REVIEW ARTICLE

Clinical and biological aspects of myeloid leukemia in Down syndrome

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Children with Down syndrome are at an elevated risk of leukemia, especially myeloid leukemia (ML-DS). This malignancy is frequently preceded by transient abnormal myelopoiesis (TAM), which is self-limited expansion of fetal liver-derived megakaryocyte progenitors. An array of international studies has led to consensus in treating ML-DS with reduced-intensity chemotherapy, leading to excellent outcomes. In addition, studies performed in the past 20 years have revealed many of the genetic and epigenetic features of the tumors, including *GATA1* mutations that are arguably associated with all cases of both TAM and ML-DS. Despite these advances in understanding the clinical and biological aspects of ML-DS, little is known about the mechanisms of relapse. Upon relapse, patients face a poor outcome, and there is no consensus on treatment. Future studies need to be focused on this challenging aspect of leukemia in children with DS.

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INTRODUCTION

For more than 6 decades we have known that children with Down syndrome (DS) have a significant risk for developing both acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) compared with the general population [1, 2]. Over the years, many groups have confirmed this finding of an increased risk of leukemia, including a Danish registry of 2814 children with DS between 1961 and1994 [2], a finish cohort of 3581 children with DS between 1978 and 1986 [3], an Australian cohort of 1442 children with DS between 1953 and 2002 [4], and an English cohort of 1453 children with DS between 1963 and1999 [5]. These studies reported a 150-fold increased risk in the development of AML for children with DS less than the age of 5, but a recent publication from 2021 of a cohort of 3.9 million children in North America demonstrated a nearly 400-fold increased risk of developing AML in this same group of children with DS who are less than 5 years of age [6].

Children with DS are not only at risk for AML, but also for transient abnormal myelopoiesis (TAM). The World Health Organization (WHO) classification recognizes these two myeloid proliferations related to Down syndrome as TAM and myeloid leukemia associated with Down syndrome (ML-DS) [7]. TAM, previously referred to as transient leukemia or transient myeloproliferative disorder, occurs in about 10% of infants with DS and often precedes ML-DS [8]. TAM and ML-DS commonly present with the clinical and hematologic features of acute megakaryoblastic leukemia (AMKL) [9]. While ML-DS and TAM share many features of AMKL, the immunophenotype of ML-DS tumors is distinct from blasts in morphologically similar related diseases in children without DS, such as AML [10]. TAM and ML-DS have overlapping immunophenotypes, including the presence of CD33, CD36, CD48, and the TPO-R, and frequently the megakaryocytic markers CD41, CD42b, and CD61 [10, 11]. Of note, the blasts were found to be negative for EPO-R, which was suggested to be a consequence of the *GATA1* mutation [10]. Several differences have been observed, however, such as the presence of the CD11b and CD13 in the AML phase [10, 11].

CLINICAL ASPECTS OF TAM AND ML-DS

Transient abnormal myelopoiesis

TAM, a transient clonal proliferation of myeloid blasts associated with the *GATA1* mutation, occurs in ~10% of infants with DS as determined by morphologic assessment of a blood smear [8]. The WHO defines TAM as an "increased peripheral blood blast cells in a neonate with DS" without specifying the minimal percentage of blasts. Screening children with DS with low numbers of circulating blasts using sensitive methods to detect a *GATA1* mutation, a genetic change unique to TAM and ML-DS, suggests that TAM may be quite common, with the incidence of *GATA1* mutations approaching 30% [8]. A Dutch surveillance study screened patients for TAM and similarly found patients with detectable *GATA1* mutation without an increase of blasts. This group suggests that the WHO criteria should be updated to "the presence of at least 5% blasts defined by immunophenotyping or morphology and/or the presence of a *GATA1* mutation in a neonate with DS" allowing for uniform diagnosis of TAM across groups going forward [12]. Alternatively, Tunstall et al. proposed that TAM (which they refer to a transient leukemia—Down syndrome TL-DS) be defined by the presence of a *GATA1* mutation in a neonate with DS with a blast count of >10% or clinical features consistent with the disorder [13]. Pine et al. investigated newborn screens of children with DS and reported that a *GATA1* mutation could be used as a possible biomarker for an increased risk to later develop

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Table 1. Clinical Studies of TAM.

