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## **Down syndrome and leukemia: from basic mechanisms to clinical advances**

Baruchel, Andre ; Bourquin, Jean-Pierre ; Crispino, John ; Cuartero, Sergi ; Hasle, Henrik ; Hitzler, Johann ; Klusmann, Jan-Henning ; Izraeli, Shai ; Lane, Andrew A ; Malinge, Sebastien ; Rabin, Karen R ; Roberts, Irene ; Ryeom, Sandra ; Tasian, Sarah K ; Wagenblast, Elvin

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# Down syndrome and leukemia: from basic mechanisms to clinical advances

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## Abstract

Children with Down syndrome (DS, trisomy 21) are at a significantly higher risk of developing acute leukemia compared to the overall population. Many studies investigating the link between trisomy 21 and leukemia initiation and progression have been conducted over the last two decades. Despite improved treatment regimens and significant progress in identifying genes on chromosome 21 and the mechanisms by which they drive leukemogenesis, there is still much that is unknown. A focused group of scientists and clinicians with expertise in leukemia and DS met in October 2022 at the Jérôme Lejeune Foundation in Paris, France for the 1<sup>st</sup> International Symposium on Down Syndrome and Leukemia. This meeting was held to discuss the most recent advances in treatment regimens and the biology underlying the initiation, progression, and relapse of acute lymphoblastic leukemia and acute myeloid leukemia in children with DS. This review provides a summary of what is known in the field, challenges in the management of DS patients with leukemia, and key questions in the field.

## Introduction

Children with Down syndrome (DS, trisomy 21) have a significantly increased risk of both myeloid and lymphoid leukemia compared to the general pediatric population.<sup>1</sup> Understanding the mechanisms of leukemia predisposition related to constitutive trisomy 21 (T21) and characterizing the genetic landscape and multistep pathogenesis of DS-associated leukemias have led to major discoveries over the last two decades<sup>2-11</sup> (Figure 1). Notably, many of the genetic, cellular, and molecular mechanisms found in DS-associated leukemias are relevant in non-DS individuals, as gain of chromosome 21 is also frequently observed in hematologic malignancies as a somatic event.<sup>12</sup> Significant progress has been made in the treatment of

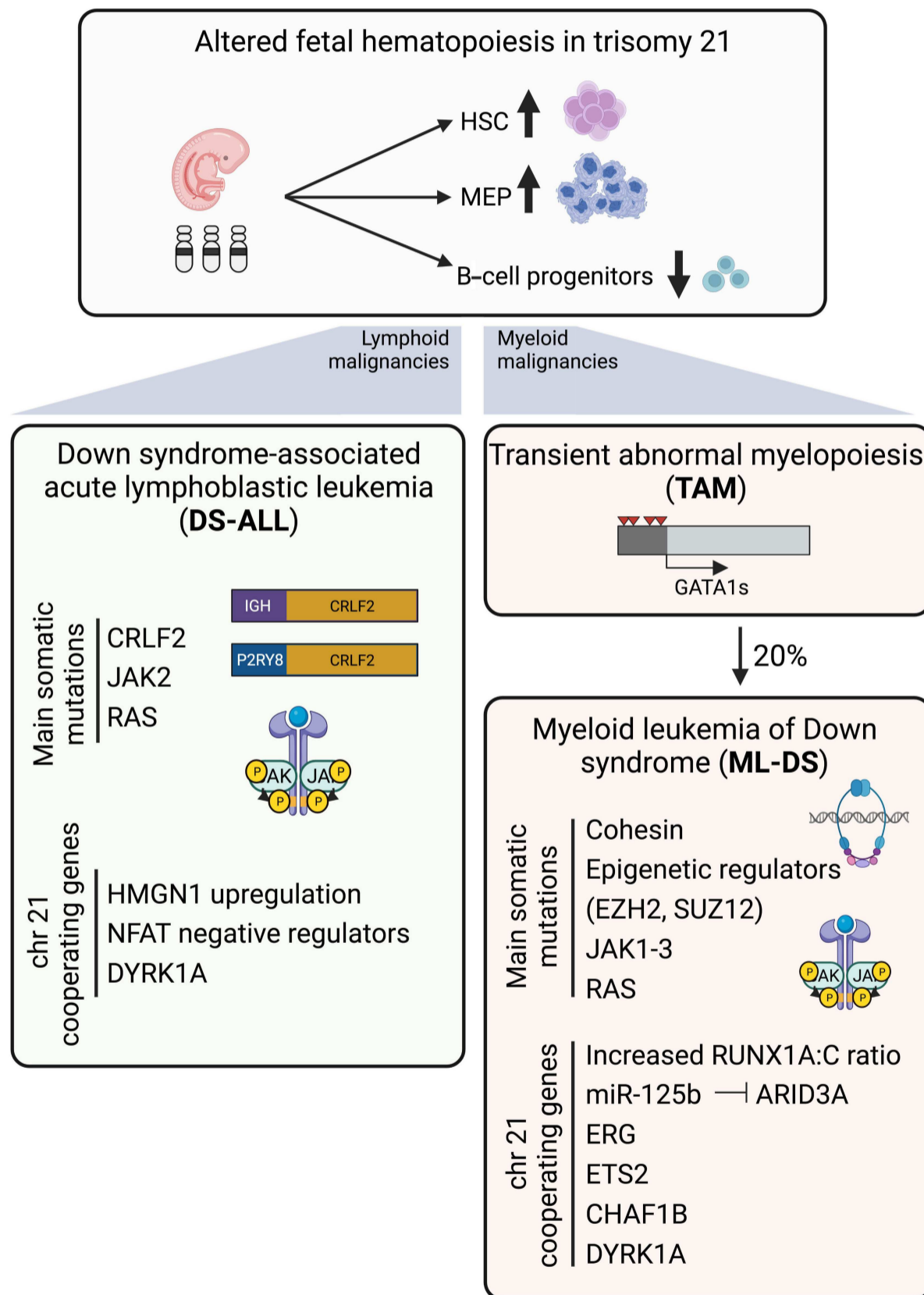
children with myeloid leukemia of Down syndrome (ML-DS) with 5-year survival now approaching 90%. In contrast, children with Down syndrome-associated B-cell acute lymphoblastic leukemia (DS-ALL) have worse outcomes than non-DS children with ALL, in part due to a high sensitivity to chemotherapy.<sup>13,14</sup> Outcomes for relapsed/refractory leukemia in children with DS are extremely poor, highlighting the need to improve quality of care for these children who have other T21-associated health issues that complicate their chemotherapy treatment. This review summarizes the current knowledge in the field and the discussions of a panel of scientists and clinicians on topics that range from treatment regimens and complications, to leukemia predisposition and initiation, from mechanistic insights to key questions in the field.

