haematologica
Journal of the Ferrata Storti Foundation

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Received: August 18, 2023.
Accepted: April 16, 2024.

Citation: Marta B. Fernandes, A. Margarida Gomes, Mariana L. Oliveira, Joana Caldas, Paulo Lúcio, Rathana Kim, Aurélie Caye-Eude, Filomena Pereira, Aida B. de Sousa, Alessia De Stefano, Matilde Y. Follo, Maria V. Soares, João F. Lacerda, Joana Desterro, Hélène Cavé, Emmanuelle Clappier, Ximo Duarte, Patrícia Ribeiro, and João T. Barata. Differential activation of basal and IL-7-induced PI3K/Akt/mTOR and JAK/STAT5 signaling distinguishes pediatric from adult acute lymphoblastic leukemia.
Haematologica. 2024 Apr 24. doi: 10.3324/haematol.2023.284102 [Epub ahead of print]

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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Author Contributions

MBF, AMG, MLO and ADS performed experiments, and analyzed and interpreted data. MBF also drafted the manuscript. JC, PL, FP, ABS, MYF, MVS, JFL, JD, XD, and PR contributed with critical patient samples and clinical information and/or crucial feedback. JTB designed the project structure, analyzed and interpreted data, coordinated the studies, and wrote the manuscript. All authors critically read and agreed to the final version of the manuscript.

Data sharing statement

For original data please contact joao_barata@medicina.ulisboa.pt

Acknowledgements

We are grateful to the patients and their families for generously providing the samples that were used in our studies. We also thank the Flow Cytometry core facility of Instituto de Medicina Molecular João Lobo Antunes for their technical support. We also gratefully acknowledge the Center for Biological Resources of the Robert Debré (CRBcancer; BB-0033-00076) and Saint-Louis (CRB BiRTH) hospitals.

Funding

This work was supported by the European Research Council, under the European Union's Horizon 2020 research and innovation programme and European Union's Horizon Europe (ERC-CoG-648455, ERC-POC-862545 and ERC-POC-101069429), by “la Caixa” Foundation (HR21-00761), and by Worldwide Cancer Research (WWCR 24-0426) to JTB. MBF was the recipient of a PhD fellowship from Fundação para a Ciência e a Tecnologia (FCT), Portugal. ADS was the recipient of a Marco Polo fellowship from the University of Bologna, Italy.

Conflicts of interest statement

The authors declare no conflicts of interest.

Age distribution of B-cell acute lymphoblastic leukemia (B-ALL) is bimodal, peaking in childhood at 2-5 years of age and in adults after the age of 50, with children displaying significantly better prognosis than adults. Signaling pathways triggered by leukemia cell-autonomous lesions or by extracellular cues, such as interleukin-7 (IL-7), have been shown to play a pivotal role in B-ALL biology and response to treatment [1-3]. However, whether age-related differences exist in signaling pathway activation between pediatric and adult B-ALL cases has not been scrutinized. Here, we characterized the basal and IL-7-induced PI3K/Akt/mTOR and JAK/STAT5 signaling profile of pediatric patients (range, 1-14 years) and adult patients (range, 29-75 years), using phospho-specific flow cytometry. We show that there are clear age-related differences in signaling activation that correlate with sensitivity to pathway-specific small molecule inhibitors. Our results underline the importance of considering the age group when predicting potential clinical benefits of signaling targeted therapies.

IL-7, a bone marrow stroma-produced cytokine, is vital for normal B-cell development [4-6]. However, IL-7 can also stimulate the proliferation of B-ALL cells [3] and aberrant IL-7/IL-7R-mediated signaling contributes to malignant transformation of developing B-cells [6]. *IL7R* gain-of-function mutations, which are able to initiate B-ALL in mice [1, 2], occur in up to 3% of human B-ALL cases [7]. *IL7R*-driven leukemias display activation of JAK/STAT and PI3K/Akt/mTOR signaling and are sensitive to pharmacological inhibitors of these pathways [1].