Study	N	Years	Treated LDAC	Treated Any*	Early Death	OS	ML-DS#
Yamato et al.	167	2011–2014	31%	41%	13%	81%	20%
Flasinski et al.	102	2007–2015	38%	38%	5%	91%	17%
Gamis et al.	135	1999–2004	18%	25%	18%	77%	20%
Klusmann et al.	146	1993–2006	19%	19%	15%	85%	23%
Muramatsu et al.	70	1985–2006	0%	33%	23%	74%	22%
Massey et al.	48	1996–1999	0%	4%	17%	64%	22%

*Any treatment includes cytarabine, steroids, or exchange transfusion.
#Percentage of patients who develop ML-DS calculated based on patients who survived TAM.

ML-DS, however, given less than 25% of infants with TAM will go on to develop AML, this information may cause more stress than benefit [14].

Although TAM resolves in the majority of cases, it is not always a benign entity. Many groups have sought to elucidate the natural history of TAM as summarized in Table 1. The Children's Oncology Group (COG) trial COG A2971 enrolled 135 infants with a 3 year overall survival (OS) of 77% and about half of deaths were attributed to TAM [15]. The AML-BFM study group enrolled 146 children with TAM and observed a 5 year OS of 85% with 23% of patients going on to develop ML-DS [16]. POG-9481 included 48 patients with TAM with 17% experiencing early death; 19% of the infants progressed to leukemia [17]. In a Japanese retrospective study, 70 children with TAM had a 23% early death rate, primarily from hepatic and cardiopulmonary failure [18]. In another study in Japan, 167 children with TAM were enrolled in an observational study (TAM-10), and early death was seen in 13% of cases with 20% developing ML-DS. They further reported that low-dose cytarabine (LDAC) reduced early mortality and that positive minimal residual disease (MRD) predicted future ML-DS, but other clinical features were not predictive of subsequent ML-DS [19]. Alford et al. determined that the specific type of *GATA1* mutation was also not a useful predictor for future development of ML-DS [20].

Treatment of TAM with LDAC was investigated in the TMD07 trial in Germany and the Netherlands to assess whether early intervention could reduce TAM-related mortality, as well as prevent the progression to ML-DS. In total, 102 patients were enrolled with treatment given to patients with symptoms at diagnosis or those with positive minimal residual disease 8 weeks after diagnosis. LDAC reduced TAM-related mortality but did not prevent progression to ML-DS [21]. This suggests that patients with TAM and a set of identified clinical risk factors such as high white blood cell count, hydrops fetalis, ascites, or liver dysfunction may benefit from treatment with low-dose cytarabine to prevent TAM-related mortality. Treatment with more targeted therapeutic approaches should be evaluated to achieve the goal of preventing progression to ML-DS.

Myeloid leukemia—Down syndrome

Enhanced cytarabine sensitivity in ML-DS. The NOPHO registry between 1984 and 2001 demonstrated that outcomes of ALL in children with DS are worse than those without DS, while ML-DS outcomes were better than myeloid leukemia in children without DS [22]. One possible explanation for the improved outcomes in ML-DS is sensitivity to chemotherapy [23]. Taub et al. studied the metabolism and sensitivity of myeloblasts from patients with DS to cytarabine. The DS myeloblasts had increased metabolism and increased sensitivity to cytarabine compared with non-DS myeloblasts [24]. Zwaan et al. performed drug-sensitivity studies on leukemia cells from patients with DS and similarly found them to be sensitive to cytarabine as well as other cytotoxic agents, including etoposide and anthracyclines [25]. Taub et al. suggest that the increased sensitivity may be due to increased activity of cystathionine-β-synthase localized to 21q22.3 [26]. Frost et al. suggest that the difference in sensitivity seen in AML vs. ALL must be cell-lineage specific rather than a factor attributable to three copies of chromosome 21 [23]. Further work is needed in this area to identify additional therapeutic targets and agents for patients with DS who develop ALL, ML-DS, and relapsed ML-DS.

