

CNL and aCML should be considered as a single entity based on molecular profiles and outcomes

Gonzalo Carreño-Tarragona,¹ Alberto Álvarez-Larrán,² Claire Harrison,³ José Carlos Martínez-Ávila,⁴ Juan Carlos Hernández-Boluda,⁵ Francisca Ferrer-Marín,⁶ Deepti H. Radia,³ Elvira Mora,⁷ Sebastian Francis,⁸ Teresa González-Martínez,⁹ Kathryn Goddard,¹⁰ Manuel Pérez-Encinas,¹¹ Srinivasan Narayanan,¹² José María Raya,¹³ Vikram Singh,¹⁴ Xabier Gutiérrez,¹ Peter Toth,¹⁵ Paula Amat-Martínez,⁵ Louisa McIlwaine,¹⁶ Magda Alobaidi,¹⁷ Karan Mayani,¹⁸ Andrew McGregor,¹⁹ Ruth Stuckey,²⁰ Bethan Psaila,^{21,22} Adrián Segura,²⁰ Caroline Alvares,²³ Kerri Davidson,²⁴ Santiago Osorio,²⁵ Robert Cutting,²⁶ Caroline P. Sweeney,²⁷ Laura Rufián,¹ Laura Moreno,¹ Isabel Cuenca,¹ Jeffery Smith,¹⁴ María Luz Morales,¹ Rodrigo Gil-Manso,¹ Ioannis Koutsavlis,²⁸ Lihui Wang,²⁹ Adam J. Mead,³⁰ María Rozman,³¹ Joaquín Martínez-López,¹ Rosa Ayala,^{1,*} and Nicholas C. P. Cross^{32,33,*}

¹Hematology Department, Hospital Universitario 12 de Octubre, I+12, Centro Nacional de Investigaciones Oncológicas, Complutense University, Centro de Investigación Biomédica en Red de Oncología, Madrid, Spain; ²Hematology Department, Hospital Clínic, Barcelona, Spain; ³Hematology Department, Guy's and St. Thomas NHS Foundation Trust, London, United Kingdom; ⁴Agricultural Economics, Statistics and Business Management Department, Escuela Técnica Superior de Ingeniería Agrónoma, Alimentaria y Biosistemas, Universidad Politécnica de Madrid, Madrid, Spain; ⁵Hematology Department, Hospital Clínic, Valencia, Spain; ⁶Hematology Department, Hospital Morales Meseguer, Centro de Investigación Biomédica en Red de Enfermedades Raras, Universidad Católica San Antonio de Murcia, Murcia, Spain; ⁷Hematology Department, Hospital Universitario La Fe, Valencia, Spain; ⁸Hematology Department, Sheffield Hospital, Sheffield, United Kingdom; ⁹Hematology Department, Hospital Universitario de Salamanca, Salamanca, Spain; ¹⁰Hematology Department, Rotherham Hospital, Rotherham, United Kingdom; ¹¹Hematology Department, Hospital Clínico Universitario, Santiago de Compostela, Spain; ¹²Hematology Department, University Hospital Southampton, Southampton, United Kingdom; ¹³Hematology Department, Hospital Universitario de Canarias, Tenerife, Spain; ¹⁴The Clatterbridge Cancer Centre, Liverpool, United Kingdom; ¹⁵Hematology Department, Sheffield Teaching Hospitals NHS Foundation Trust, Sheffield, United Kingdom; ¹⁶Hematology Department, Glasgow Royal Infirmary, Glasgow, United Kingdom; ¹⁷Department of Haematology, Chelsea and Westminster NHS Trust West Middlesex Hospital, London, United Kingdom; ¹⁸Hematology Department, Hospital General de La Palma, Santa Cruz de Tenerife, Spain; ¹⁹Department of Haematology, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle, United Kingdom; ²⁰Hematology Department, Hospital Universitario de Gran Canaria Dr. Negrín, Las Palmas de Gran Canaria, Spain; ²¹MRC Molecular Haematology Unit, MRC Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom; ²²Department of Haematology, Oxford University Hospitals NHS Trust, Oxford, United Kingdom; ²³Hematology Department, University Hospital of Wales, Cardiff, United Kingdom; ²⁴Hematology Department, Kirkcaldy Hospital, Fife, Scotland; ²⁵Hematology Department, Hospital Universitario Gregorio Marañón, Madrid, Spain; ²⁶Hematology Department, Doncaster Hospital, Doncaster, Yorkshire, England; ²⁷Hematology Department, Vale of Leven Hospital, Alexandria, West Dunbartonshire, Scotland; ²⁸Hematology Department, Western General Hospital, Edinburgh, United Kingdom; ²⁹Haemato-Oncology Diagnostic Service, Liverpool Clinical Laboratories, Liverpool University Hospital, Liverpool, United Kingdom; ³⁰Medical Research Council (MRC) Molecular Haematology Unit, MRC Weatherall Institute of Molecular Medicine, NIHR Biomedical Research Centre, University of Oxford, Oxford, United Kingdom; ³¹Hemopathology Unit, Hospital Clínic, Barcelona, Spain; ³²Wessex Regional Genetics Laboratory, Salisbury, United Kingdom; and ³³Faculty of Medicine, University of Southampton, Southampton, United Kingdom

Key Points

- There is a significant overlap between the clinical characteristics and molecular profiles of CNL and aCML.
- CNL and aCML can be classified as a single entity.

