Chapter

Juvenile Myelomonocytic Leukemia (JMML): A Mimicker of *KMT2A*-Rearranged Acute Myeloid Leukemia (AML)

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Abstract

Juvenile myelomonocytic leukemia (JMML) is the most confusing mimicker of *KMT2A*-rearranged acute myeloid leukemia (AML). Clinical presentation, age of susceptibility (infancy or early childhood) and abnormal monocytosis are common clinical features. To complicate matters, JMML morphologically resemble acute myelomonocytic leukemia (AML M4) and distinction must be made based on accurate blast and promonocyte counts. As treatment significantly varies, AML/JMML overlap can lead to catastrophic consequences that can be avoided by timely management. Therefore, meticulous knowledge of JMML is essential to treat patients with hematologic malignancies. The pathognomic feature of JMML is increased infiltration of the peripheral blood, bone marrow, and viscera by abnormal myelomonocytic cells. Molecular diagnostics has generated substantial dividends in dissecting the genetic basis of JMML. We can now molecularly confirm the diagnosis of JMML in approximately over 90% of patients who harbor driver mutations in *KRAS, NRAS, PTPN11, NF1*, or *CBL* genes. The presence of monosomy 7 is a classic feature of JMML that can support the diagnosis in many cases. On the other hand, cytogenetics and Fluorescence in situ hybridization analysis (FISH) are indispensable to differentiate *KMT2A*-rearranged AML from JMML. In particular, AML with t(9;11) is associated with monocytic features that can be easily mistaken for JMML.

Keywords: JMML, *KMT2A*, DNA hypermethylation, p-STAT5, azacitidine

1. Introduction

JMML is a rare aggressive clonal hematopoietic neoplasm of infancy and early childhood that combines excessive proliferation of the granulocytic and monocytic lineages with dysplasia making JMML analogous to chronic myelomonocytic leukemia (CMML). Therefore, the current WHO classification of myeloid neoplasms and acute leukemia envisages JMML as an overlap myeloproliferative/myelodysplastic neoplasm. The diagnosis of JMML is mainly based on the clinical presentation in combination with morphology of the peripheral blood smears and the molecular analysis of leukemic cells. The peripheral blood and bone marrow of

JMML diagnostic criteria
I. Clinical and hematologic features (all 4 features mandatory):
• PB monocyte count ≥1 x 10^9/L.
• Blast percentage in PB and BM <20%.
• Splenomegaly.
• Absence of Philadelphia chromosome (*BCR/ABL1* rearrangement).
II. Genetic studies (1 finding sufficient):
• Somatic mutation in *PTPN11* or *KRAS* or *NRAS* (Germ line mutations -indicating Noonan syndrome- need to be excluded).
• Clinical diagnosis of NF1 or *NF1* mutation.
• Germ line *CBL* mutation and loss of heterozygosity of CBL.
III. For patients without genetic features, besides the clinical and hematologic features listed under I, the following criteria must be fulfilled:
• Monosomy 7 or any other chromosomal abnormality or at least 2 of the following criteria:
• Hemoglobin F increased for age.
• Myeloid or erythroid precursors on peripheral blood smear.
• GM-CSF hypersensitivity in colony assay.
• Hyperphosphorylation of STAT5.

[1]*Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016;127(20):2391–2405. doi:10.1182/blood-2016-03-643,544*

Table 1.
The diagnostic criteria for juvenile myelomonocytic leukemia[1].

children with JMML may display features of both myeloproliferative and myelo-dysplastic disorders (ie, immature and dysplastic forms), as well as cytopenias due to marrow infiltration and/or splenomegaly. Splenomegaly, lymphadenopathy, and skin rashes are common clinical features but the clinical picture of JMML can be somewhat non-specific. JMML is frequently confused with viral infections, immunodeficiency syndromes, myelodysplastic syndromes, myeloproliferative neoplasms or other forms of leukemia [1]. Moreover, JMML has diverse molecular subtypes with contrasting features and varied clinical outcomes. The broad overlap in clinical and laboratory features in combination with the heterogeneity of JMML itself made the diagnosis of JMML a hurdle to most hematologists. Consequently, the diagnosis of JMML almost always relies on the diagnostic criteria (**Table 1**). The chief backbone of the diagnostic criteria is the associated molecular and/or cytoge-netic abnormalities. In particular, the genomic landscape of JMML had revolution-ized our understanding of leukemogenesis of this greatly equivocal disease. JMML is a predominantly fatal disease but the clinical course can be highly variable. At both extremes of the disease, a third of patients follow a relatively indolent course while approximately 15% of cases develop AML, the so called 'blast crisis' [2].

2. Clinical features

- Patients present with splenomegaly, fever, thrombocytopenia, monocytosis, and elevated HbF.

- Leukocytosis is common in JMML, but a presenting white blood count <10 x 10^9/L is occasionally noted.

- Anemia is generally not a leading symptom and rarely requires red blood cell (RBC) transfusion.

- Most patients present with evidence of infection or constitutional symptoms that may mimic viral infections.

- Polyclonal hypergammaglobulinemia and presence of autoantibodies.

- The hallmark of the disease is the overproduction of myelomonocytic cells (monocytic and granulocytic cells) that infiltrate skin and vital organs (spleen, liver, and lungs) leading to various clinical manifestations as follows [3]:

1. Lungs: dry cough, tachynoea and interstitial infiltrates on chest X-ray are signs of pulmonary infiltration.

2. Skin: Cafe-au-lait spots might be indicative of an underlying germline conditions such as Neurofibromatosis type 1 (NF1) or Noonan syndrome-like disorder. Neurofibromas occur in patients with NF1. About 25% of patients have pleomorphic skin rashes in the forms of:

   o Eczematous eruptions (cradle cap).

   o Indurated raised lesions with central clearing.

   o Sweet syndrome.

3. Gut infiltration may predispose patients to diarrhea—sometimes with bloody features—and gastrointestinal infections.

4. Lymph nodes: About half of all patients have lymphadenopathy. Leukemic infiltrates may give rise to markedly enlarged tonsils.

5. There is generally marked splenomegaly/hepatosplenomegaly:

   o Hepatomegaly is generally less prominent than splenomegaly.

   o Approximately 7–10% of JMML patients will not have splenomegaly at diagnosis but virtually all patients will develop splenomegaly within weeks to months of initial presentation (spleen size rapidly increases with time).