Early trials. Early trials, which treated ML-DS similarly to AML without DS, showed that children with ML-DS did not tolerate intense chemotherapy as well as the group without DS, which led to a greater complication rate. The AML-BFM 93 trial treated 28 patients with ML-DS and observed a higher rate of death from infection-associated complications (17.9% vs. 5.4% in non-DS), most during induction therapy [27]. Despite this higher risk of toxicity, there were some early suggestions that a higher EFS could be achieved in ML-DS compared with AML without DS. The POG AML study 8498 treated 12 patients with ML-DS and found a superior EFS compared with children without DS [28]. As some trials began to reduce the intensity for ML-DS, it became clear that infectious complications and cardiomyopathy remained relatively common in this population, especially given congenital cardiac defects that often occur in patients with DS. In the AML-BFM 2004 study, infectious complications were common in ML-DS, with high morbidity, despite reduced-intensity [29]. The POG 9421 trial treated 57 children with ML-DS with a relatively high remission rate but with a high incidence of cardiomyopathy and a 5 year OS of 78.6% [30]. Many studies showed that less intense therapy could achieve higher survival in ML-DS compared with AML without DS. The AML-BFM 93 and AML-BFM 98 trials were compared and showed that less-intense and standardized chemo in ML-DS led to less toxicity and higher survival [31]. A less-intensive regimen trial, AML-Down, performed in Japan between 1987 and 1997, using ADE (a combination of cytarabine, daunorubicin, and etoposide) showed EFS at 8 years of 80% [32]. A retrospective review of a Canadian cohort between 1990 and 2003 showed similar outcomes with a reduction in cytarabine dose [33]. The NOPHO-AML88 and NOPHO-AML93 trial comparison show that reducing intensity decreased treatment-related toxicity without changing relapse rates [34]. In the United Kingdom, the AML 10 and AML12 trials, which enrolled 46 children with ML-DS, the 5 years OS was 47% from 1988 to 1995 and increased to 75% from 1996 to 2002; treatment-related deaths and disease-free survival was higher in patients with DS [35]. JCCLSG AML9805 enrolled 24 ML-DS patients, and observed a 5 year OS 83% and 5 year EFS 87.5% [36]. Finally, a NOPHO registry between 1984 and 2001 showed that ML-DS treated after 1992 had an improved outcome with a 10 year EFS of ~83% [22].

Prognostic factors and minimal residual disease

Increasing age has been identified as a poor prognostic factor for ML-DS. The children's cancer group (CCG) trial CCG 2891 identified >4 years at diagnosis as an adverse risk factor [37]. The

Table 2. Recent clinical studies in ML-DS.

	AAML0431-COG	ML-DS 2006 – Europe	JPLSG D05 - Japan
Study Goal	Reduce anthracycline	Reduce Etoposide	Identify High Risk subgroup
N	204	170	72
Hx of TAM	31%	35%	49%
N > 4 years	0 patients	5 patients	5 patients
Years	2004–2015	2006–2015	2008–2010
Chemo cycles	6 cycles	4 cycles	5 cycles
LPIT	2 (reduced from 7)	4 (reduced from 11)	0
Dauno mg/m2	240	240	250
Ara-C mg/m2	27,800	27,400	3500
Etopo mg/m2	750	450	1350
TRM	1%	2.9%	1.4%
5 Year EFS	89.9%	87%	83%
5 Year OS	93%	89%	88%
Published	Blood 2017	Blood 2017	PBC 2016

LPIT, Lumbar puncture with intrathecal chemotherapy, *TRM*, Treatment-related mortality.

subsequent COG trial, A2971, reduced treatment intensity compared with CCG 2891 and maintained a 5 year OS of 84% but again showed worse outcome for age >4 (5-year EFS rate: 33% for >4 years vs. 81% for ages 0–4 years) [38]. To further explore the differences by age groups, Hasle et al. investigated a cohort of ML-DS > 4 years old and found these patients commonly lacked a *GATA1* mutation setting them apart from younger children with ML-DS [39]. Moreover, they detected the presence of recurrent translocations that are seen in non-DS AML, such as t(8;21), t(15;17), and t(10;11) in these older children with DS [39]. A Japanese trial between 2000 and 2004, which enrolled 72 patients, showed reduced cytarabine produced excellent outcomes overall, but found that monosomy 7 was associated with adverse outcomes [40]. MRD has also been investigated as a prognostic factor and mechanism to guide treatment. MRD by either flow cytometry or detection of a *GATA1* mutation after initial induction therapy represents a significant prognostic factor for predicting ML-DS relapse, as shown in the AML-D11 trial in Japan [41].

Recent trials

A select group of recently published ML-DS trials, shown in Table 2, highlights current therapeutic strategies that attempt to reduce intensity while maintaining excellent outcomes. The ML-DS 2006 trial was successfully performed in Europe with the goal of reducing cumulative etoposide exposure. In this study, patients achieved a 5 year EFS of 87% and 5 years OS 89% [42]. The AAML0421 study performed by COG with the goal of reducing anthracycline exposures was very successful, reporting outcomes of 90% 5 year EFS and 93% 5 year OS in patients <4 years of age [43]. The Japanese Pediatric Leukemia/Lymphoma Study Group D05 study treated standard-risk patients with a relatively low dose of cytarabine (3500 mg/m2 cumulative dose). The study had a 5 year EFS of 83% and 5 year OS of 88% [44] with reduced cytarabine doses. The most recent COG trial AAML1531 tested a similar reduction in cytarabine exposure in a subgroup of children with ML-DS (age <4, MRD < 0.05% after induction). Interim analysis of this subgroup of patients treated with reduced cytarabine showed a 2 year EFS of 85.6% and 2 year OS of 91.0%, which, while similar to the Japanese study D05, was significantly lower than the COG predecessor study AAML0431 [45]. Therefore, that arm of the COG trial closed due to inferior efficacy, revealing a threshold for cytarabine levels.