# Clinical challenges related to Down syndrome-associated leukemia

## Epidemiology of Down syndrome and leukemia

Studies on the unique epidemiological patterns of cancer in DS include those that identified an increased risk of leukemia in children with DS ranging from a 3- to 100-fold

increase with the true risk increase estimated at 10- to 20-fold. The ratio of lymphoid to myeloid leukemias is higher in non-DS children, at 5:1, than it is in children with DS, in whom the ratio is closer to 1:1. One of the largest studies investigating the incidence of leukemia in DS was a Danish study<sup>1</sup> examining 2,814 children with DS with long-term updates provided in 2016.<sup>15</sup> Overall, the cumulative risk of leukemia in children with DS is 2% by 5 years



**Figure 1. Overview of Down syndrome-associated leukemia.** Trisomy 21 affects fetal blood formation, causing an increase in hematopoietic stem cells and megakaryocyte-erythroid progenitors, but a decrease in B-cell progenitors. Mutations in *GATA1* cause transient abnormal myelopoiesis, which can lead to myeloid leukemia of Down syndrome (ML-DS) upon acquisition of additional somatic mutations. In the lymphoid branch, alterations in *CRLF2* or *JAK2* can lead to Down syndrome-associated acute lymphoblastic leukemia (DS-ALL). In both ML-DS and DS-ALL, the increased dosage of genes located on chromosome 21 cooperates with somatic mutations in disease onset and progression. Figure generated with BioRender.com. HSC: hematopoietic stem cells; MEP: megakaryocyte-erythroid progenitors; chr 21: chromosome 21.

of age and 2.5% by 30 years of age. In DS-ALL, about 50% of patients overexpress *CRLF2* and 20% have *JAK2* mutations. *GATA1* mutations are seen in almost all patients with ML-DS but are effectively absent in non-DS children. ML-DS is also associated with somatic mutations in other genes, with the next most common group of alterations found in genes encoding effectors of the cohesin complex as well as epigenetic regulators.<sup>3,4,6</sup> The unique ML-DS disease may also occur in rare individuals with a germline *GATA1* mutation and acquired T21.<sup>16</sup>

Another unusual aspect of the cancer spectrum in DS is the reduced frequency of solid tumors in this population,<sup>15,17,18</sup> with the exception of testicular cancer. Possible explanations for this decreased cancer incidence were discussed in a report by Ossuna-Marco and colleagues.<sup>19</sup> Based upon these observations, cancer screening recommendations for adults with DS<sup>20</sup> include screening for colon cancer similar to the general public, no screening for breast cancer, and screening for cervical cancer only in sexually active women 25 years and older. Annual screening for testicular cancer is recommended between the ages of 15 and 45 years.

### Myeloid leukemias and Down syndrome

The key clinical subsets of DS-associated leukemias are: (i) transient abnormal myelopoiesis (TAM); (ii) ML-DS with *GATA1s* mutation; and (iii) DS-ALL. A recent study examining leukemia risk in a cohort of 3.9 million children, of whom 4,401 had DS, documented a statistically significant risk of acute myeloid leukemia (AML) before the age of 5 years with the risk being highest for ML-DS. This raises the question of whether all children with DS should be empirically screened for *GATA1s*. Early intervention approaches, such as the TMD 2007 prevention trial (TMD07) in which 102 infants with DS and clinical symptoms of TAM were treated at diagnosis or 8 weeks after positive minimal residual disease (MRD) detection with low-dose cytarabine chemotherapy, showed a reduction in TAM-related mortality, but did not prevent disease progression to future ML-DS.<sup>21</sup>

ML-DS can be associated with low blood counts and bone marrow blasts, which may be lower than 20% in the bone marrow and mostly show a megakaryocytic phenotype (previously classified as acute megakaryocytic leukemia in children with DS). Central nervous system involvement and chromosomal translocations are observed more frequently in non-DS acute megakaryocytic leukemia than in ML-DS. DS blasts are hypersensitive to chemotherapy, especially cytarabine, etoposide, and anthracyclines. Outcomes for children with ML-DS are favorable with event-free survival approaching 90%. The early treatment experience showed that high dose-intensive chemotherapy was not beneficial in children with ML-DS due to high treatment-related mortality.<sup>22</sup> Subsequently, clinicians in-

roduced DS-specific myeloid-directed chemotherapy protocols that pursued a stepwise reduction of treatment intensity and achieved a reduction of both treatment-related mortality and cardiotoxicity. A recent study by the Children's Oncology Group (COG) in children with ML-DS reduced treatment intensity through approaches including: (i) anthracycline chemotherapy reduction; (ii) etoposide reduction; and (iii) identification of patients on the basis of flow MRD or high-risk ML-DS for treatment with high-dose cytarabine-containing cycles. However, the trial was stopped due to the inability to define standard-risk patients with ML-DS for high-dose cytarabine omission by flow cytometry MRD assessment.<sup>23</sup> While very low-dose cytarabine regimens can be curative,<sup>24</sup> an appropriate subset of patients who can be cured with therapy reduction cannot currently be identified.