3. Pathologic features

3.1 Peripheral blood

The peripheral blood (**Figure 1**) is the most important specimen for diagnosis [4]. It typically shows leukocytosis and thrombocytopenia. The vast majority of JMML patients have thrombocytopenia with the exception of children with NF1- mutated JMML, who show platelet counts within the normal range in most cases. The median reported white blood cell counts are 25–30 x 10^9/L. The leukocytosis consists mainly of neutrophils, with some immature cells (e.g. promyelocytes and myelocytes)
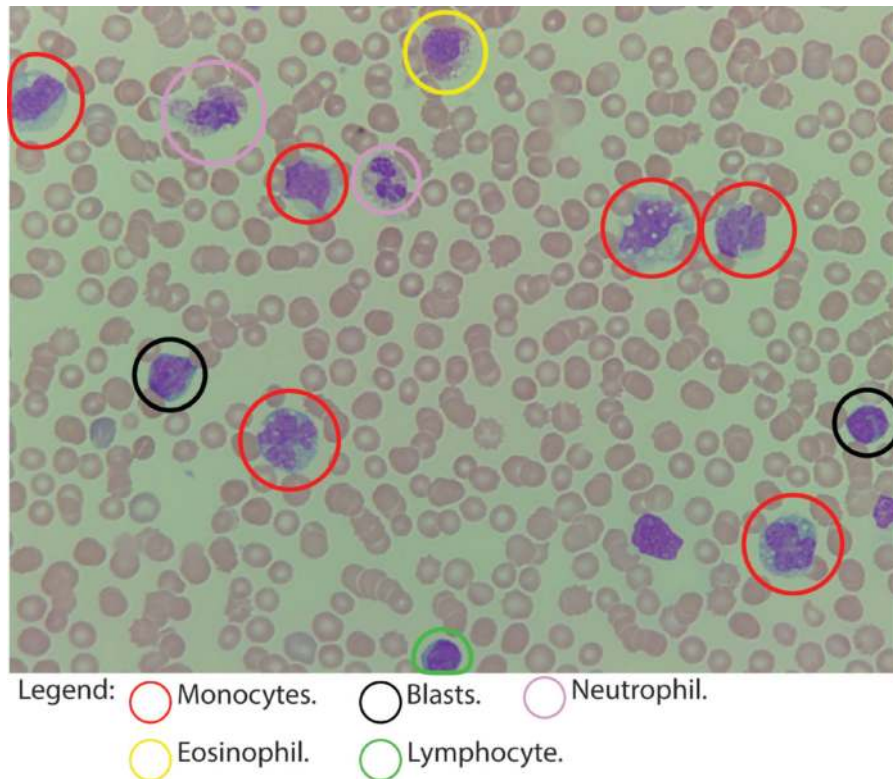
Figure 1.
Peripheral blood of a 13 months old boy with PTPN11-mutated JMML. Photomicrograph shows leukocytosis with neutrophilia and monocytosis. There is left shift, toxic granulation and vacuolation. Occasional primitive cells (~ 2%) are present. No nucleated RBCs are seen.
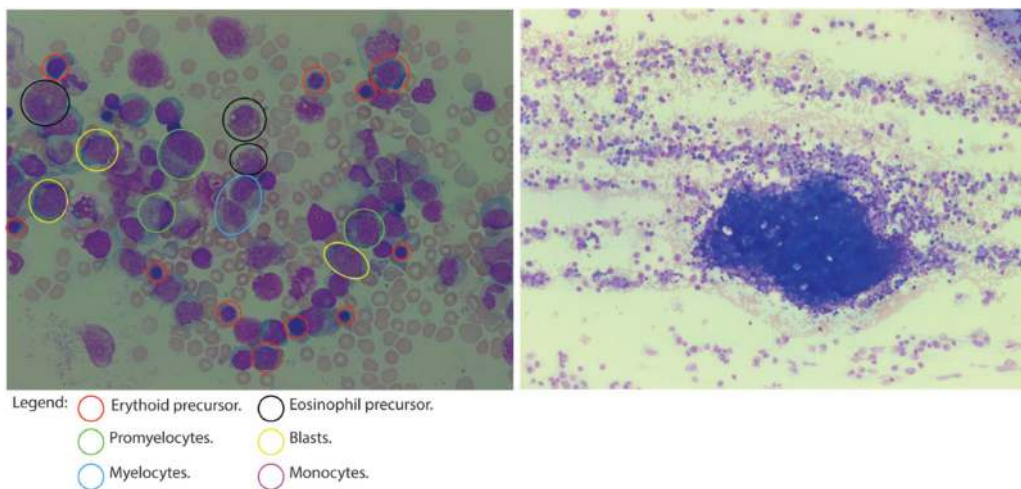

Figure 2.
Bone marrow aspirate demonstrates a hypercellular marrow (100 – Age = %normal cellularity) with left shift and increased myeloid and monocyte lineages. Blast count is about 4%. There is occasional evidence of hemophagocytosis and mild erythroid dysplastic features.

and monocytes. Although most cases show a striking monocytosis, often with dysplastic forms, the absolute monocyte count can be <1 x 10^9/L. Blasts (including promonocytes) usually account for <5% of the white blood cells, and always <20%. Eosinophilia and basophilia are observed in a minority of cases. Nucleated red blood cells are often seen. Red blood cell changes include macrocytosis (particularly in patients with monosomy 7), but normocytic red blood cells are more common.

3.2 Bone marrow

Bone marrow (**Figure 2**) findings are consistent with the diagnosis of JMML but are not per se diagnostic. However, bone marrow aspiration is necessary to exclude AML M4. The most consistent finding in bone marrow specimens is the reduced number or absence of megakaryocytes in about two third of cases. The bone marrow aspirate and biopsy are hypercellular with granulocytic proliferation, although in some patients erythroid precursors may predominate. Monocytes in the bone marrow are often less prominent than in the peripheral blood, generally accounting for 5–10% of the bone marrow cells. The marrow blasts (including promonocytes) can be moderately elevated, but does not reach the level seen in acute leukemia (account for <20% of the bone marrow cells). Auer rods are never present. Dysplasia is usually minimal; however, dysgranulopoiesis (including pseudo-Pelger-Huet neutrophils and hypogranularity) may be noted in some cases, and erythroid precursors may be enlarged. No specific immunophenotypic abnormalities have been reported in JMML.

4. Differential diagnosis

4.1 KMT2A-**rearranged AML masquerading as JMML**

Unlike in AML, the bone marrow in patients with JMML demonstrates no blockage of differentiation of myeloid elements. Rather, as is seen in chronic myeloid leukemia (CML), the bone marrow in JMML displays myeloid hyperplasia with increased production of monocytic cells along the full spectrum of differentiation, including blast forms, promonocytes, monocytes, and macrophages. The marrow blast count may be slightly elevated but in classic JMML it does not reach the counts seen in AML. Nevertheless, differentiating JMML from AML is nearly impossible on clinical grounds alone as significant hepatosplenomegaly and respiratory failure can occur in both. Moreover, blood counts and hematologic features may mimic AML. This especially holds true for infants with the lysine methyltransferase 2A (*KMT2A*) rearrangements who occasionally present with hepatosplenomegaly and low blast count resembling JMML [5]. A puzzling interface between *KMT2A*-rearranged AML and JMML therefore exists. Recent reports have validated the close mimicry between *KMT2A*-rearranged AML and JMML [6]. Unless unveiled by cytogenetics, JMML can conceal the clinical diagnosis of *KMT2A*-rearranged AML. Age of susceptibility (infancy or early childhood) and abnormal monocytosis have blurred the line between these distinct entities. Specifically, JMML may mimic AML with t(9;11). t(9;11) is the most frequent molecular subtype involving the *KMT2A* gene (*KMT2A-MLLT3*) in AML. t(9;11)-positive AML/JMML overlap was well-characterized in the medical literature [7]. Both conditions have increased immature monocytes and blasts. I reported a 14-month-old girl with t(9;11)-positive AML who died as she received JMML-directed therapy. The clinical picture, peripheral smear and the suboptimal blast count of only 10% had stealthily impersonated JMML [8].