The genomic landscape of ALL has been shown to vary with age, with favorable and unfavorable cytogenetics being less and more frequent, respectively, in adults [7]. Moreover, adult patients display increased incidence of extramedullary disease with central nervous system involvement [8]. However, whether these age-related differences correlate with the activation of key pro-tumoral signaling pathways is ill explored,

despite the knowledge that signaling inhibitors can have considerable clinical impact, as demonstrated by the use of tyrosine kinase inhibitors in Philadelphia chromosome-positive (Ph^+) B-ALL cases [9].

PI3K/Akt/mTOR pathway is frequently activated in pediatric B-ALL and associated with poor response to chemotherapy [10]. Constitutive hyperactivation of this signaling axis was also observed in adult B-ALL cases [11]. Thus, we questioned whether there are differences in activation of PI3K/Akt/mTOR signaling between the two age groups. Initially, we compared pediatric (n=40; median age: 4 years; range: 1-14 years) and adult (n=21, median age: 56 years; range, 29-75 years) patient samples collected at diagnosis after informed consent and under ethical approval of Instituto Português de Oncologia de Lisboa and Hospital Santo António dos Capuchos, Lisbon, Portugal (see **Supplementary Table 1 – Exploratory cohort** for patient information). Flow cytometry analyses of Akt (S473) and S6 (S235/236) phosphorylation levels showed that PI3K/Akt/mTOR pathway activation was higher in pediatric than in adult cases (**Figure 1A,B**). Given these age-related differences, we next analyzed the impact of the pan-PI3K inhibitor Buparlisib (BKM120) on leukemia cell viability *ex vivo*. Pharmacological inhibition of PI3K signaling pathway had a clear impact on leukemia cell survival in both age groups, although pediatric samples were significantly more sensitive to Buparlisib (**Figure 1D,E**), in agreement with higher PI3K signaling activation and suggestive of stronger reliance of childhood than adult ALL on this pathway.

In contrast, there were no significant differences between childhood and adult B-ALL regarding JAK/STAT5 pathway activation (**Figure 1C**). This is remarkable, since the frequency of Ph^+ cases (which are known to display STAT5 activation) was lower in children (2 of 18 cases, 11%) than in adults (5 of 13 analyzed, 38.4%). Interestingly,

childhood Ph^+ samples had levels of STAT5 phosphorylation similar to the remaining samples, whereas Ph^+ adult cases were among those with highest phospho-STAT5 (**Figure 1C**), confirming our previous observations [11]. These findings may suggest that other genetic alterations leading to high STAT5 phosphorylation, similar to *BCR::ABL1* in Ph^+ ALL, may be more frequent in pediatric cases. However, such alterations, known as *BCR::ABL1*/Ph-like alterations (e.g. *CRLF2* rearrangements or mutations in *IL7R* or in JAK kinases) are more frequent in adults.

Therefore, we speculated that B-ALL cells from pediatric patients may be more sensitive to IL-7 than those from adult cases, resulting in levels of STAT5 activation similar to those arising from BCR::ABL1 or other cell-autonomous lesions. In agreement with our hypothesis, we found that IL-7 promoted the viability of pediatric leukemias to a higher extent than that of adult cases (**Figure 2A**). We then sought age-related differences in IL-7Rα surface expression as potential cause for increased IL-7 responsiveness in pediatric cases. However, childhood and adult samples displayed similar IL-7Rα levels (**Figure 2B**). This notwithstanding, we questioned whether signaling responses could differentiate the two age groups. IL-7 stimulation did not promote phosphorylation of Akt or S6 in adult patients. In contrast, pediatric B-ALL samples showed Akt and S6 activation in response to IL-7 in 54% and 34.7% of the cases, respectively (**Figure 2C,D,F** and **Table 1**). STAT5 phosphorylation was upregulated by IL-7 in 50% of adult cases as opposed to 83% of pediatric cases (**Figure 2E,F and Table 1**). Within the responsive cases, the degree of STAT5 phosphorylation was clearly higher in childhood B-ALL samples (**Figure 2E**).