Chronic neutrophilic leukemia (CNL) and atypical chronic myeloid leukemia (aCML) are rare myeloid disorders that are challenging with regard to diagnosis and clinical management. To study the similarities and differences between these disorders, we undertook a multicenter international study of one of the largest case series (CNL, $n = 24$; aCML, $n = 37$ cases, respectively), focusing on the clinical and mutational profiles ($n = 53$ with molecular data) of these diseases. We found no differences in clinical presentations or outcomes of both entities. As previously described, both CNL and aCML share a complex mutational profile with mutations in genes involved in epigenetic regulation, splicing, and signaling pathways. Apart from *CSF3R*, only *EZH2* and *TET2* were differentially mutated between them. The molecular profiles support the notion of CNL and aCML being a

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*R.A. and N.C.P.C. are joint senior authors.

Sequencing data of cases centrally sequenced that have been reported in this article have been deposited in the in the NCBI Sequence Read Archive (www.ncbi.nlm.nih.gov/sra; accession number: PRJNA895737).

For those cases ($n = 16$) for whom sequencing information was extracted locally from clinical records, the sequencing methodology and panel of genes tested is detailed in the supplemental Material.

Data are available on request from the corresponding author, Gonzalo Carreño-Tarragona (gonzalo.carreno@salud.madrid.org).

The full-text version of this article contains a data supplement.

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continuum of the same disease that may fit best within the myelodysplastic/myeloproliferative neoplasms. We identified 4 high-risk mutated genes, specifically *CEBPA* ($\beta = 2.26$, hazard ratio [HR] = 9.54, $P = .003$), *EZH2* ($\beta = 1.12$, HR = 3.062, $P = .009$), *NRAS* ($\beta = 1.29$, HR = 3.63, $P = .048$), and *U2AF1* ($\beta = 1.75$, HR = 5.74, $P = .013$) using multivariate analysis. Our findings underscore the relevance of molecular-risk classification in CNL/aCML as well as the importance of *CSF3R* mutations in these diseases.

Introduction

Chronic neutrophilic leukemia (CNL) and atypical chronic myeloid leukemia (aCML) are rare *BCR::ABL1*-negative neutrophilic myeloid neoplasms characterized by leukocytosis, splenomegaly, and dismal outcomes.¹⁻³ Despite belonging to different disease categories according to the 2016 World Health Organization (WHO) classification (CNL as a myeloproliferative neoplasm [MPN] and aCML as a myelodysplastic MPN [MDS/MPN]),¹ the differential diagnosis between these entities remains a challenge because of their shared features, with the difference between them based mainly on morphology, with dysplasia as the main characteristic to distinguish aCML from CNL.^{1,4-6} The clinical relevance of this distinction, however, remains unclear.

The discovery of colony-stimulating factor 3 receptor (also known as granulocyte colony-stimulating factor receptor and encoded by *CSF3R*) mutations in up to 89% of patients with CNL was a huge step forward in the understanding of the pathogenesis of this disorder. It provided a new biomarker and diagnostic criteria as well as a therapeutic target.⁷⁻¹² However, *CSF3R* mutations are not exclusively seen in CNL, and up to 40% of aCML cases present these variants.^{5,7,13} Furthermore, both diseases share recurrent mutated genes, particularly those implicated in epigenetic regulation and splicing but also in components of signaling pathways.^{4,6,9} *SETBP1* and *ASXL1* mutations seem to have a prognostic impact and are present in 30% and 41% of aCML and CNL cases, respectively.^{2,6,9,14-16} Mutations in *SRSF2* and *U2AF1*, encoding splicing factors, are seen in 20% and 40% of CNL and aCML cases, respectively, and *U2AF1* mutations have been linked with *CSF3R*-mutated neoplasms.^{3,6,15,16} The co-occurrence of mutations in genes encoding signaling and splicing factors is a hallmark of MDS/MPN and it has been suggested that CNL might be more appropriately classified as a subtype of MDS/MPN rather than MPN.^{4,17}

Despite these advances, CNL and aCML are very infrequent diseases and large series of cases are not available. This makes it difficult to define the clinical characteristics of these disorders as well as prognostic determinants and appropriate treatment. Patients with CNL have a median overall survival (OS) of 24 months, with disease progression and hemorrhagic complications being the main causes of death.^{2,18,19} A similarly poor outcome is seen for aCML (OS of 21.4 months) with a clinical presentation of leukocytosis and splenomegaly that can progress to acute myeloid leukemia (AML) in up to 40% of the cases.^{3,5,6} Despite this poor prognosis, neither of these disorders has a standard of care treatment apart from allogeneic hematopoietic cell transplant in young patients.^{2,13,20} Hydroxyurea, hypomethylating agents, interferon, and, lately, ruxolitinib, which inhibits the action of *CSF3R* by

blocking JAK2, are currently considered as therapeutic options.^{2,12,13,20,21}

To shed light on the differences and similarities between these 2 entities with regard to their molecular pathogenesis and to identify prognostic factors, we undertook an international multicenter study. Clinical and genomic information was obtained for more than 60 cases with either CNL ($n = 24$) or aCML ($n = 37$). Our study provides new information that helps to classify, diagnose, and predict outcomes for these patients.