Chromosomal rearrangements involving the *KMT2A* gene do not exist in the genomic landscape of JMML. KMT2A gene rearrangements are common genetic mutations in pediatric AML with an incidence of 15–25% (50–60% in children younger than two years). However, both KMT2A-rearranged AML and JMML share common morphologic features. *KMT2A*-rearranged AML is usually AML M4 or M5, and both are characterized by increased numbers of monoblasts or abnormal

monocytes. JMML morphologically resemble AML M4 and distinction must be made based on accurate blast and promonocyte counts.

Differentiating AML from JMML is vital for survival. Chemotherapy regimens for JMML are mainly cytoreductive as a bridge to hematopoietic stem cell transplantation (HSCT) rather than curative as for AML. Therefore, any delay in establishing the correct diagnosis and/or administration of wrong treatment can be lethal. Molecular diagnosis has become the mainstay to distinguish between AML and JMML. Cytogenetics and FISH should be immediately performed to detect *KMT2A* gene rearrangements in every suspicious case.

4.2 JMML masquerading as RALD

JMML is closely related to RAS-associated autoimmune leukoproliferative disorder (RALD) [9]. RALD is a non-malignant, non-infectious leukoproliferative disease that resembles the autoimmune lymphoproliferative syndrome (ALPS) caused by mutations affecting the FAS/FASL pathway.

Similar to patients with ALPS, RALD patients present with lymphadenopathy, massive splenomegaly, increased circulating B cells, hypergammaglobulinemia, and autoimmunity. However, RALD was separated from ALPS as:

1. In contrast to ALPS, biomarkers such as $CD4^-/CD8^-$ double negative T-cell receptor $\alpha\beta$ ($TCR\alpha\beta^+$) T cells and serum vitamin B12 levels are not always increased.

2. Germline or somatic mutations in *FAS, FASL, or CASP10* are absent in RALD.

RALD is a RAS-associated somatic disorder characterized by myelomonocytic and lymphoid hyperplasia that shares identical somatic *KRAS* or *NRAS* mutations found in up to 25% of JMML patients. It is thought that RAS activation itself can alter selection patterns of autoreactive B cells and antibody production leading to autoimmune manifestations. Overlap features of both JMML and RALD include:

1. Cytopenias, lymphadenopathy, and splenomegaly.

2. Persistent absolute or relative monocytosis is a cardinal feature of RALD.

3. Bone marrow and peripheral blood smear findings overlap with those of JMML in children or CMML in older patients.

4. Morphologic features compatible with dysplasia, such as hyposegmented pelgeroid neutrophils, can also be seen in RALD further obscuring the distinction between RALD and JMML.

5. Autoimmunity can be noted in up to 25% of children with JMML, whereas hypergammaglobulinemia is present in more than 50% of cases.

6. Nearly all patients with RALD meet the revised diagnostic criteria for JMML.

Distinguishing RALD from JMML can be impossible on clinical grounds alone. However, this distinction has an important prognostic value as RALD is characterized by an indolent clinical course whereas JMML can be fatal if left untreated. The most definitive diagnostic distinction between RALD and JMML occurs in the

setting of a cytogenetic abnormality (eg, monosomy 7), which excludes RALD and favors a malignant process. However, normal bone marrow cytogenetics has been reported in around 65% of JMML patients.

JMML/RALD overlap can lead to inappropriate treatment decisions. In some patients, RALD was misdiagnosed as JMML and vice versa. It is the author's experience that a patient with KRAS-mutated JMML was misdiagnosed as RALD for many weeks until he developed full -blown JMML. It is noteworthy that RALD can occur as the initial presentation and transform to JMML several years later. Indeed, JMML/RALD represents a continuum of two different phenotypes of the same disorder. Although both RALD and JMML share common RAS mutations, the transition from RALD to JMML is caused by additional genetic or epigenetic events. Therefore, patients with RALD should be very closely monitored for acquisition of additional dysplastic, molecular, or clonal karyotypic abnormalties that may herald malignant transformation [10].

4.3 JMML masquerading as myeloproliferative neoplasms (MPNs)

JMML can mimic myeloproliferative malignancies with receptor tyrosine kinase translocations. Identification of these cases is crucial, because patients may benefit from receptor tyrosine kinase–targeted inhibitors.

4.4 Nonmalignant disorders mimicking JMML

- Viral infections such as human herpes virus-6 (HHV-6), parvovirus, or cytomegalovirus (CMV). Occasionally, patients with JMML may present with these viral infections in addition to their underlying hematologic malignancy. Differentiation between CMV-related disease and JMML in infants excreting CMV is sometimes difficult, because clinical and laboratory findings of CMV infection can overlap with those of JMML.

- Leukocyte adhesion deficiency variants.

- Wiskott Aldrich syndrome: need to be considered in male infants.

- Infantile malignant osteopetrosis (IMO): can mimic all clinical and hematological features of JMML.

- Hemophagoytic lymphohistiocytosis (HLH).

- Autoimmune diseases, including rheumatoid arthritis and systemic lupus erythematosus.