The most recent COG AAML1531 phase III clinical trial for children with ML-DS investigated whether it is necessary to use high-dose cytarabine as part of treatment in all patients with ML-DS given that the bulk of infectious adverse events is associated with this treatment element. Results of this study called into question whether stratification of treatment intensity for ML-DS can be based on MRD. MRD levels were measured in the bone marrow by flow cytometry after the first course of treatment. Patients with ML-DS who were MRD-negative were treated without high-dose cytarabine but turned out to have an inferior probability of survival compared to historical controls (treated with high-dose cytarabine). The conclusion was that the intensity of treatment could not be reduced by omitting high-dose cytarabine even for MRD-negative patients.<sup>23</sup> While treatment-related mortality has been successfully reduced for children with ML-DS, management of relapsed/refractory ML-DS is emerging as a therapeutic challenge. Whereas patients with primary ML-DS have a very favorable prognosis, the outlook for those with relapsed/refractory disease is dire.

Overall, challenges of ML-DS treatment include how to perform risk stratification integrating new information about molecular subgroups of ML-DS, how to reduce relapse events, how to improve outcomes for refractory and relapsed ML-DS and how to get access to new agents for patients with DS, who historically were routinely excluded from early phase trials. There is a need for a leukemia prevention strategy in children with DS if it becomes feasible to identify the subset of patients with TAM at highest risk of progression to ML-DS and to develop an early, safe, and efficacious intervention.

### Lymphoblastic leukemias and Down syndrome

Analysis of eight clinical trials across ten countries with matched DS-ALL and non-DS-ALL patient characteristics found lower event-free survival for patients with DS-ALL. Event-free survival for patients with *ETV6-RUNX1* fusions



was 79% in DS-ALL and 96% in non-DS-ALL cases. Patients with DS-ALL harboring *IKZF1* deletions were also noted to be at highest risk of MRD positivity and/or relapse. Dr. Baruchel discussed the ongoing European ALLTogether clinical trial (NCT03911128) that includes pediatric patients with DS-ALL treated with blinatumomab (a monoclonal antibody targeting CD19) replacing two chemotherapy consolidation blocks. In this trial, patients with standard-risk DS-ALL are not receiving blinatumomab, while those with intermediate-risk disease are being randomized to blinatumomab-containing therapy or standard-of-care chemotherapy. A recent study of 16 children with relapsed/refractory DS-ALL treated with CD19-directed chimeric antigen receptor (CAR) T-cell therapy found clinical outcomes and toxicities that were comparable to those of children with non-DS-ALL,<sup>25</sup> further supporting the potential use of CD19-targeted immunotherapy for children with DS-ALL in first relapse in an attempt to avoid the toxicity of hematopoietic stem cell (HSC) transplantation. In an ongoing study of the French LEA cohort with more than 6,000 children cured of acute leukemia, 67 patients with DS-ALL are being compared to 201 matched patients with non-DS ALL and also to age-matched DS patients without leukemia followed at the Fondation Jérôme Lejeune (Paris, France). This study will help to dissect what complications are related to ALL treatment and which are predictably related to DS, including overweight, cataracts, bone mineral deficiency, and hypothyroidism (L. Nizery, C. Mircher, G. Michel, A. Baruchel; personal communication). Outcome data from cases of DS-ALL (n=743) compared to non-DS-ALL (n=21,703) treated on four COG trials conducted between 2003-2019 demonstrate the challenges in the clinical management of DS-ALL.<sup>26</sup> Favorable cytogenetic alterations (*ETV6::RUNX1* and trisomy of chromosomes 4 and 10) were significantly less frequent in DS-ALL than in non-DS-ALL (14.4% vs. 46.7%,  $P<0.0001$ , as were unfavorable cytogenetic alterations (*BCR::ABL1*, *KMT2A-R*, hypodiploid, and *iAMP21*) (0.7% vs. 7.2%,  $P<0.0001$ ), whereas neutral cytogenetic alterations were more frequent (84.9% vs. 46.2%,  $P<0.0001$ ). Early treatment-related mortality was observed among DS-ALL patients; the deaths were primarily due to overwhelming sepsis during periods of neutropenia. Treatment modifications were then implemented, including reductions in anthracyclines and intravenous methotrexate, administration of leucovorin rescue after intrathecal methotrexate, shortened maintenance duration for boys, and reduction of vincristine/steroid pulses during maintenance. Enhanced supportive care measures were instituted, including inpatient observation during intensely myelosuppressive phases, antibiotic and antifungal prophylaxis, and IgG monitoring and replacement. The 5-year event free survival and overall survival were poorer for DS-ALL than for non-DS-ALL patients overall, and within the National Cancer Institute's

standard-risk and high-risk subgroups. The cumulative incidence of relapse was higher (11.7% vs. 9.3%,  $P=0.0198$ ), as were the risks of induction death (3.4% vs. 0.8%,  $P<0.0001$ ) and death in remission (4.8% vs. 1.7%,  $P<0.0001$ ). DS-ALL cases with *CRLF2* overexpression had a better 5-year event free survival and better overall survival than non-DS-ALL cases. In multivariable analysis, risk factors independently associated with inferior event-free survival in DS-ALL included age at diagnosis  $\geq 10$  years, initial white blood count  $\geq 50 \times 10^9/L$ , and end-induction MRD  $\geq 0.01\%$ . Patients with DS also exhibited increased rates of mucositis, infection, hyperglycemia, and seizures.