Outcomes for those that relapse

While the EFS for ML-DS has gradually improved over the last few decades, treatment options are limited for patients with relapsed or refractory disease and outcomes remain poor, despite novel therapies. In total, 29 patients with relapsed or refractory ML-DS were treated in Japan with 3 years OS of 26% without a clear benefit of hematopoietic stem cell transplant (HSCT) [46]. The Center for International Blood and Marrow Transplant Research reviewed outcomes of 28 ML-DS cases and found that the 3 year OS was 19%; both risk or relapse and risk of transplant-related mortality were higher than non-DS AML controls [47]. The CCG Studies 2861 and 2891 included 118 ML-DS cases, and while the EFS (69%) was higher in the children with DS, HCST offered no advantage [48].

Prospects for advanced therapies

As reduced dosing of traditional chemotherapy drugs reaches a limit, there are several novel agents that may be of interest to study. Liposomal daunorubicin may cause less treatment-related toxicity, including cardiotoxicity, in pediatric AML [49]. Liposomal daunorubicin and cytarabine together (CPX-351) are safe and effective in newly diagnosed secondary AML in adults [50] and are also safe and effective in relapsed pediatric AML [51]. CPX-351 is currently being investigated in Europe in children with ML-DS. Another new agent, the Wee1 kinase inhibitor AZD1775 (adavosertib, previously MK-1775), has been shown to enhance cytotoxicity of cytarabine in ML-DS cells [52]. Furthermore, ML-DS blasts display remarkable sensitivity to histone deacetylase in inhibitors such as vorinostat in xenotransplantation models in vivo [53]. A single case report of relapsed ML-DS noted a response to vorinostat [54]. Hypomethylating agents have also shown promise in AML. A single case report of relapsed ML-DS responded to azacytidine [55]. Finally, other agents such as the BCL2 inhibitor venetoclax and antibody-drug conjugate gemtuzumab ozogamicin show efficacy in pediatric AML and may deserve further investigation for ML-DS [56, 57].

GENETICS OF ML-DS

Trisomy 21 and GATA1s

GATA1 is an essential regulator of numerous hematopoietic lineages including red blood cells and megakaryocytes [58]. In 2002, mutations in *GATA1* were found to be a uniform event in patients with ML-DS and subsequently identified in TAM [59–61]. These mutations almost always occur in coding sequences that lie in exon 2 of the *GATA1* gene and lead to sole expression of the truncated isoform GATA1 short (GATA1s). GATA1s retains its two zinc-finger domains and the entire C terminus, but lacks the first 83 amino acids that constitute a putative transcriptional activation domain. Although it is clear that GATA1s cooperate with trisomy 21 to drive the transient phase of the disease, the mechanisms by which the loss of the N-terminal domain of GATA1 promotes the growth of TAM blasts has remained elusive. Moreover, while trisomy 21 has been shown to distort the hematopoietic stem and progenitor cell (HSPC) compartment in the fetal liver of mice with a bias toward immature megakaryocyte and erythroid lineages and also alter the repertoire of HSPCs in human fetal liver, iPSCs, and embryonic stem cells, how trisomy 21 promotes leukemia is also unclear [62–68]. Specific genes on chromosome 21 are discussed later in this review and in other reviews [69–72].

Transient leukemia resolves within the first few months of life, suggesting that the fetal microenvironment is likely an important contributor to disease progression. One possible mechanism for the spontaneous resolution of TAM was proposed by Cantor and