5. Genomics

5.1 The central role of the RAS signaling pathway

JMML is an oncogenic RAS mutant cancer where approximately 90% of patients carry either somatic or germline gain of function mutations in *PTPN11, KRAS, NRAS, CBL, or NF1* genes that lead to constitutive activation of the RAS signaling pathway (**Figure 3**). The RAS signaling pathway is a component of multi-step signal transduction pathway that controls the cellular proliferation, differentiation,
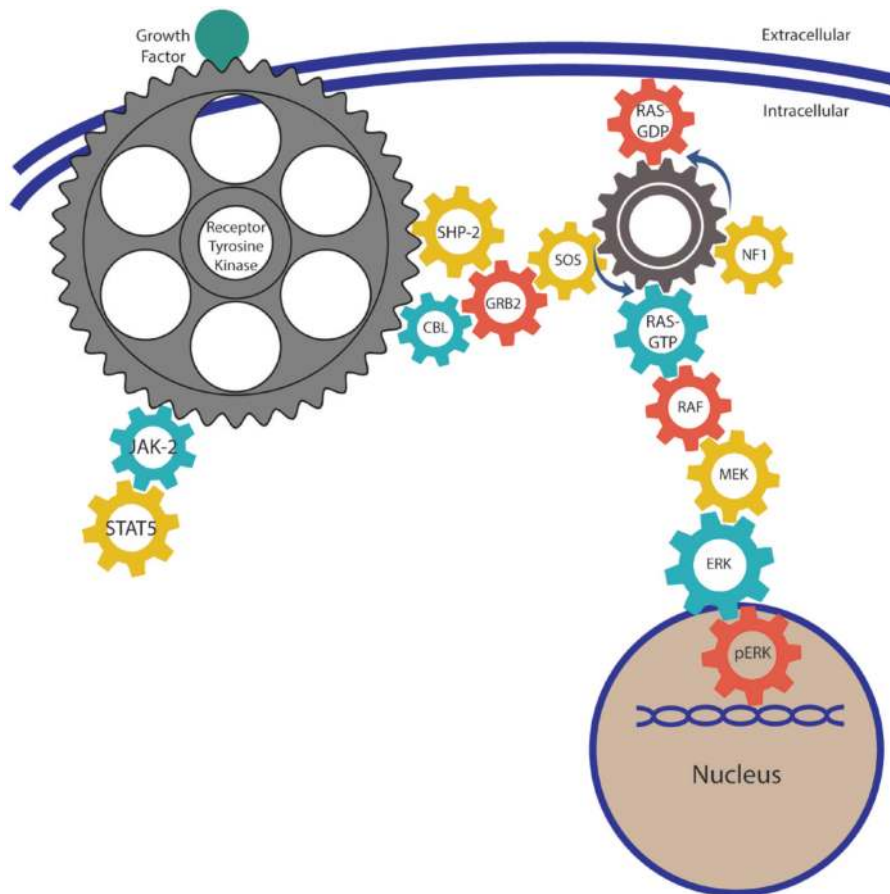
Figure 3.
Illustrative representation of the RAS signaling pathway portraying the transmission of extracellular signals in a gear train to the nucleus. Upon stimulation, a docking site for GRB2 is created which binds to GEF (SOS). The GRB2-SOS interaction converts RAS-GDP to RAS-GTP. The RAS-GTP initiates a cascade of phosphorylations on RAF, MEK and, lastly, ERK. The phospho-ERK then translocates into the nucleus. A counteracting force by GAP (neurofibromin) accelerates the conversion of RAS-GTP to RAS-GDP to restrain the activation of the RAS signaling pathway. Mutations of RAS pathway prevent the conversion of RAS-GTP to RAS-GDP to induce the constitutive activation of the RAS signaling pathway, the characteristic feature of JMML.

and survival. It relays extracellular stimuli such as growth factors to the nucleus where the response to those stimuli is executed by an induced transcriptional program [11].

The RAS family include the proto-oncogenes *HRAS, NRAS and KRAS* which represent a subset of a superfamily of small membrane-localized GTPases. Like other small GTPases, RAS proteins function as molecular switches that cycle between an inactive guanosine diphosphate (GDP)-bound and an active guanosine triphosphate (GTP)-bound conformations. RAS is activated when the receptor tyrosine kinases (RTKs, a large superfamily of receptors for a wide array of growth factors) are activated by growth factor binding leading to RTK autophosphorylation and the creation of docking sites for adaptor molecules like growth factor receptor-bound protein 2 (GRB2). The amount of GTP-RAS generated is regulated by two opposing regulatory mechanisms [12]:

a. Guanine nucleotide exchange factors (GEFs) are necessary for the conversion of GDP-RAS into GTP-RAS. The Son of Sevenless (SOS) is the GEF for the RAS signaling pathway. SOS is recruited by the adaptor protein GRB2 in response to RTK activation. The binding of SOS to GBR2 localizes it to the plasma

membrane, where it can activate the membrane bound RAS. Gain of function of the Src-homology tyrosine phosphatase 2 (SHP-2) results in activation of the GEFs and in this way to a continuous activation of RAS. SHP-2 is the encoded gene product of *PTPN11*, the most commonly mutated gene in JMML.

b. GTPase activating proteins (GAPs), like neurofibromin act antagonistically to inactivate RAS by increasing their intrinsic rate of GTP hydrolysis to the inactive form (RAS-GDP) leading to the termination of the RAS signaling. Neurofibromin is the encoded gene product of the tumor suppressor gene *NF1,* the gene implicated in Neurofibromatosis type 1.

Cancer-associated RAS mutations typically alter amino acids G12, G13 or Q61. These mutant RAS proteins display impaired GTPase activity which renders them resistant to GTPase activating proteins (GAPs). Mutant RAS proteins are therefore locked in the signal-transmitting GTP- bound form (the cytosolic ratio of GTP is much higher than GDP at 10:1). The active GTP-RAS in turn activates the RAF kinase, resulting in a downstream proliferative effect.

The *CBL* gene (11q23.3) is a proto-oncogene that encodes three distinct homologs which represent the mammalian CBL family of proteins. Members of the CBL protein family (Cbl/c-Cbl, Cbl-b, and Cbl-c/Cbl-3) are E3 ubiquitin ligases that functions as a negative regulator of many signal transduction pathways by promoting degradation by ubiquitination of activated RTKs (and nonreceptor tyrosine kinases). Studies in animal models and genetic analyses in human cancer have firmly established that CBL proteins function as tumor suppressors. The mutant CBL (lacking E3 ligase activity)- dependent oncogenesis is driven by loss of ubiquitination-dependent degradation of activated RTKs resulting in a synergistic increase in signaling. Mutant CBL proteins may indeed promote oncogenesis in JMML via the RAS signaling pathway. In addition, CBL is an adaptor protein that positively regulates signal transduction. Approximately 150 proteins are associated with or are regulated by CBL proteins. Among these proteins is the adaptor GRB2 that binds to CBL. The CBL-GRB2 interaction modulates the interaction between GRB2 and SOS and hence it is also possible that CBL-GRB2 interactions lead to activation of the RAS pathway.

5.2 GM-CSF–dependent hypersensitivity

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and the related cytokines interleukin 3 (IL-3) and interleukin 5 (IL-5) regulate hematopoietic cell survival, proliferation, differentiation, migration, and perform effector functions such as phagocytosis or reactive oxygen species release. Unlike other cytokine receptors, GM-CSF receptor (GMR) has a significant nonredundant role in macrophage-mediated acute and chronic inflammation, pulmonary homeostasis, allergic diseases, and myeloid haematologic malignancies [13]. GMR is found in myeloid cells and some non-hematopoietic cells, but it is not expressed by lymphoid cells such as T cells. GMR is composed of a ligand-specific α chain (GMR α) and a β common (βc) signaling subunit, which is shared with the IL-3 and IL-5 receptors. Both of which are members of the cytokine receptor family. The binding of GM-CSF to the ligand specific GMRα promotes the formation of a higher-order signaling complex that leads to the activation of non-receptor tyrosine kinases JAK2 and Src family kinases (c-Src and Lyn), which subsequently phosphorylate GMRβc to create docking sites for adapters and signal relay molecules (such as SHP-2) initiating downstream signaling. GM-CSF, IL-3, and IL-5 activate at least three downstream pathways: the JAK/STAT pathway, the RAS signaling pathway, and PI

3-kinase activation pathway. These pathways should not be viewed as being mutually exclusive and may have substantial overlap [14].

