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Down syndrome and leukemia: from basic mechanisms to clinical advances

André Baruchel¹, Jean-Pierre Bourquin², John Crispino³, Sergi Cuartero⁴*, Henrik Hasle⁵, Johann Hitzler⁶, Jan-Henning Klussmann⁷, Shai Izraeli⁸,9, Andrew A Lane¹⁰, Sebastien Malinge¹¹*, Karen R Rabin¹², Irene Roberts¹³, Sandra Ryeom¹⁴*, Sarah K Tasian¹⁵, Elvin Wagenblast¹⁶

¹Hôpital Universitaire Robert Debré (APHP and Université Paris Cité), Paris, France; 
²University Children’s Hospital, Zurich, Switzerland; 
³St. Jude Children’s Research Hospital, Memphis, TN, USA; 
⁴Josep Carreras Leukemia Research Institute, Barcelona, Spain; 
⁵Department of Pediatrics and Adolescent Medicine, Aarhus University Hospital, Aarhus, Denmark; 
⁶The Hospital for Sick Children, Toronto, Canada; 
⁷Department of Pediatrics, Goethe University Frankfurt, Frankfurt, Germany; 
⁸Schneider Children's Medical Center of Israel, Petah Tikva, Israel; ⁹Dept. of Human Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel Aviv University 
¹⁰Dana-Farber Cancer Institute, Boston, MA, USA; 
¹¹Telethon Kids Institute - Cancer Centre, Perth, Australia; 
¹²Baylor College of Medicine, Texas Children’s Cancer Center, Houston, TX, USA; 
¹³University of Oxford, Oxford, England; 
¹⁴Department of Surgery, Herbert Irving Comprehensive Cancer Center, Columbia University Irving Medical Center, New York, NY, USA; 
¹⁵Children’s Hospital of Philadelphia and University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA; 
¹⁶Icahn School of Medicine at Mount Sinai, New York, NY, USA

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* Co-corresponding authors: Sergi Cuartero, scuartero@carrerasresearch.org; Sébastien Malinge, sebastien.malinge@telethonkids.org.au; Sm, swr2117@cumc.columbia.edu

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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 (T21) 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 Down syndrome met in October 2022 at the Jérôme Lejeune Foundation in Paris, France for the 1st 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.\textsuperscript{1} Understanding the mechanisms of leukemia predisposition related to constitutive trisomy 21 (T21) and characterizing the genetic landscape and multistep pathogenesis of DS-associated leukemias has led to major discoveries over the last two decades\textsuperscript{2-11} (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 are also frequently observed in hematological malignancies as a somatic event.\textsuperscript{12}

Significant progress has been made in the treatment of children with myeloid leukemia of Down syndrome (ML-DS) with a 5-year survival now approaching 90%. In contrast, children with Down syndrome-associated B-acute lymphoblastic leukemia (DS-ALL) have worse outcomes than ALL in non-DS children in part due to a high sensitivity to chemotherapy.\textsuperscript{13,14} 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 trisomy 21-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 Associated with Down Syndrome-Associated Leukemia

Epidemiology of Down Syndrome and Leukemia

The unique epidemiological patterns of cancer in Down syndrome include studies that identified an increased risk of leukemia in children with DS ranging from a 3- to 100-fold increase with the true risk estimated at 10-20-fold. The fraction of lymphoid to myeloid leukemias is larger in non-DS children at a 5:1 ratio, while the ratio is closer to 1:1 in children with DS. One of the largest studies investigating the incidence of leukemia in DS was a Danish study\textsuperscript{1} examining 2,814 children with DS with long-term updates provided in 2016.\textsuperscript{15} Overall, the cumulative risk of leukemia in children with DS is 2% by 5 years of age and 2.5% by 30 years of age. In DS-ALL, about 50% of patients overexpress \textit{CRLF2} and 20% have \textit{JAK2} mutations. \textit{GATA1} mutations are seen in almost all patients with ML-DS and 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.\textsuperscript{3,4,6} The unique ML-DS disease may also occur in rare individuals with germline \textit{GATA1} mutation and acquired trisomy 21.\textsuperscript{16}
Another unusual aspect of the cancer spectrum in DS is the reduced frequency of solid tumors in this population,\textsuperscript{15,17,18} with the exception of testicular cancer. Possible explanations for this decreased cancer incidence were discussed in a report by Ossuna-Marco and colleagues.\textsuperscript{19} Based upon these observations, cancer screening recommendations for adults with DS\textsuperscript{20} 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 at 25 years and older. Screening for testicular cancer was recommended annually between the ages of 15-45.