Targeting STAT5 or STAT5 target genes, such as *BCL2* and *PIM*, was shown to reduce leukemia burden in mice and induce apoptosis of newly diagnosed and tyrosine kinase inhibitor-resistant Ph^+ ALL patient-derived cells [12]. Thus, we incubated samples

from both age groups with a STAT5 inhibitor (N9-((4-oxo-4H-chromen-3-yl)methylene) nicotinohydrazide; STAT5i) in the presence of IL-7 for 72h. B-ALL pediatric patient samples were more sensitive to STAT5 inhibition than adult patient samples as observed by a higher decrease in cell viability (**Figure 2G,H**).

Adult and pediatric B-ALL cases differ in oncogenic subtype composition. For example, Ph^+ ALL is more frequent in adults, whereas ETV6::RUNX1 cases are common in children and rare in adults [13]. To ensure that our observations were not substantially affected by age-related ALL subtype biases, we first removed the Ph^+ cases from our analyses. Exclusion of pediatric and adult Ph^+ cases did not alter our initial conclusions (**Supplementary Figure 1**).

We next evaluated a confirmatory cohort of pediatric and adult B-ALL French patients that were classified as Ph^+, ETV6::RUNX1 or KMT2A/MLL-rearranged (MLLr). Patient samples were obtained, after informed consent and ethical approval, from Hôpital Saint-Louis and Hôpital Robert Debré, Paris, France (**Supplementary Table 1 – Confirmatory cohort**). Analysis of the 3 genetic subgroups altogether (**Supplementary Figure 2A**) or comparing non-Ph^+ cases (MLLr and ETV6::RUNX1) vs. Ph^+ cases (**Supplementary Figure 2B**) confirmed the lack of differences in basal STAT5 phosphorylation levels between children and adults found in the Portuguese patient cohort (**Figure 1C**). Moreover, we also confirmed that pediatric B-ALL samples respond better to IL-7 (**Supplementary Figure 2C**), irrespective of subtype (**Supplementary Figure 2D**).

Our findings may reflect a combination of normal age-related differences and leukemia peculiarities. Mouse old B-cell progenitors have lower phospho-Akt and phospho-STAT5 than younger counterparts [14], and are poorly responsive *in vitro* to IL-7 but not to other growth factors [15]. Notably, the lower fitness resulting from impaired IL-

7-mediated signaling in ageing B-cell precursors sets the stage for the development of Ph^+ leukemias due to increased competitiveness of Ph^+ B-cells, which are selected because Bcr-Abl constitutive signaling compensates for the impaired IL-7-mediated signaling that occurs in aged precursors [14]. This contrasts with young B-lymphoid progenitors, where IL-7 responsiveness is high and Bcr-Abl provides a much smaller competitive advantage [14]. If these features are conserved in humans, they suggest that the lower ability of adult B-ALL cells to respond to IL-7 *ex vivo*, as we report herein, may at least partially reflect a normal ageing process. Moreover, the higher frequency of Ph^+ cases in adult B-ALL may, to some extent, reflect the decreased ability of adult B-cell precursors to respond to IL-7.

Overall, our studies expose age-related differences in PI3K/Akt/mTOR and JAK/STAT5 signaling pathway activation in B-ALL that are associated with differential sensitivity to signaling-specific inhibitors. These results may have important implications for clinical decision-making using targeted therapies, as pediatric B-ALL patients will likely benefit more from PI3K pathway inhibitors and anti-IL-7R signaling therapies than adult cases. Evidently, larger studies are warranted that extend and integrate the age-associated findings on signaling activation presented here within the full scope of karyotypic, genetic, epigenetic and transcriptomic features that characterize the distinct B-ALL subtypes.

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Table 1. Frequency of IL-7 induced phosphorylation of Akt (S473), S6 (S235/236) and STAT5 (Y694) in pediatric and adult patient samples. Fold-change values >1.25 were considered as a positive response.