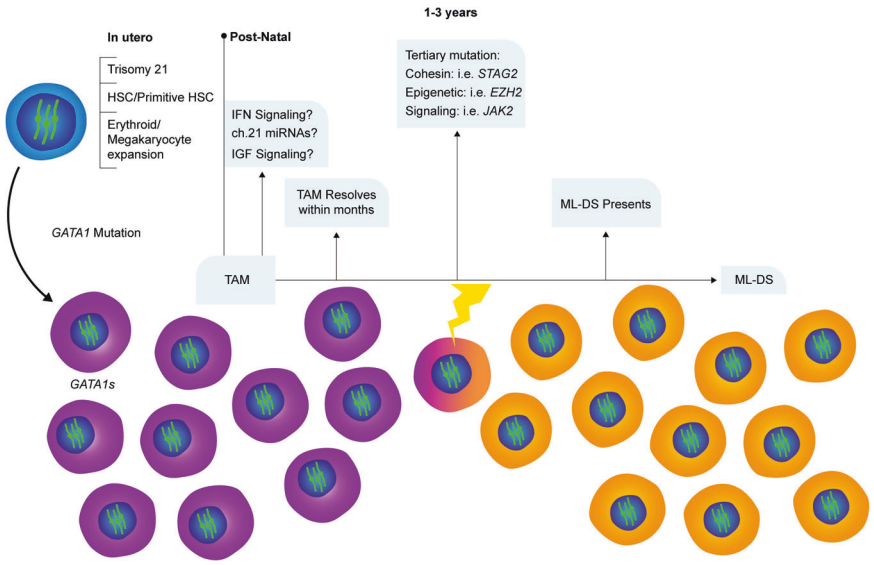

Fig. 1 Timeline of events during ML-DS progression. In utero, trisomic hematopoietic stem and progenitor cells give rise to an expansion of erythroid/megakaryocyte progenitors that frequently acquire a mutation in *GATA1*. The combination of trisomy 21 and expression of the short GATA1 isoform (GATA1s) in the absence of full-length GATA1 leads to transient abnormal hematopoiesis (TAM) in as many as 10% of newborns. After birth, TAM regresses in part due to increased IFN signaling and altered IGF signaling in the post-natal environment, however other factors almost certainly contribute. Within four years after TAM resolution, up to 20% of individuals with TAM will acquire a third mutation that promotes ML-DS. Green lines represent chromosome 21. Blue cell only trisomy 21; Purple cells, trisomy 21 with a *GATA1* mutation; Orange cells, trisomy 21 with a *GATA1* mutation and a third mutation in cohesin, epigenetic regulators, or signaling pathway genes.

colleagues. They demonstrated that elevated levels of IFN signaling outside of the fetal microenvironment significantly reduced the hyperproliferation of megakaryocytic precursors derived from GATA1s mice and that deletion of *Ifnar1* or neutralizing IFNα/β antibodies increased their proliferation [73]. In addition, there is evidence that insulin-like growth factor (IGF) signaling plays a role in the transient phenotype; it has been shown in both patient samples and mouse models of ML-DS that fetal but not adult megakaryocytic progenitors are dependent on IGF signaling in part through GATA1 coordination of an E2F transcriptional network that is disrupted by the GATA1s mutation [74]. Together, these studies highlight the importance of understanding differences between the fetal and adult microenvironment that could be leveraged to suppress ML-DS. Another possibility to consider is the tendency of fetal liver progenitors to enter a quiescent state with migration to the bone marrow. Most likely, multiple mechanisms cooperate in TAM resolution.

Cooperating mutations are necessary for leukemia progression

Recent sequencing studies have demonstrated that TAM is driven by the combination of a *GATA1* mutation and trisomy 21 without the need for additional genetic alterations [75, 76]. However, after TAM has resolved, any remaining disease-driving clones can acquire a third hit that drives TAM blasts out of quiescence leading to ML-DS (Fig. 1). There have been multiple efforts within the last decade to identify the mutations that aid in leukemia progression. In 2013, Ogawa and colleagues sequenced 41 TAM, 49 ML-DS, and 19 non-DS AMKL patient samples and identified recurrent mutations in members of the cohesin complex and its associated factors, such as *RAD21* and *STAG2* (53% in total), epigenetic regulators such as *EZH2* (45%), and regulators of common signaling pathways, such as *JAK2* (47%) [76]. In 2019, Klusmann and colleagues performed exome and targeted sequencing on 111 TAM and 141 ML-DS patient samples [75]. They found many of the same mutations as the prior study and in similar proportions, but also reported the results of a CRISPR-knockout screen of 22 putative ML-DS driver genes in a disomic model of murine fetal liver TAM. After introducing the GATA1s mutation and the sgRNAs against the 22 genes, they transplanted these mutant cells to mice and monitored for leukemia. While the leukemia potential of the majority of the known tertiary mutations was confirmed, it is curious that disruption of the cohesin complex was not associated with disease progression. The absence of an effect may be due to the disomic nature of the cells, suggesting that cohesin mutations require trisomy 21 to drive ML-DS. Alternatively, there may be a species-specific oncogenic effect of cohesin mutations, in which animal models are less susceptible to leukemia with this alteration. Additional studies are needed to clarify the contributions of cohesin mutations in ML-DS.