The most recent COG trial for children with DS incorporates blinatumomab to replace some elements of intensive chemotherapy. An increased risk of seizures was observed among DS-ALL patients over 10 years old, leading to an amendment requiring antiseizure prophylaxis during blinatumomab infusions for these patients. The trial has been temporarily suspended because it met a futility rule for inability to demonstrate a statistically significant decrease in treatment-related mortality in high-risk DS patients. The next COG trial for DS-ALL will likely employ a different approach for high-risk DS-ALL patients, extensively utilizing immune-targeted therapies such as blinatumomab and inotuzumab (a monoclonal antibody targeting CD22) to replace intensive chemotherapy blocks (K. Rabin; personal communication).

The frequency of *CRLF2* rearrangements (either *P2RY8::CRLF2* fusions or *IGH::CRLF2* translocations) is high in DS-ALL and these rearrangements are also the most common genetic alteration in approximately 50% of Philadelphia chromosome-like (Ph-like) ALL.<sup>27,28</sup> Preclinical and clinical studies demonstrated that constitutive JAK/STAT and other kinase signaling in *CRLF2*-rearranged Ph-like ALL may be targetable by tyrosine kinase inhibitors.<sup>28-31</sup> Emerging data from Dr. Tasian's laboratory suggest that preclinical *CRLF2*-rearranged DS-ALL and non-DS Ph-like ALL patient-derived xenografts (PDX) are similarly sensitive to the selective JAK1/2 inhibitor ruxolitinib *in vivo*. Following demonstration of safety and identification of a recommended phase II dose of ruxolitinib in children with relapsed/refractory cancers in the COG ADVL1011 phase I trial,<sup>32</sup> ruxolitinib in combination with multi-agent chemotherapy is under evaluation in children, adolescents, and young adults with newly-diagnosed *CRLF2*-rearranged or other JAK pathway-mutant Ph-like ALL via the recently completed COG AALL1521 phase II clinical trial that is currently awaiting outcome data to address its primary endpoint.<sup>33</sup> However, patients with DS-ALL were excluded from participation in ADVL1011 and AALL1521, so the potential clinical activity of JAK inhibitor strategies in this population remains unknown.

Given the inferior clinical outcomes of children with DS-ALL and their increased risk of toxicity, particularly with

HSC transplantation, significant interest exists in development of immunotherapeutic strategies. In addition to the aforementioned CD19- and CD22-targeting antibody-based and cellular immunotherapies, recent preclinical studies have shown potent activity of CAR T cells targeting the thymic stromal lymphopoietin receptor (TSLPR) encoded by *CRLF2*.<sup>34</sup> A first-in-human phase I clinical trial of TSLPR-CAR T-cell immunotherapy for adults and children with relapsed/refractory TSLPR/*CRLF2*-positive leukemia is planned to open in 2023. Additional preclinical studies in *CRLF2*-rearranged Ph-like and DS-ALL PDX models are also investigating the therapeutic potential of combining CAR T cells and kinase inhibitors to augment long-term remission. A recent report by Tasian and colleagues showed that co-treatment with ruxolitinib and TSLPR CAR T cells impaired T-cell functionality, although it was beneficial in reducing life-threatening inflammatory cytokine production. Conversely, delayed administration of ruxolitinib augmented anti-leukemia activity in both Ph-like ALL and DS-ALL and suggests future potential for a 'maintenance' inhibitor therapeutic strategy following CAR T-cell immunotherapy.<sup>35</sup>

The current European IntReALL clinical trial for children with relapsed ALL includes correlative biology studies of biospecimens from patients with very high-risk non-DS ALL and DS-ALL with the goal of drug response profiling. Machine learning and artificial intelligence can be used to perform such profiling in ALL cells with the ability to classify specific cell types (e.g., leukemia versus stromal) and perform cell segmentation analysis. An individual drug sensitivity profile can be developed for patient cohorts and will allow the development of new disease management strategies for patients with relapsed/refractory leukemia. Drug response profiling can also be utilized as a tool to identify appropriate precision therapies for interval stabilization of patients prior to CD19-CAR T-cell immunotherapy. Anecdotes of patients in whom drug response profiling was used as a 'bridge' until the next treatment include a highly chemorefractory ALL patient who underwent drug response profiling and was treated with cytarabine and the MEK inhibitor, trametinib due to the presence of a *KRAS*<sup>G12D</sup> mutation. Leukemia burden was markedly reduced, and the patient was ultimately able to proceed to HSC transplantation in remission. Drug response profiling was conducted in another patient with a *CNTRL::ABL* fusion and identified sensitivity to ponatinib (a tyrosine kinase inhibitor), which was used for leukemia stabilization prior to CD19 CAR T-cell therapy. Drug response profiling complements existing technology platforms for precision oncology, and an International Leukemia Board for relapsed/refractory disease is now utilizing patients' data from drug response profiling studies. A Pan-European precision tumor board is further prioritizing targets and therapy options by embedding drug

response profiling into early phase clinical trials to guide therapy or individualized therapies as well harmonizing the data dictionary and decentralizing data management.