Methods

Patients and samples

We performed a multicenter retrospective international study with the participation of 17 hospitals from the United Kingdom and 11 hospitals from Spain. Initially, a total of 69 cases were identified from clinical and laboratory records that were considered to have been diagnosed with CNL or aCML between 1986 and 2020, according to established criteria¹. Of these, 6 were excluded after the initial review because of a pre-existing myeloid neoplasm or a likely incorrect diagnosis. After molecular analysis, 21 cases, including all of those found to have pathogenic *JAK2*, *SF3B1*, and *KIT* mutations, were reviewed centrally via the microscopic evaluation of bone marrow trephines ($n = 13$) or re-evaluation of the pathology reports and case histories ($n = 8$) by an expert hematopathologist. This resulted in a further exclusion of 2 cases who were initially considered to have aCML but subsequently considered to have primary myelofibrosis (PMF) or chronic myelomonocytic leukemia (CMML). The final study group thus consisted of 61 cases. The study was approved by the ethics committee of Hospital Universitario 12 de Octubre (No 19/163) and conducted in accordance with the Declaration of Helsinki.

Sequencing

Next-generation sequencing (NGS) data using local diagnostic panels (which included *CSF3R* and a range of genes commonly mutated in myeloid malignancies) were available from 16 patients at the time of inclusion. For those patients in whom NGS sequencing had not been performed locally and DNA was available ($n = 37$), we performed targeted NGS (Ion Torrent Proton System—Life Technologies, Carlsbad, CA) of 43 genes implicated in myeloid malignancies (*ASXL1*, *BCOR*, *BCORL1*, *CALR*, *CBL*, *CEBPA*, *CSF3R*, *DNMT3A*, *EGLN1*, *EPAS1*, *EPOR*, *ETV6*, *EZH2*, *FLT3*, *IDH1*, *IDH2*, *JAK2*, *KDM6A*, *KIT*, *KMT2A*, *KRAS*, *MPL*, *NF1*, *NPM1*, *NRAS*, *PHF6*, *PRPF40B*, *RAD21*, *RUNX1*, *SETBP1*, *SF3A1*, *SF3B1*, *SH2B3*, *SMC1A*, *SRSF2*, *STAG2*, *TET2*, *THPO*, *TP53*, *U2AF1*, *VHL*, *WT1*, *ZRSR2*), as described.²² Sequencing data of cases centrally sequenced can be found in the NCBI Sequence Read Archive. For those cases ($n = 16$) for whom

sequencing information was extracted locally from clinical records, the sequencing methodology and panel of genes tested are detailed in the supplemental Material. Please contact the corresponding author for other forms of data sharing. Pathogenic and likely pathogenic variants, variant allele frequencies (VAFs), and gene classification into functional groups are summarized in the supplemental Material (supplemental Table 1). In total, we had NGS data of 53 patients (CNL, $n = 23$; and aCML, $n = 30$), not including the 2 cases who were excluded after a central morphological review.

Statistical analysis

A descriptive statistics of continuous variables was performed in which mean and standard deviation were used, except for time-to-event variables, in which median was used. Categorical variables were presented in absolute and relative frequencies and odds ratios. Comparison between quantitative variables was performed with Student t test or analysis of variance and between categorical variables with χ^2 or Fisher exact test. Time-to-event analysis, for example, OS and progression, was performed using Kaplan-Meier univariate analysis and a Cox proportional hazards model corrected for possible confounders in multivariate analysis. Continuous variables were analyzed using a linear model corrected for other variables as indicated. False discovery rate was used when multiple comparisons were made. $P < .05$ was considered statistically significant. Principal component analysis (PCA) was used in order to assess how close the diseases (CNL, aCML, polycythemia vera, essential thrombocythemia, PMF, and CMML) cluster to each other based on mutational profiles. Statistical analyses were performed using R under RStudio using ROCR, survminer, and ggplot2 packages.

Results

Clinical presentation and complications do not differ between aCML and CNL

Patient's characteristics are summarized in Table 1. Of the 61 cases, the median age at presentation was 69.4 years, and 65.6% of them were male. Splenomegaly was present in 55.7% of the patients, and the mean white blood cell (WBC) count was $62.2 \times 10^9/L$. Transformation to AML occurred in 29.5% of the patients ($n = 18$). No differences were found between both entities with the exception of monocyte counts, which were higher in aCML compared with CNL ($P = .018$). As expected, dysplasia was absent in all CNL cases. Interestingly, bleeding as a cause of death was similar between both diseases (15.4% in CNL vs 10.5% in aCML, $P = .737$ for all causes of death). We found no differences in the survival between entities (median OS of 17.7 months vs 15.2 months for aCML and CNL, respectively, supplemental Figure 1). Hydroxyurea was selected as first-line treatment in 79.6% of the patients. Second-line treatment ($n = 29$) was heterogeneous: 13.8% received hydroxyurea and 20.7% received interferon- α , whereas 27.6% received ruxolitinib (50% of patients with *CSF3R* mutations) on a compassionate basis because it has shown efficacy in some CNL and aCML cases, particularly those with *CSF3R* mutations.¹² The remaining 37.9% of the cases received other treatments, mainly hypomethylating agents. No differences were found in the first-line setting for CNL and aCML although there was a clear tendency toward the use of hypomethylating agents in aCML (22.2% vs 0% $P = .066$). In addition, patients with CNL