The cohesin complex

Cohesin is a ring-shaped protein structure that comprises of SMC1, SMC3, RAD21, and STAG1 or STAG2. Mutations in each of these genes have been detected in ML-DS, although *STAG2* and *RAD21* are the most frequently mutated [75, 76]. Mutations in these genes are mutually exclusive, suggesting they similarly influence the function of the entire complex. Cohesin is best known for its roles in sister chromatid cohesion and DNA repair; however, its role in disease by regulating higher chromatin architecture is now well appreciated [77]. In 2015, three papers investigated the role of cohesin in normal hematopoietic differentiation and reported that cohesin deficiency led to increased HSPC self-renewal and decreased differentiation, and in one model, a myeloproliferative phenotype [78–80]. ATAC sequencing of cells with different cohesin gene mutations uncovered enrichment for RUNX1, GATA1, and ETS motifs in the accessible chromatin regions. This observation is notable in that RUNX1, ERG, and ETS2 are on chromosome 21 and play key roles in acute leukemia.

Given that heterozygous mutations in cohesin genes are associated with malignancy while homozygous loss is lethal suggests that new therapies that further reduce activity of the complex may be effective in ML-DS. Indeed, a recent synthetic lethality screen in *STAG2*-mutated leukemia cell lines uncovered multiple hits in the DNA repair pathway, including polyadenosine diphosphate-ribose polymerase (PARP) 1 [81]. Furthermore, this study showed that *STAG2* mutant cells were more sensitive to PARP inhibitors such as talazoparib, suggesting a potential

therapeutic avenue for the treatment of cohesin mutated AMLs. A second group performed a similar synthetic lethality screen with 3009 FDA-approved compounds [82]. While they also found compounds that impaired DNA repair, they determined that a GSK3 inhibitor was also very effective, suggesting that WNT signaling is also critical in cohesin mutant cells. They further edited the ML-DS CMK cell line to harbor a STAG2 R614* mutation and demonstrated that these cells were highly sensitive to GSK3 inhibition. Together, these studies show that cohesin mutant ML-DS can be therapeutically exploited with targeted agents.

HSA21 gene dosage

Having an extra chromosome suggests that genes expressed on that chromosome would be more highly expressed than disomic counterparts. This "gene dosage" effect of trisomy 21 has been suggested to be critical for leukemia and overexpression of a set of genes such as *ERG*, *DYRK1A*, *CHAF1B*, and *RUNX1* has been implicated in AML and ALL [66, 83–91]. Studies to model cooperativity of these alterations with specific tertiary events have been limited, but one study demonstrated that *GATA1* and *MPL* mutations are sufficient to promote leukemia in a mouse model of DS [66].

Although parsing through genes in the DS-critical region remains a crucial area of research in the coming years, it is important to remember that the genes contributing to the ML-DS phenotype may lie outside of the minimally required genes that drive many of the DS phenotypes. For example, SON, which was shown to inhibit megakaryopoiesis by transcriptionally suppressing RUNX1, likely contributes to ML-DS [92]. SON also repressed the expression of AP-1 transcription factors that are important for megakaryopoiesis. Another gene of interest is the histone H2A deubiquitinase USP16. Trisomy of USP16 in the Ts65Dn murine model of DS was shown to decrease the self-renewal of HSCs and expansion of somatic tissues [93]. Using this same mouse model, USP16's importance in DS musculoskeletal deformities was demonstrated by impaired muscle stem cell function [94]. Further work in disomic murine HSPCs demonstrated that conditional knockout of *Usp16* impaired hematopoietic lineage commitment and differentiation [95]. This evidence for USP16 regulating stem-cell phenotypes and more specifically HSPC function implies a potential function in ML-DS.

Contributions of microRNAs to ML-DS

There are 5 microRNAs (miRNAs) encoded on chromosome 21; let-7c, miR-99a, miR-125b, miR-155, and miR-802 [96]. miR-99a, -125b, and -155 are well known to be regulated by inflammatory signaling pathways in hematopoietic cells [97–99]. Furthermore, miR-125b-2 has been demonstrated to be an oncomiR for megakaryoblastic leukemia [100]. Beyond these chr21 miRNAs, there may be others that may participate in ML-DS. For example, SON has been shown to control GATA2 by negatively regulating the Mirc11 cluster, which includes miR-23a~27a~24-2 miRNAs on chromosome 19 [101]. Mirc11 has recently been shown to influence myeloid cell fate and regulate inflammatory signals in myeloid progenitors of *mirc11*-knockout mice [102, 103]. There is also evidence suggesting that Mirc11 functions as a negative regulator in acute erythroleukemia [104]. Mirc11 is also a positive transcriptional target of RUNX1 during megakaryopoiesis [105]. Another potential contributor is miR-486, which is located on chromosome 8. Izraeli and colleagues demonstrated that this miRNA is upregulated in ML-DS patients, is regulated by GATA1s, and enhanced the survival and erythroid immunophenotype of ML-DS cells in part by regulating AKT activation [106]. Curiously, miR-486 could not induce self-renewal of E12.5 wild-type murine fetal liver cells, but it did enhance the self-renewal phenotype of E12.5 cells with a *Gata1* mutation, suggesting a transformative effect within GATA1s contexts. Importantly, this observation was independent of T21.