## Mechanisms underlying the increased incidence of leukemia in children with Down syndrome

### Susceptibility and predisposition to leukemia

The role of T21 in disrupting fetal hematopoiesis occurs through genome-wide transcriptional perturbation, including genes encoding transcription factors, pro-inflammatory cytokines, and various microRNA in fetal hematopoietic stem and progenitor cells and in stromal cells. Infants with DS display expanded prenatal HSC and myeloid progenitors, with fetal liver HSC being significantly biased toward erythro-megakaryopoiesis compared to disomic controls. The impact of T21 in early hematopoiesis has been confirmed using human induced pluripotent stem cells (iPSC).<sup>36-38</sup> Indeed, reproducing hematopoiesis from trisomic iPSC has shown that T21 alone is sufficient to enhance erythropoiesis, and that DS fetal-like hematopoietic progenitors have an increased capacity to form myeloid and megakaryoblastic colonies.

The pre-leukemic step, TAM (which precedes ML-DS) is unique to DS, is almost exclusively due to acquired *GATA1s* in T21 cells and often undergoes spontaneous remission shortly after birth (before 90 days). However, in some cases the *GATA1s* mutation persists, and children with DS develop ML-DS. The transcription factor *GATA1* is a master regulator of blood cell development, especially erythropoiesis and megakaryopoiesis, and mutations are found in virtually all TAM and ML-DS cases. This somatic mutation leads to the expression of a shorter isoform named *GATA1s*, lacking the N-terminal transactivation domain. Interestingly, these mutations have been recently identified in familial congenital anemia<sup>39</sup> and in a subset of Diamond-Blackfan anemia that displays dyserythropoiesis and dysmegakaryopoiesis,<sup>40,41</sup> as well as somatically in megakaryoblastic leukemias in children without DS who present a genetic landscape similar to that of ML-DS, including gain of chromosome 21.<sup>42-44</sup> Roberts and colleagues at Oxford have identified *GATA1s* mutations present in ~25% of neonates with DS.<sup>45</sup> Seven of these infants ultimately developed ML-DS (6%); one of these patients had a clinically 'silent' TAM, with blasts <10%, no clinical features and a low variant allele frequency of a *GATA1s* mutation. More than 90% of *GATA1* mutations and blasts resolve spontaneously, disappearing before 90 days of life with no *GATA1s* mutations acquired postnatally. It has been found that *GATA1s* blasts are much less proliferative in the postnatal environment until additional muta-

tions are acquired and ML-DS develops. Applying CRISPR/Cas9-mediated genome engineering strategies in T21 livers from normal and DS fetuses, Wagenblast and colleagues showed that *GATA1s* induces a bias toward megakaryopoiesis and cooperates with T21 to lead to a TAM phenotype.<sup>10</sup> *In vitro* analyses of human iPSC derived from TAM specimens or genetically engineered to express *GATA1s*, confirmed that DS hematopoietic progenitors expressing *GATA1s* are biased toward the myelo-megakaryocytic compartment.<sup>38,46,47</sup>

The existence of a pre-leukemic state in DS-ALL is currently unknown, but recent results suggest that T21 alone has an impact on fetal B lymphopoiesis, compatible with a prenatal origin. Jardine and colleagues described the effect of T21 on fetal bone marrow hematopoiesis with a predominance of erythroid cells, a significant loss of B progenitors, and a skewing of B and NK lymphoid output of fetal bone marrow HSC and multipotent progenitors.<sup>9</sup> These cells are unable to make B cells even *in vitro*.<sup>48</sup> T21 human iPSC also have a decreased ability to form B cells *in vitro*.<sup>49</sup> Fetal T21 cells are less efficient at making T cells and there is an activation of pro-inflammatory patterns of gene expression.<sup>9</sup> T21 also perturbs gene expression in stromal cells<sup>9</sup> and studies of stromal cell function are currently ongoing.

A genome-wide association study of inherited genetic variants associated with ALL susceptibility in children with DS<sup>7</sup> included 542 cases (children with DS-ALL) and 1,192 controls (children with DS without ALL). The study identified four susceptibility loci at genome-wide significance, at *IKZF1*, *CDKN2A*, *ARID5B*, and *GATA3*. While these loci have also been associated with ALL susceptibility in children without DS, T21 appears to modify the penetrance of inherited ALL susceptibility with a greater magnitude of effect size for *CDKN2A*. A whole genome sequencing study is in progress, which will enable identification of rare, structural, and chromosome 21 variants associated with ALL susceptibility in DS. Another study in progress is evaluating the association between co-occurring birth defects and ALL risk in children with DS. Dr. Rabin presented results of a study of somatic genomic characterization of a cohort of 295 cases of DS-ALL characterized by whole genome and whole transcriptome sequencing.<sup>50</sup> In accordance with prior reports, *CRLF2* rearrangements were identified in 54% of DS-ALL, versus 6% of a non-DS-ALL comparator cohort. *CRLF2*-rearranged cases were significantly younger at diagnosis and more frequently involved *P2RY8* versus *IGH* as the fusion partner in DS-ALL compared to non-DS-ALL.

### Impact of chromosome 21 genes on leukemia

Mouse models of DS have been utilized to 'hunt' for oncogene(s) on T21. Dr. Klusmann's laboratory performed a CRISPR screen for potential new oncogenes on T21 using

CMK cells (ML-DS) and identified *RUNX1* as a candidate.<sup>51</sup> Deletion of *RUNX1* in an ML-DS PDX model suppressed xenograft growth. *RUNX1* is transcribed from two different promoters and has three isoforms in humans: *RUNX1A*, *1B*, and *1C*. ML-DS patients present a disequilibrium of these *RUNX1* isoforms with a higher *RUNX1A/RUNX1C* ratio. *RUNX1A* blocks megakaryoblast differentiation while the *1C* isoform blocks proliferation. *RUNX1A* expression cooperates with *GATA1s* to increase megakaryocytic phenotypes and induce an ML-DS-like phenotype *in vivo*. Mechanistically, *RUNX1A* binds to the MYC binding partner MAX allowing for upregulation of a MYC/E2F-induced proliferative program. Interestingly, *GATA1s* plus *RUNX1A* synergize with *miR-125b* expression to further enhance a malignant phenotype. Recent observations suggest that the therapeutic potential of MYC inhibitors, including MYCi361, to disrupt the *RUNX1A/MAX* interaction should be explored in ML-DS, as well as in other myeloid leukemias harboring T21.<sup>52</sup>

