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

The age distribution of cases 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 cases of B-ALL has not been scrutinized. Here, we characterized the basal and IL-7-induced PI3K/Akt/mTOR and JAK/STAT5 signaling profile of pediatric patients age (range, 1-14 years) and adult patients age, (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 have an 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 has been poorly 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] The PI3K/Akt/mTOR pathway is frequently activated in pediatric B-ALL and associated with a poor response to chemotherapy.[10] Constitutive hyperactivation of this signaling axis was also observed in adult cases of B-ALL.[11] Thus, we questioned whether there are differences in activation of PI3K/Akt/mTOR signaling between the two age groups. Initially, we compared samples from children (N=40; median age, 4 years; range, 1-14 years) and adults (N=21; median age, 56 years; range, 29-75 years) collected at diagnosis after informed consent and under ethical approval of the Instituto Português de Oncologia de Lisboa and Hospital Santo António dos Capuchos, Lisbon, Portugal (see *Online Supplementary Table S1* – Exploratory cohort, for information about the patients). Flow cytometry analyses of Akt (S473) and S6 (S235/236) phosphorylation levels showed that PI3K/Akt/mTOR pathway activation was higher in pediatric cases 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 the 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 ALL 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 those of the remaining samples, whereas Ph^+ adult cases were among those with highest phospho-STAT5 (Figure 1C), confirming our previous observations.[11] These findings 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 Janus kinases) are more frequent in adults.

We, therefore, 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 a 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

