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Adult Low-Hypodiploid Acute Lymphoblastic Leukemia Emerges from Preleukemic TP53-Mutant Clonal Hematopoiesis

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ABSTRACTLow hypodiploidy defines a rare subtype of B-cell acute lymphoblastic leukemia
(B-ALL) with a dismal outcome. To investigate the genomic basis of low-hypodiploidALL (LH-ALL) in adults, we analyzed copy-number aberrations, loss of heterozygosity, mutations, and
cytogenetics data in a prospective cohort of Philadelphia (Ph)-negative B-ALL patients (*n* = 591, ages
18-84 years), allowing us to identify 80 LH-ALL cases (14%). Genomic analysis was critical for evidenc-
ing low hypodiploidy in many cases missed by cytogenetics. The proportion of LH-ALL within Ph-negative
B-ALL dramatically increased with age, from 3% in the youngest patients (under 40 years old) to 32% in the
oldest (over 55 years old). Somatic *TP53* biallelic inactivation was the hallmark of adult LH-ALL, present
in virtually all cases (98%). Strikingly, we detected *TP53* mutations in posttreatment remission samples in
34% of patients. Single-cell proteogenomics of diagnosis and remission bone marrow samples evidenced
a preleukemic, multilineage, *TP53*-mutant clone, reminiscent of age-related clonal hematopoiesis.SIGNIFICANCE: We show that low-hypodiploid ALL is a frequent entity within B-ALL in older adults,
relying on somatic *TP53* biallelic alteration. Our study unveils a link between aging and low-hypodiploid

- ALL, with TP53-mutant clonal hematopoiesis representing a preleukemic reservoir that can give rise to
- aneuploidy and B-ALL.
- 25 See related commentary by Ogawa and Saiki.

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INTRODUCTION 26

27 B-cell precursor acute lymphoblastic leukemia (B-ALL) represents a rare malignancy in adults associated with a poor prog-28 29 nosis, especially in the older range of patients (1-3). Despite 30 significant improvements provided by pediatric-inspired treatment regimens (4), there is still an important discrepancy 31 32 between B-ALL outcomes in children and adults, likely owing 33 to two major factors. First, treatment-related toxicity increases 34 with age, which prevents the use of intensive chemotherapy 35 (1, 5). Second, adult B-ALL have decreased sensitivity to treat-36 ments when compared with children due to different leuke-37 mia genomic backgrounds. Hence, large genomic studies have 38 highlighted the age-related prevalence of distinct genetic aber-39 rations, with a drop in low-risk abnormalities (i.e., high hyper-40 diploidy and ETV6::RUNX1) beyond the age of 10, together with the progressive increase in high-risk subtypes, i.e., BCR::ABL1, 41 BCR::ABL1-like, KMT2A-rearranged and low hypodiploidy (6-9). 42 Therefore, a better understanding of the pathogenesis of high-43 44 risk adult B-ALL is needed to improve clinical management. B-ALL with low hypodiploidy (LH-ALL) is a distinct 45 entity primarily defined by karyotype harboring 32 to 39 46

chromosomes with a nonrandom pattern of chromosome losses (10). Afterward, this entity was renamed "low hypodiploidy/near triploidy" to include cases with duplicated 50 low-hypodiploid genome sharing the same poor prognosis 51 (11). Within childhood B-ALL, LH-ALL represents a very small subset associated with a dismal outcome despite being 52 53 treated with the most intensive procedures (12). A previous study in pediatric ALL showed that most LH-ALL harbored 54 TP53 mutations, with half of them being of germline origin, connecting LH-ALL to the spectrum of the Li-Fraumeni cancer-predisposing syndrome (13). In adults, LH-ALL is also associated with poor outcomes (8) and frequent TP53 muta-58 tions (14-16), but few data addressed the somatic origin of 59 TP53 mutations. 60

Clonal hematopoiesis is a condition characterized by the 61 clonal expansion of hematopoietic stem/progenitor cells 62 (HSPC) carrying somatic mutations at detectable levels. 63 Sequencing of large cohorts of healthy subjects has shown 64 that the prevalence of clonal hematopoiesis increased con-65 tinuously with age, defining age-related clonal hematopoiesis 66 (ARCH; refs. 17-19). Importantly, TP53 is one of the most 67

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frequent genes involved in ARCH. In addition, TP53-mutant clonal hematopoiesis is associated with an increased risk of developing myeloid malignancies, including secondary and treatment-related myeloid neoplasms (20-22).

72 Here, we identified and characterized a large cohort of 73 adults with LH-ALL. Then, considering the dramatically 74 increased proportion of LH-ALL with age, we sought to inves-75 tigate the possible role of TP53-mutant clonal hematopoiesis 76 in the pathogenesis of adult LH-ALL. Using paired diagnosis/ 77 remission samples and a single-cell multiomics platform, we 78 demonstrate that LH-ALL can arise from a preleukemic TP53-79 mutant HSPC clone.