*ERG* is an ETS-family transcription factor very similar to *FLI1*, and both are involved in oncogenic fusions found in Ewing sarcoma. As *ERG* is located on chromosome 21, studies have addressed a possible role for *ERG* overexpression in fetal liver hematopoiesis, which could explain the aberrant hematopoietic differentiation of T21 and possible involvement in subsequent TAM or ML-DS. Dr. Izraeli and colleagues showed that *ERG* overexpression transforms mouse fetal liver megakaryocytic progenitors and increases the production of megakaryocyte-erythroid progenitors, especially in cooperation with *GATA1s*. High *ERG* expression correlates with poor prognosis in AML and is associated with an increased fraction of leukemic stem cells. Forced expression of *ERG* represses myeloid differentiation genes and the activation of HSC self-renewal genes. It was found that a single amino acid substitution (proline 199 to leucine: P199L) at the end of the 'pointed' (PNT) domain is involved in protein-protein interactions. In pre-leukemic conditions, the PNT domain has a role in preserving the stem cell phenotype associated with *ERG*, thereby preventing premature differentiation.<sup>53</sup> Although it does not cause leukemia on its own or change the secondary structure of *ERG*, P199L is sufficient to disrupt *ERG*'s transforming ability in fetal liver transplantation assays and self-renewal in colony-forming assays. Interestingly, the myeloid differentiation block normally induced by *ERG* was partially reversed by the P199L mutant. Using Bio-ID proximity ligation mass spectrometry, Dr. Izraeli found that the P199L mutant was unable to interact with the HDAC3/NCoR repressive complex. While H3K27 acetylation at myeloid differentiation genes was lost in *ERG*-transduced cells, this did not happen in cells with the *ERG* P199L mutant. In AML cell lines that were dependent on *ERG* for growth, targeting HDAC3 blocked their growth *in vitro* and *in vivo*, but did not in AML



cells that were ERG-independent, suggesting that HDAC3 inhibition may be a therapeutic strategy for ERG-dependent AML (*S. Izraeli, unpublished data*). Future challenges include the investigation of the role of epigenetic interactors with ERG in leukemia and other malignancies.

Dr. Klusmann and colleagues have also investigated three chromosome 21 microRNA (miRNA) for their contribution to ML-DS. Notably, these three miRNA are located in a region of chromosome 21 that is not included in the commonly used Ts65Dn mouse model of DS. The three candidate miRNA: *miR-125b*, *miR-99a* and *let-7c* were investigated *in vitro* using mouse fetal liver cells genetically engineered by CRISPR to generate a *GATA1s* mutation. Studies by Dr. Klusmann's group investigating each of the miRNA in different combinations found that only *miR-125b* cooperates with *GATA1s* to increase the pool of megakaryocyte progenitors *in vitro* and led to aggressive acute megakaryocytic leukemia when transplanted into recipient mice.<sup>54</sup> They identified the epigenetic regulator *ARID3A* as the main target of *miR-125b* and showed that *ARID3A* is highly expressed during normal megakaryopoiesis. This study also showed that *ARID3A* is post-transcriptionally repressed by *mir-125b*, which is highly expressed in TAM and ML-DS. Loss or knock-down of *ARID3A* leads to enhanced proliferation and prevents differentiation of megakaryocyte progenitors promoting an ML-DS-like signature, as well as enrichment of *GATA1s*-related E2F and MYC targets. Their data show that *ARID3A* is a megakaryocytic transcription factor cooperating with *GATA1s* and is a novel tumor suppressor gene in TAM/ML-DS.

Global chromatin alterations are associated with T21 in both myeloid and lymphoid leukemias. Lane and colleagues have shown transcriptional similarities between DS and other B-ALL with gain of chromosome 21. They identified *HMGN1*, located on 21q22, as an oncogene in DS-ALL and other B-ALL with somatic +21.<sup>8</sup> *HMGN1* is a chromatin structural protein that de-compacts chromatin and acts in opposition to histone H1. Modulation of *HMGN1* expression levels induces a genome-wide increase in transcription<sup>55</sup> and a specific de-repression of genes controlled by the PRC2 complex in B-ALL. This is due to an increase in H3K27 acetylation and an increase of B-cell factors. Trisomy of *HMGN1* in a DS mouse model (Ts1Rhr) or its ectopic expression promotes pre-B-cell colony expansion and self-renewal, a phenotype that can be reversed with GSK-J4, which targets *HMGN1* via inhibition of histone demethylases. Loss-of-function models demonstrated that *HMGN1* is necessary for a pro-B phenotype. Interestingly, *HMGN1* has been shown to upregulate the expression of *CRLF2*, a gene that is somatically altered in about 50% of DS-ALL.<sup>56</sup>