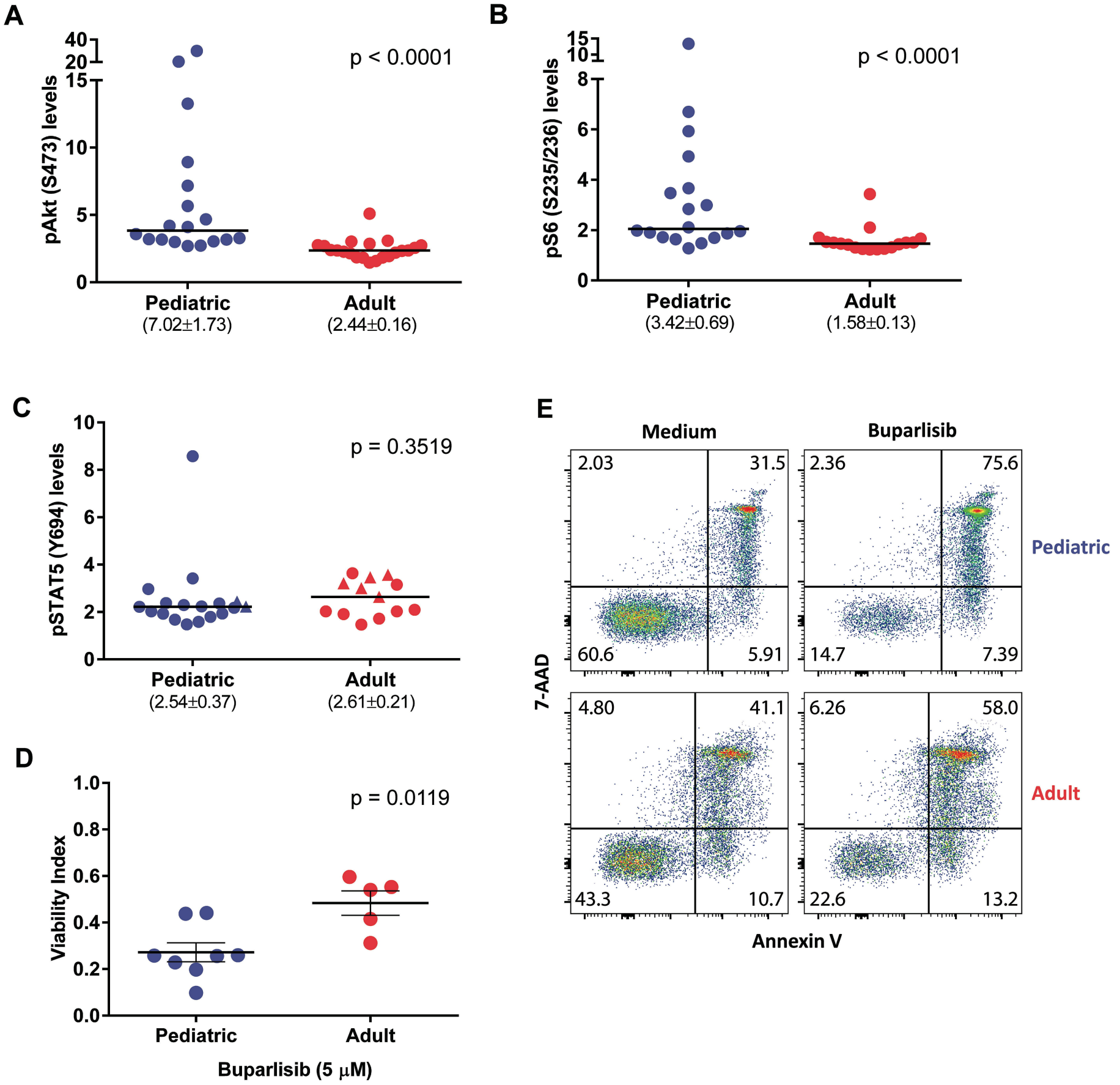
	IL-7 response (Fold change >1.25)		
	pAkt (S473)	**pS6 (S235/236)**	**pSTAT5 (Y694)**
Pediatric	54% (13/24)	34.7% (8/23)	83% (20/24)
Adult	0% (0/6)	0% (0/6)	50% (3/6)