were more frequently treated with ruxolitinib as second-line treatment (46.6% vs 7.3% $P = .038$). Only 17% of the patients underwent allogeneic hematopoietic stem cell transplant, which was associated with improved survival (20.3 vs 79.3 months, $P = .018$, supplemental Figure 2).

Mutational profile of aCML and CNL

Of the 61 aCML/CNL cases, 53 had an NGS myeloid panel performed (Table 2; Figure 1). The mean number of mutated genes per patient was 3.47 (1-10), with 88.7% of the patients showing mutations in epigenetic pathways, 71.7% in signaling pathways, and 56.6% in splicing genes (Table 1). Most patients had mutations in multiple pathways, with 32.0% of them having mutations in 2 of these pathways and 41.5% in 3 of them. In addition, 22.6% of the patients had mutations in transcription factors, but very few patients, 1.9%, had mutations in tumor suppressor genes. Apart from *CSF3R* (49.1%), the most common mutated genes were *SETBP1* (43.3%), *SRSF2* and *ASXL1* (both 41.5%), *EZH2* (34.0%), and *TET2* (28.3%). No differences were found between CNL and aCML in the pathways affected, but at the individual gene level, *CSF3R* was more commonly mutated in CNL cases (87% vs 20% $P < .001$), whereas *EZH2* and *TET2* were more commonly mutated in aCML (50% vs 13% $P = .012$ and 43.3% vs 8.7%, $P = .014$). As for cytogenetic abnormalities, an altered karyotype was presented in 19.4% of aCML cases vs 4.8% of CNL cases ($P = .130$) (Table 1; supplemental Table 2).

As some authors have suggested that aCML is a *JAK2* V617F-negative neoplasm²³ (despite some series describing up to 26% of *JAK2*-mutated aCML cases⁴), bone marrow biopsies and aspirates were reviewed for all 4 *JAK2*-mutated cases. Three showed a morphology compatible with aCML with hypercellularity (>90%) and dysplasia; the single *JAK2*-positive CNL case showed no dysplasia. This case (number 27 in supplemental Table 1) presented with a complex mutational profile with variants in *CSF3R*, *NRAS*, *TET2*, *EZH2*, *JAK2* plus a double mutation in *CEBPA* (biallelic *CEBPA*-mutated AML have been associated with *CSF3R* mutations)²⁴ and died from bleeding after extreme progression of leukocytosis. The other 2 *CEBPA*-mutated cases presented with monoallelic mutations. A central review of the 4 cases with *SF3B1* or *KIT* mutations excluded alternative diagnoses and specifically refractory anemia with ring sideroblasts associated with thrombocytosis and mastocytosis, respectively.

PCA of molecular and genetic profiles of CNL and aCML

To assess how these molecular and genetic characteristics cluster CNL and aCML in relation to other MPN and CMML, the most common MDS/MPN subtype, we performed in silico PCA (supplemental Figures 3 and 4; supplemental Tables 3 and 4) using our data in combination with previously published data.²⁵⁻²⁷ This analysis showed that CNL and aCML cluster close to each other between classic MPN and CMML, largely because of abundant mutations in signaling, splicing factors, and epigenetic regulator genes and a lack of mutations in tumor suppressor genes.

Genotype-phenotype associations

We evaluated associations between clinical presentation features and mutations in different genes (supplemental Figures 5-10;

