# Differential activation of basal and IL-7-induced PI3K/Akt/mTOR and JAK/STAT5 signaling distinguishes pediatric from adult acute lymphoblastic leukemia

## Authors

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# Differential Activation of Basal and IL-7-induced PI3K/Akt/mTOR and JAK/STAT5 Signaling Distinguishes Pediatric from Adult Acute Lymphoblastic Leukemia

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## Materials and Methods

**Patients and samples.** Primary leukemia samples from pediatric and adult patients diagnosed with B-ALL were collected from Instituto Português de Oncologia de Lisboa and Santo António dos Capuchos Hospital, Lisbon, Portugal (Exploratory cohort), or from Saint-Louis Hospital and Robert Debré Hospital, Paris, France (Confirmatory cohort). All samples were collected after informed consent was obtained in accordance with the Declaration of Helsinki and under ethical approval. In the exploratory cohort, median age of the pediatric patients (n=40) was 4 years (range, 1-14 years) and the median age of adult cases (n=21) was 56 years (range, 29-75 years). In the confirmatory cohort, median age of the pediatric patients (n=16) was 3 years (range, 1-12 years) and the median age of adult cases (n=16) was 37 years (range, 20-53 years). Features of B-ALL patients are summarized in Supplementary Table 1. 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). Samples were processed within 24 h of collection.

