(HR/IR) first relapse of B-acute lymphoblastic leukemia (B-ALL) in children and adolescents/young adults (AYAs) demonstrates superior efficacy and tolerability of blinatumomab: a report from Children's Oncology Group Study AALL1331 [abstract]. *Blood*. 2019;134(suppl 2). Abstract LBA-1.

4. Masurekar AN, Parker CA, Shanyinde M, et al. Outcome of central nervous system relapses in childhood acute lymphoblastic leukaemia–prospective open cohort analyses of the ALLR3 trial. *PLoS One*. 2014;9(10):e108107.
5. Dobson SM, García-Prat L, Vanner RJ, et al. Relapse-fated latent diagnosis subclones in acute B lineage leukemia are drug tolerant and possess distinct metabolic programs. *Cancer Discov*. 2020;10(4):568-587.
6. Shlush LI, Mitchell A, Heisler L, et al. Tracing the origins of relapse in acute myeloid leukaemia to stem cells. *Nature*. 2017;547(7661):104-108.
7. Young AL, Challen GA, Birmann BM, Druley TE. Clonal haematopoiesis harbouring AML-associated mutations is ubiquitous in healthy adults. *Nat Commun*. 2016;7:12484.
8. Choi S, Henderson MJ, Kwan E, et al. Relapse in children with acute lymphoblastic leukemia involving selection of a preexisting drug-resistant subclone. *Blood*. 2007;110(2):632-639.
9. Anderson K, Lutz C, van Delft FW, et al. Genetic variegation of clonal architecture and propagating cells in leukaemia. *Nature*. 2011;469(7330):356-361.
10. Williams R, Gordon PM. Editor's pick: the role and regulation of quiescence in acute lymphoblastic leukaemia. *EMJ Hematol*. 2017;5(1):72-79.
11. Tsurusawa M, Aoyama M, Saeki K, Fujimoto T. Cell cycle kinetics in childhood acute leukemia studied with in vitro bromodeoxyuridine labeling, Ki67-reactivity, and flow cytometry. *Leukemia*. 1995;9(11):1921-1925.
12. Nicholson MD, Antal T. Competing evolutionary paths in growing populations with applications to multidrug resistance. *PLoS Comput Biol*. 2019;15(4):e1006866.
13. Dieck CL, Ferrando A. Genetics and mechanisms of NT5C2-driven chemotherapy resistance in relapsed ALL. *Blood*. 2019;133(21):2263-2268.
14. Szikriszt B, Póti Á, Pipek O, et al. A comprehensive survey of the mutagenic impact of common cancer cytotoxics. *Genome Biol*. 2016;17:99.
15. McFaul K, Liptrott N, Cox A, et al. Cytotoxic chemotherapy and the evolution of cellular and viral resistance to antiretroviral therapy in HIV-infected individuals with lymphoma. *HIV Clin Trials*. 2016;17(5):197-203.
16. Shanmuganathan N, Hughes TP. Molecular monitoring in CML: how deep? How often? How should it influence therapy? *Hematology Am Soc Hematol Educ Program*. 2018;2018:168-176.
17. Gaynon PS, Sun W. Oligoclonality and new agent evaluation in acute lymphoblastic leukaemia. *Br J Haematol*. 2016;173(6):950-957.
18. Chabner BA. Antineoplastic agents. In: Hardman JG, Limbird LE, eds. Goodman & Gilman's The Pharmacological Basis of Therapeutics, New York, NY: McGraw-Hill; 1996:
19. Lutz C, Woll PS, Hall G, et al. Quiescent leukaemic cells account for minimal residual disease in childhood lymphoblastic leukaemia. *Leukemia*. 2013;27(5):1204-1207.
20. van Delft FW, Horsley S, Colman S, et al. Clonal origins of relapse in ETV6-RUNX1 acute lymphoblastic leukemia. *Blood*. 2011;117(23):6247-6254.