RESULTS 80

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DNA Sequencing-Based Assessment of Copy-81 Number Aberrations and Loss-of-Heterozygosity 82 Is Critical for Identifying LH-ALL 83

84 We studied a prospective cohort of adults with newly diag-85 nosed Philadelphia (Ph)-negative B-ALL with available diagnostic sample (n = 591; Fig. 1A). We used targeted-capture 86 sequencing to carry out copy-number aberrations (CNA) 87 88 analysis and classified cases by a modal chromosomal num-89 ber based on both karyotype and CNA data (Fig. 1B; Supplementary Fig. S1A and Supplementary Table S1). Noticeably, 90 91 hypodiploid leukemic cells can sometimes undergo endorep-92 lication, leading to a high number of chromosomes. There-93 fore, we examined all the cases presenting with either ≤ 40 (n = 41) or ≥ 50 chromosomes (n = 87). All cases with ≤ 40 chro-94 mosomes corresponded to LH-ALL, except one having near 95 haploidy, confirming that the latter corresponds to a subtype 96 virtually not found in adult ALL. Within cases with ≥50 chro-97 mosomes, loss-of-heterozygosity (LOH) analysis enabled to 98 99 identify cases with a duplicated low-hypodiploid genome, as 100 harboring multiple uniparental disomies corresponding to the same pattern as that of chromosome losses in classic low hypodiploidy (Supplementary Fig. S1B). Importantly, LOH 103 analysis unequivocally distinguished duplicated low hypo-104 diploidy from high hyperdiploidy, the latter corresponding to a distinct good-risk B-ALL subtype.

Nearly half (40/87, 46%) of all cases with \geq 50 chromosomes 106 turned out to have a duplicated low-hypodiploid genome (Fig. 1C). The ancestral low-hypodiploid clone was detectable by karyotype in only a few patients (5/40, 13%; Fig. 1D), suggesting that they could have been misclassified as high hyperdiploidy based on cytogenetics only. Moreover, in 26 of 80 cases (33%) 111 112 eventually classified as LH-ALL, conventional cytogenetics concluded to culture failure or normal karyotype, suggesting that 113 114 leukemic cells failed to grow in vitro. We also identified two 115 LH-ALL cases as having the canonical pattern of chromosome 116 losses, albeit with a modal chromosome number >39 due to 117 trisomy 21. Therefore, our data illustrate that CNA and LOH analyses are critical to allocate correctly B-ALL to distinct ane-118 119 uploidies representing clinically relevant B-ALL subtypes.

LH-ALL Represents a Major B-ALL Subtype in Older Adults

Overall, combined cytogenetic and molecular analyses of 122 591 Ph-negative B-ALL adult patients identified 80 patients 123 with LH-ALL (13.5%), either harboring classic low hypodiploidy (n = 40) or duplicated low hypodiploidy (n = 40). We analyzed the baseline characteristics of LH-ALL patients with regard to all 126 the other Ph-negative B-ALL from adult patients (Table 1). LH-127 ALL patients were significantly older than other B-ALL patients (median 59 vs. 39 years, P < 0.0001) and there was a dramatic increase in LH-ALL prevalence with age, ranging from 3% (8/267) 130 in patients below the age of 40 to 32% (55/171) in patients over 131 55 years. Other distinct features of LH-ALL included a lower 132 white blood cell count (3.2 vs. 7.5 G/L, P < 0.001) and a lower 133 marrow blast infiltration (79 vs. 92%, P < 0.001). Overall, LH-134 ALL represents a large fraction of adult Ph-negative B-ALL and 135 has an age-related distribution, from being rare in young adults 136 to become very common in older patients.

Adult LH-ALL Exhibits TP53 Biallelic Alteration and a Distinct Pattern of Chromosomal and Gene Abnormalities

We performed targeted DNA sequencing (DNA-seq) of a panel of genes recurrently altered in B-ALL and integrated karyotype, CNA, LOH, and mutation data to characterize the full spectrum of genomic alterations in adult LH-ALL. The pattern of chromosome losses was highly recurrent, including chromosomes 3, 7, 16, and 17 in virtually all cases and chromosomes 13 and 15 in over 80% of cases, whereas chromosomes 2, 4, 9, 12, and 20 were more variably lost, in half of cases each (Fig. 1D; Supplementary Fig. S1C). Of note, chromosome 21 was retained in all cases. In addition, structural abnormalities were present in 31 of 80 (39%) cases.

We found TP53 mutations in 78 of 80 (98%) cases (Fig. 2A; 152 Supplementary Table S2). They included missense, nonsense, 153 splice-site mutations, and short insertions/deletions, all pre-154 dicted to be pathogenic (Fig. 2B). Monosomy 17 or uniparental disomy led to the loss of the second TP53 allele 156 in all cases but one having biallelic TP53 mutations. TP53 157 mutations were present at variant allele frequencies (VAF) correlated with leukemic cell infiltration (Fig. 2C), suggest-159 ing that alterations of both alleles were present in the major 160 clone. Noticeably, considering the whole cohort of adult Phnegative B-ALL, 76% (78/102) of patients with TP53 mutation 162 had LH-ALL, whereas TP53 mutations were rarely detected 163 in other B-ALL (5% vs. 98%, P < 0.001, two-tailed Fisher test; 164 Fig. 2D). Therefore, biallelic alteration of TP53 is a hallmark 165 of LH-ALL within adult B-ALL.