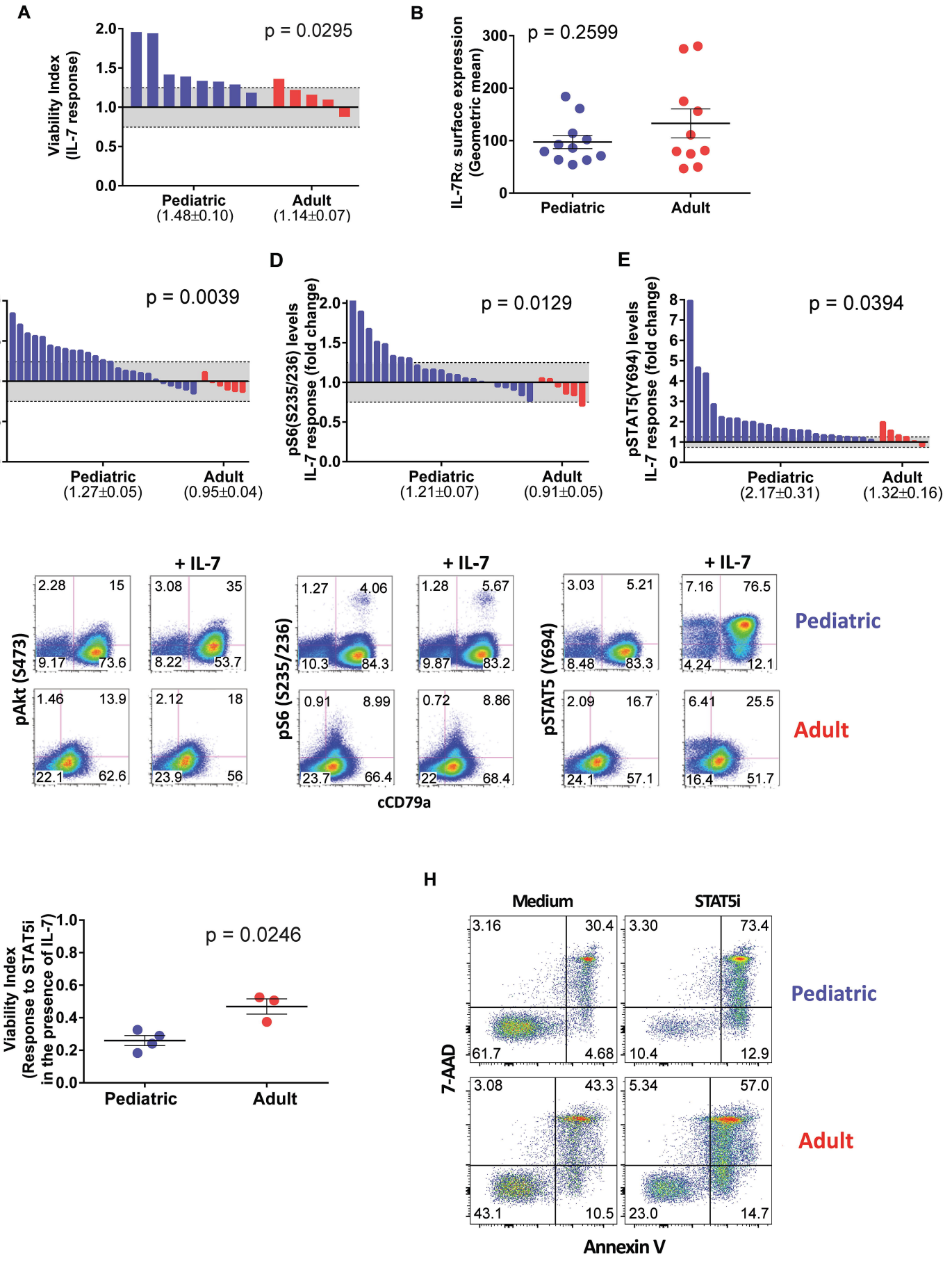
Figure Legends

Figure 1. Higher PI3K/Akt/mTOR signaling pathway activation in pediatric B-ALL is associated with higher sensitivity to the PI3K inhibitor Buparlisib. (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. In **(C)**, Ph^+ patients are indicated by a triangle. Mean ± SEM is shown in parentheses. Statistical analysis was performed by 2-tailed Mann-Whitney test. **(D,E)** B-ALL samples were cultured for 72h in medium alone or with Buparlisib (5 μM), collected and stained with Annexin V and 7-AAD for cell viability assessment by flow cytometry. **(D)** Viability index, calculated as the ratio of viability of cells cultured with Buparlisib over that of cells cultured in medium alone, is indicated. Points represent individual samples, and horizontal bars denote mean ± SEM. Statistical analysis was performed by unpaired t-test with Welch's correction. **(E)** Annexin V by 7-AAD dot plots of 2 representative cases. The percentage of live (bottom left), early apoptotic (bottom right), and late apoptotic/necrotic (top right) cells is indicated in the respective quadrants.

Figure 2. Pediatric B-ALL patient samples have stronger signaling and functional responses to IL-7, and are more sensitive to STAT5 inhibition in the presence of IL-7, than adult cases. (A) Bone marrow 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. Statistical analysis was performed by 2-tailed Mann-Whitney test. **(B)** Cell surface levels of IL-7Rα were determined by flow cytometry analysis. Points represent individual samples, horizontal bars denote mean ± SEM. Statistical analysis was performed by unpaired t-test with Welch's correction. **(C-E)** B-ALL samples were stimulated with 50 ng/mL IL-7 for 30 min and the levels of phospho-Akt S473 **(C)**, phospho-S6 S235/236 **(D)** and phospho-STAT5 Y694 **(E)** were analyzed by flow cytometry. **(F)** Flow cytometry dot plots representative of data in (C-E). 