The Promise of Single-cell Technology in Providing New Insights Into the Molecular Heterogeneity and Management of Acute Lymphoblastic Leukemia

Holly C.L. Pearson1,2, Kooper V. Hunt3,4, Toby N. Trahair1,2,5, Richard B. Lock1,2, Heather J. Lee3,4, Charles E. de Bock1,2

Correspondence: Heather J. Lee (Heather.Lee@newcastle.edu.au); Charles E. de Bock (cdebock@ccia.org.au).

ABSTRACT
Drug resistance and treatment failure in pediatric acute lymphoblastic leukemia (ALL) are in part driven by tumor heterogeneity and clonal evolution. Although bulk tumor genomic analyses have provided some insight into these processes, single-cell sequencing has emerged as a powerful technique to profile individual cells in unprecedented detail. Since the introduction of single-cell RNA sequencing, we now have the capability to capture not only transcriptomic, but also genomic, epigenetic, and proteomic variation between single cells separately and in combination. This rapidly evolving field has the potential to transform our understanding of the fundamental biology of pediatric ALL and guide the management of ALL patients to improve their clinical outcome. Here, we discuss the impact single-cell sequencing has had on our understanding of tumor heterogeneity and clonal evolution in ALL and provide examples of how single-cell technology can be integrated into the clinic to inform treatment decisions for children with high-risk disease.

INTRODUCTION
Acute lymphoblastic leukemia (ALL) is the most common cancer in children, characterized by the clonal proliferation of progenitor B and T cells in the bone marrow (BM), blood, and extramedullary sites.1 Once considered an incurable disease, the 5-year survival rate of pediatric ALL has increased substantially over the past 50 years and now exceeds 90% in most developed countries.2,3 This marked improvement can be attributed to risk-stratified and response-adapted multiagent chemotherapy, substantial changes in our understanding of genetic risk and improvements in supportive care.4 Although significant advances have been made in treating pediatric ALL, approximately 10%–20% of children will relapse, and their prognosis is poor. Children who experience more than one relapse have an overall survival rate between 25% and 40%, and this has remained relatively static for the past two decades.5,6

The molecular mechanisms underlying drug resistance, treatment failure, and disease relapse are in part due to the clonal diversity of ALL that evolves over time.7,8 In recent years, much of our understanding of this diversity has been shaped by bulk genomic sequencing. Analyses of paired diagnosis and relapse samples from patients with pediatric ALL identified a range of genomic changes as the disease progressed, including the loss of diagnosis-specific alterations and the gain of new mutations and deletions.9-11 Relapse-specific mutations include genes regulating lymphoid development (IKZF1),9,11 tumor suppression (TP53),12 kinase signaling (NRAS, KRAS),13,14 epigenetic modifications (KDM6A),15 and drug resistance (NT5C2, CREBBP).16,17 Many of these mutations have been associated with high-risk traits in pediatric ALL, such as early relapse and chemoresistance.14,17,18 Interestingly, many of these mutations and deletions present at relapse were also detected at diagnosis, but at much lower levels.6,11 This finding indicates that relapse may arise from the outgrowth of a minor subclone present at diagnosis, which acquires additional mutations that offer a survival advantage. Although bulk sequencing has undoubtedly contributed to our understanding of the clonal nature of pediatric ALL, it alone is insufficient to fully dissect the genetic, transcriptomic, and epigenetic heterogeneity of ALL.

This challenge is now in part overcome by the study of single cells that has rapidly evolved since the introduction of single-cell RNA sequencing (scRNA-seq) in 2009. Today, there is a growing demand for single-cell technology, with nearly 200 different methods to profile not only transcriptomic but genetic, epigenetic, and proteomic information in individual cells. In this review, we highlight the impact single-cell techniques have had on our understanding of pediatric ALL and discuss the potential clinical applications of single-cell methods for the treatment and management of pediatric ALL.

AN OVERVIEW OF SINGLE-CELL TECHNOLOGIES
Single-cell sequencing technologies have empowered researchers to characterize rare and heterogeneous cell populations...
in unprecedented detail, revealing new insights in many fields including development,\textsuperscript{19,20} immunology,\textsuperscript{21} and cancer.\textsuperscript{22,23} The enormous potential of single-cell analyses has stimulated the prolific growth of experimental and analytical methods,\textsuperscript{24} accompanied by continued expansion of the molecular modalities that can be assessed using these techniques.\textsuperscript{25} Single-cell technologies can be broadly divided into analysis of either DNA (genomics, epigenomics) or RNA (transcriptomics) with newer applications moving to combine both within the same cell (Figure 1).

### Single-cell genomics

Of relevance to cancer biology, is the ability to study genetic variations in individual cells. Although bulk DNA sequencing (DNA-seq) can be used to infer clonal subpopulations based on variant allele frequency analysis, it cannot be used to definitively test the co-occurrence of specific mutations in individual cells.\textsuperscript{26} Thus single-cell DNA sequencing (scDNA-seq) can reveal cancer clonal architecture in far greater detail.\textsuperscript{27} For example, single-cell analysis of acute myeloid leukemia (AML) identified rare subclones with mutations missed in bulk analysis and allowed mapping of tumor evolution and identification of initial driver mutations based on co-occurrence of mutations.\textsuperscript{28} ScDNA-seq methods can identify copy number variant and point mutations (eg, single nucleotide variants [SNV], insertions, and deletions)\textsuperscript{27} but are associated with a very high-sequencing demand. To overcome the cost of sequencing whole genomes from many single cells, methods for targeted sequencing of commonly mutated genes have been developed. These methods combine droplet encapsulation and barcoding of single cells with targeted amplification and sequencing of regions of interest. Customized panels are commercially available for >20 cancer types (including AML, ALL, and chronic lymphocytic leukemia), with each panel covering 50–2000 commonly mutated genes. In AML, targeted scDNA-seq revealed diverse patterns of clonal evolution and selection of drug-resistance clones in response to FLT3 inhibition.\textsuperscript{29} Similarly, targeted scDNA-seq in ALL has been used to demonstrate that minor subclones at diagnosis can become clinically relevant major clones at relapse.\textsuperscript{30}