Alterations of genes involved in cell-cycle regulation defined the second most frequently altered pathway. Specifically, focal deletions and loss-of-function mutations in the CDKN2A and RB1 genes (Fig. 2A; Supplementary Fig. S2A), associated with monosomies 9 and 13, led to biallelic inactivation in 21 (26%) and 18 (23%) cases, respectively, in a mutually exclusive 172

Figure 1. Identification of a cohort of 80 LH-ALL cases by karyotyping and sequencing-based assessment of CNA and LOH. A, Flow diagram describing the study cohort of Ph-negative B-ALL adult patients. B, Distribution of B-ALL cases according to their modal chromosome number assessed by karyotyping and/or sequencing-based CNA analysis. C, Proportion of LH-ALL within cases with ≤40 chromosomes and cases with ≥50 chromosomes after integration of sequencing-based LOH analysis. D, Heat map of chromosome anomalies as determined by karyotyping or CNA/LOH analysis in the 80 LH-ALL cases.



Table 1. Demographic and clinical characteristics ofpatients at baseline.

		LH-ALL	Non LH-ALL	Р
	Patients, n	80	511	
	Sex, n (%) Male Female	42 (53) 38 (47)	269 (53) 242 (47)	1
	Age, year Median Range	59 18-84	39 18-84	<0.0001
	Age group, n (%) 18-24 y 25-39 y 40-54 y ≥ 55 y	5 (6) 3 (4) 17 (21) 55 (69)	123 (24) 136 (27) 136 (27) 116 (23)	<0.0001
	White blood cell cou Median Range	ntª, Giga per lit 3.2 0.4–87.1	er 7.5 0.4-712.0	<0.0001
	Marrow blast infiltra Median Range	ation ^ь , % 79 21-99	92 12-100	<0.0001
	CD10 expression ^c , <i>n</i> Positive Negative	(%) 62 (79) 16 (21)	382 (76) 118 (24)	0.67

^aWhite blood cell counts were available for 582 patients. ^bMarrow blast cell counts were assessed for 569 patients. ^cCD10 expression was assessed by flow cytometry for 578 patients. Two-tailed independent t tests and two-tailed Fisher tests were used for continuous and categorical variables, respectively, and statistical significance was defined as a P < 0.05.

pattern. The lymphoid transcription factor gene *IKZF2* was
recurrently affected by focal deletions, observed in 15 cases
(19%). By contrast, no intragenic deletion or mutation of *IKZF1*, one of the most frequently altered genes in adult
B-ALL, was found, although hemizygosity was observed in all
cases as a result of monosomy 7. Similarly, *PAX5* was rarely
targeted by focal deletions or mutations (8%).

180 Aberrations involving cell signaling genes were present in 25 (31%) LH-ALL cases, including loss-of-function mutations 181 and focal deletions of NF1 in 18 (23%) cases (Supplemen-182 183 tary Fig. S2B). We also identified FLT3 mutations in 6 cases 184 (8%; Supplementary Fig. S2C), NRAS mutations in 3 cases 185 (4%), and remarkably, JAK2 p.V617F mutation in one case 186 (EI_046). Finally, we detected TET2 and DNMT3A mutations 187 corresponding to classic mutations reported in ARCH and myeloid malignancies in 11% and 10% of cases, respectively 188 189 (Supplementary Fig. S2D-S2E).

Altogether, adult LH-ALL is characterized by a distinct
pattern of aneuploidy, consistent biallelic alteration of *TP53*,
and a constellation of additional gene alterations, the most
frequent involving *CDKN2A*, *RB1*, *NF1*, and *IKZF2*.

TP53 Mutations Are Detected at Remission in a194**Substantial Proportion of Adults with LH-ALL**195

Given the age-related distribution of LH-ALL and the role 196 of TP53 mutations in ARCH, we hypothesized that LH-ALL 197 may be secondary to TP53-mutant ARCH. To test this hypothesis, we looked for TP53 mutations in posttreatment remis-199 sion bone marrow samples assessed for minimal residual 200 disease (MRD). Among the 73 patients with available samples, 201 we observed persistence of the TP53 mutation identified at 202 diagnosis in 25 patients (34%), at VAF ranging from 2.6% to 203 51.2% (cutoff value for positivity set at 2%; Fig. 3A and B; Sup-204 plementary Table S3). DNMT3A and TET2 mutations were 205 also detected in remission samples from 9 and 6 patients, 206 respectively. MRD levels based on the quantification of clonal 207 rearrangements of immunoglobulin or T-cell receptor genes 208 (IG/TR) were either undetectable or measured at much lower 209 levels (Supplementary Table S4), indicating that those mutations were not related to residual B-ALL leukemic cells. 211

It should be noticed that 5 patients had a *TP53* mutation with a VAF >40%, raising the possibility of a germline origin, as previously observed in pediatric LH-ALL. This could be ruled out in two patients for whom *TP53* mutation was not detected in nonleukemic cells (Supplementary Table S3). In the three remaining patients, including one with detectable *TP53* mutation in sorted T cells and two without available nonleukemic material, a germline origin could not be ruled out.

