

Chronic neutrophilic leukemia: a clinical perspective

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Abstract: Chronic neutrophilic leukemia (CNL) is a rare myeloproliferative neoplasm (MPN) that includes only 150 patients described to date meeting the latest World Health Organization (WHO) criteria and the recently reported *CSF3R* mutations. The diagnosis is based on morphological criteria of granulocytic cells and the exclusion of genetic drivers that are known to occur in others MPNs, such as *BCR-ABL1*, *PDGFRA/B*, or *FGFR1* rearrangements. However, this scenario changed with the identification of oncogenic mutations in the *CSF3R* gene in approximately 83% of WHO-defined and no monoclonal gammopathy-associated CNL patients. *CSF3R* T618I is a highly specific molecular marker for CNL that is sensitive to inhibition in vitro and in vivo by currently approved protein kinase inhibitors. In addition to *CSF3R* mutations, other genetic alterations have been found, notably mutations in *SETBP1*, which may be used as prognostic markers to guide therapeutic decisions. These findings will help to understand the pathogenesis of CNL and greatly impact the clinical management of this disease. In this review, we discuss the new genetic alterations recently found in CNL and the clinical perspectives in its diagnosis and treatment. Fortunately, since the diagnosis of CNL is not based on exclusion anymore, the molecular characterization of the *CSF3R* gene must be included in the WHO criteria for CNL diagnosis.

Keywords: *CSF3R*, *SETBP1*, CNL, neutrophilic, WHO, PTK inhibitors

Current diagnosis criteria and treatment for chronic neutrophilic leukemia (CNL)

CNL is an uncommon myeloid malignancy characterized by a high number of mature neutrophils in the peripheral blood (PB), a hyperplastic bone marrow (BM), and hepatosplenomegaly.¹ Applying the 2008 World Health Organization (WHO) criteria for CNL, the diagnosis could be confirmed in only 51% of the clinically suspected patients with CNL. For the “true” CNL cases, the median age was 65 years (26–83 years) at diagnosis of which 67% were male.²

The disease course of CNL is variable, but acceleration is typically characterized by refractory neutrophilia, worsening organomegaly, and blastic transformation. Median time to acute myeloid leukemia (AML) transformation is 21 months (3–94 months) and median survival is 23.5 months (1–106 months). The most frequent causes of death are intracranial hemorrhage, progressive disease/blastic transformation, and regimen-related toxicity from induction chemotherapy or transplantation.^{1,3}

Clinical presentation

No specific symptom is observed at diagnosis presentation, and leukocytosis is detected incidentally in routine laboratory tests. The most frequent symptom is hepatosplenomegaly and some patients present with fatigue, weight loss, and bruise. Lymphadenopathy is uncommon at CNL presentation.¹

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Laboratory findings

The laboratory features of CNL include persistent neutrophilic leukocytosis with minimal left-shift, often characterized by toxic granulation and Döhle bodies, and elevated leukocyte alkaline phosphatase (LAP) and vitamin B12 levels.^{1,3-5}

According to the 2008 WHO diagnostic criteria for CNL, the PB leukocytosis is $\geq 25 \times 10^9/L$ (median $57 \times 10^9/L$, but as high as $138 \times 10^9/L$);² where more than 80% of leukocytes are segmented neutrophils/band forms; $< 10\%$ are immature granulocytes (promyelocytes, myelocytes, and metamyelocytes); and $< 1\%$ myeloblasts.⁵ Granulocytic dysplasia is not present, and there is no monocytosis, eosinophilia, or basophilia. The hemoglobin levels are low (more or less 11.0 g/dL) and the platelet numbers are normal but often they decrease in the advanced stages of the disease.¹

BM morphology

BM aspirates and biopsies show a myeloid hyperplasia ($> 90\%$ cellularity) where myeloblasts represent less than 5% of the cells. Erythro and megakaryopoiesis are typically normal, and dyspoiesis are not present in any cell lineage. Reticulin fibrosis is not significantly increased.