Table 1. Characteristics of 61 patients with aCML or CNL

	Whole series (61)	aCML (37)	CNL (24)	P
Gender = % male (n)	65.6% (40)	67.6% (25)	62.5% (15)	.765
Age (mean, range)	69.4 (32.9-92.5)	68.6 (40.6-91.4)	71.2 (33.0-92.5)	.692
Median OS (mo)	17.2	17.7	15.2	.86
Transformation to AML	29.5% (18)	32.4% (12)	25% (6)	.534
Median time to transformation (mo)	14.3	13.6	15.2	.52
HSCT	17% (9)	25.8% (8)	4.5% (1)	.097
Cause of death				.737
Progression	65.6% (21)	63.2% (12)	69.2% (9)	
Bleeding	12.5% (4)	10.5% (2)	15.4% (2)	
Other	21.9% (7)	26.3% (5)	15.4% (2)	
Splenomegaly	55.7% (34)	48.6% (18)	66.7% (16)	.263
Hepatomegaly	21.7% (10)	14.7% (5)	41.7% (5)	.124
Cutaneous bleeding	10.6% (5)	8.6% (3)	16.7% (2)	.808
Mucosal bleeding	4.3% (2)	2.9% (1)	8.3% (1)	1.000
Hemoglobin (g/L)	83.32	85.73	79.81	.669
Platelets ($\times 10^9/L$)	303.65	332.83	259.88	.387
WBCs ($\times 10^9/L$)	62.23	68.15	52.70	.284
Neutrophils ($\times 10^9/L$)	53.46	57.23	47.89	.486
Lymphocytes ($\times 10^9/L$)	3.70	4.16	2.96	.069
Monocytes ($\times 10^9/L$)	1.88	2.43	1.02	.018
Abnormal cytogenetics	13.5% (7)	19.4% (6)	4.8% (1)	.130
Mutations detected by NGS	100% (53)	100% (30)	100% (23)	NA
Number of mutated genes (mean)	3.47	3.77	3.09	.118
Mutated <i>CSF3R</i>	59.1% (26)	27.3% (6)	90.9% (20)	<.001
Mutated <i>SETBP1</i>	52.2% (24)	56% (14)	47.6% (10)	.787
Mutated signaling genes	71.7% (38)	63.3% (19)	82.6% (19)	.216
Mutated transcription factor genes	22.6% (12)	30% (9)	13.0% (3)	.258
Mutated tumor suppressor genes	1.9% (1)	3.3% (1)	0% (0)	1.000
Mutated epigenetic modifier genes	88.7% (47)	93.3% (28)	82.6% (19)	.433
Mutated splicing factor genes	56.6% (30)	53.3% (16)	60.9% (14)	.788
Mutated Cohesins and other genes	17% (9)	16.7% (5)	17.4% (4)	1.000

P for differences between aCML and CNL. Statistically significant differences are in bold. HSCT, hematopoietic stem cell transplant.

supplemental Tables 5-10). Initial analysis indicated that higher WBC was associated with mutations in *CSF3R* ($P = .035$), *JAK2* ($P < .001$), *TET2* ($P = .013$), and *RAD21* ($P = .008$). After correction for multiple comparisons, this association was maintained for *JAK2* mutations ($P = .011$). Other statistically significant associations after adjustment for multiple comparisons were *JAK2* ($P = .02$) and *TET2* mutations ($P = .023$) for higher neutrophil counts, *RAD21* mutations ($P = .016$) for higher monocytes, and *FLT3* mutations ($P < .001$) for higher lymphocytes. Finally, higher platelets were seen in patients with *ETV6* mutations ($P = .034$). No associations were noted with hemoglobin levels or splenomegaly or between any clinical feature and *MPL* mutations.

Co-occurrence of mutations and exclusion analysis

In order to understand the biology of these diseases, we analyzed the co-occurrence and exclusivity of mutations (Figure 2; supplemental Tables 11 and 12). A number of associations were

noted: *ASXL1* mutations were associated with *ZRSR2* mutations, *CEBPA* with *EZH2*, *CSF3R* with *SETBP1*, *EZH2* with *NRAS* and *TET2*, *JAK2* with *TET2*, *KRAS* with *NF1*, and *SETBP1* mutations with *SRSF2*. On the other hand, mutated *CSF3R* meant the exclusion of *EZH2* and *TET2* mutations, mutated *EZH2* excluded *SETBP1* and *SRSF2* mutations, and *NRAS* variants excluded *STAG2* mutations.

Characterization of *CSF3R*-mutated patients

As *CSF3R* mutations are a hallmark of CNL but are not an exclusive marker of this disorder, we analyzed the *CSF3R*-mutated population as a single group and compared them with *CSF3R*-unmutated cases. Clinical characteristics and outcomes between the 2 groups were indistinguishable (supplemental Table 13). We found no molecular differences between *CSF3R*-mutated and unmutated cases (supplemental Table 14) apart from the already mentioned exclusivity between *CSF3R* mutations and *EZH2* and

Table 2. Molecular characterization by NGS in 53 patients with aCML or CNL

% Mutated gene (n)	Whole series, % (53)	aCML, % (30)	CNL, % (23)	P
<i>ASXL1</i>	41.5 (22)	43.3 (13)	39.1 (9)	.979
<i>BCORL1</i>	3.8 (2)	6.7 (2)	0 (0)	.593
<i>CBL</i>	15.1 (8)	20 (6)	8.7 (2)	.452
<i>CEBPA</i>	5.7 (3)	6.7 (2)	4.3 (1)	1.000
<i>CSF3R</i>	49.1 (26)	20 (6)	87 (20)	<.001
<i>DNMT3A</i>	1.9 (1)	0 (0)	4.3 (1)	.893
<i>ETV6</i>	3.8 (2)	3.3 (1)	4.3 (1)	1.000
<i>EZH2</i>	34 (18)	50 (15)	13 (3)	.012
<i>FLT3</i>	3.8 (2)	6.7 (2)	0 (0)	.593
<i>IDH2</i>	3.8 (2)	6.7 (2)	0 (0)	.593
<i>JAK2</i>	7.5 (4)	10 (3)	4.3 (1)	.805
<i>KIT</i>	3.8 (2)	6.7 (2)	0 (0)	.593
<i>KMT2A</i>	1.9 (1)	3.3 (1)	0 (0)	1.000
<i>KRAS</i>	1.9 (1)	3.3 (1)	0 (0)	1.000
<i>MPL</i>	1.9 (1)	3.3 (1)	0 (0)	1.000
<i>NF1</i>	3.8 (2)	6.7 (2)	0 (0)	.593
<i>NRAS</i>	9.4 (5)	10 (3)	8.7 (2)	1.000
<i>PTPN11</i>	20 (2)	12.5 (1)	50 (1)	.843
<i>RAD21</i>	3.8 (2)	6.7 (2)	0 (0)	.593
<i>RUNX1</i>	13.2 (7)	20.0 (6)	4.3 (1)	.208
<i>SETBP1</i>	43.4 (23)	36.7 (11)	52.2 (12)	.396
<i>SF3B1</i>	3.8 (2)	3.3 (1)	4.3 (1)	1.000
<i>SRSF2</i>	41.5 (22)	40.0 (12)	43.5 (10)	1.000
<i>STAG2</i>	9.4 (5)	6.7 (2)	13 (3)	.754
<i>TET2</i>	28.3 (15)	43.3 (13)	8.7 (2)	.014
<i>U2AF1</i>	7.5 (4)	3.3 (1)	13 (3)	.423
<i>ZRSR2</i>	7.5 (4)	10 (3)	4.3 (1)	.805