RAS signaling pathway mutations might potentiate JAK/STAT signaling by stabilizing or directly activating the GM-CSF receptor or its associated signaling molecules. In particular, the dysregulated SHP-2 (a non-receptor protein tyrosine phosphatase) by JMML-associated mutations is normally essential for efficient STAT5 activation in myeloid cells that are stimulated with IL-3 (SHP-2 is recruited to phosphorylated tyrosine residues on the activated β subunit of the GMR). Elevated levels of RAS-GTP might also increase the degree and/or duration of JAK2 kinase activity. The disturbed GMR signaling in JMML leads to an aberrant response to GM-CSF which can be tested by 2 laboratory assays:

5.2.1 CFU-GM assay (the traditional methylcellulose assay)

The selective hypersensitivity of myeloid progenitor cells of JMML to the GM-CSF in hematopoietic colony formation assays (CFAs) results in spontaneous growth of colony-forming units -granulocyte/macrophage (CFU-GM) in the absence of exogenous growth factors. JMML cells are cultured in semisolid methylcellulose media producing excess number of monocyte–macrophage colonies in the absence of added exogenous growth factors. This spontaneous proliferation of JMML myeloid progenitor cells is primarily due to the striking hypersensitivity of progenitors to GM-CSF in vitro.

This phenomenon, and hallmark of the disease, was first described in 1991. The CFU-GM assay has 2 main pitfalls. The assay is not standardized across diagnostic laboratories and although sensitive it is rather a non-specific assay. Viral infections such as HHV-6 and CMV have also been reported to cause GM-CSF hypersensitivity. Although the hypersensitive pattern of CFU-GM colony growth in methylcellulose is neither necessary nor sufficient to establish a diagnosis of JMML, it used as a minor diagnostic criterion for those 10 percent of JMML patients who do not have identifiable molecular abnormalities affecting the RAS signaling pathway.

5.2.2 p-STAT5 phospho-specific flowcytometry assay

Low-dose GM-CSF can induce hyperphosphorylation of STAT5 in either $CD33^+$/$CD34^+$ (myeloid precursor cells) or $CD33^+$/$CD34^-$/$CD14^+$/$CD38^{low}$ population (represents the more mature monocytic cells) in patients with JMML. In contrast to $CD34^+$/$CD33^+$ cells, neither $CD34^-$/$CD33^+$ (mature myeloid cells) nor $CD34^+$/$CD33^-$ (non-myeloid precursors), demonstrated any STAT5 hyperphosphorylation in response to GM-CSF. These results indicate that the peculiar STAT5 hyperphosphorylation signature specifically resides in the myeloid compartment of hematopoietic progenitor cells [15].

After binding of GM-CSF to its receptor, Janus-kinase-2 (JAK-2) is recruited to the cytoplasmic domain of the β chain, and activation of JAK-2 occurs, which subsequently induces STAT-5 phosphorylation. This signaling pathway induces migration of STAT-5 dimers to the nucleus and promotes the transcription of various genes such as pim-1 and CIS to induce cell differentiation. This cytokine-specific response is exclusively present in JMML patients compared with healthy controls or other pediatric MPNs, indicating that it is critical for disease pathogenesis. The aberrant response of phospho-STAT5 (p-STAT5) to sub-saturating doses of GM-CSF is demonstrated by phospho-specific flowcytometry assay. This assay is superior to the traditional CFU-GM assay in the following aspects [16]:

1. As compared to CFU-GM assay, p-STAT5 profiling is a rapid diagnostic tool for JMML. CFU-GM assay takes up to several weeks of culture as it requires monocyte depletion.

2. p-STAT5 profiling could discriminate JMML from diseases mimicking JMML, such as CMV infection and transient myeloproliferative disorder in Noonan syndrome (NS/MPD) that also exhibit GM-CSF hypersensitivity by in vitro colony assay but not by phospho-specific flowcytometry.

Interestingly, CMML (the adult equivalent of JMML) also displays GM-CSF-dependent hypersensitivity. Primary CMML samples demonstrate GM-CSF–dependent hypersensitivity by hematopoietic CFAs and phospho-STAT5 (pSTAT5) flowcytometry compared with healthy donors. Among CMML patients, the pSTAT5 hypersensitive response positively correlated with high-risk disease, peripheral leukocytes, monocytes, and signaling associated mutations.

The p-STAT5 response to low doses of GM-CSF was also detectable in AML M4/M5. JMML, CMML, and M4/M5 AML are related entities in which hyperactive RAS and aberrant JAK2/STAT5 signaling are early or initiating events. As such, M4/M5 AML might be distinct from other subtypes of AML, in which aberrant transcription factor fusions such as PML-RARA and AML1-ETO likely represent primary leukemogenic events. This has important therapeutic implications, as M4/M5 AML might be highly dependent on RAS and JAK2/STAT5 signaling, and therefore sensitive to inhibitors of these pathways. Because GM-CSF signaling is critical for monocyte differentiation and survival, targeting GM-CSF in the therapeutics of JMML in vitro and AML in vivo has been reported, with varying degrees of success [17].

The absence of the p-STAT5 phospho-flow signature in conditions that are phenotypically similar to JMML highlights the importance of the signaling disruptions that we observe specifically in JMML. The flow cytometric p-STAT5 profiling is a reliable, sensitive and specific diagnostic tool for identifying patients with JMML that was added in the 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia as a minor diagnostic criterion for those 10 percent of JMML patients who do not have identifiable molecular abnormalities affecting the RAS signaling pathway.

In fact, hyperphosphorylation of STAT5 does not only add to the genetic mapping of JMML but it has significant therapeutic implications. Kotecha et al. demonstrated that p-STAT5 phospho-flow signature may identify children with NRAS and PTPN11 mutations who will have a benign clinical course and can be observed closely without aggressive treatment. p-STAT5 phospho-flow signature was also absent in infants with NS/MPD which indicates that phospho-flow cytometry provides a more specific readout of the rewired signaling networks found in JMML. Remarkably, p-STAT5 signaling response can indicate disease status as the p-STAT5 responsive population was shown to disappear in remission and reappear during relapse. Differences in GM-CSF-stimulated phospho-STAT5 levels might be utilized to identify targeted agents with potential efficacy as well as to follow response to treatment, relapse, or transformation to AML [18].