DOI 10.1182/blood.2020005258





**RESPONSE**

# Therapy-induced mutagenesis in relapsed ALL is supported by mutational signature analysis

Samuel W. Brady,[1] Xiaotu Ma,[1] Bin-Bing S. Zhou,[2,3] Ching-Hon Pui,[4] Jun J. Yang,[5] and Jinghui Zhang[1]

[1]Department of Computational Biology, St. Jude Children's Research Hospital, Memphis, TN; [2]Key Laboratory of Pediatric Hematology and Oncology Ministry of Health, Department of Hematology and Oncology, Shanghai Children's Medical Center and National Children's Medical Center and [3]Pediatric Translational Medicine Institute, Department of Pharmacology and Chemical Biology, Shanghai Jiao Tong University School of Medicine, Shanghai, China; and [4]Department of Oncology and [5]Department of Pharmaceutical Sciences, St. Jude Children's Research Hospital, Memphis, TN

We appreciate the interest of Gaynon et al[1] in our study on acute lymphoblastic leukemia (ALL) relapse.[2] They argued against our finding that therapy-induced drug resistance mutations occur in ALL and stated that these mutations were potentially preexisting at diagnosis but undetectable. They focused primarily on our mathematical modeling of ALL doubling times, while ignoring other important biological evidence from our study.

First, our strongest evidence of therapy-induced drug resistance mutations came from mutational signature analysis, not the modeling of doubling times. We showed that >25% of relapsed ALLs bore 1 or 2 treatment-induced mutational signatures, referred as novel signatures A and B. We showed experimentally that novel signature B was induced by thiopurine treatment, and the therapy-induced mutations were clonal in most relapses (supplemental Figure 10); hence therapy does not simply induce minor subclonal variants.

Second, drug resistance mutations in relapsed ALL preferentially occurred at trinucleotide contexts mutated by therapy. For example, relapse-specific *NT5C2* R367Q mutations were C>T mutations occurring at the center of TCG trinucleotides. We showed experimentally that thiopurines cause this type of mutation, and *NT5C2* R367Q mutations were significantly enriched in relapses that bore the thiopurine signature (supplemental Figure 16). We further analyzed the probability that each drug resistance mutation was caused by individual signatures present in each leukemia sample, based on each signature's preference to mutate at specific trinucleotides, using a published method.[3] This indicated that *NT5C2*, *TP53*, *NR3C1*, and *PRPS1* drug resistance mutations were likely induced by treatment in a subset of patients. A separate manuscript in preparation strongly reinforces this paradigm.

Gaynon et al also misinterpreted our conclusions; we did not dismiss the contribution of preexisting clones to relapse. They cited the study of Dobson et al,[4] in which we participated as collaborators, including part of the genomic analysis, as evidence that a minor subclone is usually present at diagnosis, which leads to the eventual relapse. Similarly, we also showed in supplemental Figure 17 that in 80% of patients the relapse-fated clone was detectable at diagnosis. Indeed, our Visual Abstract