***In vitro* culture.** Primary B-ALL cells isolated by density centrifugation over Ficoll-Paque were cultured in a round bottom 96-well plate at 2 x $10^6$ cells/mL at 37ºC with 5% $CO_2$ with RPMI 10 (control medium) or 10 ng/mL IL-7. After 72h cells were harvested, and viability of leukemic cells was quantitatively determined by staining with Annexin V (R&D systems) and Propidium iodide (PI) or 7-AAD. Samples were at least 20% viable in medium alone (no IL-7) at the time point of analysis (72h).

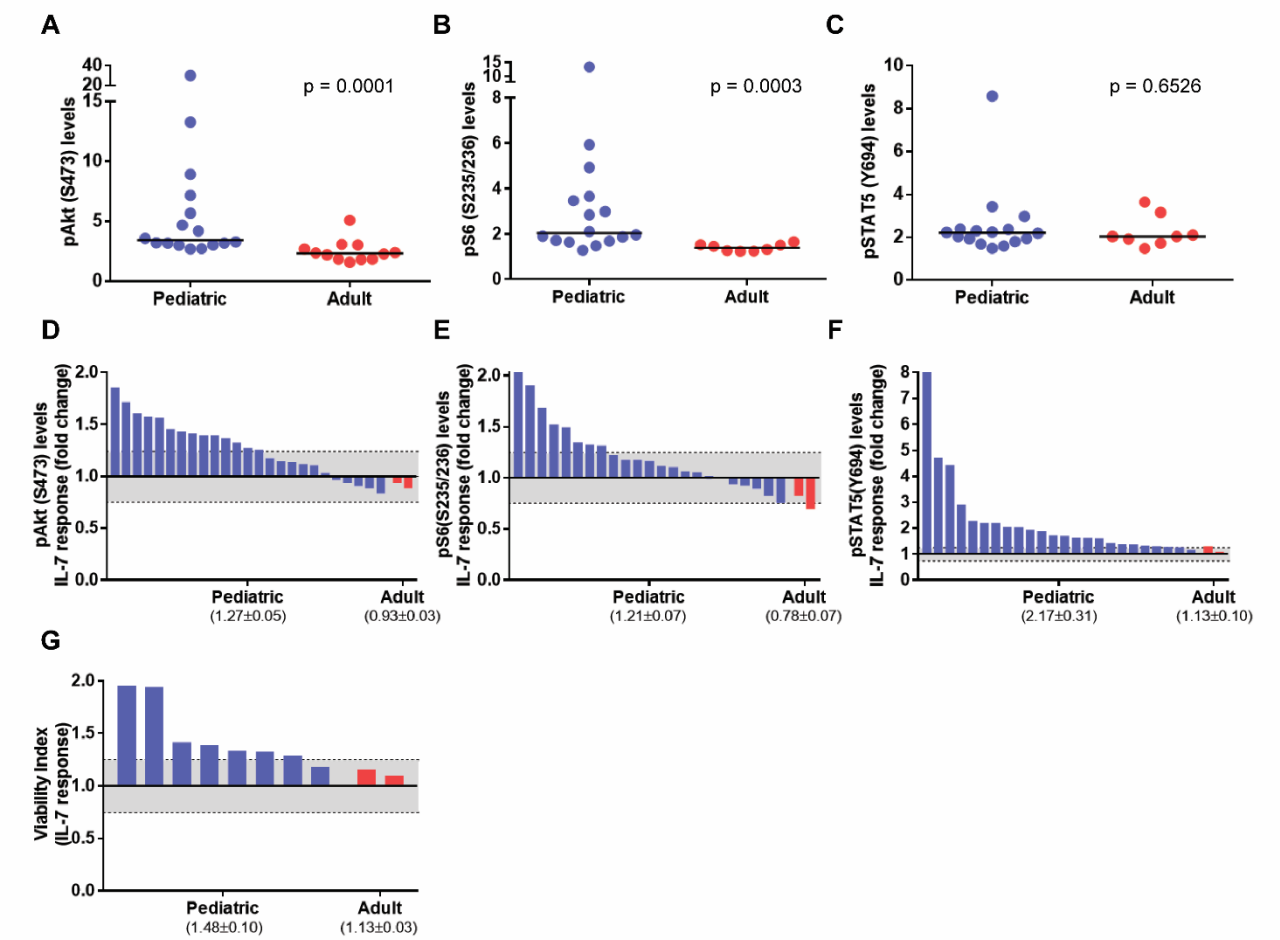
**Intracellular phospho-specific flow cytometry.** To assess the phosphorylation status of PI3K/Akt/mTOR and JAK/STAT5 pathways in primary B-ALL cells, intracellular phospho-flow cytometric analyses were performed as previously described (Gomes., *et al* Haematologica 2014; doi: 10.3324/haematol.2013.096438). Primary B-ALL cell viability after thawing was at least 80%. Cells were washed twice with 1x PBS (Gibco; hereafter PBS) and incubated for 30 min at 37ºC with IL-7 (50 ng/mL). Afterwards, cells were washed with PBS, pelleted down by centrifugation, and fixed with Cytofix buffer (BD Biosciences) for 10 min at 37ºC. Cell permeabilization was done with ice-cold Perm buffer III (BD Biosciences) for 30 min on ice. Cells were first washed in PBS and then in Stain buffer (BD Biosciences), and stained with CD79a-