CNL co-occurs with monoclonal gammopathy (MG) of undetermined significance in approximately 33% of the cases.² This phenomenon has been reported in the literature with this subset of 12 patients presenting a MG associated with lambda light chain excess.^{6,7} However, it remains unclear whether the neutrophilic leukocytosis is a leukemoid response to the underlying MG, or if the presence of the two diseases represents a real entity.⁸ In cases where the BM shows a plasma cell dyscrasia, it is important to prove the neutrophilic clonality by cytogenetic and/or molecular tests.⁶

Molecular cytogenetics and clonality

Molecular cytogenetics should be negative for the well-defined markers of other neoplasms such as the Philadelphia chromosome and the *BCR-ABL1* fusion gene (characteristic of chronic myeloid leukemia – CML); and rearrangements in *PDGFRA/B* or *FGFR1* (characteristic of eosinophilic leukemia). *Janus kinase 2 (JAK2)* mutations are not specific for any myeloproliferative neoplasm (MPN) but can provide evidence that the proliferation is clonal.⁵ Although clonality has been demonstrated in CNL,^{9,10} the majority of patients exhibit normal cytogenetics.^{1,3,4} In CNL, trisomy 8 and del(20q) are the most common nonspecific chromosomal abnormalities observed at diagnosis or at the time of progressive disease.

Differential diagnosis

Exclusionary criteria include no evidence of a reactive neutrophilia (inflammatory, infectious, or malignant disease) or other MPNs, such as primary myelofibrosis (PMF), polycythemia vera (PV), essential thrombocythemia (ET), myelodysplastic syndrome (MDS), chronic myelomonocytic leukemia (CMML), and atypical CML (aCML).⁵

PV diagnosis requires hemoglobin levels > 18.5 g/dL in men and > 16.5 g/dL in women or the presence of *JAK2* V617F or *JAK2* exon 12 mutation. In the case of ET, a platelet count $> 450 \times 10^9/L$ is necessary, and we observe megakaryocyte proliferation with large and mature morphology and demonstration of *JAK2* V617F or other clonal marker or no evidence of reactive thrombocytosis. In the case of PMF, megakaryocyte proliferation and atypia are observed, accompanied by either reticulin and/or collagen fibrosis; or demonstration of *JAK2* V617F or other clonal marker or no evidence of reactive BM fibrosis. In CMML, we observe a persistent (> 3 months) PB monocytosis ($> 1 \times 10^9/L$), no *BCR-ABL1*, no *PDGFRA/B* mutations, $< 20\%$ blast or promonocytes in BM or PB, and dysplasia or clonal cytogenetic or molecular abnormality. Finally, for aCML, PB leukocytosis $> 13 \times 10^9/L$, increased neutrophils/precursors with dysgranulopoiesis, $> 10\%$ immature granulocytes, $< 20\%$ PB myeloblasts, no *BCR-ABL1*, no *PDGFRA/B* mutations, $< 2\%$ PB basophilia, no monocytosis and $< 10\%$ PB monocytes; and BM hypercellular with increased granulocyte proliferation and granulocytic dysplasia in erythroid or megakaryocytic lineages; $< 20\%$ myeloblasts are observed.⁵

In particular, in cases of plasma cell dyscrasia, it is important to prove the neutrophilic clonality by cytogenetic and/or molecular tests. The current diagnostic criteria for CNL (WHO 2008) are summarized in Table 1.

Treatment and stem-cell transplantation (SCT)

No standard of care exists for CNL. Therapy has primarily consisted of hydroxyurea or other oral chemotherapeutics, as well as interferon-alpha.^{1,3,4,11-15} These agents can elicit an improvement in blood counts, but exhibit no proven disease-modifying benefit. Although splenic irradiation and splenectomy may provide transient palliation of symptomatic splenomegaly, the latter has been associated with the worsening of neutrophilic leukocytosis in CNL. The limited experience with induction-type chemotherapy for blastic transformation is generally poor, with death related to resistant disease or regimen-related toxicities.