\textit{Myeloid Leukemias and Down Syndrome}

The key clinical subsets of DS-associated leukemias include: (1) Transient Abnormal Myelopoiesis (TAM), (2) myeloid leukemia (ML)-DS with GATA1\textsubscript{s} mutation, and (3) DS-ALL. A recent study examining leukemia risk in a cohort of 3.9 million children of whom 4,401 had DS, reported a statistically significant risk for AML before age 5 with the highest risk for ML-DS. This raises the question of whether all children with DS should be empirically screened for GATA1\textsubscript{s}. Early intervention approaches, such as 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) with low-dose cytarabine chemotherapy, showed a reduction in TAM-related mortality, but did not prevent disease progression to future ML-DS.\textsuperscript{21}

ML-DS can be characterized by low blood counts and bone marrow blasts that may be lower than 20% in the bone marrow, which mostly show a megakaryocytic phenotype (previously classified as acute megakaryocytic leukemia [AMKL] in children with DS). Central nervous system involvement and chromosomal translocations are observed more frequently in non-DS AMKL as compared to 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 (EFS) 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 (TRM).\textsuperscript{22} Subsequently, clinicians introduced DS-specific myeloid-directed chemotherapy protocols that pursued a stepwise reduction of treatment intensity and achieved a reduction of both TRM and cardiotoxicity. A recent study by the Children’s Oncology Group (COG) in children with ML-DS reduced treatment intensity through approaches including (1) anthracycline chemotherapy reduction, (2) etoposide reduction, and (3) 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.\textsuperscript{23} While very low dose cytarabine regimens can be curative,\textsuperscript{24} an appropriate subset of patients who can be cured with therapy reduction cannot be currently identified.
The most recent COG AAML1531 phase 3 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. While TRM 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 subgroup 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 were feasible to identify the subset of patients with TAM at highest risk of progression to ML and to develop an early, safe, and efficacious intervention.

**Lymphoblastic Leukemias and Down Syndrome**

Analysis of eight clinical trials across 10 countries with matched DS-ALL and non-DS-ALL patient characteristics found lower EFS for patients with DS-ALL. EFS for patients with ETV6-RUNX1 fusions was 79% in DS-ALL and 96% in non-DS-ALL. 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. Patients with standard-risk DS-ALL do not receive blinatumomab, while those with intermediate risk disease are randomized to blinatumomab-containing therapy or standard of care chemotherapy. A recent study of 16 children with relapsed/refractory DS-B-ALL treated with CD19-directed chimeric antigen receptor T cell (CD19-CART) therapy found clinical outcomes and toxicities that were comparable to those of children with non-DS-ALL, further supporting the potential use of CD19-targeted immunotherapy for children with DS-ALL in first relapse in an attempt to avoid toxic hematopoietic stem cell (HSC) transplantation. In an ongoing study with the French LEA Cohort with more than 6000 children cured of acute leukemia, a comparison of 67 patients with DS-ALL to 201 matched patients with non-DS ALL and also to age-matched DS patients without leukemia are being followed at the Fondation Jerôme
Lejeune in Paris, France. This study will help dissect what is related to ALL treatment and what is 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 4 recent COG trials between 2003-2019 demonstrate the challenges in clinical management of DS-ALL\textsuperscript{26}. Favorable cytogenetic alterations were significantly less frequent in DS versus non-DS ALL (14.4% vs 46.7%, p <0.0001, consisting of ETV6::RUNX1 and trisomy of chromosomes 4 and 10), as were unfavorable cytogenetic alterations (0.7% vs 7.2%, p <0.0001, consisting of BCR::ABL1, KMT2A-R, hypodiploid, and iAMP21), whereas neutral cytogenetics was more frequent (84.9% vs 46.2%, p <0.0001). Early TRM was observed among DS-ALL patients, primarily due to overwhelming sepsis during periods of neutropenia. Treatment modifications were then implemented, including reductions in anthracyclines and IV 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 five-year EFS and overall survival (OS) were poorer for DS versus non-DS ALL overall, and within the NCI standard risk and high-risk subgroups. The cumulative incidence of relapse was higher (11.7% vs 9.3%, p = 0.0198), as well as the risk 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 EFS and OS than non-DS ALL cases. In multivariable analysis, risk factors independently associated with inferior EFS in DS-ALL included age at diagnosis >10 years, initial white blood count ≥50,000/uL, and end-induction minimal residual disease ≥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 TRM 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 high frequency of CRLF2 rearrangements (either P2RY8::CRLF2 fusions or IGH::CRLF2 translocations) in DS-ALL are also the most common genetic alteration in approximately 50% of Philadelphia chromosome-like (Ph-like) ALL\textsuperscript{27,28} Preclinical and clinical studies demonstrated that
constitutive JAK/STAT and other kinase signaling in \textit{CRLF2}-rearranged Ph-like ALL may be targetable by tyrosine kinase inhibitors\textsuperscript{28–31}. Emerging data from the Tasian laboratory suggest that preclinical \textit{CRLF2}-rearranged DS-ALL and non-DS Ph-like ALL patient-derived xenografts (PDXs) are similarly sensitive to the selective JAK1/2 inhibitor ruxolitinib in vivo. Following demonstration of safety and identification of a recommended phase 2 dose of ruxolitinib in children with relapsed/refractory cancers in the COG ADVL1011 phase 1 trial,\textsuperscript{32} ruxolitinib in combination with multi-agent chemotherapy is under evaluation in children, adolescents, and young adults with newly-diagnosed \textit{CRLF2}-rearranged or other JAK pathway-mutant Ph-like ALL via the recently-completed COG AALL1521 phase 2 clinical trial that is currently awaiting outcomes data to address its primary endpoint.\textsuperscript{33} 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 toxicity risk, particularly with HSC transplantation (HSCT), 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 \textit{CRLF2}.\textsuperscript{34} A first-in-human phase 1 clinical trial of TSLPR-CART immunotherapy for adults and children with relapsed/refractory TSLPR/CRLF2+ leukemia is planned to open in 2023. Additional preclinical studies in \textit{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 of ruxolitinib with TSLPR CAR-T cells impaired T cell functionality, although was beneficial in reducing life-threatening inflammatory cytokine production. Conversely, delayed administration of ruxolitinib augmented anti-leukemia activity in both Ph-like and DS-ALL and suggests future potential for a ‘maintenance’ inhibitor therapeutic strategy following CAR T cell immunotherapy.\textsuperscript{35}