Circulating miRNAs have been proposed to act as biomarkers for various cancers as they are frequently contained in exosomes that protect them from degradation and deliver them to other cells to exert their function [107–109]. It has recently been suggested that miRNAs are candidates for biomarkers of diagnosis and prognosis, as well as therapeutic targets for both AML and chronic lymphocytic leukemia [110, 111]. One recent study demonstrated an upregulation of circulating miR-16-5p, miR-99b-5p, and miR-144-3p in the nanoparticle-enriched fraction of plasma from DS patients that correlated with nervous-system development, neuronal cell body, and certain manifestations of leukemia upon gene ontology analysis [112]. Similar effects of other circulating miRNAs may be instrumental in ML-DS.

Other drivers of malignancy

Several studies have implicated NFAT signaling in ML-DS. Multiple regulators of NFAT signaling, including DYRK1A and DSCR1, are present on chromosome 21, and studies have revealed that NFAT signaling is dysregulated in the trisomic setting [66, 113]. Furthermore, overexpression of *Dscr1*, a calcineurin inhibitor encoded on HSA21, in mice resulted in thrombocytosis and increased numbers of megakaryocytes [114]. Similarly, overexpression of DYRK1A, which phosphorylates and inactivates NFATs, is associated with increased megakaryopoiesis, suggesting that restoration of NFAT activity may be therapeutically relevant to ML-DS [66].

An intriguing new study revealed that children with DS display evidence of clonal hematopoiesis and harbor mutations in leukemia-associated genes, most prominently *TET2* [115]. However, oncogenic mutations in *TET2* were relatively rare in ML-DS [75, 76]. Moreover, *DNMT3A* mutations were rarely seen in clonal hematopoiesis in the DS population, despite this gene being one of the most commonly mutated genes in clonal hematopoiesis in adults. *DNMT3A* mutations are also extremely rare in ML-DS, with none reported in the Klusmann study and only one in the Ogawa study [75, 76]. These observations are consistent with the low incidence of *DNMT3A* mutations in the pediatric leukemia population [116].

OUTSTANDING QUESTIONS AND FUTURE DIRECTIONS

Despite the substantial progress that has been made in understanding the landscape and mechanisms of ML-DS progression, major questions remain unanswered. For example, what is the cell of origin for TAM and ML-DS and what is the role of trisomy 21? There are currently no single-cell datasets from TAM/ML-DS to aid in this discussion, however, a recent report by Wagenblast et al. aimed to shed light on this question. They introduced *GATA1* and *STAG2* mutations into human fetal HSPCs from disomic and trisomic backgrounds and characterized these cells both in vitro and in vivo [117]. Their work suggests that TAM is derived from the LT-HSC and requires trisomy 21, while ML-DS can arise from nearly all HSC and myeloid progenitor subsets independent of trisomy. With respect to the timing of the mutations, they found that GATA1s and the *STAG2* mutation could transform both fetal liver and cord blood progenitors but not bone marrow cells. This observation is consistent with a stage-specific requirement of *GATA1* mutations and the possibility of *GATA1* mutations occurring in a unique fetal liver progenitor cell [118].

The Wagenblast study also addressed the critical question of the importance of trisomy 21 to TAM and ML-DS. They found that trisomy 21 is essential for development of TAM but not required for leukemia driven by the combination of *GATA1* and *STAG2* mutations [117]. They also implicated three chromosome 21 miRNAs (miR-99a, miR-125-b, and miR-155) as potential drivers of TAM but dispensable for ML-DS. This suggests that trisomy 21 may be necessary for the survival and/or expansion of human *GATA1* mutant fetal liver clones, while this requirement is lost following