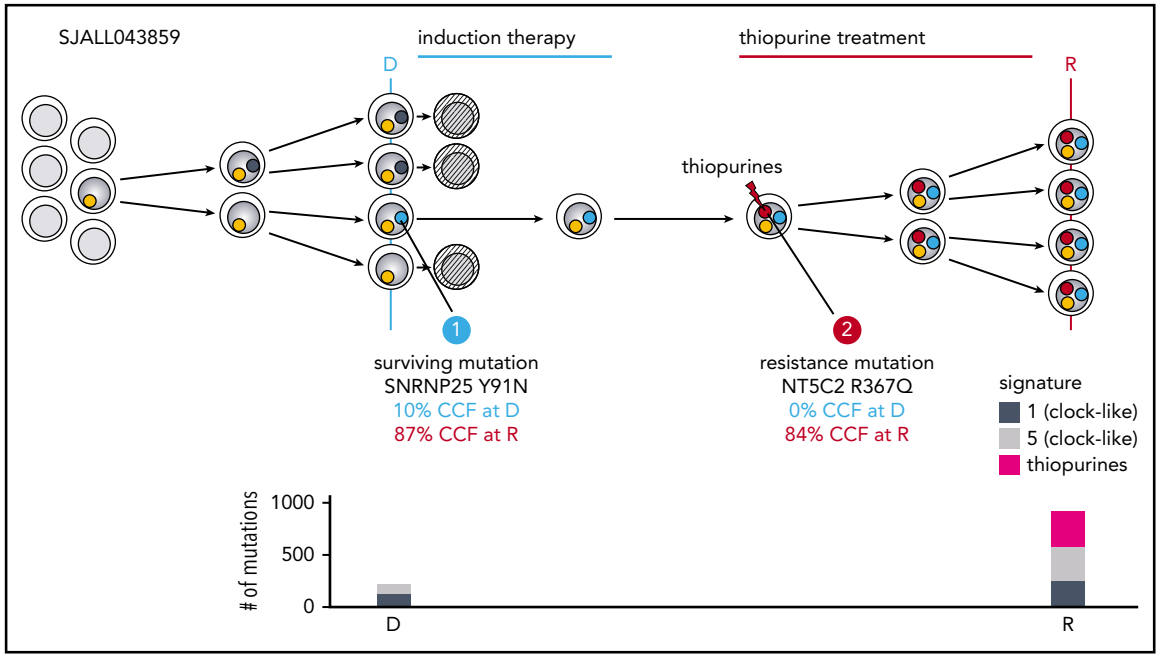


**Figure 1. Example showing the multistep acquisition of resistance in ALL.** Cells are represented with mutations shown as small colored circles. At diagnosis (D), patient SJALL043859 had a subclonal mutation in *SNRNP25* of uncertain significance in 10% of leukemic cells (10% cancer cell fraction or CCF), which increased to 87% at relapse (R). At relapse, an *NT5C2* R367Q mutation was detected at 84% CCF within the *SNRNP25* lineage, but was not found at diagnosis at 747× coverage, indicating that the *NT5C2* variant descended from the *SNRNP25* clone. The relapse sample also acquired the thiopurine signature (bottom), and the *NT5C2* mutations had >50% probability of having been induced by thiopurines because it occurred at a thiopurine-preferred trinucleotide context. These findings are based on whole-genome sequencing and targeted deep sequencing (484 to 1284× coverage of the *SNRNP25* and *NT5C2* mutations).

and Figure 7 showed that even in cases where drug resistance mutations were likely therapy induced, this mutagenesis *occurred within a minor clone present at diagnosis*, which further evolved as a result of therapy (Figure 1). We referred to this minor-to-major clone as a "persistent clone" because it was not fully drug resistant but later acquired a bona fide drug resistance mutation, such as in *NT5C2*, which we never detected at diagnosis using 500× capture sequencing across the cohort nor with 4000 to 50 000× sequencing in 3 patients. Minor survivor subclones lacking drug resistance mutations can be readily detected at diagnosis, whereas the resistance mutations evolving from these subclones, such as in *NT5C2* and *PRPS1*, are rarely if ever detected using high-depth sequencing at diagnosis (Figure 3 of original paper,[2] and Figure 1), including in Dobson et al[4] and other studies,[5,6] consistent with multistep acquisition of frank resistance. Furthermore, Dobson et al were not able to detect therapy-induced mutational signatures because they used exome sequencing of 14 patients, yielding an insufficient number of mutations to analyze mutational signatures (median of only 25 in diagnostic and 39.5 mutations in relapsed samples), compared with our whole-genome sequencing of >100 patients (median 332 at diagnosis and 810 mutations at relapse).

Finally, Gaynon et al misstated our conclusions from our mathematical modeling, when they noted: "With relapses outside these limits (later than 213 to 374 days), the authors conclude that the mutations could not have been present in even a single cell at diagnosis." Our actual statement was: "Early relapses [9 to 36 months after diagnosis], by contrast, may have occurred through a 2-step process in which a "persister" clone survives treatment yet cannot proliferate until acquiring a bona fide resistance mutation during treatment … which is also supported by the mutational signature analysis presented later. Alternatively, early relapses may have occurred through delayed proliferation (>9-day doubling time), perhaps during specific treatment regimens."[2] Thus, we clearly noted that relapses after 9 months could have been due to a preexisting resistant clone with slower proliferation. The statement of Gaynon et al that "[d]oubling times are not constant or uniform among the heterogeneous leukemic population" agrees with what we stated in the quotation above. Thus, we did not conclude that post-374-day relapses *had to be* from therapy-induced clones, but only claimed that at least 20% of relapses after 9 months arose from clones harboring therapy-induced mutations based on mutational signature data (see Visual Abstract and Figure 7). The only firm conclusion we made from the modeling was that very early relapses (<9 months) likely arose from ready-made resistant clones present at diagnosis (see Figure 3B).