P for differences between aCML and CNL. *ATRX*, *BCOR*, *CALR*, *EGLN1*, *EPAS1*, *EPOR*, *IDH1*, *KDM6A*, *NPM1*, *PHF6*, *PRPF40B*, *SF3A1*, *SH2B3*, *SMC1A*, *THPO*, *TP53*, *VHL*, and *WT1* were not mutated in any CNL or aCML case. Statistically significant differences are in bold.

TET2 mutations and the known association with *SETBP1*. Twenty-four cases had 1 *CSF3R* mutation plus a mutation in at least 1 other gene; VAF were available for 23 of these cases. In 14 cases (65.2%), the VAF was comparable between *CSF3R* and the other genes, which we considered to be within 10% of each other and with the VAF ~50%. For 4 cases (17.4%), the *CSF3R* VAF was higher than that of other genes. Interestingly, all 4 had a *CSF3R* VAF of >80%, suggesting loss of heterozygosity. In 4 cases (17.4%), the *CSF3R* VAF was more than 15% lower than that of other mutations, suggesting the possibility that mutated *CSF3R* might have been a secondary event in this case.

Patients with compound *CSF3R* mutations (n = 6; supplemental Table 15) did not show any differential clinical features.

Mutational landscape and outcomes

Next, we analyzed the influence of the molecular profile on patient outcome. There was neither association between transformation to AML and any mutated gene or pathway nor the time to

transformation. When OS analysis was performed, mutations in *CBL*, *CEBPA*, *EZH2*, *NRAS*, *TET2*, and *U2AF1* were associated with shorter survival in the whole series (Figure 3; supplemental Table 16). These differences were maintained for *CEBPA* ($\beta = 2.26$, hazard ratio [HR] = 9.54, $P = .003$), *EZH2* ($\beta = 1.12$, HR = 3.062, $P = .009$), *NRAS* ($\beta = 1.29$, HR = 3.63, $P = .048$), and *U2AF1* ($\beta = 1.75$, HR = 5.74, $P = .013$) under a Cox model adjusted for WBC, gender, and age (supplemental Table 17). *SRSF2* mutations were associated with better survival under this model ($\beta = -1.17$, HR = 0.312, $P = .026$) as has been previously shown for aCML.¹⁷

Molecular-risk classification of CNL and aCML

Considering the high-risk mutated genes of univariate survival analysis and the relevance of *CSF3R* in the biology, diagnosis, and treatment of these diseases, we classified patients based on mutational status irrespective of whether they were diagnosed with CNL or aCML. Group 1 was defined by the absence of mutations in *CBL*, *CEBPA*, *EZH2*, *NRAS*, *TET2*, *U2AF1*, and *CSF3R*, group 2 presented with mutations in *CSF3R* but not high-risk genes, group 3 with mutations in any of the high-risk genes but wild-type for *CSF3R*, and group 4 presented mutations in *CSF3R* plus any of the high-risk genes (Figure 4; Table 3). The median time to acute transformation was significantly shorter in high-risk groups (Table 3). On multivariate analysis adjusted for age, sex, and leukocyte count, groups 3 (HR: 18.0, $P = .012$) and 4 (HR: 15.6, $P = .003$), but not 2 (HR: 4.5, $P = .192$), were associated with a higher risk of death in comparison with group 1 (Figure 4; supplemental Table 18). The presence of mutations in *CSF3R* worsened the prognosis in the low-risk group (42.8 vs 34.67 months, respectively), but the differences were not significant.

Discussion

CNL and aCML (now renamed as MDS/MPN with neutrophilia in the fifth WHO classification but still called aCML in the International Consensus Classification)^{28,29} are rare myeloid neoplasms with overlapping characteristics.^{2,5,6,30} Despite the inclusion of molecular criteria in the 2016 WHO classification,¹ differentiation between these entities remains problematical, and effective prognostication and treatment remain undefined. Here, we report one of the largest published cohorts of CNL and aCML cases, with 53 sequenced cases from a multicenter international study.