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. **(G,H)** B-ALL samples were cultured for 72h in the presence of 10 ng/mL IL-7 alone or with STAT5i (200 µM), stained with Annexin V and 7-AAD and cell viability determined by flow cytometry. **(G)** Viability index, calculated as the ratio of viability of cells cultured with IL-7 plus STAT5i over that of cells cultured with IL-7 alone, is indicated. Points represent individual samples, and horizontal bars denote mean ± SEM. Statistical analysis was performed by unpaired t-test with Welch's correction. **(H)** Annexin V by 7-AAD dot plots of 2 representative cases. The percentage of live (bottom left), early apoptotic (bottom right), or late apoptotic/necrotic (top right) cells is indicated in the respective quadrant.


A
pAkt (S473) levels
p < 0.0001
Pediatric
(7.02±1.73)
Adult
(2.44±0.16)
B
pS6 (S235/236) levels
p < 0.0001
Pediatric
(3.42±0.69)
Adult
(1.58±0.13)
C
pSTAT5 (Y694) levels
p = 0.3519
Pediatric
(2.54±0.37)
Adult
(2.61±0.21)
D
Viability Index
p = 0.0119
Pediatric
Adult
Buparlisib (5 μM)
E
Medium
Buparlisib
2.03
31.5
60.6
5.91
2.36
75.6
14.7
7.39
4.80
41.1
43.3
10.7
6.26
58.0
22.6
13.2
7-AAD
Annexin V
Pediatric
Adult


A
Viability Index (IL-7 response)
p = 0.0295
Pediatric (1.48±0.10)
Adult (1.14±0.07)
B
IL-7Rα surface expression (Geometric mean)
p = 0.2599
Pediatric
Adult
C
pAkt (S473) levels IL-7 response (fold change)
p = 0.0039
Pediatric (1.27±0.05)
Adult (0.95±0.04)
D
pS6(S235/236) levels IL-7 response (fold change)
p = 0.0129
Pediatric (1.21±0.07)
Adult (0.91±0.05)
E
pSTAT5(Y694) levels IL-7 response (fold change)
p = 0.0394
Pediatric (2.17±0.31)
Adult (1.32±0.16)
F
+ IL-7
pAkt (S473)
pS6 (S235/236)
pSTAT5 (Y694)
cCD79a
Pediatric
Adult
G
Viability Index (Response to STAT5i in the presence of IL-7)
p = 0.0246
Pediatric
Adult
H
Medium
STAT5i
7-AAD
Annexin V
Pediatric
Adult

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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SUPPLEMENTARY DATA

Materials and Methods

Supplementary Figures 1 and 2

Supplementary Table 1

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).