Table 1 2008 WHO diagnostic criteria for CNL

- Leukocytosis (WBC $\geq 25 \times 10^9/L$)
 - Segmented neutrophils/band $> 80\%$
 - Immature granulocytes $< 10\%$
 - Myeloblasts $< 1\%$
- Hypercellular BM
 - Neutrophilic granulocytes increased in number and percentage
 - Myeloblasts $< 5\%$
 - Neutrophilic maturation pattern normal
 - Megakaryocytes normal or left shifted
- Hepatosplenomegaly
- No cause for neutrophilia or, if so, clonality
 - No infectious/inflammatory process
 - No underlying tumor
- No *BCR-ABL1*
- No *PDGFRA*, *PDGFRB*, or *FGFR1* rearrangements
- No evidence of PV, ET, or PMF
- No evidence of MDS or MDS/MPN
 - No granulocytic dysplasia
 - No myelodysplastic changes
 - Monocytes $< 1 \times 10^9/L$

Notes: Diagnosis requires leukocytosis ($\geq 25 \times 10^9/L$), $> 80\%$ neutrophils, $< 10\%$ immature granulocytes, and 1% myeloblasts. No dysplasia, monocytosis, *BCR-ABL1*, *PDGFRA*, *PDGFRB*, or *FGFR1* rearrangements, and no underlying process that can cause neutrophilia.

Abbreviations: WHO, World Health Organization; CNL, chronic neutrophilic leukemia; WBC, white blood cells; BM, bone marrow; PV, polycythemia vera; ET, essential thrombocythemia; PMF, primary myelofibrosis; MDS, myelodysplastic syndromes; MPN, myeloproliferative neoplasms.

As CNL frequently progresses to blast crises and to be refractory to therapy, allogeneic hematopoietic SCT represents the only possibility to cure these patients. Revisiting SCT in CNL patients, it is observed that the 71% of the patients who received the transplant at the chronic phase have an ongoing remission of more than 7 months, in contrast with those who received it at the accelerated phase and died after the procedure.^{3,16–18} To summarize, SCT may result in favorable long-term outcomes in selected patients, particularly when undertaken in the chronic phase of disease.^{1,3,4,11,13}

Genetic alterations in CNL

As stated above, due to the lack of either specific or prognostic molecular markers, the diagnosis of CNL has been considered of exclusion. However, in 2013, a disease-defining mutation in *CSF3R* and a potentially prognostic mutation in *set binding protein 1* (*SETBP1*) were found.^{2,19,20} Since then, the scientific community has considerably progressed in the molecular pathogenesis of CNL and an additional genetic alteration has been reported.²¹ In the following sections, we will revisit the main molecular alterations in CNL.

CSF3R mutations

Mutations in *CSF3R* have been recently defined as the common genetic event in patients with CNL by Maxson et al,¹⁹

becoming a potentially useful biomarker for diagnosing and therapy target.^{2,22} *CSF3R* encodes the transmembrane receptor for the granulocyte colony-stimulating factor (G-CSF; CSF3), which provides the proliferative and survival signal for granulocytes and also contributes to their differentiation and function.^{23–25}

These mutations were present in approximately 83% of patients with WHO-defined/no MG-associated CNL (Figure 1) and fall into two classes: nonsense or frameshift mutations (D771fs, S783fs, and Y752X) that lead to the premature truncation of the cytoplasmic tail of the receptor (same as the secondary *CSF3R* mutation in severe congenital neutropenia [SCN]); and point mutations in the extracellular domain of *CSF3R* (T615A and T618I). The most common *CSF3R* alteration in CNL is the membrane proximal mutation T618I. *CSF3R* is known to signal downstream through both *Janus kinase* (JAK) and SRC tyrosine kinase pathways, and the two classes of *CSF3R* mutations exhibit different downstream signaling and kinase inhibitor sensitivities. *CSF3R* truncation mutations operate predominantly through SRC kinases, and exhibit drug sensitivity to SRC kinase inhibitors, such as dasatinib. In contrast, *CSF3R* membrane proximal mutations strongly activate the JAK/STAT pathways and are sensitive to JAK kinase inhibitors, such as ruxolitinib in vitro (Figure 1).