The role of *HMGN1* in AML has also been investigated.<sup>57</sup> *HMGN1* is highly expressed in stem/progenitor cells and is necessary for differentiation of myeloid progenitors. Its

overexpression in myeloid progenitors increases chromatin accessibility and transcription in the *HOXA* cluster. The *HMGN1* nucleosome binding domain is sufficient for the differentiation phenotype, and *HMGN1* promotes hematopoietic and leukemia stem cell activity. This *HMGN1* overexpression phenotype can be reversed by targeting the histone acetyl transferases CBP/p300. A newly developed mouse model of conditional *Hmgn1* deletion shows a possible effect on *Kmt2a-Mllt3* (formerly *Mll-AF9*) induced leukemogenesis. Global chromatin profiling studies during normal and malignant myeloid differentiation have revealed H3K23 acetylation as one of the most dramatic changes during normal *versus* AML differentiation. Since histone H3K23 acetylation is catalyzed by the lysine acetyltransferase KAT6A/6B and with recent reports showing that KAT6A and ENL form an epigenetic transcriptional control to drive leukemogenesis,<sup>58</sup> studies should explore whether there is a common T21 epigenetic phenotype and whether DS, hematopoietic differentiation, and leukemia are connected in the epigenome. Therapeutic approaches targeting epigenetic regulators have been investigated in pre-clinical models and in patients. Klusmann and colleagues tested LSD1 inhibition in combination with JAK/STAT inhibition in a preclinical ML-DS model and proposed this combination for future clinical trials.<sup>59</sup> Other epigenetic therapies, such as histone deacetylase inhibitors or azacytidine, have been administered to individual patients with good responses,<sup>3,60-63</sup> further supporting a role for epigenetic therapies in the treatment of DS-associated leukemias.

Interestingly, chromosome 21 encoded genes may also play a role in the treatment-related mortality observed in DS-ALL patients as well as the decreased incidence of solid tumors associated with DS. Four inhibitors of the calcineurin-NFAT signaling pathway, *S100b*, *PCP4*, *DSCR1*, and *DYRK1A*, are located on chromosome 21 and all function to negatively regulate the calcium-sensitive ser/thr phosphatase calcineurin. The calcineurin pathway is important for neutrophil and macrophage function. Dr. Ryeom has hypothesized that attenuation of this pathway in innate immune cells blocks their ability to effectively eliminate infections observed in patients with DS-ALL, leading to increased treatment-related mortality. Dr. Ryeom's laboratory has developed a mouse model of DS with attenuation of calcineurin-NFAT signaling by the expression of a third copy of *Dscr1* and can model treatment-related mortality in these mice after treatment with dexamethasone followed by challenge with the bacterial endotoxin, lipopolysaccharides. Ongoing studies in her laboratory are investigating neutrophil and macrophage dysfunction in this DS mouse model with chromosome 21 genes suppressing calcineurin signaling in these immune cells with the intent of identifying biomarkers of early treatment-related mortality in patients with DS-ALL. Pre-



vious studies by Ryeom and colleagues identified suppression of calcineurin signaling in endothelial cells as a mechanism underlying the reduced incidence of solid tumors observed in individuals with DS.<sup>64</sup> They and others found defects in tumor angiogenesis in DS mouse models and teratomas derived from T21 iPSC, suggesting that attenuation of calcineurin signaling blocks endothelial cell activation and suppresses tumor angiogenesis, thereby preventing the expansion of microscopic dormant tumors. Other studies investigating cancer protection in the DS population involve the contribution of chromosome 21 encoded tumor suppressor genes, such as *ETS2*.<sup>64-67</sup>

### Key somatic mutations in Down syndrome-associated leukemia

Dr. Crispino's laboratory has been instrumental in determining the role of *GATA1s* mutations and T21 in driving DS leukemogenesis and has discovered key chromosome 21 genes (*ERG*, *CHAF1B* and *DYRK1A*) that are responsible for the increased risk of ML-DS in children with DS. Crispino and colleagues have also demonstrated that beyond the known expansion of megakaryopoiesis, *GATA1s* expression also impairs erythropoiesis.<sup>68</sup> The erythroid phenotype can be seen during fetal life through at least 20 months, with the knock-in *Gata1s* mouse model displaying macrocytic anemia and bone marrow fibrosis.<sup>68,69</sup> Mechanistically, *GATA1s* expression in erythroid cells is associated with major deficiencies in gene regulation which can be rescued by loss of one copy of *GATA2*, as shown in collaboration with Dr. Izraeli's laboratory.<sup>70</sup> These data re-emphasize the key role of *GATA1* and of its alteration in the erythromegakaryocytic compartment.

Previous large-scale sequencing studies found that the most frequently mutated protein complex in ML-DS is cohesin, one of the main drivers of three-dimensional genome folding. Interestingly, the main partner of cohesin, the DNA-binding protein CTCF, is also recurrently mutated among patients with ML-DS.<sup>3,4</sup> While both proteins are also frequently mutated in other cancers, it is unusual to find both mutated at such high frequencies in the same cancer type. Cohesin and CTCF are responsible for partitioning the genome into topologically associating domains, which are functional units of gene regulation that both facilitate and limit the range of action of enhancer-promoter interactions.<sup>71</sup> However, only a minor fraction of genes are highly sensitive to perturbations in the levels of cohesin or CTCF. By depleting cohesin in mouse macrophages and hematopoietic progenitors, studies by Cuartero and colleagues showed a dramatic reduction in inflammatory gene expression, indicating a key role for cohesin in the activation of inducible genes.<sup>72</sup> Interestingly, a similar phenotype was observed after depletion of CTCF.<sup>73</sup> The inability to respond to inflammatory signals was linked to the incapacity of AML cells to differentiate, as normal HSPC

differentiate in response to inflammation.<sup>74</sup> However, how the control of inducible gene expression plays a role in the transition from TAM to ML-DS remains an unresolved issue, as does the possibility of interplay between cohesin/CTCF and mutant *GATA1* chromatin binding.