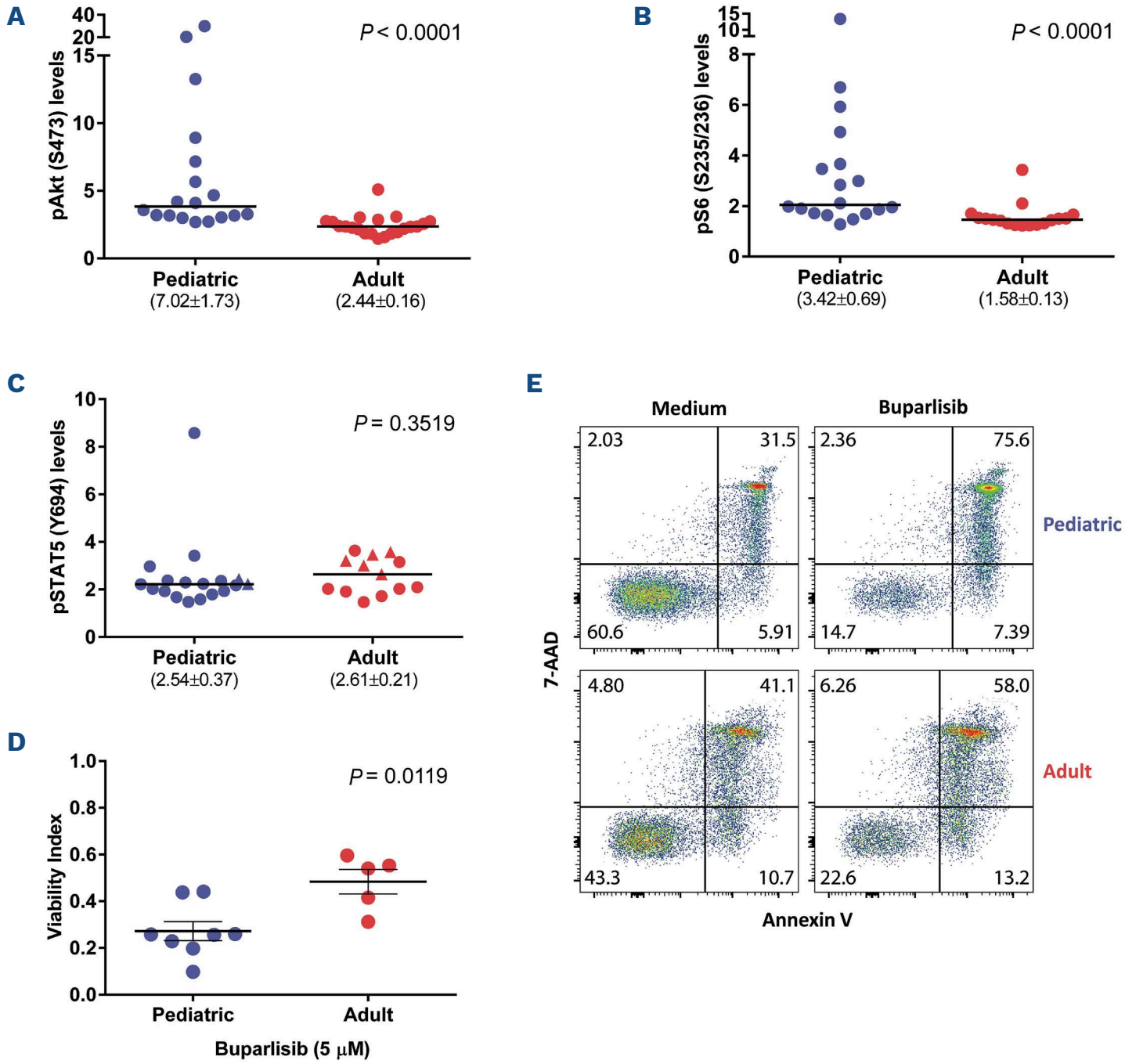

Figure 1. Higher PI3K/Akt/mTOR signaling pathway activation in pediatric B-cell acute lymphoblastic leukemia is associated with greater sensitivity to the PI3K inhibitor buparlisib. (A-C) Levels of phosphorylated Akt S473 (A), S6 S235/236 (B) and STAT5 Y694 (C) in bone marrow cells from pediatric and adult B-cell acute lymphoblastic leukemia (B-ALL) samples were quantified by flow cytometry analysis using phospho-specific antibodies. Points represent individual samples and horizontal bars denote the median. In (C), Philadelphia chromosome-positive patients are indicated by triangles. The mean ± standard of error of mean (SEM) is shown in parentheses. The statistical analysis was performed using a two-tailed Mann-Whitney test. (D, E) B-ALL samples were cultured for 72 h in medium alone or with buparlisib (5 μM), collected and stained with annexin V and 7-aminoactinomycin D (7-AAD) for cell viability assessment by flow cytometry. (D) The 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 using an unpaired *t* test with Welch correction. (E) Annexin V by 7-AAD dot plots of two representative cases. The percentages of live (bottom left), early apoptotic (bottom right), and late apoptotic/necrotic (top right) cells are indicated in the respective quadrants.

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

Table 1. Frequency of IL-7-induced phosphorylation of Akt (S473), S6 (S235/236) and STAT5 (Y694) in samples from pediatric and adult patients.

	Response to interleukin-7* N of positive cases/N of tested cases (%)		
	pAkt (S473)	pS6 (S235/236)	pSTAT5 (Y694)
Pediatric	13/24 (54)	8/23 (34.7)	20/24 (83)
Adult	0/6 (0)	0/6 (0)	3/6 (50)

*Fold-change values >1.25 were considered as a positive response.