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 (DRP). Machine learning and artificial intelligence can be used to perform automated DRP in ALL cells with the ability to classify specific cell types (eg, 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. DRP can also be utilized as a tool to identify appropriate precision therapies for interval stabilization of patients prior to CD19-CART immunotherapy. Anecdotes of patients who utilized DRP to ‘bridge’ until the next treatment include a highly chemorefractory ALL patient who underwent DRP and was treated with cytarabine and the MEK inhibitor, trametinib due to the presence of a \textit{KRAS}\textsuperscript{G12D} mutation. Leukemia burden was markedly reduced, and the patient was ultimately able to proceed to

\textit{CRLF2}
HSCT in remission. Another patient with a $CNTRL::ABL$ fusion used DRP to identify sensitivity to ponatinib (tyrosine kinase inhibitor) that was used for leukemia stabilization prior to CD19 CAR T therapy. DRP complements existing technology platforms for precision oncology, and an International Leukemia Board for relapsed/refractory disease is now utilizing patient data from DRP studies. A Pan-European precision tumor board is further prioritizing targets and therapy options by embedding DRP into early phase clinical trials to guide therapy or individualized therapies as well harmonizing the data dictionary and decentralized data management. Dr. Bourquin further emphasized the role of CD19-CART in the treatment of patients with relapsed/refractory DS-ALL with studies showing many patients in continued remission at up to 24 months post-therapy, congruent with remission rates reported in patients with relapsed/refractory non-DS-ALL.\textsuperscript{53}

**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, proinflammatory cytokines, and microRNAs in fetal hematopoietic stem and progenitor cells and in stromal cells. Infants with DS display expanded prenatal HSC and myeloid progenitors, with fetal liver HSCs being significantly biased toward erythro-megakaryopoiesis compared to disomic controls. The impact of T21 in early hematopoiesis has also been confirmed using human induced pluripotent stem cells (hiPSCs)\textsuperscript{36–38}. Indeed, reproducing hematopoiesis from trisomic iPSCs 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 (that 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 (and 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\textsuperscript{39} and in a subset of Diamond-Blackfan Anemia that displays dyserythropoiesis and dysmegakaryopoiesis,\textsuperscript{40,41} as well as somatically in megakaryoblastic leukemias developed in children without DS that present a genetic landscape similar to ML-DS, including gain of chromosome 21\textsuperscript{42–44}. Roberts and colleagues at Oxford have identified $GATA1s$ mutations present in \textasciitilde25% of neonates with DS\textsuperscript{45}. 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. Greater than 90% of GATA1 mutations and blasts spontaneously resolve, disappearing before 90 days of life with no GATA1s mutations acquired postnatally. They find that GATA1s blasts are much less proliferative in the postnatal environment until additional mutations 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. In vitro analyses of hiPSCs derived from TAM specimens or genetically engineered to express GATA1s, confirmed that DS hematopoietic progenitors expressing GATA1s are biased toward the myelo-megakaryocytic compartment.