APC, P-Akt S473-Alexa Fluor 488, P-S6 S235/S236-Alexa Fluor 488 and P-STAT5 Y694-Alexa Fluor 488 for 30 min at room temperature. Cells were washed with Stain buffer and analyzed by flow cytometry. Phospho-flow analyses were performed within a live cell gate and, therein, within $CD79a^+$. Thus, phospho-flow results reflect differences strictly within the live cell population of B-ALL cells. The fold change in Mean Fluorescence Intensity (MFI) was calculated by dividing the MFI of stimulated cells with that of unstimulated cells. Fold change >1.25 values were considered as a positive response to IL-7.

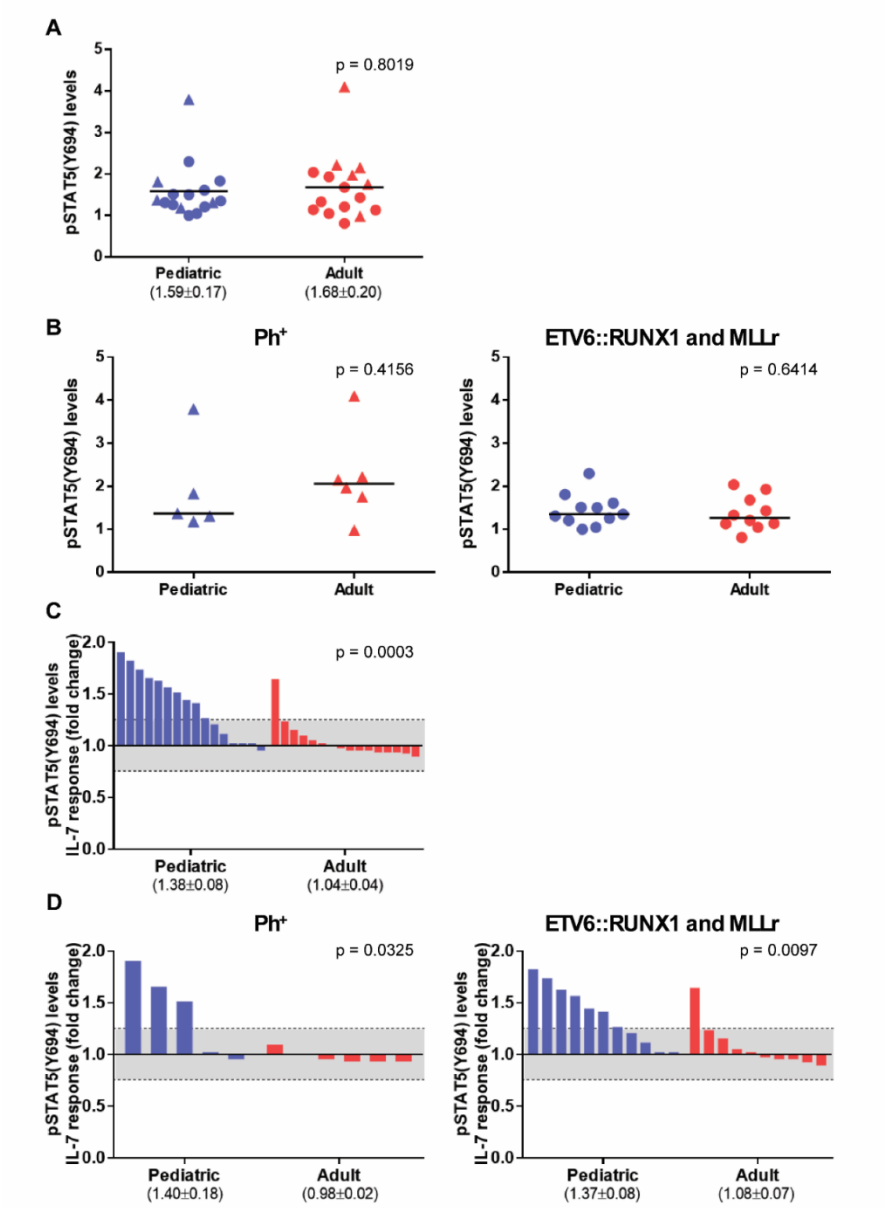
**Flow cytometry analysis of IL-7Rα surface expression.** For cell surface assessment of interleukin 7 receptor α chain (IL-7Rα or CD127) expression, cells were washed in ice-cold PBS and incubated for 30 min on ice with α-human IL-7R PE-conjugated antibody (R&D Systems). Cells were then washed in ice-cold PBS and immediately analyzed by flow cytometry. Expression of surface IL-7Rα was assessed within the live cell population, as determined by the forward scatter (FSC) *versus* side scatter (SSC) distribution.

***Ex vivo* drug testing.** Primary B-ALL cells isolated by density centrifugation over Ficoll-Paque were cultured in a round bottom 96-well plate as 2 x $10^6$ cells/mL with pan-class I PI3K inhibitor Buparlisib (BKM120) or STAT5 inhibitor (N9-((4-oxo-4H-chromen-3-yl)methylene) nicotinohydrazide; STAT5i) at 37ºC with 5% $CO_2$ with RPMI 10 (control medium) or 10 ng/mL IL-7. DMSO concentration was normalized in each well. Cell viability was assessed after 72 h by flow cytometry analysis of FSC *versus* SSC discrimination or annexin V/7-AAD staining.

**Statistical analysis.** GraphPad Prism version 6.01 (GraphPad Software) was used to perform statistical analysis. Differences between groups were calculated using unpaired two-tailed t-tests. Welsh's correction was used in t-tests when variances were found different. When appropriate, two-tailed Mann-Whitney test was used. *p*-values lower than 0.05 were considered statistically significant. The tests used are mentioned in figure legends.