### Single-cell transcriptomics

Single-cell transcriptomics has been used to study cancer stem cells,\textsuperscript{31} metastasis-initiating cells,\textsuperscript{32} chemotherapy resistance,\textsuperscript{32} and cancer immune responses.\textsuperscript{33} For example, van Galen et al\textsuperscript{31} showed that cell types determined by scRNA-seq in AML revealed extensive diversity with some tumors harboring up to six malignant myeloid cell types. Leukemic stem cells that drive tumor progression were also shown to express signaling pathways normally exclusive to less differentiated progenitor cells as well as pathways exclusive to more differentiated myeloid cells. Similar to DNA-seq, single-cell transcriptomics requires amplification of template molecules before library preparation. This is typically achieved using poly-A primers and template-switching biochemistry.\textsuperscript{34,35} However, the limited sensitivity of these methods means that genes expressed at low levels are unrepresented in the sequencing library. ScRNA-seq methods were the first to be implemented in droplet-encapsulation platforms,\textsuperscript{36–38} allowing their rapid and widespread uptake in all fields of biology.\textsuperscript{34} These methods typically sequence only the 3′ end of transcripts and are associated with very low sequencing demand, allowing tens of thousands of cells to be profiled in individual studies.\textsuperscript{39,40} Other methods are integrating long-read sequencing of full-length transcripts to investigate isoform expression and DNA rearrangements in antigen receptor loci.\textsuperscript{41,42}

### Single-cell epigenomics

Many epigenetic processes (including DNA methylation, histone modifications, and chromatin accessibility) become dysregulated in cancer, and this fact has been exploited in various clinical applications.\textsuperscript{43} Single-cell epigenomics can reveal the regulatory processes that lead to transcriptional heterogeneity in cancer, with important clinical implications. For example, single-cell analysis of chromatin accessibility more clearly defines

---

**Figure 1. Clonal heterogeneity in ALL.** ALL arises through the acquisition of somatic mutations over time resulting in extensive subclonal diversity at diagnosis. Using specific single-cell isolation strategies, such as FACS, MACS, microfluidics or microwell partitioning, different single-cell analyses can now be applied to understand the clonal heterogeneity resident in the bone marrow. This includes (i) single-cell DNA analysis of the genome to identify INDELS and SNVs that in turn can provide information on the order of mutation acquisition and clonal evolutionary trajectories; (ii) single-cell ATAC and DNA methylation analyses to measure epigenetic changes in different clones; (iii) single-cell RNA analysis to define the transcriptome and cell state of different clones; and (iv) single-cell proteomics which most often uses antibodies targeting cell-surface proteins to immunophenotype cells. Increasingly, these single-cell methodologies are now being combined to provide a rich multiomic view of the different clones present in ALL. ALL = acute lymphoblastic leukemia; ATAC = assay for transposase-accessible chromatin; FACS = fluorescence-activated cell sorting; INDELS = insertions/deletions; MACS = magnetic-activated cell sorting; SNVs = single nucleotide variants.
hematological cell types than scRNA-seq. In AML, open chromatin analysis identified distinct regulatory sites active in preleukemic stem cells that may represent the earliest steps in leukemic transition. Single-cell DNA methylation analysis has also been applied to characterize circulating tumor cells and response to epigenetic therapies. In leukemia cells, treatment with hypomethylating agents (azacytidine and decitabine) induces DNA methylation heterogeneity, which could contribute to the variable response seen in AML patients.

Single-cell multiomics

It is also possible to combine analysis of the genome, transcriptome, epigenome, and other modalities using single-cell multiomic analyses. These combinatorial approaches allow genetic regulation to be studied in incredible detail and were named the 2019 “Method of the Year” by Nature Methods. For example, by modifying current droplet-based scRNA-seq methods, it is possible to integrate single-cell gene expression profiles and genotyping of somatic mutations from thousands of cells as well as retrospectively identify fusion genes. In cancer, single-cell multiomic methods have been used to investigate cancer evolution and to reveal epigenetic changes in genetic subclones. In chronic myeloid leukemia (CML), Gaiti et al showed that reduced conformity between promoter methylation and transcription observed in bulk analysis may be the result of increased intratumor heterogeneity. Furthermore, treatment with ibrutinib caused over-representation of clones that had distinct epimutation patterns and were enriched for expression of cell-cycle, proliferation, and toll-like receptor pathways independent of genetic subclonal drivers.

Emerging single-cell analyses

The repertoire of molecular modalities that can be profiled in single cells continues to expand, and spatial profiling, lineage tracing and single-cell proteomics are adding further dimensions to these analyses. Spatial transcriptomics, Nature’s 2020 “Method of the Year,” can be performed on tissue sections using barcode arrays that record the coordinates of mRNA molecules in a sample. This technology was first applied in prostate cancer where it revealed differences in gene expression between the core and periphery of the cancer, as well as in the stroma adjacent to the tumor. In ALL, analysis of tissues such as the BM, spleen, and thymus will help to determine how the tumor microenvironment promotes disease progression and identify therapeutic approaches to prevent drug resistance.

To place cells in the temporal context of development or disease progression, lineage tracing approaches have been used. These are typically based on introduced cell barcodes but can also use natural barcodes provided by somatic mutations in mitochondrial DNA. Since barcodes can be recovered from the same cell as transcriptomic data, it is possible to link cell histories with molecular profiles. For example, lineage tracing has been used to reveal the processes of cancer evolution and acquisition of therapy resistance.

Recent studies have used high-sensitivity mass spectrometry to achieve single-cell proteomics, and another report has coupled click chemistry with mass spectrometry to study lipid metabolism in single cells. Thus, it will soon be possible to study cell signaling pathways and altered metabolism in single cancer cells.

APPLICATION OF SINGLE-CELL TECHNOLOGIES IN PEDIATRIC ALL

Single-cell technologies are a powerful tool to study the genetic, transcriptomic, and epigenetic heterogeneity of many cancers, including pediatric ALL. Here, we discuss recent studies that have utilized single-cell technologies to reconstruct the clonal architecture and investigate the heterogeneity of pediatric ALL and characterize the bone marrow microenvironment (BMM) and its role in leukemia development and treatment resistance.

Dissecting tumor heterogeneity and clonal evolution

Tumor heterogeneity and order of mutation acquisition is important in understanding the etiology of ALL. One of the earliest studies in ALL to investigate clonal structure at the single-cell level was published by Gawad et al. Here, targeted single-cell sequencing, complemented by bulk genomic sequencing, was used to identify regions of interest that contain SNVs, deletions, and IgH sequences in 6 pediatric B-ALL patients. This analysis revealed that most structural variants, such as large deletions, occurred before SNV acquisition in these patients and that KRAS mutations were acquired late in leukemia development. This study provided an unprecedented insight into the development of pediatric ALL and led the way for future studies focusing on clonal structure and evolution in pediatric ALL.

