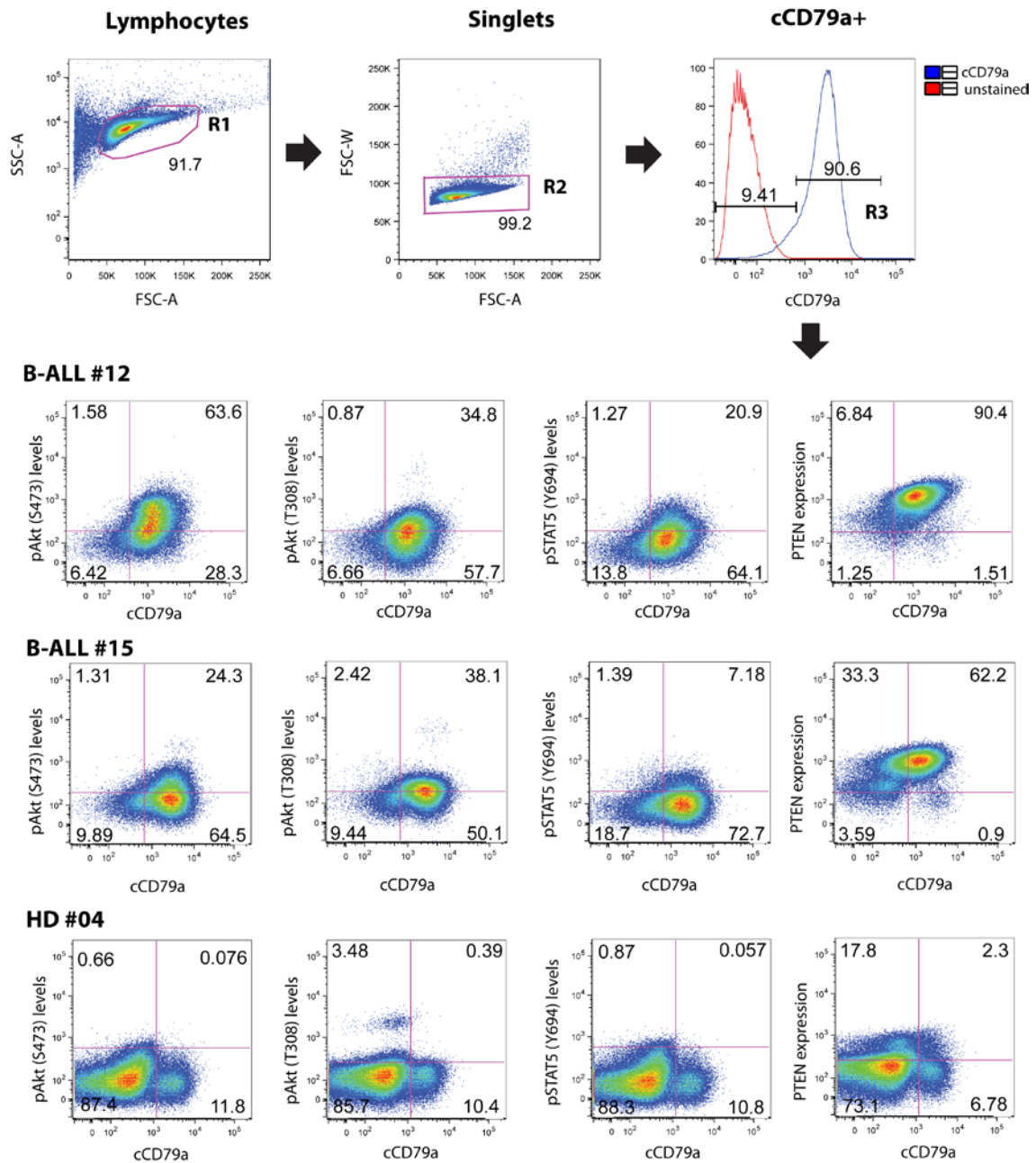
**Figure S1. Gating strategy for phospho-flow cytometry analysis.** Debris were excluded, and lymphocytes included, using a forward scatter area (FSC-A) *versus* side scatter area (SSC-A) gate (R1). Single cells (singlets) were then selected on a FSC-A *versus* FSC-W plot (R2) to exclude signaling data from doublets. Cytoplasmic CD79a (cCD79a)+ cells (R3) were selected on a histogram of cCD79a staining and analysis of individual phosphoproteins was then performed in this cell population. Examples of phospho-Akt (S473), phospho-Akt (T308), phospho-STAT5 (Y694) and total PTEN *versus* cCD79a stainings for the two individual B-ALL samples and one healthy donor are presented. Background fluorescences from unstained cells (equivalent to irrelevant isotypic controls) were used as negative controls to define positivity in each channel.