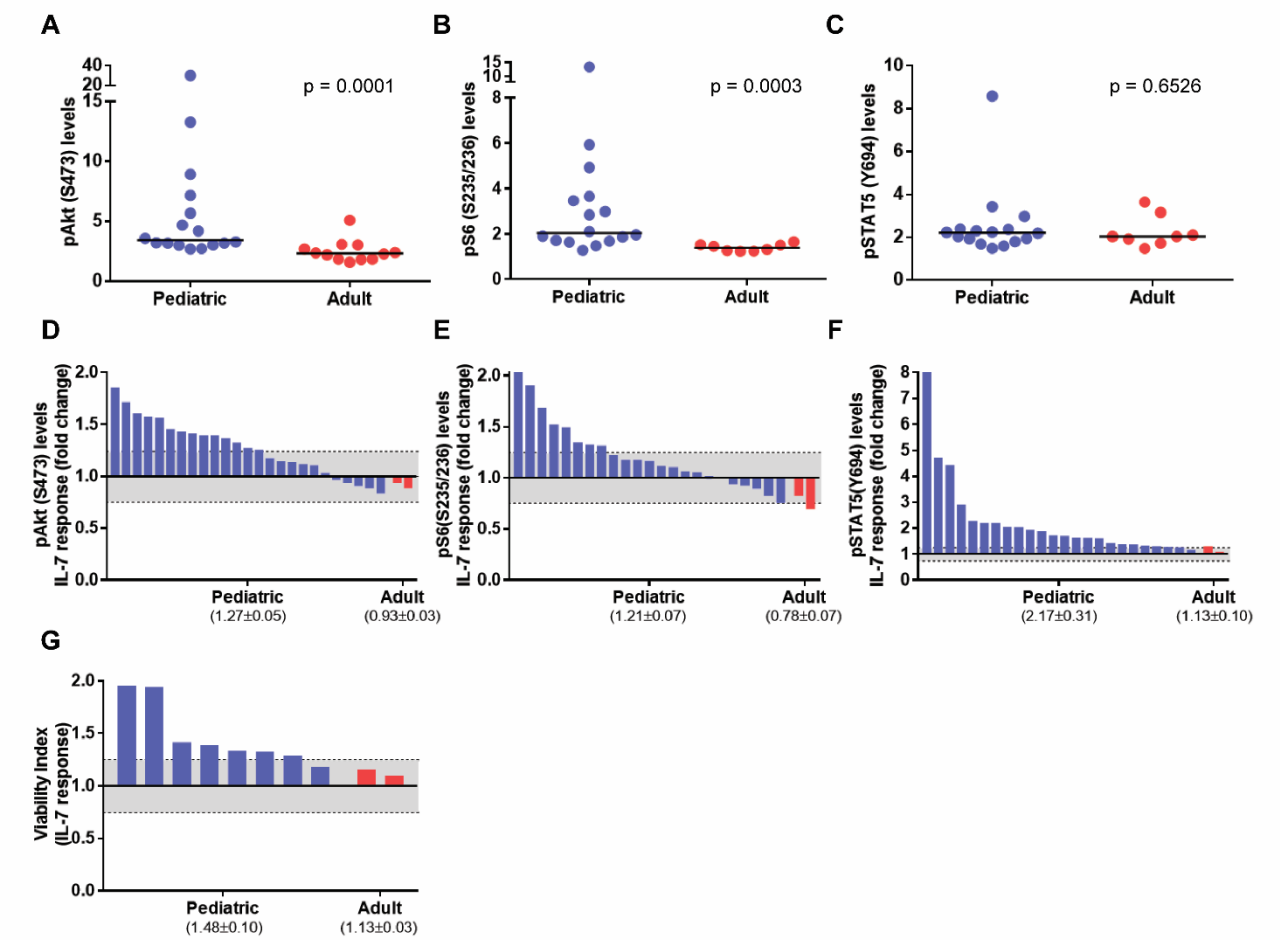
Intracellular phospho-specific flow cytometry. To assess the phosphorylation status of PI3K/Akt/mTOR and JAK/STAT5 pathways in primary B-ALL cells, intracellular phospho-flow cytometric analyses were performed as previously described (Gomes., *et al* Haematologica 2014; doi: 10.3324/haematol.2013.096438). 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.