Next, to evaluate the dynamics of the TP53-mutant cell fraction, we used digital droplet PCR (ddPCR) to quantify 221 TP53 mutations in 9 patients with available longitudinal 222 postremission bone marrow samples (Fig. 3C; Supplementary 223 Fig. S3). In two patients with confirmed somatic TP53 muta-224 225 tion, the TP53-mutant clone remained in a steady state at high rates at all time points. Four patients displayed a reduc-226 tion of the TP53-mutant fraction over time, yet uncoupled 227 from lower or undetectable IG/TR MRD levels. Noticeably, in 228 two of these patients, the higher sensitivity of ddPCR allowed 229 to detect a low fraction of persistent TP53-mutant cells not 230 detected by sequencing. In three other patients, no TP53mutant cells were detected. Overall, these results reveal that a 232 substantial proportion of adults with LH-ALL carry somatic 2.3.3 TP53 mutations in nonleukemic cells repopulating bone mar-234 row after treatment. 235

Integrated Single-Cell Genotyping and Immunophenotyping Reveals Multilineage TP53-Mutant Clonal Population at Remission Stage

We sought to investigate the contribution of the *TP53*-239 mutant clone to the different hematopoietic lineages and 240 infer the oncogenic route toward LH-ALL. Using antibody-241 derived tag (ADT) sequencing and a custom single-cell DNA-242 seq (scDNA-seq) panel (23–25), we performed simultaneous 243 single-cell immunophenotyping and genotyping on three 244 bone marrow specimens obtained after induction. The three 245

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Figure 2. Landscape of recurrent genomic alterations in adult LH-ALL. **A**, Heat map of recurrent CNA and mutations in the 80 LH-ALL cases. Focal alterations refer to CNA not involving whole chromosome arms and mutations. Cases with several mutations are referred to as multihit. **B**, Lollipop plot depicting *TP53* mutations detected in both LH-ALL and other Ph-negative B-ALL. **C**, Correlation between *TP53* mutation VAF and blast percentage, as assessed by flow cytometry after ficoll or morphology on marrow smear, using Pearson correlation coefficient. **D**, Proportion of *TP53* mutations in LH-ALL and other Ph-negative B-ALL with available data from the study cohort (*n* = 499). Het, heterozygous; Hom: homozygous; indel: insertion/deletion.

RESEARCH ARTICLE





Figure 3. Tracking of ARCH-related mutations in remission samples of LH-ALL patients. **A**, Heat map of mutations in paired diagnosis and remission samples for 71 patients. Data are based on sequencing data with a threshold for positivity at 2% VAF. **B**, Box plot for VAF of mutations detected by sequencing at remission. **C**, Longitudinal assessment of *TP53*-mutant cell fraction as determined by ddPCR (0.05–0.1% sensitivity), along with clonal *IG/TR*-based MRD quantification (0.01%–0.001% sensitivity), for six patients with *TP53* mutation detected in remission samples, including two below the sequencing detection threshold.



Figure 4. Integrated single-cell genotyping and immunophenotyping reveal a multilineage *TP53*-mutant clonal population at the remission stage in LH-ALL patients. **A**, Uniform Manifold Approximation and Projection (UMAP) plot of 10,598 cells from remission samples of three patients (EI_047, EI_031, and EI_035 having 1,017, 5,450, and 4,131 cells available for analysis, respectively). Cells are clustered by expression of cell-surface markers and colored according to assigned clusters. **B**, Same UMAP plots with cells colored by expression of specific cell-surface markers used to assign cell clusters. (continued on next page)

patients were treated in the EWALL-INO trial and had a *TP53*mutation detected at postinduction remission, whereas MRD
measured on the same sample was either low or undetectable.
Protein expression data allowed discrimination of 7 distinct clusters (Fig. 4A). Based on lineage-specific markers, we

were able to identify confidently T cells, B cells, monocytes, 251 dendritic cells (DC), and myeloid and erythroid cell clusters 252 (Fig. 4B; Supplementary Fig. S4A and S4B). Cell identity 253 could not be assigned to the remaining cluster owing to 254 unspecific and weak labeling of frail cells (thereafter named 255



Figure 4. (Continued) C, Same UMAP plots (top) for individual patient's remission samples, with cells colored according to TP53 genotype. Histograms (bottom) show the proportion of each TP53 genotype within each cell cluster. D, UMAP plots for the EI_035 remission sample, with cells colored according to genotype for several heterozygous SNPs allowing LOH assessment. ref, reference allele; alt, alternative allele.

"unspecific labeling"). Of note, few B cells were detected, which may be related to therapy including B cell-directed antibodies. Cell type composition was comparable across patients, but analyses on each sample individually allowed better identification of discrete cell populations not detected in all patients, such as natural killer (NK) cells and plasmacytoid dendritic cells (pDC; Supplementary Fig. S5).

Projection of the TP53 genotype on the protein uniform 264 manifold approximation and projection (UMAP) representation allowed us to describe the cell architecture of 265 the TP53-mutant clone (Fig. 4C; Supplementary Fig. S6). Patients EI_031 and EI_047 had similar patterns with TP53heterozygous mutant cells detected in substantial fractions of myeloid, erythroid, and monocyte/DC clusters (hereafter col-270 lectively named myeloid clusters). The "unspecific labeling" cluster was likely related to myeloid populations according 271 to the similar proportion of mutant cells. T-cell populations 272 273 also contained mutant cells, yet at lower rates. For patient 274 EI_031, NK cells, pDC, and B-cell clusters could be identi-275 fied and also harbored mutant cells. Of note, the observation 276 of rare cells with a homozygous genotype was likely related