Following on from this work, De Bie et al reconstructed the clonal architecture of T-ALL using targeted scDNA-seq to investigate the heterogeneity of T-ALL at diagnosis. Detection of a dominant leukemia clone in four T-ALL patients, accompanied by smaller subclones with fewer mutations, uncovered a degree of genetic heterogeneity in T-ALL at diagnosis. Based on this single-cell data, De Bie et al inferred the order of mutations acquired during T-ALL development. Inactivation of CDKN2A/B, T-cell receptor deletions and fusion genes were observed as intermediate events, whereas NOTCH1 mutations occurred after the bulk of genetic mutations as late events in three of the four patients. Interestingly, they found that most early mutations occurred in genes of unknown significance. This suggests that while these genes may not be definitive oncogenic drivers, they may confer a permissive state for the acquisition of additional mutations that drive leukemia development and require further research to establish their significance. The order of mutation acquisition and degree of heterogeneity in leukemia has important clinical implications for the design and development of new precision medicine strategies, highlighting the importance of this study.

There are additional studies that have investigated the evolution of structural variants in both B- and T-ALL but using a single-cell technique known as multicolor fluorescence in situ hybridization (mFISH). Potter et al revealed that CRLF2 rearrangements in B-ALL occur as both early and late events during the development of leukemia, whereas Furness et al identified the STIL-TAL1 fusion and loss of CDKN2A/B as early subclonal events in STIL-TAL1 T-ALL. Although the work from Gawad et al, De Bie et al, Potter et al, and Furness et al has undoubtedly contributed to our understanding of clonal structure during leukemia development, these studies are limited by the number of cells captured for each patient and the number of genetic markers used, and as such, the true extent of genetic heterogeneity may not have been uncovered.

The limitation of low cell number is now in part overcome, with more recent studies taking advantage of new technology, which utilize high-throughput methods to capture thousands of cells per sample. For example, targeted scDNA-seq combined with high-throughput droplet-based microfluidics technology accurately identified SNVs and small insertion-deletion mutations in 305 genomic regions in over 100,000 cells from both T-ALL and B-ALL patients. Both studies provided a comprehensive insight into the clonal diversity of pediatric ALL at diagnosis. Interestingly, whereas the majority of T-ALL patients accumulated subclonal mutations, clonal heterogeneity in B-ALL appeared to be more subtype-specific, where patients with high hyperdiploidy or PAX5 alterations had a higher number of subclones. Additionally, Alberti-Servera et al highlighted a high degree of heterogeneity of NOTCH1 mutations in T-ALL. NOTCH1 was the most frequently mutated gene, with...
33 variants identified across 8 patients. Half of the patients had more than 2 NOTCH1 variants that in some, were present in different clones, and in others, accumulated in the same cell. Consistent with previous findings by De Bie et al., NOTCH1 mutations emerged as late subclonal events, suggesting a reliance of leukemia cells on NOTCH1 for leukemia progression, rather than initiation. Analysis of longitudinal samples from three T-ALL patients at single-cell resolution identified minor subclones at diagnosis that evolved to become clinically relevant major clones at relapse. In line with previous studies, this supports the current hypothesis of classic Darwinian branching evolution of genetically diverse subclones as a mechanism of treatment evasion and disease relapse in pediatric ALL. That is, that chemotherapy deterministically selects for a pre-existing rare subclone that is genetically distinct and has the potential to acquire additional mutations that offer a survival advantage to drive relapse.

However, this hypothesis was recently challenged in a study by Turati et al. that argued phenotypic heterogeneity rather than genotypic heterogeneity is the major driver of relapse. Turati et al. suggests that changes in clonal composition between samples taken at diagnosis and relapse could also be explained by evolutionary events that occur after chemotherapy. To investigate mechanisms of clonal selection in pediatric ALL, Turati et al. established multiple xenografts from B-ALL patient samples and administered induction chemotherapy to induce maximum cytoreduction. Reconstruction of the immediate post-treatment BM residuum at single-cell resolution using mFISH and single-cell whole-genome sequencing revealed that chemotherapy has minimal effect on the clonal complexity of residual disease, suggesting that treated cells are as genetically heterogeneous as untreated cells. Transcriptional analysis using scRNA-seq revealed that these polyclonal residual xenograft cells were in fact transcriptionally homogeneous, characterized by signatures associated with primitive developmental states, quiescence, and downregulated expression of MYC and E2F and their target genes. Importantly, small numbers of cells with the same transcriptional phenotype were detected in untreated samples using scRNA-seq, suggesting that chemotherapy applies selective pressure to the phenotypic heterogeneity of B-ALL, rather than genetic.

ScRNA-seq has also been used to better understand mechanisms of intrathymic transcriptional heterogeneity in pediatric ALL at diagnosis. Caron et al. suggested that the developmental state of leukemia cells and their expression of ribosomal proteins may be a source of transcriptional heterogeneity seen within pediatric ALL patients at diagnosis. The use of scRNA-seq is particularly important here because bulk RNA sequencing cannot differentiate cells based on their transcriptional profile. Analysis of transcriptomes of 39,375 cells identified at least two distinct transcriptional clusters in 5 of the 8 patients. Differential gene expression analysis revealed the most significantly deregulated genes were genes involved in B- and T-cell maturation and genes coding ribosomal proteins. An inverse relationship between predicted developmental stages and ribosomal protein expression was observed, where highly differentiated cells within transcriptional clusters had lower expression of ribosomal proteins. It remains unclear whether the intrathymic transcriptional heterogeneity seen here has any clinical significance for these patients. However, given the work by Turati et al., it may be possible that the various stages of development or maturation potential defining these transcriptional clusters have different fitness advantages during treatment.

Understanding normal hematopoietic ontogeny is an important step to better understand the origin of pediatric leukemia, given the prenatal origins of many pediatric ALL cases, particularly those occurring in infants. A recent study integrated the transcriptomes of healthy hematopoietic cells with leukemia cells from B-ALL patients to identify healthy cellular counterparts within leukemic subpopulations. Each leukemia cell subpopulation had a different healthy counterpart consisting of pro-B, pre-B, or immature-B cells, revealing various degrees of differentiation between subpopulations. This suggests that leukemogenesis maintains a similar developmental trajectory to normal hematopoiesis, with early developmental arrest leading to lineage-defined subpopulations. Analyses of diagnosis, refractory, remission, and relapse samples from a single patient revealed changes in leukemia cell subpopulations during treatment and disease progression. At diagnosis, the majority of cells were identified as leukemic pre-B and immature-B cells, although relapse was driven by leukemic pro-B cells, suggesting that relapse leukemia cells have increased stemness and stronger differentiation potential.