5.3 JMML interlacing with RASopathies

As opposed to the somatic mutations found in cancer, germline mutations of genes that encode components or regulators of the RAS signal transduction pathway result in clinically defined group of human genetic syndromes collectively termed as "RASopathies" or "neuro- cardio-facio cutaneous syndromes (NCFCS) [19]. The RASopathies are one of the largest known groups of malformation syndromes with

an incidence of around 1 in 1000 individuals. They exhibit numerous overlapping phenotypic features such as neurodevelopmental dysmorphic features, an increased risk of autoimmunity and predisposition to cancer and abnormal myelopoiesis in infancy. The RAS signal transduction pathway plays an essential role in regulating the cell cycle and cellular growth, differentiation, and senescence, all of which are critical to normal development. Therefore, it is not surprising that RAS signal transduction pathway dysregulation has detrimental effects on both embryonic and later stages of development. RASopathies include:

1. Noonan syndrome: PTPN11, KRAS, SOS1, RAF1, NRAS.

2. Costello: HRAS.

3. Cardiofacio-cutaneous syndromes (KRAS, BRAF, MEK1/2).

4. Neurofibromatosis type 1: NF1.

5. NS-like syndromes: CBL-syndrome (CBL) and NS-like disorder with loose anagen hair (SHOC2).

6. Legius syndrome (NF-1-like syndrome): SPRED1.

Noonan syndrome (NS) is the most genetically diverse and most common RASopathy occurring in 1 of 1000 to 2500 births. NS is characterized by developmental disorders, short stature, a typical facial appearance (facial dysmorphism), congenital heart defects, and skeletal anomalies. So far, heterozygous mutations in nine genes (*PTPN11, SOS1, KRAS, NRAS, RAF1, BRAF, SHOC2, MEK1* and *CBL*) have been documented to underlie this disorder or clinically related phenotypes. Germline mutations in the *PTPN11* gene have been described in 50% of the Noonan syndrome cases. Somatically acquired JMML-associated PTPN11 mutations are not seen in NS. Somatic mutations differ from germline PTPN11 mutations which are predicted to result in a weaker gain-of-function than the somatic mutations [20].

The most common hematopoietic disorder in NS is a transient myeloproliferative disorder (MPD) estimated to occur in up to 10% of all children with NS. Nearly all patients with NS/MPD have mutations in *PTPN11*. The transient MPD in Noonan syndrome is diagnosed in the neonatal period or early infancy. In contrast to JMML, NS/MPD is thought to be of polyclonal origin. It generally resolves spontaneously over months or years. However, leukocytosis and tissue invasion by monocytes and immature myeloid cells can have deleterious effects. An estimated 10% of cases of NS/MPD acquire a cytogenetic abnormality and progress to JMML. Thus, NS/MPD is a tumor predisposition syndrome and affected patients should be followed closely. JMML in *PTPN11*-mutated NS seems to behave differently from sporadic JMML as it occurs at a very young age (infancy) and tends to regress spontaneously. Although the hematological picture can be indistinguishable from JMML in some cases, the myeloproliferation is polyclonal in most instances, and thus, the disorder has not been recognized by the World Health Organization (WHO) as a separate JMML category. Therefore, recognizing NS in a JMML patient is important in order to identify those patients who might benefit from a watch-and-wait strategy. Similar to *PTPN11*-mutated NS, a transient MPD was noted in some children with NS and *KRAS* or *NRAS* germline mutations.

NF1 gene is a negative modulator that normally restricts RAS activation. Loss of heterozygosity (LOH) with loss of the normal NF1 allele in leukemic cells leads to activation of RAS signaling pathway. In most cases, the diagnosis of NF1 can be

established clinically by the presence of ≥6 caf'e au lait macules >0.5 cm in diameter. In addition, one-half of these children have a parent affected by NF1.The risk of developing JMML for the patient with NF1 is estimated 200- to 350-fold higher than in patients without NF1.

Germ line mutations of the *CBL* gene cause CBL syndrome characterized by a high frequency of neurologic features/vasculopathy, mild NS-like features, and a high risk of JMML. The germline mutation represents the first hit, with somatic LOH being the second hit positively selected in JMML cells. Indeed, all children with JMML and *CBL* mutations were found to have a germline *CBL* missense mutation on one allele and acquired LOH on the other allele in leukemic cells. The development of vasculopathies occurs in the second decade of life unless allogeneic HSCT is successful. The most frequently observed vasculopathies are optic atrophy, optic neuritis, hypertension, cardiomyopathy, and arteritis.

5.4 Cytogenetic studies

JMML cells show a normal karyotype in 65% of cases, sole monosomy 7 in about 25%, and other aberrations in 10%. A remarkable feature of many JMML cases with normal karyotype is a markedly increased synthesis of fetal hemoglobin (HbF). The likelihood of an abnormal karyotype is dependent on the genetic subtype, with monosomy 7 being noted most often in *KRAS*-mutated disease. Monosomy 7 is the most frequent chromosomal aberration in both MDS and JMML. It is likely that haploinsufficiency of genes located on chromosome 7 contributes to clonal evolution.

6. JMML genetic subtypes

Deregulated RAS signaling is the main driving event in JMML. The 5 canonical RAS pathway mutations (*PTPN11, KRAS, NRAS, CBL*, and *NF1* genes) represent five genetically and clinically distinct subtypes in JMML. Mutations in the respective genes can either occur as germline ("syndromic") or as somatic lesion in hematopoietic cells ("nonsyndromic"). *PTPN11-, NRAS-,* and *KRAS*-mutated JMML are characterized by heterozygous somatic gain-of-function mutations in nonsyndromic children, whereas JMML in neurofibromatosis type 1 and JMML in children with CBL syndrome are defined by germline RAS disease and acquired biallelic inactivation of the respective genes in hematopoietic cells. Their clinical features are presented in **Table 2**.

The concept that mutations associated with JMML are mutually exclusive within a given individual implies that a mutation in any one of these genes is sufficient to activate the signaling pathway and drive the proliferation of JMML cells. However, this does not mean that two or more genetic mutations cannot occur at the same time as one would comprehend from the term 'mutually exclusive'. In fact, coexisting mutations in *NRAS, KRAS, PTPN11, CBL and NF1* were found in 11% of JMML patients [21]. Analysis of single colonies for two patients with compound RAS pathway mutations demonstrated that both mutations occurred in the same colony. PTPN11 and NF1 lesions were the most frequent of these cooperative events. In addition, one patient harbored two *NRAS* lesions (p.G13D and p.Q61K).

The five JMML genetic subtypes are key determinants for treatment adaptation in patients with JMML. However, their use in a risk stratification algorithm is hindered by their failure to independently prognosticate the clinical outcome. In fact, the prognostic relevance is owned by the number of mutations at diagnosis rather than the type of mutation itself. Patients with JMML who harbor two or