to allelic dropout and biologically not relevant, in agree-
ment with similar proportions observed for constitutional
heterozygous single-nucleotide polymorphisms (SNP; Sup-
plementary Fig. S7).277
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Patient EI_035 harbored a large fraction of cells in 281 myeloid clusters carrying TP53-homozygous mutation, 282 pinpointing a concomitant CNA or copy-neutral LOH in 283 those cells. By contrast, T cells were mainly wild-type, with 284 a minority being heterozygous. Including amplicons cover-285 ing common SNPs in the scDNA-seq panel allowed us to 286 investigate allelic imbalance throughout the genome at the 287 single-cell level. Thus, we evidenced LOH at 17p (but not 288 at 17q) in myeloid clusters, suggesting a cytogenetic aber-289 ration at 17p resulting in the loss of the wild-type allele 290 (Fig. 4D; Supplementary Fig. S8). Those cells also had 291 LOH at 5q and 16q, whereas no other LOH possibly related 2.92 to the LH-ALL clone was observed. Thus, the presence of 293 those aberrations restricted to the myeloid compartments 294 may be related to a distinct, myelodysplastic clone, only 295 sharing the TP53 alteration with the LH-ALL clone. Alto-296 gether, these results show that patients with LH-ALL carry 297

TP53-mutant HSPCs able to repopulate bone marrow after 299 intensive treatment.

LH-ALL Arises from Preexisting TP53-Mutant 300 301 Clonal Hematopoiesis

302 We next aimed to address whether the TP53-mutant multi-303 lineage cell population arose from clonal selection under the stress of cytotoxic therapy for B-ALL or preexisted at diagno-304 sis. We profiled 14,518 cells from three diagnostic samples, 305 306 including two paired with remission samples already presented 307 (EI_047 and EI_035). ADT-sequencing and IG/TR clono-specific sequences enabled to recognize B-ALL leukemic cells and 308 309 to identify minor nonleukemic cell populations (Fig. 5A and B; Supplementary Fig. S9). In B-ALL cells from the 3 patients, 310 TP53 and SNP single-cell genotyping showed homozygous 312 (hemizygous) TP53 mutation and LOH at multiple loci (Fig. 5C; Supplementary Fig. S10), in agreement with bulk 313 data (Fig. 1D; Supplementary Table S2). In EI_046 and EI_047, 314 substantial fractions of TP53-heterozygous mutant cells were 315 observed within myeloid and NK cell populations, as observed 317 in the paired-remission sample of EI_047. Similarly, in EI_035, 318 the myeloid population was predominantly TP53-homozygous mutant. By contrast, the small subset of TP53-heterozygous 319 mutant T cells was likely related to the cell doublet artifact, in agreement with a similar rate of detection of clonal IG/TR 321 rearrangements (Supplementary Table S5). Consistent results 323 were obtained through analyses of the fluorescence-activated cell sorting (FACS) of cell fractions (Supplementary Table S6). 324 325 Overall, these results confirm that the TP53-mutant mye-326 loid population preexisted at B-ALL diagnosis and indicate that TP53-mutant ARCH likely preceded the onset of B-ALL. 327

Other ARCH-Related Genes Can Be Involved in the 329 Pathogenesis of LH-ALL

Because other ARCH-related mutations were identified in 330 331 bulk diagnostic samples from several patients with LH-ALL, we aimed to investigate their relation with TP53-mutant 333 clonal hematopoiesis and LH-ALL and decipher the sequence of mutation acquisition. 334

EI_047 had a TET2 mutation detected in bulk sequencing at 336 diagnosis at 48% VAF. Unexpectedly, single-cell analysis of the diagnosis sample revealed that this mutation was restricted 337 338 to B-ALL cells and was not carried by myeloid cells (Fig. 6A). Accordingly, it was not detected in the bulk remission sample. 339 EI_046 harbored a JAK2 V617F detected at 7% VAF in the 340 341 bulk diagnosis sample. Single-cell analysis revealed that it was 342 restricted to myeloid and erythroid cells, in a homozygous 343 state, reminiscent of myeloproliferative neoplasms (MPN). 344 Moreover, the TP53 and JAK2 mutations appeared to be in 345 independent clones, as no cell carried both mutations (Supplementary Fig. S11). Therefore, single-cell analysis uniquely 346 evidenced a JAK2-mutant ARCH or MPN-like clone, concomi-347 tant but clonally unrelated to B-ALL. 348

349 EI_035 had a DNMT3A mutation at diagnosis (VAF 45%), 350 still detected at remission (VAF 42%). Single-cell analysis of the diagnosis sample showed that almost all B-ALL and 351 nonleukemic cells carried heterozygous DNMT3A mutation. 352 The presence of TP53-wild-type DNMT3A-mutant cells in the 353 354 remission sample (Supplementary Fig. S11) indicated that DNMT3A mutation occurred earlier than TP53 mutation. In 355

addition, and as observed at remission, myeloid cells had LOH 356 at 5q, 16q, and 17p, suggesting a premalignant, concurrent 357 clone at B-ALL diagnosis (Fig. 6B). All B-ALL cells had LOH at 358 these loci and at additional loci in relation with low hypodip-359 loidy. However, the discordant homozygous genotype at 16q between B-ALL and myeloid cells indicated a branched rather 361 than linear relation between the myeloid and B-ALL clones.

Overall, these data allow to reconstruct the sequence of mutation acquisition (Fig. 6C) and show that adult LH-ALL can develop on a background of clonal hematopoiesis resembling myelodysplastic syndrome (MDS) or MPN.