Understanding the extent of leukemia-induced bone marrow microenvironment remodeling

Increasingly the role of the bone marrow microenvironment (BMM) has been found to play an important role in the progress of leukemia and evasion of treatment. Single-cell technology has now provided unprecedented resolution of the BMM and its role in supporting ALL. In B-ALL, the myeloid compartment of the BM was extensively remodeled at different stages of disease. Using scRNA-seq and cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), Witkowska et al. showed that the resident monocyte population in the BM was the most sensitive to the presence of leukemia, with an abundance of nonclassical monocytes at diagnosis and relapse, at the expense of the classical monocyte population. Monocyte abundance was associated with inferior relapse-free and overall survival in pediatric ALL, suggesting a role for leukemia-associated monocytes in promoting B-ALL progression.

Chronic immune activation and exhaustion/dysfunction of T cells was recently observed in patient B-ALL BM. Bailur et al. used a combination of single-cell transcriptomics and mass cytometry to compare the BMM of pediatric B-ALL patients to healthy donors. In the BM of high-risk B-ALL patients, naïve T-cell depletion was accompanied by increased activation of effector T cells. In further support of leukemia-induced BMM remodeling, Anderson et al. revealed substantial changes in gene expression in normal pre-B and pro-B cells during the early stages of leukemia development in a BCR-ABL1-positive B-ALL mouse model. This included reduced expression of genes regulated by the activating protein 1 transcription factor complex, overexpression of CDKN2A and 16 and enrichment of RNA-binding protein NELFE and transcription factors MYC and BCL11A. Together, these studies extend our knowledge of BMM remodeling in B-ALL.

CLINICAL TRANSLATION OF SINGLE-CELL TECHNOLOGY IN PEDIATRIC ALL

Informing prognosis and complementing standard-of-care MRD

Despite remarkable improvements in the prognosis of pediatric ALL, the treatment of high-risk and relapsed ALL remains challenging. This is compounded by the definitions of remission and treatment failure not being well standardized. Nevertheless, minimal residual disease (MRD) is currently the strongest prognostic indicator for pediatric ALL and is used to refine risk stratification and treatment for newly diagnosed and relapsed patients and identify patients who have a higher risk of disease relapse. However, current MRD monitoring can only inform on overall tumor burden and any clonal heterogeneity of the disease remains unknown. Understanding the degree of tumor heterogeneity is important due to its prognostic implications. Therefore, although the current high costs associated
with single-cell analysis precludes it from being performed routinely for every child in the clinic, the application of single-cell technologies for patients identified to be at high risk of relapse has the opportunity to change how high-risk pediatric ALL is treated and managed (Figure 2).

The integration of single-cell analyses with routine MRD monitoring was demonstrated when Alberti-Servera et al\(^{10}\) used targeted scDNA-seq to reconstruct the clonal architecture of an MRD positive patient sample. As part of routine MRD follow-up, molecular remission failure was detected by the presence of TLX3 using reverse transcription-quantitative PCR.\(^{10}\) The clonal complexity of the disease was then revealed by targeted scDNA-seq, which identified two clones harboring WT1 and NOTCH1 mutations.\(^{10}\) Here, the stable expression of TLX3 throughout disease progression enabled ultrasensitive monitoring of overall tumor burden using PCR-based MRD assays, whereas single-cell analysis monitored tumor heterogeneity, providing an opportunity for improved prognostic evaluation of pediatric ALL patients, regardless of their MRD marker.

Single-cell profiling of MRD samples could identify clonal populations harboring genetic alterations associated with therapy resistance and the early detection of these clones could be used to inform prognosis and treatment decisions. There are specific subgroups of patients with a much higher risk of treatment failure due to their genetic subtype (eg, MLL-rearranged, hypodiploid, TCF3-HLF-positive) who would benefit the most from this analysis. Additionally, patients with BCR-ABL1-positive ALL or ALL with ABL-class fusions\(^{11}\) receiving tyrosine kinase inhibitor (TKI) therapy often develop resistance caused by the acquisition of secondary mutations in ABL1.\(^{12}\) There are over 70 known TKI-resistant mutations\(^{13}\) and the early detection of one could prompt a change in treatment, whether that be treatment intensification, a stem cell transplantation or a new targeted therapy. Relapse following CD19-directed chimeric antigen receptor T-cell (CAR-T) therapy is common and 30\%–50\% of relapses are CD19 negative (CD19\(^{-}\)).\(^{10,4}\) A recent study demonstrated that transcriptomic profiling using scRNA-seq can identify rare CD19\(^{+}\) leukemia cells that were present before CAR-T therapy and led to CD19\(^{-}\) relapse.\(^{12}\) This suggests that single-cell methods can be used to predict the risk of CAR-T therapy failure by identifying CD19\(^{-}\) cells before treatment. However, it is important to note that while single-cell sequencing methods, such as scRNA-seq, are readily available in research, the use of flow cytometry as a single-cell method may be the better choice in the clinic for the analysis of patient samples before and during CAR-T therapy.

A new subgroup of BCR-ABL1-positive pediatric ALL patients who present with CML-like disease manifesting in “lymphoid blast crisis” may be more amenable to single-cell analysis. These patients have multilineage involvement, leading to discordance between the Ig/TCR and BCR-ABL1 MRD levels.\(^{11}\) Using single-cell methods, such as scRNA-seq or CITE-seq to understand the role of biological heterogeneity in CML-like BCR-ABL1-positive ALL will help to define prognosis and guide appropriate treatment decisions for these patients.

These case studies highlight that the molecular modality of single-cell analyses will likely be unique for each patient, depending on the question being asked. For example, patients with MLL-rearranged ALL, a high-risk infant leukemia characterized by aberrant DNA methylation,\(^{25}\) may benefit from single-cell methylation analysis. Similarly, it would be important to consider how best to maximize the information obtained from finite samples, and whether we need to enrich for rare leukemia cell populations. Even in children with poor responses and high-risk disease, there is often a very dramatic reduction in leukemia burden during the first few weeks of treatment, so the timing of sampling would be critical. Sampling and single-cell analysis at diagnosis will likely capture the major clonal leukemia populations, which may be destined to respond, and so serial longitudinal analyses during treatment would allow the progressive enrichment and identification of resistant cell populations and genetic heterogeneity (Figure 2). As normal hematopoiesis is restored; however, it is important to consider methods to further enrich for the ever-decreasing proportion of leukemia cells in the BM milieu. For example, methods such as fluorescence-activated cell sorting and magnetic cell separation can enrich for leukemia cells using the expression of patient-specific cell-surface markers. Together, this provides a distinct advantage over bulk sequencing methodology whereby single-cell analysis can inform prognosis by complementing standard-of-care MRD monitoring and identify markers therapy resistance and guide treatment decisions based on the molecular heterogeneity of the disease (Figure 2).