There were no differences in clinical presentations and outcomes of both diseases. Only the monocyte count was higher in aCML cases, which is not surprising given the fact that monocytosis is one of the exclusion criteria for CNL.¹ The number of mutated genes was similar, and we found very few differences in the mutational profile of CNL and aCML. In fact, only 3 genes, *CSF3R* (one of the diagnostic criteria for CNL¹), *EZH2*, and *TET2*, were differentially mutated. The prevalence of *TET2* and *EZH2* mutations plus the high rate of cytogenetic aberrations may partly explain the monocytosis and dysplasia seen in aCML.³¹⁻³³ Unfortunately, our panels did not include *ETNK1*, a gene reported to be mutated in aCML.³⁴ When we looked into the affected pathways, no differences were found. This highlights the common molecular pathophysiology of these diseases and supports the notion of CNL and aCML being a continuum of the same disease.^{4-6,9} Of course, the minimal difference between these 2 entities might, in part, be

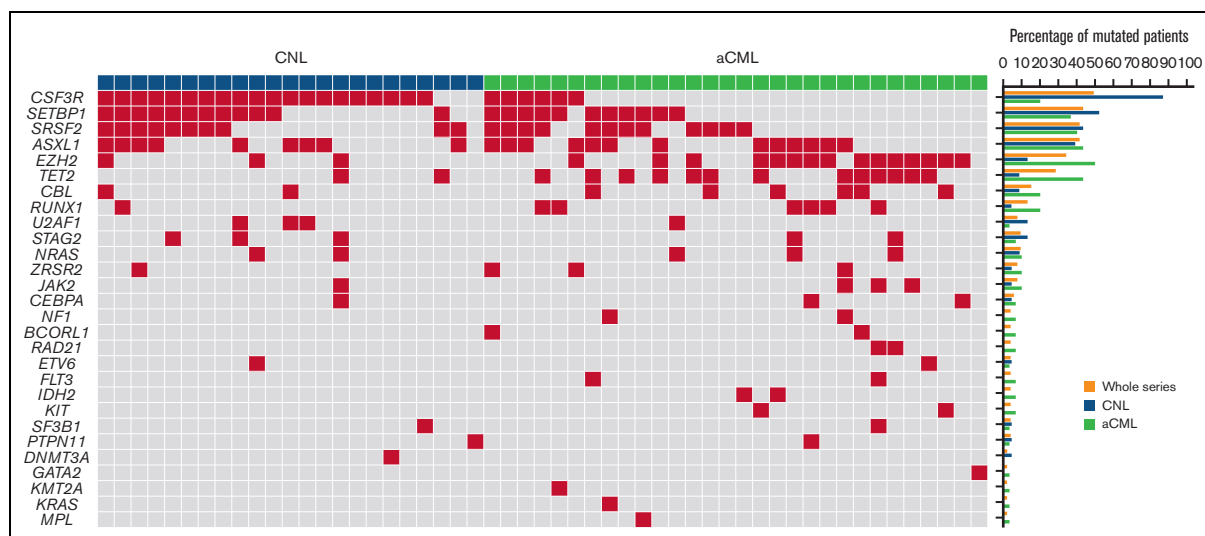


Figure 1. Mutational profile of patients with CNL and aCML. Right panel shows all the samples and their mutated genes (blue CNL cases and green aCML cases). Left panel represents the percentage of patients with mutation for each gene in the whole series and for patients with CNL and aCML, respectively.

attributable to the lack of central review of all cases, which was not possible because of the retrospective nature of our study and the long period of case collection (1986-2020). However, all cases with *JAK2*, *SF3B1*, and *KIT* mutations plus several others were centrally reviewed and the diagnosis confirmed. Furthermore, our results are comparable to those described in other studies^{4,17} and, of note, none of the cases classified as CNL showed any evidence of dysplasia.

When we investigated the co-occurrence and exclusivity of gene mutations, we found associations between different tyrosine kinase, epigenetic, and splicing regulator genes. Some of them, such as *CSF3R*/*SETBP1*, *EZH2*/*TET2*, and *SETBP1*/*SRSF2*, have been described previously,^{4,16,17} but some novel associations emerged. Neither *SETBP1* nor *SRSF2* were associated with aCML despite the *SETBP1*/*SRSF2* combination, having recently been proposed to delineate aCML from other MDS/MPN.¹⁷ Thus,

our findings, along with those from other groups, support the notion that CNL and aCML are highly related in terms of somatic genomic profiles, despite their morphological differences.^{4,6}

We evaluated the prognostic significance of mutational profiles of our patients. The presence of any of the high-risk mutations (*CBL*, *CEBPA*, *EZH2*, *NRAS*, *TET2*, or *U2AF1*) conferred a dismal outcome, and *CSF3R* mutations worsened the prognosis. However, we found no significant differences between *CSF3R*-mutated and unmutated cases, thus providing no support for *CSF3R* mutations as a disease-defining characteristic. These results do not fully agree with those of other studies.^{4,6,13,16,17,35} For example, *NRAS* mutations have been consistently described as a bad prognostic factor in patients with aCML/CNL, but we did not observe the adverse effect of *ASXL1* mutations shown in some studies.^{4,17} The reason behind this may be the rarity and heterogeneity of these entities, which makes the assembly of large