DISCUSSION

Our study establishes a comprehensive genomic characterization of the largest series of LH-ALL reported so far. By combining sequencing-based CNA and LOH analyses to cytogenetics data in a prospective cohort of 591 Ph-negative adult ALL, we identified a high proportion of LH-ALL, reaching 32% in the older range of patients. This increased prevalence in older adults is in agreement with, but higher than, that observed in previous studies, including the recent UKALL study of older adult ALL (9). Although the diagnostic pitfall of duplicated hypodiploidy/near triploidy is recognized, our data suggest that a number of LH-ALL cases are still being missed or misclassified owing to misleading normal or hyperdiploid karyotypes. Hence, CNA/LOH analyses in our study allowed to rescue more than a quarter of LH-ALL cases with normal/failed karyotype, whereas duplicated low hypodiploidy represented up to half of all LH-ALL. Of note, we also showed that LH-ALL patients present with lower blast counts in bone marrow and peripheral blood at diagnosis, which may explain underrepresentation in retrospective molecular studies relying on frozen samples. These findings warrant the implementation of CNA/LOH analysis in diagnostic laboratory practices, because genetic classification is critical for riskadapted treatment stratification in most modern pediatric and adult clinical trials. The improved genetic assignment will also allow the refinement of future clinical correlation studies.

Our single-cell sequencing data on diagnostic and remission samples indicate that TP53 mutation is a preleukemic event preceding aneuploidy with monosomy 17 leading to the loss of the TP53 wild-type allele. Although a dominant-negative effect of TP53 missense mutations has been proposed to mediate the 397 nonmutational inactivation of wild-type TP53 (26), it is noteworthy that genomic alteration of the second allele seems to be mandatory in the pathogenesis of LH-ALL. The specific pattern of chromosomal losses in adult LH-ALL, including the core association of 3, 7, 16, and 17 monosomies, is consistent with that observed in childhood LH-ALL and may inform about key genes involved in LH-ALL oncogenesis. Loss of chromosome 7 results in haploinsufficiency for IKZF1, a critical gene for normal 405 B-cell differentiation, the recurrent loss of which in B-ALL drives 406 a pejorative impact (27). Chromosome 16 contains CREBBP, 407 which is also targeted by focal alterations in B-ALL, especially in 408 relapsed cases (28). Noteworthy, as the CD19 gene is also located 409 on chromosome 16, LH-ALL having only one allele may be 410 prone to CD19 expression loss in the context of selective pres-411 sure with CD19-directed therapeutic agents (29). Chromosome 412 3 contains several tumor suppressor genes previously shown 413



Figure 5. Single-cell analysis at LH-ALL diagnosis identifies a preexisting multilineage *TP53*-mutant clonal population. **A**, UMAP plots for individual diagnostic samples from three patients (EI_47, EI_46, and EI_035), having 5,228, 4,947, and 4,343 cells available for analysis, respectively. Cells are clustered by expression of cell-surface markers and colored according to assigned clusters. **B**, Same UMAP plots for patients EI_047 and EI_035, with cells colored according to the presence of specific clonal *IG/TR* sequences allowing to identify B-ALL cells. Histograms show the proportion of positive cells within each cell cluster. **C**, Same UMAP plots (top) with cells colored according to *TP53* genotype. Histograms (bottom) show the proportion of each *TP53* genotype within each cell cluster.

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Figure 6. Integration of ARCH mutations into the clonal architecture of LH-ALL. **A**, UMAP plots for individual diagnostic samples from three patients with cells clustered by expression of cell-surface markers and colored according to genotypes of *TET2*, *JAK2*, and *DNMT3A* mutations in El_047, El_046, and El_035, respectively. Histograms show the proportion of each genotype within each cell cluster. **B**, UMAP plots for the El_035 diagnostic sample, with cells colored according to genotype for several heterozygous SNPs allowing LOH assessment. (*continued on next page*)

to be involved in B-ALL, i.e., *CD200/BTLA* locus and *SETD2*(27, 30, 31). Thus, by occurring at once, all these monosomies
resulting in the loss of several key tumor suppressor genes may
drive transformation and shape the tumor biology of LH-ALL.

418 Additionally, this combinatorial pattern could also be the result

of the counter-selection of other aneuploidies possibly lethal to the target cell.

The genomic profiling of LH-ALL evidenced additional421recurrent alterations in LH-ALL. Frequent and mutually422exclusive alterations of the cell-cycle genes CDKN2A and RB1423

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suggest their functional redundancy and an oncogenic cooperation with a *TP53*-deficient background. We identified *NF1*as a frequent target gene in adult LH-ALL, whereas it was not
observed in their pediatric counterpart (13).

428 DNMT3A and TET2 were among the top mutated genes, found in 10% of patients. As these genes are the most fre-429 quently mutated in ARCH (17), these mutations may simply 430 431 reflect the older age of LH-ALL patients. Single-cell analy-432 sis revealed different possible clonal involvement of those 433 mutations not inferred by bulk sequencing. In one patient, 434 DNMT3A mutation was the earliest somatic event, found in 435 an ancestral multilineage hematopoietic clone, as previously shown in acute myeloid leukemia (AML; ref. 32). By contrast, 436 437 in another patient, TET2 mutation was found as a secondary genetic event restricted to leukemic cells, suggesting an unan-438 ticipated oncogenic role of TET2 in B-ALL. In addition, in one 439 440 patient with JAK2 V617F mutation detected in bulk diagnosis, the single-cell analysis demonstrated that it was clonally 441 442 unrelated to TP53-mutant ALL cells, which is reminiscent