**Identifying mechanisms of therapy resistance and therapeutic targets**

Resistance to therapy and subsequent relapse remains a significant barrier to cure in pediatric ALL.\(^{97,98}\) Using single-cell methods to reconstruct the clonal architecture of pediatric ALL may provide an opportunity to identify mechanisms of relapse and develop novel treatment strategies targeting clonal diversity in newly diagnosed high-risk and relapsed ALL patients. Single-cell studies in AML have already given us an insight into the clinical application of single-cell technologies, by characterizing evolutionary processes of resistance in large cohorts of patients using scDNA-seq and identifying mutations responsible for inferior treatment response.\(^{99–101}\) However, to date, there are only a few studies that have published this information in the setting of pediatric ALL. One such study has been discussed above, where Alberti-Servera et al\(^{99}\) traced the clonal evolution of patient T-ALL cells through standard-of-care chemotherapy regimens using targeted scDNA-seq. Clones harboring mutations in NOTCH1 and PTEN were identified as drivers of relapse; however, their clinical significance remains unknown. Understanding the clonal evolution of pediatric ALL at single-cell resolution may identify genetic mutations associated with relapse, drug resistance, and inferior outcome. Identification of these mutations in the future may predict poor response to chemotherapy, prompting early modifications to treatment or intensification of treatment.

As standard-of-care chemotherapy reaches its limit of intensification, the focus has turned to targeted molecular therapeutics and immunotherapies to treat relapsed/refractory pediatric ALL.\(^{102}\) However, this has created additional challenges with new mechanisms of treatment resistance to identify. For example, the use of TKIs (such as imatinib and dasatinib) is an attractive therapeutic strategy for patients with BCR-ABL1-positive ALL and Ph-like ALL with ABL-class fusions (eg, ABL1, ABL2, CSF1R, PDGFR, and PDGFRB).\(^{103,104}\) However, TKI resistance is common and is often caused by the acquisition of secondary mutations in ABL1.\(^{12}\) These mutations do not account for all cases of TKI resistance though\(^{105}\) and play a limited role in the persistence of residual disease in good responders.\(^{106}\) As such, there remains aspects of TKI therapy that are poorly understood. Analysis before, during and after TKI therapy using single-cell methods could identify novel TKI-resistant mutations and other mechanisms of resistance or disease persistence (eg, transcriptomic or epigenomic).

Similarly, up to half of relapsed/refractory B-ALL patients treated with anti-CD19 CAR-T-cell therapy will relapse as either CD19 positive (CD19\(^{+}\)) or CD19\(^{-}\).\(^{107}\) CD19\(^{-}\) relapse is associated with limited CAR-T-cell persistence; however, the factors determining CAR-T-cell persistence are yet to be fully understood\(^{108}\) and patients with poor persistence of CAR-T cells would likely benefit from single-cell analysis. For patients who experience CD19\(^{-}\) relapse, multiple studies have shown that the loss of antigen expression results from impaired expression of CD19 mRNA.\(^{108–110}\) However, it is not clear whether CAR-T therapy
directly dysregulates CD19 transcription or allows the emergence of CD19neg clones to drive relapse. In fact, the exact mechanism may vary between patients. A greater understanding of the underlying mechanisms of response and immune evasion using single-cell methods will inform current strategies to overcome resistance and to improve the long-term efficacy of anti-CD19 CAR-T therapy.

Acquired resistance is one of the challenges associated with venetoclax (ABT-199), a BCL-2 inhibitor. Di Grande et al identified residual leukemic cells in the spleen after venetoclax monotherapy in an ETP-ALL cell xenograft model and using scRNA-seq showed that genes involved in T-cell differentiation (GATA3, c-MYB) were upregulated. GATA3 and c-MYB are known to play important roles in early lymphoid cell development, and the late acquisition may limit the efficacy of targeted therapies against NOTCH1. Similarly, CRLF2 has identified mechanisms of relapse and novel molecular targets for the treatment of relapsed pediatric ALL. Future studies with larger cohorts of patients, even more cells and the integration of multiple single-cell states will provide a deeper understanding of clonal diversity, therapy resistance, and relapse.

Figure 2. Single-cell technology can complement current MRD monitoring in high-risk pediatric ALL. The kinetics of leukemia burden throughout treatment can be measured through MRD methodologies including flow cytometry and RT-qPCR of IgH/TCR gene rearrangement (IG/TCR). Single-cell analyses of BMA have the potential to provide orthogonal information on the clonal nature of disease and help inform rational treatment decisions. This schematic represents a fictional case study of a high-risk Ph−like ALL patient to show the potential benefit of complimenting MRD monitoring with serial single-cell analyses of both the genome and transcriptome. At diagnosis, a BMA is taken for MRD monitoring of IG/TCR rearrangements and enriched for leukemia cells (e.g., using FACS) for single-cell analysis. ScDNA-seq identifies three major clones (C1, C2, and C3) with a common CRLF2-IGH translocation and IKZF1 deletion. Following standard induction therapy, the patient remains MRD positive indicative of a poor response. Combining scDNA-seq and scRNA-seq, three clones (C3, C4, and C5) are identified, linked by a common JAK2 mutation, and a recommendation is put forward to add ruxolitinib to the standard-of-care chemotherapy. This patient then moves into clinical remission (MRD negative); however, later relapses with scRNA-seq revealing two clones (C5 and C6), both with BCL-2 overexpression. The BCL-2 inhibitor venetoclax is administered in combination with standard-of-care chemotherapeutic agents and leads to a second remission.
been suggested as a promising therapeutic target in B-ALL, but the occurrence of CRLF2 rearrangements as both early and late events indicates that not all patients with CRLF2 rearrangements may respond to CRLF2 targeted therapy. This highlights how single-cell technology cannot only identify novel molecular targets but can also deconvolute the order of mutation acquisition to inform the development of targeted therapeutics.