Genetic subtype	Characteristics:
1- *NF1*-mutated JMML:	• **Incidence: 5–10% of cases.**
	• **Higher platelet count.**
	• **Higher percentage of blasts in bone marrow.**
	• **More often diagnosed after the age of 5 years than other subtypes.**
	• **Although some of the younger children can initially enjoy a relatively unaffected clinical course, *NF1*-mutated JMML is invariably fatal unless allogeneic HSCT can successfully be performed.**
2- *PTPN11*-mutated JMML:	• **Incidence: 35% of cases.**
	• **A rapidly fatal disorder unless allogeneic HSCT can successfully be performed.**
	• **Significantly worse outcome with higher probability of relapse rates when compared with other subtypes.**
	• **Frequent acquisition of *NF1* haploinsufficiency.**
	• **The p.(Glu76Lys) is the most frequently observed *PTPN11* finding in JMML.**
3- *NRAS*-mutated JMML:	• **Incidence: 18% of cases.**
	• **A heterogeneous course.**
	• **A considerable percentage of patients relapse after HSCT (typically older children with high levels of HbF).**
	• **Some patients enjoy an indolent course with spontaneous regression (typically infants or cases with G12S mutation). Clinically, these children are well and show a normal or only slightly elevated HbF.**
4- *KRAS*-mutated JMML:	• **Incidence: 14% of cases.**
	• **Most children are diagnosed below the age of 1 year (i.e, infants). They often present with particularly severe disease.**
	• **Monosomy 7 is frequently noted in leukemic cells.**
	• **In some cases, an impressive treatment response to azacitidine has been observed.**
	• **Although Aggressive at presentation, *KRAS*-mutated JMML has a low relapse rate after allogeneic HSCT and may benefit from less intensive preparative regimens.**
	• ***KRAS*-mutated JMML shares many features with RALD.**
5- *CBL*-mutated JMML:	• **Incidence: 12 to 18% of cases.**
	• **Patients display several congenital anomalies that overlap with those observed in NF-1, NS, and Legius syndrome.**
	• **Self-limiting disease: most children experience spontaneous regression of their myeloproliferation despite the persistence of LOH of the CBL locus in hematopoietic cells.**
	• **Observation without therapeutic intervention is generally advised, but in some instances grossly enlarged spleens and thrombocytopenia require therapeutic intervention.**
	• **The value of allogeneic HSCT is uncertain.**
	• **Frequent occurrence of partial rejection with stable mixed chimerism after allogeneic HSCT.**
	• **The only recurrent variant is copy-neutral isodisomy (LOH) at 11q23.3 where CBL is located. No other concomitant mutations are found.**

Table 2.
Clincal features of genetic subtypes of JMML.

Clinical risk factors:	1- **Age ≥ 2 years.**
	2- **Platelet count ≤ 40 x 10⁹/L.**
	3- **Increased fetal hemoglobin (HbF) for age.**
	4- **Male sex (in some references).**
Molecular risk factors:	1- **Two or more somatic mutations.**
	2- **Secondary genetic mutations.**
	3- **DNA hypermethylation signature defines an aggressive JMML subgroup with high risk of relapse.**
	4- *PTPN11* **mutation.**
	5- **Monosomy 7.**

Table 3.
Poor prognostic factors.

more somatic mutations at diagnosis have significantly worse event-free and overall survival rates than those with one or no event. In harmony with these findings, the known clinical risk factors predictive of poor outcome (**Table 3**) are only weakly associated with the type of index mutation but they would rather fit patients with two or more underlying somatic mutations.

In addition to the mutations in RAS pathway genes (*PTPN11, NRAS, KRAS, CBL, or NF1*), other driver mutations thought to be the initiating events of JMML have been recently identified such as *RRAS, RRAS2*, or *SH2B3* mutations. *RRAS* and *RRAS2* genes are both members of the RAS GTPase family and hence expanded the spectrum of RAS pathway mutations in JMML. Activating mutations in *RRAS* underlie a phenotype within the RASopathy spectrum. Children with *RRAS*-mutated JMML can have an atypical clinical course with rapid progression to AML [22]. Somatic *RRAS* mutations co-occurred with acquired *NRAS* lesions in atypical JMML characterized by late onset and rapid progression to AML as well.

7. Secondary genetic mutations in JMML

In addition to the initiating canonical RAS pathway mutations, secondary clonal abnormalities were detected in about one-half of the patients. The importance of these mutations relies in how secondary mutations alter the behavior of cells in contrast to cells harboring only the primary lesion. Secondary mutations are often subclonal and may be involved in disease progression rather than initiation of leukemia. It appears that such mutations characterize patients with the highest risk of progression and poor outcome. The acquisition of the second mutation would thus also explain the continuum between RALD and leukemia. The clone harboring the secondary event frequently expands at the time of relapse post HSCT. This phenomenon has important therapeutic implications. Combination of therapies with agents that target the RAS pathway as well as the secondary genetic event could prove more efficacious in the correct genetic context than monotherapy alone.

The secondary mutational events occur inside or outside the canonical RAS pathway axis. They include second hits targeting the RAS pathway (so-called 'RAS double mutants') as well as mutations in *SETBP1, JAK3, SH283*, components of the polycomb repressive complex 2 (like *EZH2* and A*SXL1*), and occasionally, spliceosome genes. Some authors linked differential expression of key regulatory noncoding RNAs, such as let-7100 or miR-150-5p,101 to the various genetic subgroups

of JMML [23]. Secondary mutations of *SETBP1* and *JAK3* were the most frequent mutations (around 15% of children) and were presumed to be involved in tumor progression and poor clinical outcomes.

8. Epigenetic landscape of JMML

Aberrant DNA methylation was found to be an initiating event in JMML. This finding has instigated the research in the field of epigenetics and led to the landmark discovery of methylation classes in 2017. In addition, epigenetic modifications are implicated in JMML disease progression and together with established clinical and genetic markers fully recapitulate the clinical and biological heterogeneity of JMML. The genome-wide DNA methylation analysis of JMML patients identified three biologically distinct JMML subgroups [24]:

1. The high-methylation group (HM) was dominated by older children and cases with somatic *PTPN11* mutations and poor clinical outcome.

2. The intermediate methylation group (IM) showed enrichment for somatic *KRAS* mutations and monosomy 7.

3. The low methylation group (LM) is enriched for somatic *NRAS* and *CBL* mutations, as well as for Noonan patients, and has a good prognosis.

The results of all DNA methylome studies in JMML were exceptionally consistent with the fact that DNA hypermethylation is a recurrent feature of JMML cells that confers treatment resistance. The dysregulated genomic DNA methylation is a crucial component of RAS-driven malignant cell transformation proposing a possible functional links between RAS pathway mutational patterns and methylation classes. However, DNA hypermethylation was only weakly associated with the canonical genotypes or cytogenetic aberrations. Instead, it correlated strongly with the known clinical risk factors predictive of aggressive disease and poor outcome, especially older age and increased HbF level (**Table 3**). In fact, DNA methylation status is an independent prognostic factor and better predictor of clinical outcome than JMML genetic types. Frequent hypermethylation in 4 genes (*BMP4, CALCA, CDKN2B*, and *RARB*) were associated with a poor prognosis. Moreover, *RASA4* hypermethylation was related to poor prognosis and disease relapse after HSCT. DNA methylation could be used as a biomarker that can both identify patients who are predicted to fail HSCT as well as those who are most likely to experience spontaneous resolution and could be observed to avoid the acute and late side effects of HSCT [25].

9. Treatment

The heterogeneity of the disease is reflected by the varied clinical outcomes. Although the current standard of care for JMML is allogeneic HSCT, continued controversy exists about identifying those patients who need to be moved quickly to HSCT versus those rare patients who might be observed [26]. The majority of children with *CBL*-mutated JMML and some *NRAS*- mutated patients experience spontaneous disease regression. These children are clinically well with a low HbF. A careful watch-and-wait strategy may be indicated as the treatment of choice. On the other hand, JMML with somatic *PTPN11* mutations appear to represent cases with aggressive biology with a high risk of relapse even after HSCT.