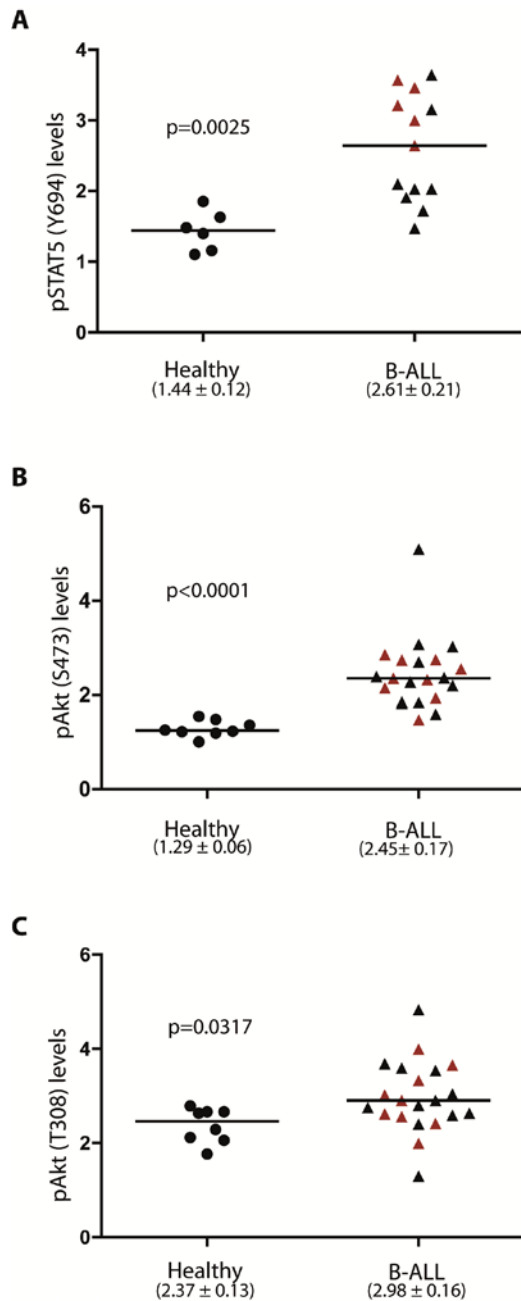
**Figure S2. PI3K/Akt and JAK/STAT pathways are constitutively hyperactivated in primary adult B-ALL cells.** Levels of phosphorylated **(A)** STAT5 (Y694), **(B)** Akt (S473), and **(C)** Akt (T308) in bone marrow cells from healthy individuals and adult B-ALL samples were quantified by flow cytometry analysis using phospho-specific antibodies. Points represent individual samples and Ph+ patients are indicated in red. Horizontal bars denote median. Mean ± SEM is shown in parentheses. Statistical analysis was performed by 2-tailed Mann Whitney test.
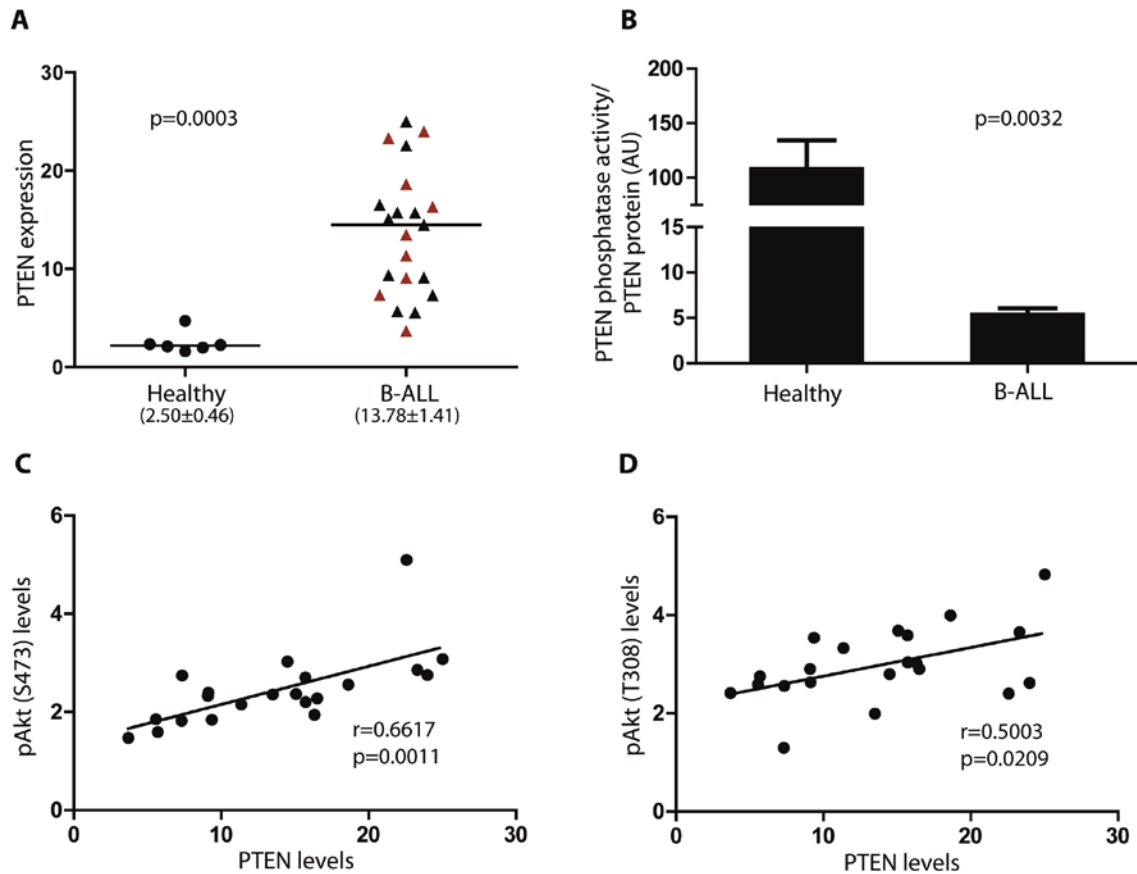
**Figure S3. PTEN lipid phosphatase activity is downregulated in adult B-ALL cases despite increased protein expression. (A)** PTEN levels in normal bone marrow cells (n= 6) and adult B-ALL primary cells (n= 21) were quantified by flow cytometry. Points represent individual samples, Ph+ patients are indicated in red, horizontal bars denote median, and mean ± SEM is shown in parentheses. **(B)** PTEN *in vitro* lipid