We thank Gaynon et al for their provocative discussion and agree that more needs to be done to fully understand the biology of leukemia relapse.

## Authorship

Contribution: S.W.B., X.M., B.-B.S.Z., C.-H.P., J.J.Y., and J.Z. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profiles: S.W.B., 0000-0001-5518-7800; C.-H.P., 0000-0003-0303-5658; J.Z., 0000-0003-3350-9682.

Correspondence: Jinghui Zhang, Department of Computational Biology, MS 1135, Room IA6038, St. Jude Children's Research Hospital, 262 Danny Thomas Pl, Memphis, TN 38105; e-mail: jinghui.zhang@stjude.org.

## Footnote

Submitted 14 July 2020; accepted 14 July 2020; prepublished online on *Blood* First Edition 20 July 2020.

### REFERENCES

1. Gaynon PS, Orgel E, Ji L. Preexisting or therapy-induced mutations in relapsed acute lymphoblastic leukemia? *Blood*. 2020;136(19):2233-2235.

2. Li B, Brady SW, Ma X, et al. Therapy-induced mutations drive the genomic landscape of relapsed acute lymphoblastic leukemia. *Blood*. 2020;135(1): 41-55.

3. Morganella S, Alexandrov LB, Glodzik D, et al. The topography of mutational processes in breast cancer genomes. *Nat Commun*. 2016;7(1):11383.

4. Dobson SM, García-Prat L, Vanner RJ, et al. Relapse-fated latent diagnosis subclones in acute B lineage leukemia are drug tolerant and possess distinct metabolic programs. *Cancer Discov*. 2020;10(4): 568-587.

5. Tzoneva G, Dieck CL, Oshima K, et al. Clonal evolution mechanisms in NT5C2 mutant-relapsed acute lymphoblastic leukaemia. *Nature*. 2018; 553(7689):511-514.

6. Li B, Li H, Bai Y, et al. Negative feedback-defective PRPS1 mutants drive thiopurine resistance in relapsed childhood ALL. *Nat Med*. 2015;21(6): 563-571.

DOI 10.1182/blood.2020008107





**TO THE EDITOR:**

# Increased tumor burden in patients with chronic myeloid leukemia after 36 months of imatinib discontinuation

Elisa Diral,[1,2] Silvia Mori,[1] Laura Antolini,[1] Elisabetta Abruzzese,[3] Philipp Le Coutre,[4] Bruno Martino,[5] Ester Pungolino,[6] Chiara Elena,[7,8] Micaela Bergamaschi,[9] Sarit Assouline,[10] Eros Di Bona,[11] Antonella Gozzini,[12] Marcio Andrade-Campos,[13] Fabio Stagno,[14] Alessandra Iurlo,[15] Alessandra Pirola,[16] Diletta Fontana,[1] Jessica Petiti,[17] Maria Luisa Bonanomi,[18] Patrizia Crivori,[18] Rocco Piazza,[1,2] Carmen Fava,[17] and Carlo Gambacorti-Passerini[1,2]