of post-MPN *TP53*-mutant AML lacking the MPN driver 443 mutation (33). 444

The major finding of our study is that TP53 mutations are not 445 only frequently associated with LH-ALL, but they are indispen-446 sable genetic events preceding aneuploidy. Hence, we detected 447 TP53 mutations in virtually all LH-ALL cases and demonstrated 448 that TP53-mutant heterozygous cells constituted a preleukemic 449 compartment. This oncogenic route relying on initiating TP53 450 mutation is thus distinct from that observed in other malignan-451 cies, including ALL (34), where TP53 mutations often occur 452 during tumor progression as a late event. In LH-ALL, TP53 453 mutation likely acts as the triggering event for genome instabil-454 ity, allowing the emergence of the low-hypodiploid clone respon-455 sible for leukemia onset. The further step of whole-genome 456 duplication, whereas otherwise rare in ALL, may be also related 457 to TP53 deficiency, as reported in other tumors (35). 458

Moreover, we show that somatic *TP53* mutations in LH-ALL 459 affect preleukemic HSPCs that retain their ability to generate mature blood cells, defining clonal hematopoiesis. This 461

connects LH-ALL to the spectrum of hematologic malignancies 462 463 related to TP53-mutant clonal hematopoiesis, namely, secondary and therapy-related AML and MDS having TP53 biallelic 464 alteration and complex karyotype (20-22). We also show that, 465 at least in some cases, a myeloid premalignant clone may evolve 466 467 concurrently to LH-ALL, which may support treatments aiming 468 at eradicating not only B-ALL but TP53-mutant clonal hematopoiesis. Altogether, our study sheds light on an unsuspected 469 470 link between LH-ALL, TP53-mutant clonal hematopoiesis, and

- related myeloid malignancies and paves the way for future inves-471
- 472 tigations regarding its clinical and therapeutic relevance.

METHODS 473

Patients and Samples 474

475 The study cohort included patients with Ph-negative B-ALL ages 18 476 to 59 years enrolled in the GRAALL-2014 trial between 2016 and 2020 477 and French patients ages ≥55 years enrolled in the EWALL-INO trial 478 between 2017 and 2022 (ClinicalTrials.gov number NCT02617004 and 479 NCT03249870, respectively). The GRAALL-2014 protocol was an intensive pediatric-inspired treatment similar to the GRAALL-2005 (5), but 480 481 with age adaptation of doses for patients 45 to 59 years old. The standard 482 induction comprised prednisone, daunorubicin, vincristine, cyclophos-483 phamide, L-asparaginase, and intrathecal prophylaxis. The EWALL-INO 484 protocol was a treatment based on a low-intensity chemotherapy back-485 bone and intrathecal prophylaxis with the addition of inotuzumab ozo-486 gamicin (INO) during the two-phase induction. The first induction phase 487 comprised 3 doses of INO (D1: 0.8 mg/m², D8 and D15: 0.5 mg/m²) 488 in combination with weekly vincristine 2 mg i.v. and dexamethasone 489 40 mg for 4 weeks, and the second phase comprised 2 doses of INO at 490 0.5 mg/m^2 in combination with dexamethasone 20 mg (D1 and D8) 491 and cyclophosphamide 300 mg/m²/day i.v. D1-3. All the patients pro-492 vided written informed consent for sample banking and analyses. The 493 study was in accordance with the Declaration of Helsinki. Cytogenetic 494 analyses at diagnosis were performed by local laboratories using standard 495 procedures. Molecular analyses were performed centrally as previously 496 described (36). Briefly, mononuclear cells from pretreatment bone mar-497 row or peripheral blood samples were isolated by Ficoll centrifugation, 498 and blast percentage was assessed by flow cytometry in most cases before 499 nucleic acid extraction. MRD was assessed by the quantification of clonal 500 IG/TR rearrangements, according to the EuroMRD guidelines.

Targeted DNA Sequencing 501

502 A custom panel of genes previously known to be targeted by recurrent mutations or CNA in B-ALL was analyzed by pan-exon capture-based 503 504 target enrichment (SureSelectXT Low Input Target Enrichment System, 505 Agilent) followed by library sequencing on the Illumina NextSeq500 platform (Illumina) as previously described (gene list in Supplementary 507 Table S7; ref. 36). Data were analyzed for variant calling using Varscan (37) and Pindel (38), and CNA analysis was conducted using Viscap (39) 509 and Facets (40). Lollipop plots were drawn with ProteinPaint (41).

B-ALL Classification Based on Cytogenetic and Molecular 510 511 Data

512 B-ALL cases were first classified by the modal chromosome number of 513 leukemia cells into three categories ($\leq 40, 41-49, and \geq 50$ chromosomes), 514 referring to the major abnormal clone at karyotype analysis when it was 515 informative (i.e., presence of abnormal mitosis) and DNA sequencing-516 based CNA analysis. Cases with ≤40 or ≥50 chromosomes were further examined as possibly being LH-ALL. By definition, all cases with 32 to 517 518 39 chromosomes (corresponding to 7-14 chromosome losses) were con-519 sidered LH-ALL. Two additional cases having 40 chromosomes with a 520 similar pattern of 7 chromosome losses and trisomy 21 were considered LH-ALL. Near haploidy was defined as 24 to 31 chromosomes. Within 521

cases with ≥50 chromosomes, LOH analysis allowed us to identify 522 duplicated LH-ALL cases as cases with LOH affecting at 7 to 14 chromo-523 524 somes. One case with LOH affecting 20 chromosomes was considered as duplicated near haploidy. High hyperdiploidy was defined as 51 to 65 chromosomes in the absence of other subtype-defining alteration. 526