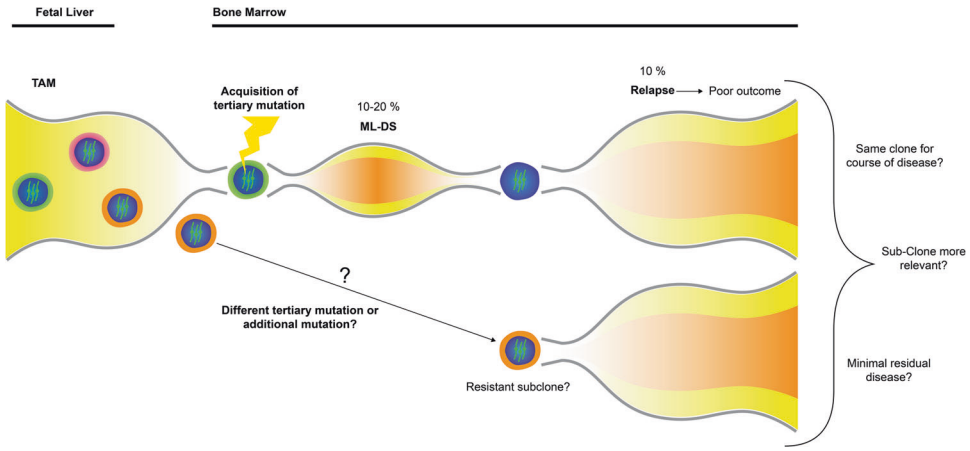

Fig. 2 Clonal evolution of ML-DS and relapse. The clonal evolution of ML-DS is currently poorly understood. It is likely that a single TAM clone with trisomy 21 and a *GATA1* mutation acquires a tertiary mutation to drive progression to ML-DS. However, it is not clear if this same clone is responsible for eventual relapse seen in 10% of ML-DS cases (top). It is possible that an independent TAM clone acquires a different tertiary mutation or numerous additional mutations to promote clones that emerge at relapse (bottom). Orange, green, and pink cells represent different *GATA1* mutant clones.

the acquisition of tertiary mutations such as *STAG2*. Similarly, animal models in which GATA1 is replaced by GATA1s developed leukemia resembling ML-DS upon the introduction of a variety of cooperating mutations, although as mentioned, not cohesin [75].

Despite these important advances in human cell and animal models, the question of the role of trisomy 21 in the ML-DS in patients remains murky. In two recent clinical studies, Cantor and Hasle independently discovered familial cases in which individuals had inherited GATA1s mutations. These children developed hematological malignancies that harbored a third copy of chromosome 21 (personal communication). Although chromosome 21 aneuploidy is seen in de novo AML [69], the disease in these children was reported to be ML-DS, suggesting that trisomy 21, whether acquired pre- or post-natal, remains essential for disease progression. Finally, one fascinating recent case report described an infant with TAM who lacked trisomy 21 [119]. This case was associated with a novel mutation in *GATA1* that affected both the N-terminal activation domain and the N-terminal zinc-finger and by the presence of additional mutations, including one in *JAK1*. It is possible that this novel GATA1 isoform alone or in cooperation with other mutations drives TAM. Together, these cases further complicate our understanding of the basis of TAM and ML-DS.

A second major area to address in ML-DS in the coming years is relapse. As we noted, ML-DS is generally associated with a favorable outcome, but despite a good response to initial therapy, a subset of ML-DS patients relapse, and when they do, the prognosis is extremely poor with 3 year OS rates around 20–25% with or without HSCT [46, 47]. Outcomes are even worse for patients that relapse early, within the 1st year after treatment. While patients with DS often have increased toxicity to chemotherapy, the deaths in these relapsed patients are commonly attributed to disease progression and not treatment-related toxicities [46]. Unfortunately, little is known about the molecular basis for this relapse or mechanisms of resistance to salvage therapies. To gain these insights, efforts need to be made to acquire paired diagnostic and relapse samples to better understand the genetics of disease progression (Fig. 2). Given the rarity of these cases, this must be performed through a large collaborative effort. Further understanding of the mechanisms of resistance for this cohort of patients may lead to novel therapeutic targets and improved therapies.

Together, the progress made over the last two decades to address the challenges of ML-DS has been excellent, but there is still much more work to do to fully elucidate the mechanisms of disease and develop less toxic and more effective treatments, especially in relapsed ML-DS. In order to address some of the major concerns in the field, single-cell sequencing from every stage of disease progression will need to be performed. Single-cell profiling, especially within the fetal liver progenitor cell population, will shed light on the cell of origin, the clonal evolution that occurs from TAM to ML-DS, and the clonal complexity that accompanies the development of chemotherapy resistance. This methodology has greatly increased our knowledge of AML progression [120–122] and will certainly be helpful in understanding AML in DS.

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