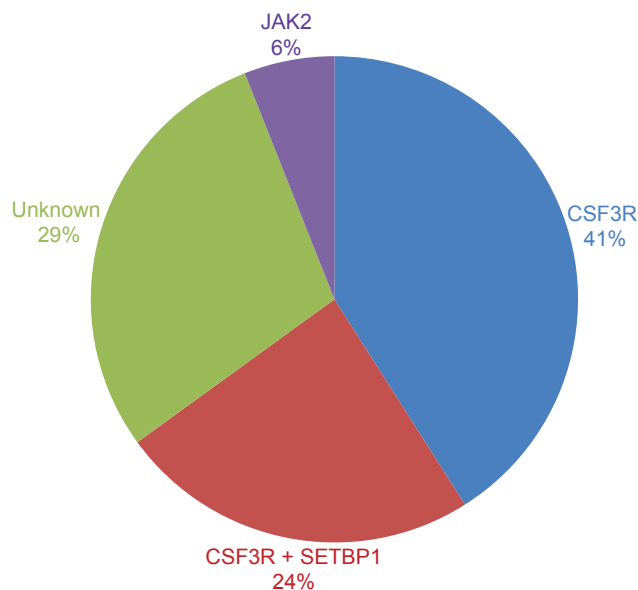


Figure 1 CNL mutations frequencies.

Notes: Percentages of *CSF3R*, *SETBP1*, and *JAK2* V617F mutations in 18 CNL WHO-defined patients are shown. Six out of 18 were MGUS/lymphoma-associated, and none had *CSF3R* or *SETBP1* mutations and only one presented *JAK2* V617F.

Abbreviations: CNL, chronic neutrophilic leukemia; WHO, World Health Organization; *SETBP1*, set binding protein 1; *JAK2*, Janus kinase 2.