**Supplementary Figure 1. Higher PI3K/Akt/mTOR signaling pathway activation and stronger signaling and functional responses to IL-7 in pediatric B-ALL, even after removal of Ph$^+$ cases in Portuguese patient cohorts. (A-C)** Levels of phosphorylated Akt S473 **(A)**, S6 S235/236 **(B)** and STAT5 Y694 **(C)** in bone marrow cells from pediatric and adult B-ALL samples were quantified by flow cytometry analysis using phospho-specific antibodies. Points represent individual samples and horizontal bars denote median. Statistical analysis was performed by 2-tailed Mann-Whitney test. Related to Figure 1A-C. **(D-F)** B-ALL samples were stimulated with 50 ng/mL IL-7 for 30 min and the levels of phospho-Akt S473 **(D)**, phospho-S6 S235/236 **(E)** and phospho-STAT5 Y694 **(F)** were analyzed by flow cytometry. IL-7-induced phosphorylation levels are expressed as the ratio of the stimulated over the unstimulated conditions. Bars represent individual samples, horizontal dashed lines and shaded area represent lack of response, defined as ≤ 1.25-fold change. Mean ± SEM is shown in parentheses. Related to Figure 2C-E. **(G)** Cells from pediatric and adult B-ALL patients were cultured for 72h in the presence or absence of 10 ng/mL IL-7, stained with Annexin V and 7-AAD and cell viability was determined by flow cytometry analysis. Viability index, calculated as the ratio between viable cells in medium with IL-7 over medium alone, is indicated. Bars represent individual samples, horizontal dashed lines and shaded area represent lack of response, defined as ≤ 1.25-fold change. Mean ± SEM is shown in parentheses. Related to Figure 2A.




**Supplementary Figure 2. French patient cohorts from defined B-ALL subgroups (ETV6::RUNX1, MLLr and Ph$^+$) confirm results from Portuguese patient cohorts: basal STAT5 phosphorylation does not differ between pediatric and adult cases, whereas IL-7-mediated STAT5 phosphorylation is higher in pediatric samples.** Pediatric and adult ETV6::RUNX1, KMT2A/MLLr (MLLr) or BCR::ABL1 (Ph$^+$) B-ALL samples were compared. **(A-B)** Basal and **(C-D)** IL-7-induced (50 ng/mL, 30 min) levels of phosphorylated STAT5 Y694 were quantified by flow cytometry analysis. In **(A-B),** Ph$^+$ patients are indicated by a triangle and ETV6::RUNX1 and MLLr are indicated by a circle. Points represent individual samples and horizontal bars denote median. Statistical analysis was performed by 2-tailed Mann-Whitney test. **(C-D)** IL-7-induced phosphorylation levels are expressed as the ratio of the stimulated over the unstimulated conditions. Bars represent individual samples, horizontal dashed lines and shaded area represent lack of response, defined as ≤ 1.25-fold change. Mean ± SEM is shown in parentheses. Statistical analysis was performed by 2-tailed Mann-Whitney test.

**Supplementary Table 1.** Features of pediatric (#1-40 and #62-77) and adult (#41-61 and #78-93) B-ALL patients from exploratory and confirmatory cohorts. IPO: Instituto Português de Oncologia; HRD: Hôpital Robert Debré; HSAC: Hospital de Santo António dos Capuchos; HSL: Hôpital Saint-Louis; Negative* – Negative for BCR::ABL1, TCF3::PBX1, MLLr and ETV6::RUNX1; n.d. – not determined.

| | Patient # | Age (years) | Sex | Cytogenetics | Hospital |
|---|---|---|---|---|---|
| Exploratory cohort | 1 | 14 | F | TCF3::PBX1 | IPO |
| | 2 | 4 | F | ETV6::RUNX1 | IPO |
| | 3 | 4 | M | Negative* | IPO |
| | 4 | 2 | F | Negative* | IPO |
| | 5 | 3 | M | Negative* | IPO |
| | 6 | 4 | M | Negative* | IPO |
| | 7 | 5 | F | ETV6::RUNX1 | IPO |
| | 8 | 2 | M | Negative* | IPO |
| | 9 | 3 | M | Negative* | IPO |
| | 10 | 3 | F | BCR::ABL1 | IPO |
| | 11 | 3 | F | ETV6::RUNX1 | IPO |
| | 12 | 7 | M | ETV6::RUNX1 | IPO |
| | 13 | 13 | M | TCF3::PBX1 | IPO |
| | 14 | 5 | M | ETV6::RUNX1 | IPO |
| | 15 | 7 | F | BCR::ABL1 | IPO |
| | 16 | n.d. | M | n.d. | IPO |
| | 17 | 2 | M | Negative* | IPO |
| | 18 | 2 | M | Negative* | IPO |
| | 19 | 6 | M | Negative* | IPO |
| | 20 | 9 | M | Negative* | IPO |
| | 21 | n.d. | M | Negative* | IPO |
| | 22 | 8 | F | Negative* | IPO |
| | 23 | 7 | M | n.d. | IPO |
| | 24 | 11 | F | Negative* | IPO |
| | 25 | 5 | M | Negative* | IPO |
| | 26 | 3 | F | Negative* | IPO |
| | 27 | 8 | M | Negative* | IPO |
| | 28 | 10 | F | Negative* | IPO |
| | 29 | 1 | M | Negative* | IPO |
| | 30 | 3 | M | Negative* | IPO |
| | 31 | 9 | M | Negative* | IPO |
| | 32 | 3 | M | Negative* | IPO |
| | 33 | 6 | F | Negative* | IPO |
| | 34 | 2 | F | Abnormal karyotype | IPO |
| | 35 | 3 | M | Negative* | IPO |
| | 36 | 3 | M | ETV6::RUNX1 | IPO |