In DS-ALL, the existence of a pre-leukemic state is currently unknown, but recent results suggest that T21 alone impacts fetal B lymphopoiesis, compatible with a prenatal origin. Jardine and colleagues described the T21 impact 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 HSCs and multipotent progenitors. These cells are unable to make B cells even in vitro. T21 hiPSC also have a decreased ability to form B cell in vitro. Fetal T21 cells are less efficient in making T cells and there is an activation of pro-inflammatory patterns of gene expression. T21 also perturbs gene expression in stromal cells with studies of stromal cell function currently ongoing.

Notably, a genome-wide association study of inherited genetic variants associated with ALL susceptibility in children with DS included 542 cases (children with DS-ALL) and 1,192 controls (children with DS without ALL). The study identified 4 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. 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 had a significantly younger age 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. Klusmann’s lab performed a CRISPR screen for potential new oncogenes on T21 using CMK cells (ML-DS) and identified RUNX1 as a candidate. 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 a 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 + 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 leukemia harboring T21.

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 of ERG overexpression in fetal liver hematopoiesis, which could explain the aberrant hematopoietic differentiation of T21 and a possible involvement in subsequent TAM or ML-DS. 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 causes the repression of myeloid differentiation genes and the activation of HSC self-renewal genes. They 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. 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 CFU 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 H3K27ac at myeloid differentiation genes was lost in ERG-transduced cells, this did not happen in 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 not AML cells that were ERG-independent, suggesting that HDAC3 inhibition may be a therapeutic strategy for ERG-dependent AMLs (unpublished data, S Izraeli). Future challenges include the investigation of the role of epigenetic interactors with ERG in leukemia and other malignancies.
Klusmann and colleagues have also investigated three chromosome 21 microRNAs (miRNAs) for their contribution to ML-DS. Notably, these three miRNAs are located in a region of chromosome 21 that is not included in the commonly used Ts65Dn mouse model of DS. The three candidate miRNAs: 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 from Klusmann’s group investigating each of the miRNAs in different combinations found that only miR-125b cooperates with GATA1s to increase the pool of megakaryocyte progenitors in vitro and led to aggressive AMKL when transplanted into recipient mice. They identified the epigenetic regulator ARID3A as the main target of miR-125b and show that ARID3A is highly expressed during normal megakaryopoiesis. Their studies show 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 a ML-DS-like signature, as well as an 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-ALLs with gain of chromosome 21. They identified HMGN1, located on 21q22, as an oncogene in DS-ALL and other B-ALL with somatic +21. HMGN1 is a chromatin structural protein that decompacts chromatin and acts in opposition to histone H1. Modulation of HMGN1 expression levels induces a genome wide increase in transcription 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 CFU-pre-B 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 CRLF2 expression, a gene that is somatically altered in about 50% of DS-ALL.

The role of HMGN1 in AML has also been investigated. 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 reveal 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, studies should explore whether there is a common T21 epigenetic phenotype and if 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. Other epigenetic therapies, such as HDAC inhibitors or Azacytidine, have been administered to individual patients with good responses, 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 TRM 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 with a proposed hypothesis from Dr. Ryeom 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 TRM. The Ryeom lab 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 TRM in these mice after treatment with dexamethasone followed by challenge with the bacterial endotoxin, lipopolysaccharides. Ongoing studies in her lab 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 TRM in patients with DS-ALL. Previous studies from 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. They and others show defects in tumor angiogenesis in DS mouse models and teratomas derived from T21 IPSCs, 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 include the contribution of chromosome 21 encoded tumor suppressor genes, such as ETS2.