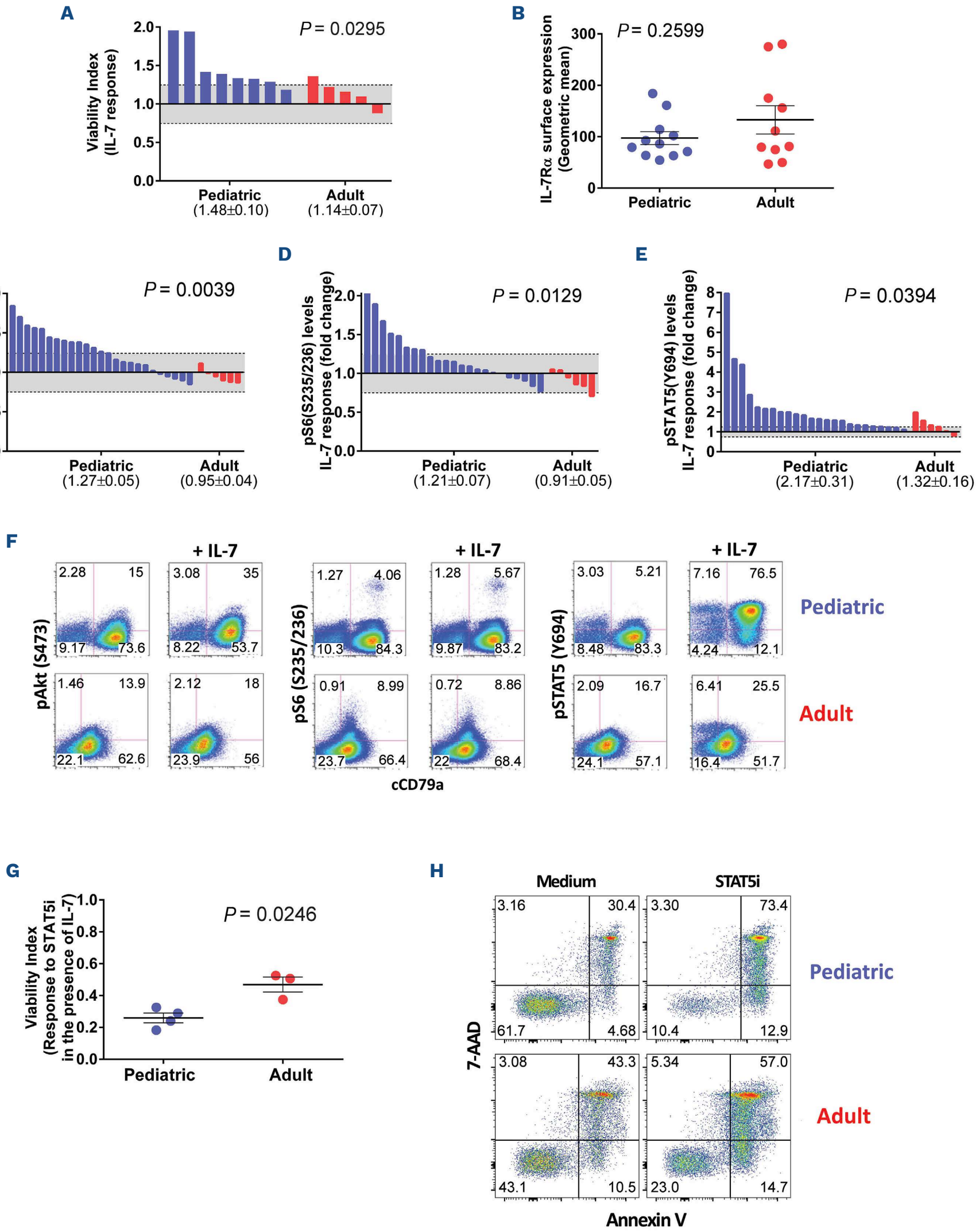

Figure 2. Samples from children with B-cell acute lymphoblastic leukemia have stronger signaling and functional responses to IL-7, and are more sensitive to STAT5 inhibition in the presence of IL-7, than samples from adults. (A) Bone marrow cells from pediatric and adult patients with B-cell acute lymphoblastic leukemia (B-ALL) were cultured for 72 h in the presence or absence of 10 ng/mL IL-7, stained with annexin V and 7-aminoactinomycin D (7-AAD) and cell viability was determined by flow cytometry analysis. The viability index, calculated as the ratio between viable cells in medium with IL-7 over medium alone, is indicated.

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Bars represent individual samples, horizontal dashed lines and the shaded area represent lack of response, defined as ≤1.25-fold change. The mean ± standard error of mean (SEM) is shown in parentheses. Statistical analysis was performed using a two-tailed Mann-Whitney test. (B) Cell surface levels of IL-7Rα were determined by flow cytometry analysis. Points represent individual samples, horizontal bars denote the mean ± SEM. Statistical analysis was performed using an unpaired *t* test with Welch 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 the shaded area represent lack of response, defined as ≤1.25-fold change. The mean ± SEM is shown in parentheses. Statistical analysis was performed using a two-tailed Mann-Whitney test. (G, H) B-ALL samples were cultured for 72 h in the presence of 10 ng/mL IL-7 alone or with a STAT5 inhibitor (STAT5i, 200 μM), stained with annexin V and 7-AAD and cell viability determined by flow cytometry. (G) The 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 the mean ± SEM. Statistical analysis was performed using an unpaired *t* test with Welch correction. (H) Annexin V by 7-AAD dot plots of two representative cases. The percentages of live (bottom left), early apoptotic (bottom right), and late apoptotic/necrotic (top right) cells are indicated in the respective quadrants.

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) in the presence of IL-7 for 72 h. B-ALL pediatric patient samples were more sensitive to STAT5 inhibition than adult patient samples as shown by a greater 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 (*Online Supplementary Figure S1*).

We next evaluated a confirmatory cohort of pediatric and adult French patients who were classified as having Ph⁺, *ETV6::RUNX1* or *KMT2A/MLL*-rearranged B-ALL. Samples were obtained, after informed consent and ethical approval, from patients at Hôpital Saint-Louis and Hôpital Robert Debré, Paris, France (*Online Supplementary Table S1* – Confirmatory cohort). Analysis of the three genetic subgroups altogether (*Online Supplementary Figure S2A*) or comparing non-Ph⁺ cases (*KMT2A/MLL*-rearranged and *ETV6::RUNX1*) *versus* Ph⁺ cases (*Online Supplementary Figure S2B*) confirmed the lack of differences in basal STAT5 phosphorylation levels between children and adults found in the Portuguese cohort of patients (Figure 1C). Moreover, we also confirmed that pediatric B-ALL samples respond better to IL-7 (*Online Supplementary Figure S2C*), irrespective of subtype (*Online Supplementary Figure S2D*).

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, *in vitro*, respond poorly to IL-7 but not to other growth factors.[15] Notably, the lesser fitness resulting from impaired IL-7-mediated signaling in aging B cells 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, in which 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 lesser ability of adult B-ALL cells to respond to IL-7 *ex vivo*, as we report herein, may at least partially reflect a normal aging 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 to 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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Disclosures

No conflicts of interest of interest to disclose.

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 patients' 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.

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Data-sharing statement

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

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