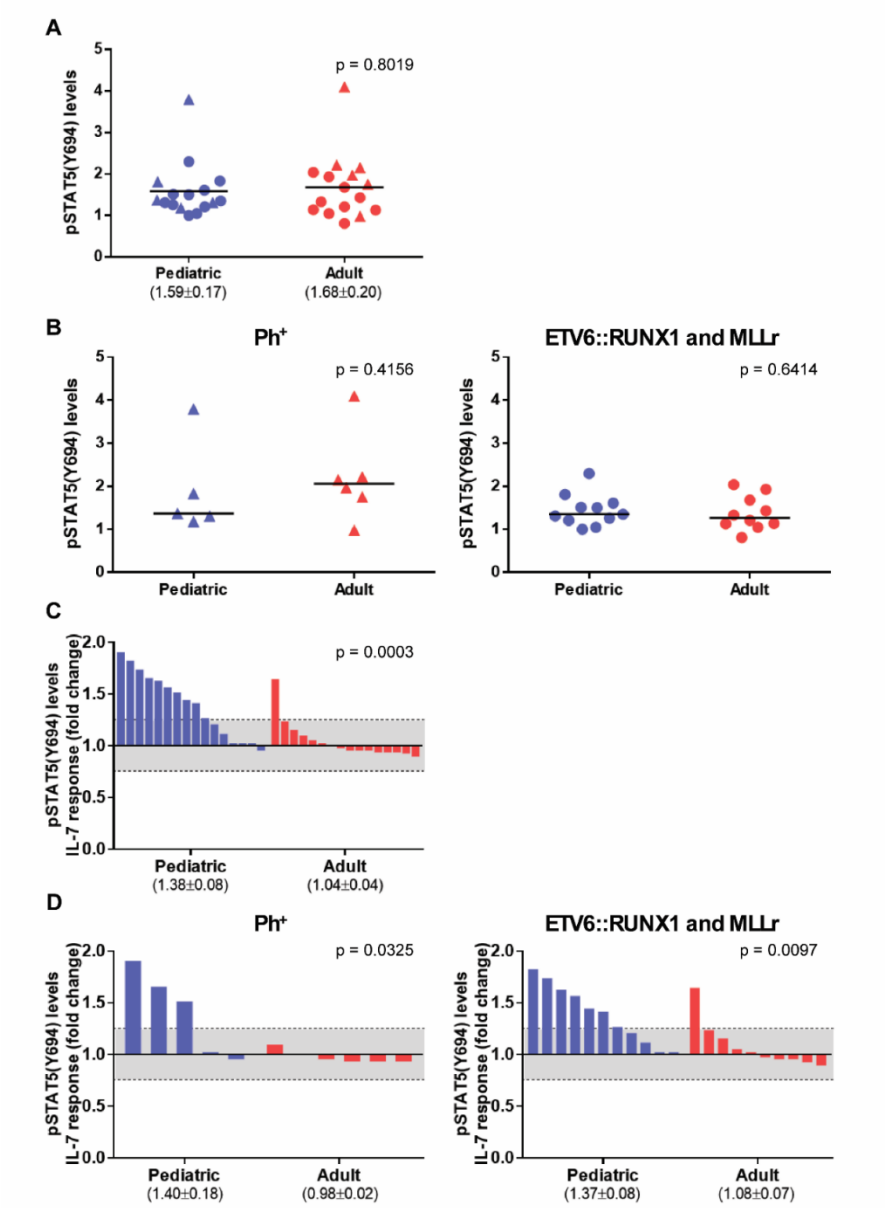
Flow cytometry analysis of IL-7Rα surface expression. For cell surface assessment of interleukin 7 receptor α chain (IL-7Rα or CD127) expression, cells were washed in ice-cold PBS and incubated for 30 min on ice with α-human IL-7R PE-conjugated antibody (R&D Systems). Cells were then washed in ice-cold 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