### Down syndrome and leukemia models

DS leukemogenesis can be modeled using primary human HSPC and xenotransplantation in mice. Utilizing fetal liver from normal and DS fetuses, Wagenblast and colleagues found that T21 HSC are immunophenotypically expanded.<sup>10</sup> Applying CRISPR approaches to functionally interrogate the impact of *GATA1s* and *STAG2*, alone or in cooperation, they found a lower CD45<sup>+</sup> engraftment for T21 fetal liver long-term HSC as compared to non-DS HSC except when *GATA1s* is expressed and *STAG2* deleted. Interestingly in this model, T21 is required for pre-leukemia initiation but seems to be dispensable for leukemia progression upon *STAG2* mutation. Blasts recapitulate the immunophenotype of TAM and ML-DS with *STAG2* knockout cells in DS fetal liver HSC surviving long-term. Recently, a separate study showed that loss of the cohesin effector SMC3 and knock in of MPL-activating mutations gradually transformed T21/*GATA1s* human iPSC into ML-DS blasts by promoting a differentiation blockade and proliferation.<sup>47</sup> Dr. Wagenblast showed that the predisposition/initiation step is at least partly controlled by chromosome 21 miRNA (*miR-99*, *miR-125b-2* and *miR-155a*). Simultaneous overexpression of all three miRNA recapitulates a T21-like hematopoietic state based on differentiation potential, transcriptional and open chromatin accessibility profiles. Furthermore, knock-out of these three miRNA led to reduced blast accumulation in mice. Dr. Wagenblast also demonstrated that expression of CD117 in the CD34<sup>+</sup> compartment marks pre-leukemia and leukemia-initiating cells, and that TAM can be therapeutically targeted with KIT inhibitors.

Wagenblast and colleagues are now using a similar CRISPR approach in which a 320 kb interstitial deletion is induced in cord blood and fetal liver HSPC to model *P2RY8::CRLF2* fusions in DS-ALL, which leads to pre-leukemia development upon xenotransplantation in mice (*E. Wagenblast, unpublished data*).

PDX models from primary patients' specimens (peripheral blood or bone marrow samples obtained from children with DS) that are propagated in immune-deficient NSG mice are being developed by Dr. Malinge and colleagues to facilitate preclinical studies of DS-ALL. These new models are undergoing comprehensive characterization at genetic, transcriptional, phenotypic and functional levels and are representative of several molecular subtypes of DS-ALL. Using these models, it was found that RAS/MAPK activation cooperates with T21 in DS-ALL. *NRAS* and *KRAS* mutations are frequently found in high hyperdiploid ALL,

a subtype of B-ALL that often harbors trisomy, tetrasomy, or even pentasomy of chromosome 21. Treatment of DS-ALL PDX models with MEK inhibitors (e.g., selumetinib, trametinib) decreased cell growth *in vitro* and leukemia burden *in vivo*, prolonging survival of DS-ALL PDX, as well as other models of B-ALL with somatic +21 (e.g., iAMP21, high hyperdiploid). *In vitro* and *in vivo* drug combinations show that RAS/MAPK inhibition in combination with vincristine and dexamethasone is effective in several DS-ALL PDX models with various genetic backgrounds (eg, *KRAS*- or *CRLF2/JAK2*-mutant). Malinge and colleagues work suggests that RAS/MAPK inhibition may be a promising therapeutic strategy in DS-ALL and other B-ALL with +21.<sup>12</sup> Recent studies showed that the chromosome 21 kinase *DYRK1A* is not only overexpressed in DS-ALL, but also in other subtypes of B-ALL with +21.<sup>75</sup> *DYRK1A* was inhibited by the small molecule EHT1610 in preclinical DS-ALL models and demonstrated that *DYRK1A* phosphorylates key targets in B-ALL cells (e.g., cyclin D3, FOXO1, STAT3). Inhibiting *DYRK1A* activity directly or one of its downstream targets prolongs survival of non-DS-ALL and non-DS B-ALL +21 models.

## Key questions in the field

Despite the significant progress made over the past two decades in understanding the mechanisms driving leukemia in DS individuals, as well as improvements in treatment regimens, there is still much to be learned. Led by Dr. Crispino, the final session of the symposium was a thought-provoking discussion on key questions in the field of DS and leukemia. Somatic *GATA1* mutations are probably the most distinctive trait of ML-DS, but why are these mutations so frequent in neonates with DS? A very strong selective pressure is undoubtedly in place, but the highly proliferative nature of fetal liver progenitors may also increase the rate of DNA lesions, which would help to explain this unusual frequency (~25%) of *GATA1* mutations. This raises another important, yet not fully resolved question: what are the most important *GATA1* target genes that are dysregulated in TAM/ML-DS? *GATA1* is a transcription factor, and any selective advantage acquired by cells is most likely through dysregulated gene expression. Yet, full-length *GATA1* and the shorter *GATA1s* share the same DNA-binding domain, and the mechanistic differences in how they control gene expression are still unclear. More broadly, identifying with precision the chromosome 21-encoded genes responsible for ML-DS and DS-ALL leukemogenesis would open up significant therapeutic opportunities. Despite numerous studies seeking such genes, the most likely explanation is a combined effect rather than a single gene. Finally, it was widely agreed that PDX models will be key in investigating these issues and identifying new treatments

for children with ML-DS and DS-ALL. Expanding and sharing the existing repertoire of DS leukemia-specific PDX resources for research is a high scientific priority.

Our understanding of the multi-step progression of leukemia in DS will undoubtedly improve in the upcoming years, and such an advancement will offer important insights into the development of all leukemias beyond those occurring in children with T21. One of the main goals of the meeting in France was the establishment of new collaborations with the intent of bringing together DS and leukemia experts in 2024 for a 2<sup>nd</sup> International Symposium for DS and Leukemia to discuss progress made and the most recent knowledge regarding the causes of DS-associated leukemias, the optimal treatment regimens and best practices for affected patients.

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