Nevertheless, allogeneic HSCT remains the most effective means of stopping the uncontrolled production of monocytic cells in the majority of patients. If left untreated, survival for most children is less than one year. The predominant cause of JMML-related death is respiratory failure as a result of pulmonary infiltration by leukemic cells (blastic transformation is infrequent in JMML). In fact, HSCT early in the course of disease significantly improved the dismal prognosis of JMML patients. However, the 5-year event-free survival (EFS) rate after HSCT is only 44–53%. Disease recurrence is the most important cause of failure, occurring with a cumulative incidence of 35%. Of note, patients with JMML who transform into AML (defined as >20% blasts in bone marrow) generally have dismal outcomes following HSCT.

Pre-transplant chemotherapy for JMML had no benefit on EFS or overall survival. A variety of pre-HSCT treatments have been employed to control symptoms of JMML (such as high white blood cell count, pulmonary problems, and/or prominent organomegaly) as well as theoretically improve outcomes. However, none of these agents induced durable responses or reduced the relapse rate. The main aim of the pre-HSCT treatments remains to bridge HSCT. Given the current lack of convincing evidence for traditional myelosuppressive chemotherapy pre-HSCT, other treatment modalities has been sought. In particular, molecular classification of cancer based on DNA methylomes has revolutionized the diagnostic and prognostic parameters of JMML and aid our understanding of the mechanistic link between epigenetic dysregulation and resistance to treatment. A DNA methyltransferase–inhibiting azanucleoside is assumed to reverse the epigenetic dysregulation in malignant cells. This has led to a European protocol to employ DNA hypomethylating agents such as azacitidine for therapy in JMML [27]. The first case report of azacitidine for JMML before HSCT was published in 2009. The child achieved a complete clinical, hematologic, cytogenetic (monosomy 7) and molecular (*KRAS* index mutation) remission during eight monthly cycles of 5-day azacitidine. The reduction of *BMP4* promoter DNA methylation preceded the disappearance of leukemic cells, demonstrating the DNA-hypomethylating activity of azacitidine in JMML cells. Although azacytidine may induce complete clinical, cytogenetic and/or molecular remission before allogeneic HSCT, complete remission has not been sustained without transplant. On the other hand, the acceptable toxicity of low-dose azacitidine and its cytoreductive potential make it an attractive option as a bridging therapy before HSCT or as palliation after 1 or more transplants have failed [28].

10. Conclusion and future perspectives

JMML is a puzzling disease with blurring clinical presentation that can commonly mimic a wide variety of other diseases. Overall, the most consistent features of the JMML phenotype are young patient age, splenomegaly and increased synthesis of hemoglobin F (HbF). The molecular basis of JMML is closely linked to Rasopathies, a family of inherited cancer predisposition syndromes characterized by cardiac defects, defective growth, facial dysmorphism and variable cognitive deficits. Defining aberrant RAS signaling transduction pathway as the common denominator linking RASopathies to JMML and a transient JMML-like disease made JMML a fundamentally a disease of uncontrolled hyperactivation of RAS signaling. The identification of RAS pathway mutations in JMML has advanced the understanding of molecular mechanisms underlying the progression from cancer predisposition to neoplasia. Deciphering of the mutational spectrum had led to the classification of JMML into 5 genetic subtypes which have distinctive genotype–phenotype characteristics. However, the clinical outcome of JMML

is not completely explained by the clinical and genetic markers which lent the epigenetic landscape of JMML a particular interest. There is hardly any pediatric oncology entity where research has benefited as much from epigenetics as JMML. Genome-wide interrogation of DNA methylation patterns has led to a classification of the disease into three distinct JMML subgroups that have clear pathogenetic and prognostic relationships. The DNA hypermethylation signature is associated with poor clinical outcome and increased risk for relapse following HSCT. Evidence suggests that DNA methylation changes could be used as a potential biomarker in a combined risk stratification algorithm in future clinical trials. Although the clinical activity of azacitidine as a DNA hypomethylating agent appears to be promising, it is unlikely that azacitidine alone have the potential to cure JMML [29]. However, azacitidine as monotherapy is safe and effective in controlling disease both in upfront and relapsed patients in order to proceed to HSCT [30]. In fact, azacitidine is currently the gold standard bridge to HSCT in JMML.

Despite the lack of associated mutations in the genes coding for the GMR, JMML is characterized by aberrant GMR signal transduction. This aberrant signaling is integral in the pathogenesis of JMML that underlines the dysregulated myelopoiesis of the disease. The resultant selective GM-CSF hypersensitivity was exploited as a diagnostic tool for JMML and it is especially useful for those diagnostically challenging cases with RAS pathway mutation-negative JMML. This entity represents the remaining 10% of cases where disease might be due to chromosomal translocations, other gene mutations, specific microRNAs (miRNA) or long non-coding RNAs (lncRNAs) [31]. GMR signal transduction is located upstream to both RAS signaling pathway and the JAK/STAT pathway. STAT5 is recruited to phosphorylated tyrosine residues on the activated β subunit of the GM-CSF receptor, indicating that STAT5 signaling profile reflects JMML hypersensitivity to GM-CSF. The p-STAT5 phospho-specific flowcytometry assay which largely replaced the traditional colony assay had also recently gained promising utility in monitoring the disease status. As disease worsens, a greater percentage of cells get hyper-responsive. Therefore, p-STAT5 phospho-flow signature could prove enticing as a marker of disease progression and indicator of relapse. As JMML lacks tractable markers, both DNA methylation and p-STAT5 phospho-flow signature are potential new armamentarium that could serve as futuristic checkpoints in order to promulgate uniform protocols designed to follow patients on therapy. Interestingly, the shared p-STAT5 phospho-flow signature in JMML, AML M4/M5 and CMML could explain the unique overlap of their clinical features.

So far HSCT represents the only therapy with a clear impact on the outcome of JMML patients. However, HSCT is replete with significant morbidities and deleterious late effects in this young population. The search for more effective and less toxic strategies coincided with advances in molecular oncology that opened up a realm of novel and targeted molecules capable of improving therapeutic tactics in JMML. In particular, scoping into the genetic basis of hyper-responsiveness in JMML could provide a means of assessing the efficacy of emerging kinase inhibitors such as those blocking the RAS (e.g., the oral MEK inhibitor trametinib) or JAK2-signaling pathways for the treatment of this disease. Notably, a combination of drugs targeting two different pathways might be more effective than a single drug. However, the choice of drugs used for treatment should be entirely based on patient's mutation status [32, 33].

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Conflict of interest

The author declares no competing financial interests.

Abbreviations

KMT2A	Histone-lysine N-methyltransferase 2A
JAK	Janus-activated kinase
STAT	signal transducer and activator of transcription factor
p-STAT5	phospho-signal transducer and activator of transcription factor 5
PI 3-kinase	phosphatidylinositol-3 kinase
HSCT	Hematopoietic stem cell transplantation
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMR	GM-CSF receptor
CFA	hematopoietic colony formation assay
CFU-GM	colony-forming units -granulocyte/macrophage
CBL	Casitas B-lineage lymphoma
HHV-6	Human Herpesvirus 6
CMV	cytomegalovirus
CMML	chronic myelomonocytic leukemia
CML	Chronic myeloid leukemia

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