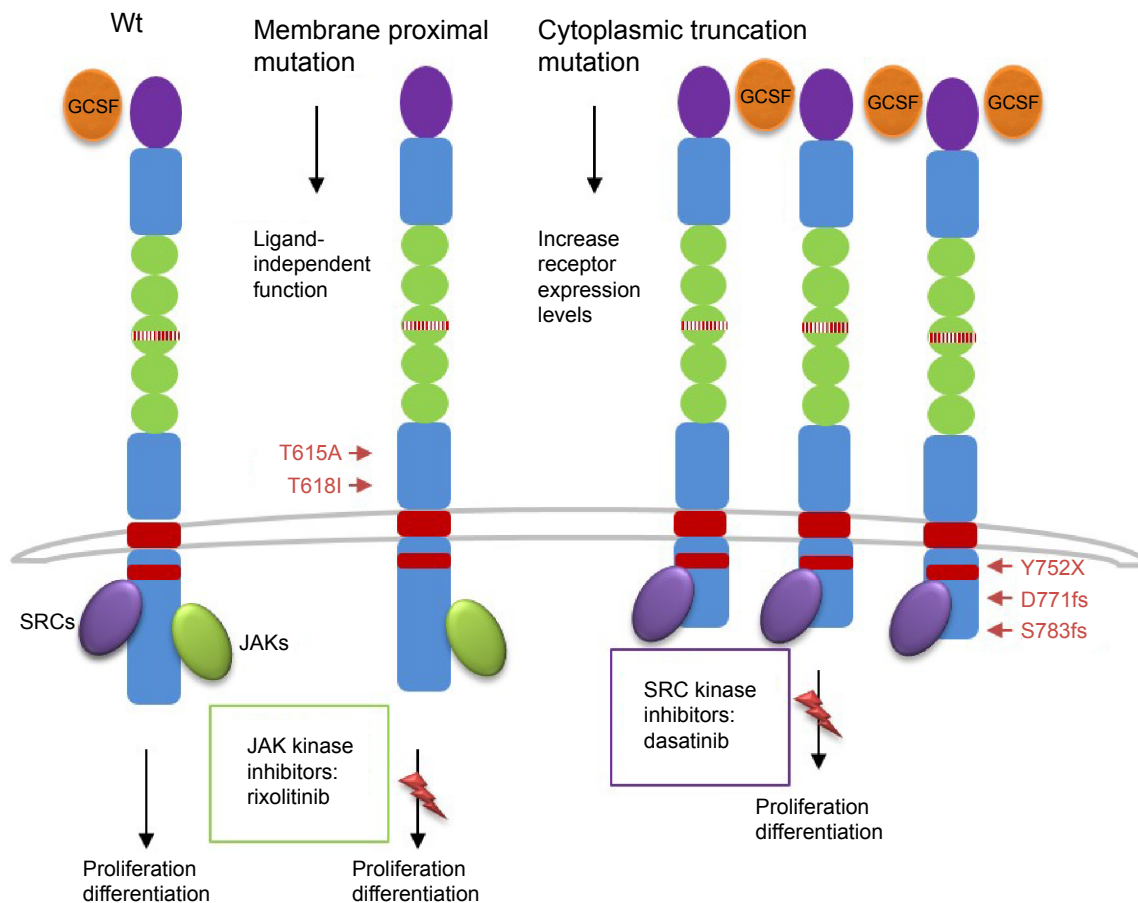


Figure 2 Schematic representation of *CSF3R* mutations and pathways activation.

Notes: *CSF3R* is known to signal downstream through both JAK and SRC tyrosine kinase pathways, and the two classes of *CSF3R* mutations exhibit different downstream signaling and kinase inhibitor sensitivities. *CSF3R* truncation mutations operate predominantly through SRC kinases and exhibit drug sensitivity to SRC kinase inhibitors, such as dasatinib. In contrast, *CSF3R* membrane proximal mutations strongly activate the JAK/signal transducer and activator of transcription pathways, and are sensitive to JAK kinase inhibitors, such as ruxolitinib.

Abbreviation: JAK, Janus kinase.

CSF3R mutations have been first described in patients with SCN, which can evolve into AML if a secondary *CSF3R* mutation develops at the time of transformation.^{26–29} These new nonsense or frameshift mutations truncate the cytoplasmic tail of *CSF3R*, impair its internalization, and alter its interactions with proteins such as SOCS family members.^{30–32} These structural and functional alterations are thought to perturb the ability of *CSF3R* to regulate granulocyte differentiation and to increase granulocytic proliferative capacity.^{33–35}

SETBP1 mutations

SETBP1 interacts with SET, a negative regulator of the tumor suppressor protein phosphatase 2A (PP2A).³⁶ *SETBP1* protects SET from protease cleavage, thus increasing the amount of SET available to repress the activity of PP2A.³⁷ In AML, *SETBP1* overexpression is significantly associated with reduced survival, indicating that *SETBP1* may be relevant

to leukemia oncogenesis.³⁷ *SETBP1* mutations were recently identified in 25% of aCML patients, and in lower frequencies in unclassified MDS/MPN (10%),²⁰ CMML (14.5%), and AML (<1%),³⁸ but no mutation was identified in lymphoid leukemia or solid tumors.²⁰ *SETBP1* mutations in aCML were associated with a higher white blood cells (WBC) count at diagnosis and poorer survival.²⁰ The prevalence of *SETBP1* mutations in CNL coexpressing *CSF3R* T618I is 24% (Figure 2).² Although these cases showed a trend to reduce survival, the analysis is limited by the small number of cases. Further follow-up studies are necessary to confirm these findings so that *SETBP1* could be used as a prognostic marker to guide therapeutic decisions.

JAK2-V617F

Mutations in the *JAK2* gene were first reported in 2005, and *JAK2* V617F became the most frequent mutation in patients with *BCR-ABL1*-negative MPN, such as PV, ET,

and PMF.^{39–42} This mutation changes a valine to a phenylalanine at position 617 and is specific to patients with myeloid neoplasm.⁴³ The presence of this alteration is extremely important to establish the clonality and to make a differential diagnosis with reactive myeloproliferation. As observed in other myeloid neoplasms, the value of *JAK2* V617F mutation founded in the 13 CNL cases was to corroborate clonality. Until now, *JAK2* V617F and *CSF3R* T618I seems to be mutually exclusive (Figure 1).² Further molecular studies of well-defined CNL, such as the one performed by Makishima et al³⁸ will elucidate the role of *JAK2* V617F mutation in the pathogenesis of CNL or will determine a new subgroup of MPN that does not coexpress *CSF3R* T618I mutation.