**FUTURE DIRECTIONS**

Recent single-cell studies in pediatric ALL have provided an insight into tumor heterogeneity, clonal evolution, the role of the BMM, and mechanisms of treatment resistance and relapse. The clinical application of single-cell technology has promising potential to inform prognosis and treatment decisions for children with ALL. Although single-cell technologies are providing unique insights into ALL biology and treatment response, the “cost per cell” currently remains prohibitive for routine analysis. However, it is to be expected that these costs will fall with time as they did for bulk sequencing, allowing this technology to eventually be used in routine patient care. So far, single-cell studies in pediatric ALL have focused on the characterization of the leukemic genome and transcriptome. Future studies focusing on the epigenome and its integration with other omics will further contribute to our understanding of pediatric ALL heterogeneity and mechanisms of chemoresistance and relapse. However, regardless of the “omic,” to really understand the biology of ALL, we will need samples from thousands of children with and without ALL and to sequence thousands of cells from each sample. Perhaps it does seem impossible, overwhelming, or ambitious to talk of these numbers, but that’s what was said about the human genome project over 20 years ago!

**AUTHOR CONTRIBUTIONS**

HCLP and KH wrote the manuscript and were involved in the conception of the article. TT and RBL provided critical revision and interpretation of the concepts presented in the article. HL and CEDB provided critical revisions, were involved in conceptual design of the review, supervised the writing and gave final approval of the version to be published. All authors approved the final manuscript.

**DISCLOSURES**

The authors have no conflicts of interest to disclose.

**REFERENCES**

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin. 2019;69:7–34.
2. Puhi CH, Yang JJ, Bhakta N, et al. Global efforts toward the cure of childhood acute lymphoblastic leukemia. Lancet Child Adolesc Health. 2018;2:440–454.
3. Inaba H, Mullighan CG. Pediatric acute lymphoblastic leukemia. Haematologica. 2020;105:2524–2539.
4. Locatelli F, Schrappe M, Bernardo ME, et al. How I treat relapsed childhood acute lymphoblastic leukemia. Blood. 2012;120:2807–2816.
5. Nguyen K, Devidas M, Cheng SC, et al; Children’s Oncology Group. Landscape, clonal evolution patterns, and role of RAS mutations in relapsed acute lymphoblastic leukemia. Proc Natl Acad Sci U S A. 2016;113:11306–11311.
6. Hunger SP, Lu X, Devidas M, et al. Improved survival for children with ALL. Blood. 2012;120:1349–1360.
7. Hunger SP, Lu X, Devidas M, et al. Improved survival for children and adolescents with acute lymphoblastic leukemia between 1990 and 2005: a report from the children’s oncology group. J Clin Oncol. 2012;30:1663–1669.
8. Hunger SP, Mullighan CG. Redefining ALL classification: toward detecting high-risk ALL and implementing precision medicine. Blood. 2015;125:3977–3987.
9. Roberts KG, Mullighan CG. Genomics in acute lymphoblastic leukemia: insights and treatment implications. Nat Rev Clin Oncol. 2015;12:344–357.
10. Mullighan CG, Phillips LA, Su X, et al. Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia. Science. 2008;322:1377–1380.
11. Ma X, Edmonson M, Vezina D, et al. Rise and fall of subclones from diagnosis to relapse in pediatric B-acute lymphoblastic leukemia. Nat Commun. 2015;6:6604.
12. Hof J, Krentz S, van Schewick C, et al. Mutations and deletions of the TP53 gene predict nonresponse to treatment and poor outcome in first relapse of childhood acute lymphoblastic leukemia. J Clin Oncol. 2011;29:3185–3193.
13. Oshima K, Khiabani H, da Silva-Almeida AC, et al. Mutational landscape, clonal evolution patterns, and role of RAS mutations in relapsed acute lymphoblastic leukemia. Proc Natl Acad Sci U S A. 2016;113:11306–11311.
14. Irving J, Matheson E, Minto L, et al. Ras pathway mutations are prevalent in relapsed childhood acute lymphoblastic leukemia and confer sensitivity to MEK inhibition. Blood. 2014;124:3420–3430.
15. Mar BG, Bullinger LB, McLean KM, et al. Mutations in epigenetic regulators including SETD2 are gained during relapse in paediatric acute lymphoblastic leukemia. Nat Commun. 2015;6:3469.
16. Meyer JA, Wang J, Hogan LE, et al. Relapse-specific mutations in NOTCH2 in childhood acute lymphoblastic leukemia. Nat Genet. 2013;45:290–294.
17. Mullighan CG, Zhang J, Kasper LH, et al. CRELBBP mutations in relapsed acute lymphoblastic leukaemia. Nature. 2011;471:235–239.
18. Tzouycheva G, Perez-Garcia A, Carpenter Z, et al. Activating mutations in the NOTCH2 nucleotide gene drive chemotherapy resistance in relapsed ALL. Nat Med. 2013;19:368–371.
19. Pijuan-Sala B, Guibentif C, Göttingers B. Single-cell transcriptional profiling: a window into embryonic cell-type specification. Nat Rev Mol Cell Biol. 2018;19:399–412.
20. Argelaguet R, Clark SJ, Mohammed H, et al. Multi-omics profiling of mouse gastrulation at single-cell resolution. Nature. 2019;576:487–491.
21. Giladi A, Amit I. Single-cell genomics: a stepping stone for future immunology discoveries. Cell. 2018;172:14–21.
22. Lawson DA, Kessenbrock K, Davis RT, et al. Tumour heterogeneity and mutational diversity at single-cell resolution. Nat Cell Biol. 2018;20:1349–1360.
23. Nam AS, Chaligere R, Landau DA. Integrating genetic and non-genetic determinants of cancer evolution by single-cell multi-omics. Nat Rev Genet. 2021;22:3–18.
24. Svensson V, Vento-Tormo R, Teichmann SA. Exponential scaling of single-cell RNA-seq in the past decade. Nat Protoc. 2018;13:599–604.
25. Stuart T, Satija R. Integrative single-cell analysis. Nat Rev Genet. 2019;20:257–272.
26. Malikic S, Jahn K, Kuipers J, et al. Integrative inference of subclonal tumour evolution from single-cell and bulk sequencing data. Nat Commun. 2019;10:2750.
27. Gerstung M, Jolly C, Leshchiner I, et al. The evolutionary history of 2,658 cancers. Nature. 2020;578:122–128.
28. Miles LA, Bowman RL, Merlinsky TR, et al. Single-cell mutation analysis of clonal evolution in myeloid malignancies. Nature. 2020;587:477–482.
29. McMahon CM, Ferring T, Canaani J, et al. Clonal selection with RAS pathway activation mediates secondary clinical resistance to selective FLT3 inhibition in acute myeloid leukemia. Cancer Discov. 2020;9:1050–1063.
30. Alberti-Servera L, Demeyer S, Govaerts I, et al. Single-cell RNA amplification sequencing reveals clonal heterogeneity and evolution in T-cell acute lymphoblastic leukemia. Blood. 2021;137:801–811.
31. van Galen F, Hovestadt V, Wadsworth Li MH, et al. Single-cell RNA-Seq reveals AML hierarchies relevant to disease progression and immunity. Cell. 2019;176:1265–1281.e24.
32. Rabilloud T, Potter D, Pankawa S, et al. Single-cell profiling identifies pre-existing CD19-negative subclones in a B-ALL patient with CD19-negative relapse after CAR-T therapy. Nat Commun. 2021;12:865.
33. Chen YP, Yin JH, Li WF, et al. Single-cell transcriptomics applied to embryonic stem cells. Nat Methods. 2013;10:368–371.
34. Tzoneva G, Perez-Garcia A, Carpenter Z, et al. Activating mutations in the NOTCH2 nucleotide gene drive chemotherapy resistance in relapsed ALL. Nat Med. 2013;19:368–371.
35. Stuart T, Satija R. Integrative single-cell analysis. Nat Rev Genet. 2019;20:257–272.
36. Malikic S, Jahn K, Kuipers J, et al. Integrative inference of subclonal tumour evolution from single-cell and bulk sequencing data. Nat Commun. 2019;10:2750.
37. Gerstung M, Jolly C, Leshchiner I, et al. The evolutionary history of 2,658 cancers. Nature. 2020;578:122–128.
38. Miles LA, Bowman RL, Merlinsky TR, et al. Single-cell mutation analysis of clonal evolution in myeloid malignancies. Nature. 2020;587:477–482.
39. McMahon CM, Ferring T, Canaani J, et al. Clonal selection with RAS pathway activation mediates secondary clinical resistance to selective FLT3 inhibition in acute myeloid leukemia. Cancer Discov. 2020;9:1050–1063.
40. Alberti-Servera L, Demeyer S, Govaerts I, et al. Single-cell RNA amplification sequencing reveals clonal heterogeneity and evolution in T-cell acute lymphoblastic leukemia. Blood. 2021;137:801–811.
37. Macosko EZ, Basu A, Satija R, et al. Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. Cell. 2015;161:1202–1214.