Key Somatic Mutations in Down Syndrome-Associated Leukemia

The Crispino 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. This erythroid phenotype can be seen during fetal life through at least 20 months, where the Knock-In (KI) Gata1s mouse model displays macrocytic anemia and bone marrow fibrosis. Mechanistically, GATA1s expression in erythroid cells is associated with major deficiencies in gene regulation that can be rescued by loss of one copy of GATA2, as shown in collaboration with the Izraeli laboratory. These data re-emphasize the key role of GATA1 and of its alteration in the erythro-megakaryocytic 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 3D genome folding. Interestingly, the main partner of cohesin, the DNA-binding protein CTCF, is also recurrently genetically mutated among patients with ML-DS. 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. 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. Interestingly, a similar phenotype was observed after depletion of CTCF. The inability to respond to inflammatory signals was linked to the incapacity of AML cells to differentiate, as normal HSPCs differentiate in response to inflammation. However, how the control of inducible gene expression plays a role in the transition from TAM to ML-DS remains an open question, as well as 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 HSPCs and xenotransplantation in mice. Utilizing fetal liver from normal and DS fetuses, Wagenblast and colleagues found that T21 HSCs are immunophenotypically expanded. Using CRISPR approaches to functionally interrogate the impact of GATA1s and STAG2, alone or in cooperation, they show a lower CD45+ engraftment for T21 fetal liver long-term HSCs as compared to non-DS HSCs 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 HSCs surviving long-term. Recently, a separate study reported that loss of the cohesin effector SMC3 and KI of MPL activating mutations gradually transformed T21/GATA1s hiPSC into ML-DS blasts by promoting a differentiation blockade and...
Wagenblast shows that the predisposition/initiation step is at least partly controlled by chromosome 21 miRNAs (miR-99, miR-125b-2 and miR-155a). Simultaneous overexpression of all three miRNAs recapitulate a T21-like hematopoietic state based on its differentiation potential, transcriptional and open chromatin accessibility profiles. Further, knock-out of these three miRNAs led to reduced blast accumulation in mice. He also demonstrated that expression of CD117 in the CD34+ compartment marks pre-leukemia and leukemia initiating cells, and that TAM can therapeutically be targeted with KIT inhibitors.

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

PDX models from primary patient specimens (peripheral blood or bone marrow samples obtained from children with DS) that are propagated in immune deficient NSG mice are being developed by Malinge and colleagues to facilitate preclinical studies of DS-ALL. These new models are undergoing comprehensive characterization at the genetic, transcriptional, phenotypic and functional levels and are representative of several molecular subtypes of DS-ALL. Using these models, it was reported that RAS/MAPK activation cooperates with T21 in DS-ALL. NRAS and KRAS mutations are also frequently found in high hyperdiploid ALL, a subtype of B-ALL that frequently harbors trisomy, tetrasomy, or even pentasomy of chromosome 21. Treatment of DS-ALL PDX models with MEK inhibitors (eg, selumetinib, trametinib) decreased cell growth in vitro and leukemia burden in vivo prolonging survival of DS-ALL PDXs, as well as other models of B-ALL with somatic +21 (eg, 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 comprised of 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 +2112. 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 +2175. DYRK1A was inhibited with the small molecule EHT1610 in preclinical DS-ALL models and demonstrated that DYRK1A phosphorylates key targets in B-ALL cells (eg, cyclin D3, FOXO1, STAT3). Inhibiting DYRK1A activity directly or one of its downstream targets prolongs survival of 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 the main open 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 explain this unusual (~25%) frequency 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 these 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 questions 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 progress 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 this meeting was the establishment of new collaborations with the intent of bringing together DS and leukemia experts in 2024 for a 2nd International Symposium for DS and Leukemia to discuss progress made and the most recent knowledge regarding the causes, optimal treatment regimens and best practices for patients with DS-associated leukemias.
References


**Figure 1. Overview of Down syndrome leukemia.** Trisomy 21 impacts fetal blood formation, causing an increase in hematopoietic stem cells (HSC) and megakaryocyte-erythroid progenitors (MEP), but a decrease in B cell progenitors. Mutations in GATA1 can cause transient abnormal myelopoiesis (TAM), which can lead to myeloid leukemia of Down syndrome (ML-DS) upon acquisition of additional somatic mutations. On 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 certain genes located in chromosome 21 cooperates with somatic mutations in the disease onset and progression.
Altered fetal hematopoiesis in trisomy 21

Lymphoid malignancies

Myeloid malignancies

Down syndrome-associated acute lymphoblastic leukemia (DS-ALL)

Main somatic mutations

- CRLF2
- JAK2
- RAS

Cooperating genes

- HMGN1 upregulation
- NFAT negative regulators
- DYRK1A

Transient abnormal myelopoiesis (TAM)

- GATA1s
- 20%

Myeloid leukemia of Down syndrome (ML-DS)

Main somatic mutations

- Cohesin
- Epigenetic regulators (EZH2, SUZ12)
- JAK1-3
- RAS

Cooperating genes

- Increased RUNX1A:C ratio
- miR-125b → ARID3A
- ERG
- ETS2
- CHAF1B
- DYRK1A