Additional alterations

In 2013, somatic mutations in calreticulin (*CALR* gene) were observed in *JAK2*- and *MPL*-negative patients (ET ~50% and PMF ~75%), making *CALR* mutations the second most common in MPN.^{44,45} Reduced frequencies were found in MDS, CMML, and aCML.^{46,47} Regarding CNL, only one case was reported with a novel *CALR* point mutation, different from

the ones found in ET and PMF, and the biological significance is unknown.⁴⁸

By using exome and RNA sequencing, Menezes et al²¹ demonstrated that the CNL genome has a combination of alterations – in addition to *CSF3R* T618I – that affects epigenetics (*ASXL1* and *TET2*), spliceosome genes (*LUC7L2* and *U2AF1*), and protein kinase (*PIM3-SCO2* fusion gene). Epigenetic modifiers provide new targets for therapeutic intervention and targeting these enzymatic activities are currently being explored from a therapeutic standpoint in several types of leukemia.^{49,50} Interestingly, the inhibition of PIM kinases by PIM kinase inhibitors in Myc-induced lymphoma resulted in cell death.⁵¹ In this complex scenario, a combination of new targeted therapies may be considered as reasonable options for the therapeutic management of this aggressive and rare subtype of leukemia.

Clinical perspectives

The recent discovery of *CSF3R* mutations and their almost invariable association with the WHO-defined CNL presents the opportunity to make significant changes in the