38. Hashimshony T, Senderovich N, Avital G, et al. CEL-Seq2: sensitive highly-multiplied single-cell RNA-Seq. Genome Biol. 2016;17:77.

39. Fawker-Corbett D, Antanaviciute A, Parikh K, et al. Spatiotemporal analysis of human intestinal development at single-cell resolution. Cell. 2021;184:810–826.e23.

40. Litvivuková M, Talavera-López C, Maatz H, Reichart D, Worth CI, Lindberg EL, et al. Cells of the adult human heart. Nature. 2020;588:466–472.

41. Joglekar A, Prijibelski A, Mahfouz A, et al. A spatially resolved brain region- and cell-type-specific histone atlas of the postnatal mouse brain. Nat Commun. 2021;12:463.

42. Singh M, Al-Eryani G, Carswell S, et al. High-throughput targeted long-read single cell sequencing reveals the clonal and transcriptional landscape of lymphocytes. Nat Commun. 2019;10:3120.

43. Jones PA, Isa JP, Baylin S. Targeting the cancer epigenome for therapy. Nat Rev Genet. 2016;17:630–641.

44. Corces MR, Buenrostro JD, Wu B, et al. Lineage-specific and single-cell chromatin accessibility charts human hematopoiesis and leukemia evolution. Nat Genet. 2016;48:1193–1203.

45. Gkountela S, Castro-Gner F, Szczerba BM, et al. Circulating tumor cell clustering shapes DNA methylation to enable metastasis seeding. Cell. 2019;176:111–122.

46. Farlik M, Sheffield NC, Nuzzo A, et al. Single-cell DNA methylome sequencing and bioinformatic inference of epigenomic cell-state dynamics. Cell Rep. 2015;10:1386–1397.

47. Hunt KV, BURNARD SM, ROEP EA, et al. scTREM-seq: single-cell analysis of transposable element methylation to link global epigenetic heterogeneity with transcriptional programs. Scientific Reports. 2022;12:5776.

48. Kuendgen A, Müller-Thomas C, Lauseker M, et al. Efficacy of azacitidine enabled by mitochondrial mutations and single-cell genomics. Cell. 2019;176:1325–1339.e22.

49. De Bie J, Demeyer S, Alberti-Servera L, et al. Single-cell sequencing reveals the origin and the order of mutation acquisition in T-cell acute lymphoblastic leukemia. Leukemia. 2018;32:1358–1369.

50. Thiele C, Wunderling K, Leyendecker P. Multiplexed and single cell tracking of lipid metabolism. Nat Methods. 2019;16:1123–1130.

51. Gavard C, Koh W, Quake SR. Dissecting the clonal origins of childhood acute lymphoblastic leukemia by single-cell genomics. Proc Natl Acad Sci U S A. 2014;111:17947–17952.

52. De Bie J, Demeyer S, Alberti-Servera L, et al. Single-cell sequencing identifies CRLF2 rearrangements as both early and late events in Down syndrome and non-Down syndrome acute lymphoblastic leukaemia. Leukemia. 2019;33:893–904.

53. Furness CL, Mansur MB, Weston VJ, et al. The subclonal complexity of STIL-TAL1+ T-cell acute lymphoblastic leukaemia. Leukemia. 2018;32:1984–1993.

54. Meyers S, Alberti-Servera L, Gielen O, et al. Monitoring of leukemia clones in B-cell acute lymphoblastic leukaemia at diagnosis and during treatment by single-cell DNA amplicon sequencing. Hemasphere. 2022;6:e780.

55. Anderson K, Lutz C, van Delft FW, et al. Genetic variegation of clonal architecture and propagating cells in leukaemia. Nature. 2011;469:356–361.

56. Turati VA, Guerra-Assunção J, Potter NE, et al. Chemotherapy induces canalization of cell state in childhood B-cell precursor acute lymphoblastic leukaemia. Nat Cancer. 2021;12:835–832.

57. Caron M, St-Onge P, Sontag T, et al. Single-cell analysis of childhood leukemia reveals a link between developmental states and ribosomal protein expression as a source of intra-individual heterogeneity. Sci Rep. 2020;10:8079.