TP53 Mutation Quantitation by Digital Droplet PCR

We performed ddPCR using the QX200 Droplet Digital PCR System (Bio-Rad) to track TP53 mutations detected in bulk sequencing of diagnosis samples, in serial bone marrow posttreatment remission samples. Commercial assays for TP53 p.R175H (dHsaMDV2010105), p.H214R (dHsaMDS2510824), and p.R248Q (dHsaMDV2010127) were used on the QX200 Droplet Digital PCR System (Bio-Rad). Reaction mix was prepared using 2× ddPCR TM Supermix for Probes (no dUTP), primers and probe mix (20x), restriction enzyme MseI 2U and 100 ng of genomic DNA as a template for ddPCR assay in a 96-well plate according to the manufacturer's protocol. Patients' diagnosis DNAs were used as positive control, peripheral blood mononuclear cells from healthy donors were used as negative control, and water was used instead of DNA for the nontemplate control reaction. Data were analyzed using QX Manager Software Standard edition, version 1.2 (Bio-Rad).

Combined Single-Cell Immunophenotyping and Genotyping

A custom scDNAseq panel was designed to cover relevant somatic mutations and common SNPs allowing the detection of LOH (Supplementary Table S8). Additional primers for amplicons of clone-specific IG/TR rearrangements were also included (Supplementary Table S9). Frozen bone marrow mononuclear cells were thawed, stained using the ADT panel TotalSeq-D Human Heme Oncology Cocktail, V1.0 (BioLegend; antibody list in Supplementary Table S10), and loaded into the Tapestri single-cell DNA genotyping platform (Mission Bio) to perform microfluidic encapsulation, lysis, and barcoding according to the manufacturer's protocol (Chemistry V2, PN_3360A). To improve erythroid cells identification, TotalSeq-D0574 anti-human CD235a (Glycophorin A) Antibody (BioLegend) was spiked at 1 µg during the resuspension procedure (available only for EI_046 diagnostic sample). DNA and ADT libraries were prepared and underwent 2 × 150 bp paired-end sequencing on a NextSeq 500 platform (Illumina).

Bioinformatics Analysis

Raw data were processed using Mission Bio's Tapestri Pipeline (DNA pipeline version 2.0.2 and Protein pipeline version 2.0.1) for preprocessing, alignment (reference genome hg19), cell barcode correction, cell identification, variant calling of DNA amplicons, and ADT-seq reads counting. Multiomics h5 files were then analyzed on R version 4.1.2 (R Foundation for Statistical Computing). TapestriR package was used to annotate variants: a genotype was considered informative if the singlecell sequencing depth was ≥5 reads. Variants were categorized as wildtype (WT) if VAF <10%, heterozygous (het) if VAF comprised between 10% and 90%, and homozygous (hom) if VAF ≥90%. Variants covered by <5 reads were considered noninformative and labeled as "missing genotype." No genotype filtering was performed before integration with ADT-seq data, in order to avoid data attrition, as all genotypes (i.e., WT, heterozygous and hemizygous/homozygous mutations) are expected. The rate of the allelic dropout was evaluated based on heterozygous SNPs. False homozygous genotypes related to allelic dropout were observed in 1% to 18% of cells (Supplementary Fig. S7). Patientspecific amplicons targeting clone-specific IG/TR rearrangements were considered informative if detected in the diagnostic sample. The IG/TR amplicons were considered positive if covered by ≥5 reads.

ADT read counts and cell barcodes were extracted from h5 files and 579 were analyzed using Seurat package V4.1.0 (42). Remission samples 581 were merged before proceeding to protein expression analysis as similar cell compositions were expected. Diagnostic samples were analyzed 582 separately. Cells with total ADT-seq read counts ≥100k were excluded 583

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to minimize the presence of doublet cells in further analyses (Supplementary Table S11). Centered log-ratio transformation was performed to normalize reads across cells before scaling, and then principal component analysis was performed on the normalized and scaled data. Dimensional reduction of protein expression data was performed using UMAP embedding and K-nearest neighbors and Louvain clustering algorithms to identify cell populations based on ADT sequencing before undergoing

manual cell population labeling based on cell-surface marker expression.

- 592 Genotype data were then merged, and relative mutated proportions were
- 593 calculated for each cluster having at least 10 cells for interpretation.

594 FACS of Cell Populations

As a cross-validation experiment, diagnostic samples from two patients were subjected to FACS in order to characterize genetic alterations in distinct cell populations. Briefly, frozen cells were thawed, stained with lineage-specific antibodies, and sorted on FAC-SAria III (BD Biosciences). Cell fractions underwent DNA extraction using QIAamp DNA Microkit (Qiagen) followed by targeted sequencing for somatic mutations and ddPCR quantitation for *IG/TR* clonal rearrangements.

603 Data Availability

Single-cell and bulk targeted sequencing data are accessible through
 the EGA database (https://www.ega-archive.org) under accession num bers EGAS00001006784 and EGAS00001006901, respectively. Other
 data are available upon reasonable request to the principal investigator.

608 Authors' Disclosures

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619 Authors' Contributions

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