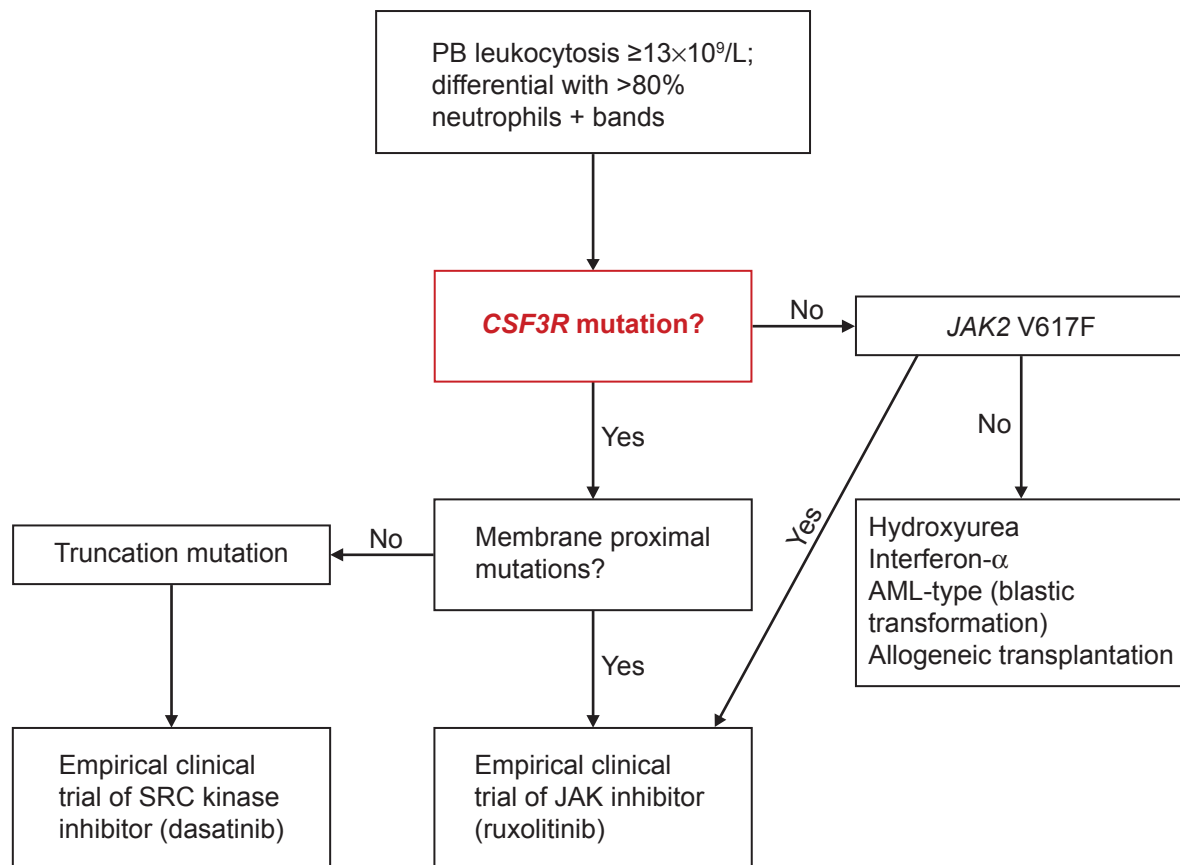


Figure 3 Algorithm for CNL diagnosis and treatment.

Notes: The presence of a membrane proximal *CSF3R* mutation in a patient with predominantly neutrophilic granulocytosis should be sufficient for the diagnosis of CNL.

Abbreviations: CNL, chronic neutrophilic leukemia; PB, peripheral blood; *JAK2*, Janus kinase 2; AML, acute myeloid leukemia.

diagnostic approach of CNL. The 2008 WHO diagnosis criteria are primarily driven by the absence of a clonal marker. Although, they require a relatively high leukocyte threshold and a list of exclusion criteria, it is equally possible to misdiagnose some cases of CNL as aCML or CMML (Table 1).

Very recently, Tefferi et al⁵² proposed a classification system for ET/PMF and CNL, based on the new genetic findings to be incorporated in the new WHO classification criteria for MPN. Such availability of a clonal marker for the majority of patients with CNL should allow lowering of the leukocyte level to $13 \times 10^9/L$, consistent with that is currently being used for the diagnosis of WHO-defined aCML (Figure 3).⁵ In addition, the authors proposed separate sets of major and minor criteria to accommodate the diagnostic possibility in both *CSF3R*-mutated and unmutated CNL. In this algorithm, diagnosis requires the presence of all three major criteria (leukocytosis $WBC \geq 13 \times 10^9/L$; segmented neutrophils/band $>80\%$ and *CSF3R* mutations) or, in the absence of *CSF3R* mutations, all minor criteria (hypercellular BM; immature granulocytes $<10\%$ in PB; no cause for neutrophilia or, if so, demonstration of clonality; no BCR-ABL1 rearrangements; and no meeting WHO diagnostic criteria for other myeloid neoplasm).

Novel therapeutic approaches

Revisiting the literature, only three CNL patients, all carrying *CSF3R* T618I mutation, received ruxolitinib therapy.^{19,53,54} Interestingly, only the case who coexpressed *SETBP1* mutation did not respond to this JAK inhibitor therapy. Until now, there is no evidence that the coexpression of *SETBP1* is responsible for treatment failure. Defining a safe profile and clinical benefits of these treatments is extremely important to establish a prospective clinical trial of ruxolitinib and other JAK inhibitors in CNL and aCML patients. In detail, the trial will address the frequency, durability, depth, and genetic modifiers of clinical responses, such as coexisting *SETBP1*² or *TET2*²¹ mutations. A prospective, multicenter phase II clinical trial investigating the safety and efficacy of ruxolitinib in this patient population is registered at ClinicalTrials.gov (NCT02092324) and is open for participant recruitment.

Conclusion

The discovery of the high-frequency *CSF3R* T618I mutation in CNL identifies a new disease-defining marker, suggesting that the molecular characterization of this gene should be included in the diagnostic criteria for this disease. Given the poor prognosis of this disorder, the potential applicability of

JAK or SRC kinase inhibitors is another important implication of the discovery of activating *CSF3R* mutation.

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Author contributions

All authors contributed toward drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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