58. Gale KB, Ford AM, Repp R, et al. Backtracking leukemia to birth: identification of clonotypic gene fusion sequences in neonatal blood spots. Proc Natl Acad Sci U S A. 1997;94:13950–13954.

59. Wiemels JL, Cazzaniga G, Danioi M, et al. Prenatal origin of acute lymphoblastic leukaemia in children. Lancet. 1999;354:1499–1503.

60. Qin P, Pang Y, Hou W, et al. Integrated decoding hematopoiesis and leukemogenesis using single-cell sequencing and its medical implication. Cell Discov. 2021;7:2.

61. Zamora AE, Crawford JC, Allen EK, et al. Pediatric patients with acute lymphoblastic leukemia generate abundant and functional neoantigen-specific CD8+ T cell responses. Sci Transl Med. 2019;11 eaat8549.

62. Das RK, vernau L, Grupp SA, et al. Naive T-cell deficits at diagnosis and after chemotherapy for impeding T cell potential in pediatric cancers. Cancer Discov. 2019;9:492–499.

63. Lindseth DS, Lemes R, Patos DM. Immunosuppressive monocytes (CD14+/HLA-DRlow/) increase in childhood precursor B-cell acute lymphoblastic leukemia after induction chemotherapy. Med Oncol. 2018;35:36.

64. Witkowski MT, Dolgalev I, Eversen NA, et al. Extensive remodeling of the immune microenvironment in B cell acute lymphoblastic leukaemia. Cancer Cell. 2020;37:867–882.e12.

65. Schoof EM, Furtwangler B, Uresin N, et al. Quantitative single-cell proteomics as a tool to characterize cellular hierarchies. Nat Commun. 2021;12:3341.

66. Specht H, Emmott E, Petelski AA, et al. Single-cell proteomic and transcriptomic analysis of macrophage heterogeneity using SC0PE2. Genome Biol. 2021;22:50.

67. Thiele C, Wunderling K, Leyendecker P. Multiplexed and single cell tracking of lipid metabolism. Nat Methods. 2019;16:1123–1130.

68. Sutton R, Venn NC, Tolosano J, et al. Clinical significance of minimal residual disease at day 15 and at the end of therapy in childhood acute lymphoblastic leukaemia. Br J Haematol. 2009;146:292–299.
89. Antic Z, Yu J, Bornhauser BC, et al. Clonal dynamics in pediatric B-cell precursor acute lymphoblastic leukemia with very early relapse. *Pediatr Blood Cancer*. 2022;69:e29361.

90. Edirwickrema A, Aleshin A, Reiter JG, et al. Single-cell mutational profiling enhances the clinical evaluation of AML MRD. *Blood Adv*. 2020;4:943–952.

91. Moorman AW, Schwab C, Wintember E, et al. Adjuvant tyrosine kinase inhibitor therapy improves outcome for children and adolescents with acute lymphoblastic leukaemia who have an ABL-class fusion. *Br J Haematol.* 2020;191:844–851.

92. Chang BH, Willis SG, Stork L, et al. Imatinib resistant BCR-ABL1 mutations at relapse in children with Ph+ ALL: a Children's Oncology Group (COG) study. *Br J Haematol.* 2012;157:507–510.

93. Apperley JF. Part I: mechanisms of resistance to imatinib in chronic myeloid leukaemia. *Lancet Oncol.* 2007;8:1018–1029.

94. Maude SL, Frey N, Shaw PA, et al. Chimeric antigen receptor T cells for leukemia. *Blood*. 2020;14:689–700.

95. Hovorkova L, Zaliova M, Venn NC, et al. Monitoring of childhood ALL using BCR-ABL1 genomic breakpoints identifies a subgroup with CML-like biology. *Blood*. 2017;129:2771–2781.

96. Stumpel DJ, Schneider P, van Roon EH, et al. Specific promoter methylation identifies different subgroups of MLL-rearranged infant acute lymphoblastic leukaemia, influences clinical outcome, and provides therapeutic options. *Blood*. 2009;114:5490–5498.

97. Bhojwani D, Pui CH. Relapsed childhood acute lymphoblastic leukaemia harboring ABL-class rearrangements. *Blood*. 2022;6:6 www.hemaspherejournal.com

98. Teachey DT, Hunger SP. Predicting relapse risk in childhood acute lymphoblastic leukemia. *Br J Haematol.* 2013;162:606–620.

99. Bhojwani D, Pui CH. Relapsed childhood acute lymphoblastic leukaemia. *Lancet*. 2014;371:1507–1517.

100. Fischer J, Paret C, El Malki K, et al. CD19 isoforms enabling resistance to CART-19 immunotherapy are expressed in B-ALL patients at initial diagnosis. *J Immunother.* 2017;40:187–195.

101. Orlando EJ, Han X, Tribouley C, et al. Genetic mechanisms of target antigen loss in CAR19 therapy of acute lymphoblastic leukaemia. *Nat Med*. 2018;24:1504–1506.

102. Sotillo E, Barrett DM, Black KL, et al. Convergence of acquired mutations and alternative splicing of CD19 enables resistance to CART-19 immunotherapy. *Cancer Discov*. 2015;5:1282–1295.

103. Di Grande A, Peirs S, Donovan PD, et al. The spleen as a sanctuary site for residual leukemic cells following ABT-199 monotherapy in ETP-ALL. *Blood Adv*. 2021;5:1963–1976.

104. Ho IC, Tai TS, Pai SY. GATA3 and the T-cell lineage: essential functions before and after T-helper-2-cell differentiation. *Nat Rev Immunol.* 2009;9:125–135.

105. Maurice D, Hooper J, Lang G, Weston K. c-Myb regulates lineage choice in developing thymocytes via its target gene Gata3. *EMBO J*. 2007;26:3629–3640.

106. Ortmann CA, Kent DG, Nagalj J, et al. Effect of mutation order on myeloproliferative neoplasms. *N Engl J Med*. 2015;372:601–612.

107. Weng AP, Ferrando AA, Lee W, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science*. 2004;306:269–271.

108. Russell LJ, Capasso M, Vater I, et al. Deregulated expression of cytokine receptor gene, CRLF2, is involved in lymphoid transformation in B-cell precursor acute lymphoblastic leukemia. *Blood*. 2009;114:2688–2698.

109. Harvey RC, Mulhaghan CG, Chen IM, et al. Rearrangement of CRLF2 is associated with mutation of JAK kinases, alteration of IKZF1, hispanic/latino ethnicity, and a poor outcome in pediatric B-progenitor acute lymphoblastic leukemia. *Blood*. 2010;115:5312–5321.