[1]Department of Medicine and Surgery, University of Milano-Bicocca, Monza, Italy; [2]Division of Hematology, San Gerardo Hospital, Monza, Italy; [3]San Eugenio Hospital, Rome, Italy; [4]Department of Hematology and Oncology, Charité - Humboldt-Universität, Campus Virchow, Berlin, Germany; [5]Hematology, Bianchi Melacrino Morelli Hospital, Reggio Calabria, Italy; [6]Hematology, Niguarda Ca' Granda Hospital, Milan, Italy; [7]Department of Molecular Medicine, University of Pavia, Pavia, Italy; [8]Department of Hematology Oncology, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Policlinico San Matteo, Pavia, Italy; [9]Hematology Clinic, IRCSS AOU S. Martino-IST, University of Genoa, Genoa, Italy; [10]Division of Haematology, Jewish General Hospital, McGill University, Montreal, QC, Canada; [11]Hematology, San Bortolo Hospital, Vicenza, Italy; [12]Hematology, AOU Careggi, University of Firenze, Firenze, Italy; [13]Haematology, Hospital Universitario Miguel Servet, Zaragoza, Spain; [14]Hematology, University of Catania, Catania, Italy; [15]Oncohematology Division, IRCCS Ca' Granda Maggiore Policlinico Hospital Foundation, University of Milan, Milan, Italy; [16]GalSeq s.r.l., Bresso, Italy; [17]University of Turin, Turin, Italy; and [18]Clioss s.r.l., Nerviano, Italy

The Imatinib Suspension and Validation (ISAV) study[1] is a multicenter trial of imatinib discontinuation (ID) among patients with chronic myeloid leukemia (CML) in undetectable deep molecular remission (U-DMR). After 12 months of follow-up, 48% of patients relapsed (total n = 107), with the majority of relapses occurring within the first 9 months. An inverse relationship between patient age and risk of relapse was also observed at this timepoint. Here we report the final results of ISAV after a median follow-up of 49 months, as well as the dynamics of leukemic tumor load as determined by digital polymerase chain reaction (dPCR) in nonrelapsed patients. This trial is registered at www.clinicaltrials.gov (NCT01578213).

Eligible patients were 18 years and older and had CML, either in chronic or accelerated phase, with U-DMR of at least 18 months' duration and at least 3 consecutive negative quantitative real-time PCR (Q-RT-PCR) just before study entry (supplemental Figure 1, available on the *Blood* Web site). A total of 107 patients were enrolled at 15 centers worldwide between 2011 and 2013. U-DMR was defined as an undetectable BCR/ABL1 by Q-RT-PCR as determined by local laboratories. Between 10 000 and 32 000 copies of the ABL1 control gene molecules were amplified, corresponding to a sensitivity of MR4 and MR4.5, respectively. All but 1 laboratory used the International Scale.

Within 7 days of providing informed consent, 20 mL of blood were collected from the patient in PAX gene tubes (PreAnalytiX GmbH, Switzerland) for dPCR analysis and the patient discontinued imatinib therapy. Q-RT-PCR tests were performed[1] monthly for the first 6 months, then every 2 months until up to 36 months from ID to assess for the maintenance of the major molecular remission (MMR) (BCR-ABL/ABL <0.1%). Patients still in MMR at 36 months entered the follow-up phase, during which Q-RT-PCR monitoring was performed every 6 months for an additional 2 years. Loss of MMR was defined as at least 1 BCR-ABL1/ABL1 value above 0.1% among 2 consecutive positive Q-RT-PCR tests. Patients with a loss of MMR resumed imatinib at the same dose used before treatment interruption and were monitored by standard Q-RT-PCR for 2 additional years.[1]

dPCR.RNA (3 μg) was reverse transcribed to complementary DNA as previously described.[2] The final concentration of the reagents were: $MgCl_2$ (5 mM); PCR Buffer (1X); DTT (100 nM); dNTPs (10 nM each); M-MLV reverse transcriptase (16 U); RNAse Inhibitor (0.2 U); and Random Hexamers Primers (12.5 mM) (Thermo Fisher Scientific). Droplet dPCR experiments were performed by the QX200 system (BioRad). The BCR-ABL1 fusion and ABL1 transcripts were quantified using DigiDropP210 MasterMix and DigiDropP210 Positive Control (Bioclarma), according to the manufacturer's protocol and using appropriate negative controls (no template). The target concentration in each sample was expressed as percentage of BCR-ABL1/ABL1, and represents the mean of 3 replicates; negative wells were counted as "0."