| | | | | | |
|---|---|---|---|---|---|
| | 37 | 3 | M | Abnormal karyotype | IPO |
| | 38 | n.d. | M | MLL rearrangements | IPO |
| | 39 | 6 | M | n.d. | IPO |
| | 40 | 11 | F | Negative* | IPO |
| | 41 | 64 | F | BCR::ABL1 | HSAC |
| | 42 | 67 | F | BCR::ABL1 | HSAC |
| | 43 | 53 | F | Negative* | HSAC |
| | 44 | 52 | M | Negative* | HSAC |
| | 45 | 46 | F | Negative* | HSAC |
| | 46 | 35 | F | BCR::ABL1 | HSAC |
| | 47 | 29 | F | MLL::AF4 | HSAC |
| | 48 | 59 | M | BCR::ABL1 | HSAC |
| | 49 | 50 | M | Negative* | HSAC |
| | 50 | 61 | F | Negative* | HSAC |
| | 51 | 64 | F | BCR::ABL1 | HSAC |
| | 52 | 60 | F | BCR::ABL1 | HSAC |
| | 53 | 57 | F | Negative* | HSAC |
| | 54 | 75 | M | BCR::ABL1 | HSAC |
| | 55 | 41 | M | BCR::ABL1 | HSAC |
| | 56 | 47 | F | BCR::ABL1 | HSAC |
| | 57 | 47 | F | Negative* | HSAC |
| | 58 | 51 | F | n.d. | HSAC |
| | 59 | 73 | M | Hyperdiploidy | HSAC |
| | 60 | 56 | F | Negative* | HSAC |
| | 61 | 65 | F | Negative* | HSAC |
| **Confirmatory cohort** | 62 | 9 | M | ETV6::RUNX1 | HRD |
| | 63 | 9 | M | BCR::ABL1 | HRD |
| | 64 | 10 | M | BCR::ABL1 | HRD |
| | 65 | 2 | M | ETV6::RUNX1 | HRD |
| | 66 | 9 | F | ETV6::RUNX1 | HRD |
| | 67 | 3 | F | BCR::ABL1 | HRD |
| | 68 | 1 | M | BCR::ABL1 | HRD |
| | 69 | 1 | F | MLL::AF4 | HRD |
| | 70 | 3 | M | MLL::AF4 | HRD |
| | 71 | 12 | F | BCR::ABL1 | HRD |
| | 72 | 2 | F | ETV6::RUNX1 | HRD |
| | 73 | 3 | M | ETV6::RUNX1 | HRD |
| | 74 | 1 | M | MLL::AF4 | HRD |
| | 75 | 5 | F | MLL::AF4 | HRD |
| | 76 | 5 | F | ETV6::RUNX1 | HRD |
| | 77 | 2 | M | ETV6::RUNX1 | HRD |
| | 78 | 26 | M | MLL::AF4 | HSL |
| | 79 | 53 | F | MLL::AF4 | HSL |
| | 80 | 49 | F | BCR::ABL1 | HSL |

| | | | | | |
|---|---|---|---|---|---|
| | 81 | 22 | M | BCR::ABL1 | HSL |
| | 82 | 46 | M | BCR::ABL1 | HSL |
| | 83 | 47 | F | BCR::ABL1 | HSL |
| | 84 | 32 | M | ETV::RUNX1 | HSL |
| | 85 | 21 | M | ETV::RUNX1 | HSL |
| | 86 | 20 | F | ETV::RUNX1 | HSL |
| | 87 | 48 | M | BCR::ABL1 | HSL |
| | 88 | 33 | M | MLL::AF4 | HSL |
| | 89 | 48 | M | MLL::AF4 | HSL |
| | 90 | 22 | M | MLL::AF4 | HSL |
| | 91 | 39 | F | MLL::ENL | HSL |
| | 92 | 50 | F | MLL::ENL | HSL |
| | 93 | 34 | F | BCR::ABL1 | HSL |