phosphatase activity was determined after immunoprecipitation of endogenous PTEN from normal bone marrow cells (n=2) and diagnostic adult B-ALL cells (n=4). PTEN activity was normalized to the levels of immunoprecipitated PTEN in each sample. (C,D) Correlation between PTEN expression levels and Akt phosphorylation at S473 (C) and T308 (D). Statistical analyses were performed by 2-tailed Mann Whitney (**A**) or Student's t (**B**) tests; or by Pearson's correlation analysis (**C,D**).
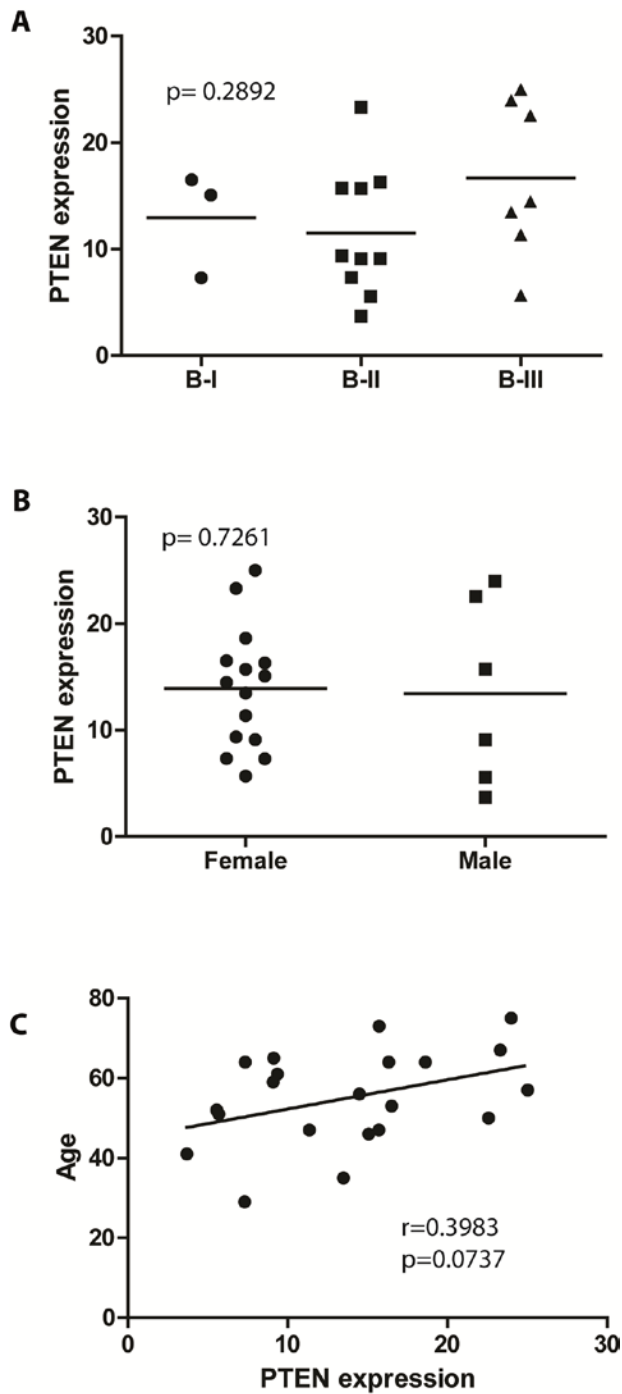
**Figure S4. PTEN expression does not appear to correlate with clinical parameters in adult B-ALL patients. (A)** Association between PTEN expression levels and B-ALL maturation stage, as defined by EGIL (B-I, B-II and B-III). Statistical analysis was performed by One-Way ANOVA. **(B)** Association between PTEN levels and gender. Statistical analysis was performed by 2-tailed Mann Whitney test. **(C)** Correlation between PTEN expression levels and age. Statistical analysis was performed by Pearson´s correlation analysis.