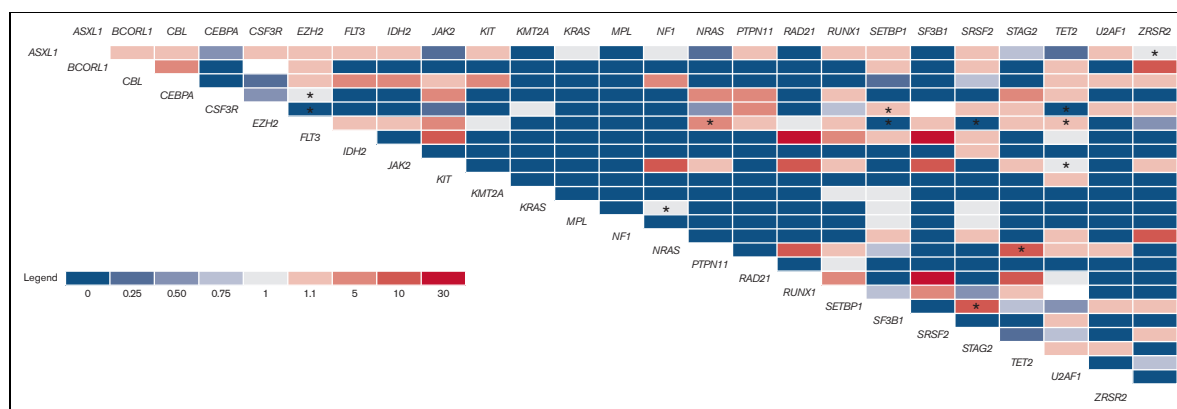


Figure 2. Co-occurrence and exclusion between mutated genes. Warm (red shaded) colors represent a pairwise association between mutated genes (with red having the highest OR); cold (blue shaded) colors indicate mutual exclusion of mutations in gene pairs (with dark blue having the lowest OR). * indicates a statistically significant association. OR, odds ratio.

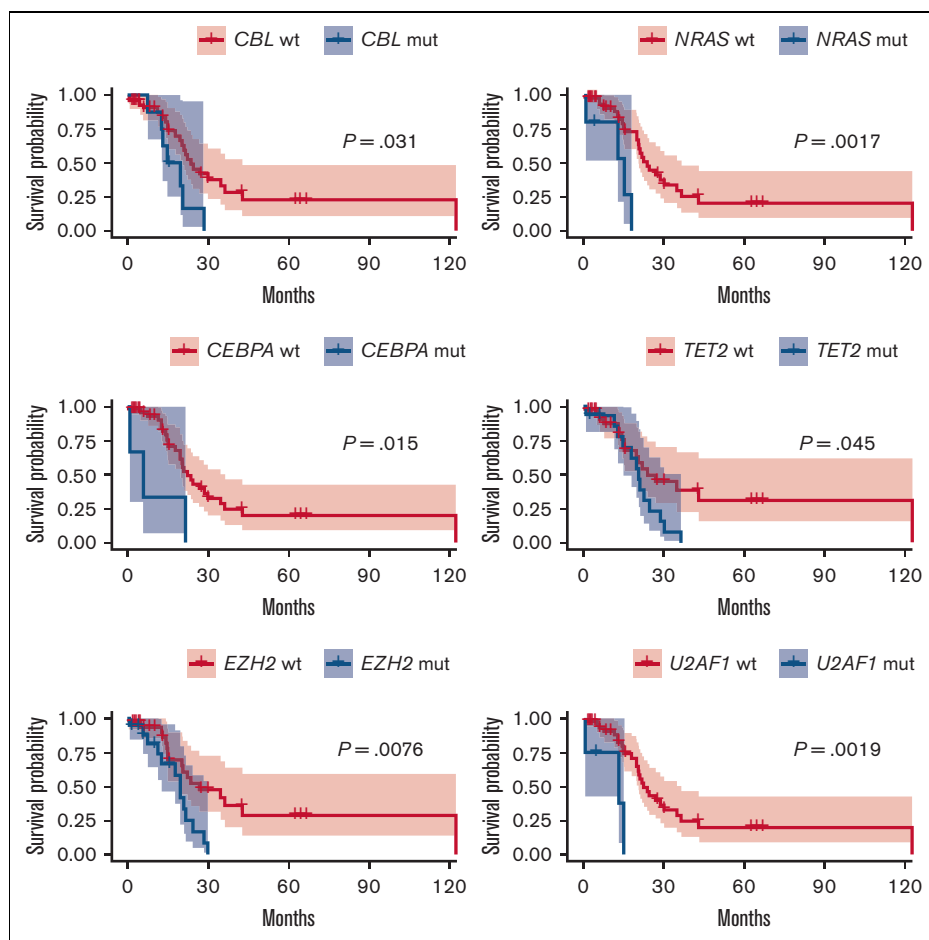


Figure 3. *CBL*, *CEBPA*, *EZH2*, *NRAS*, *TET2*, and *U2AF1* are associated with poor survival in patients with CNL/aCML on univariate analysis.

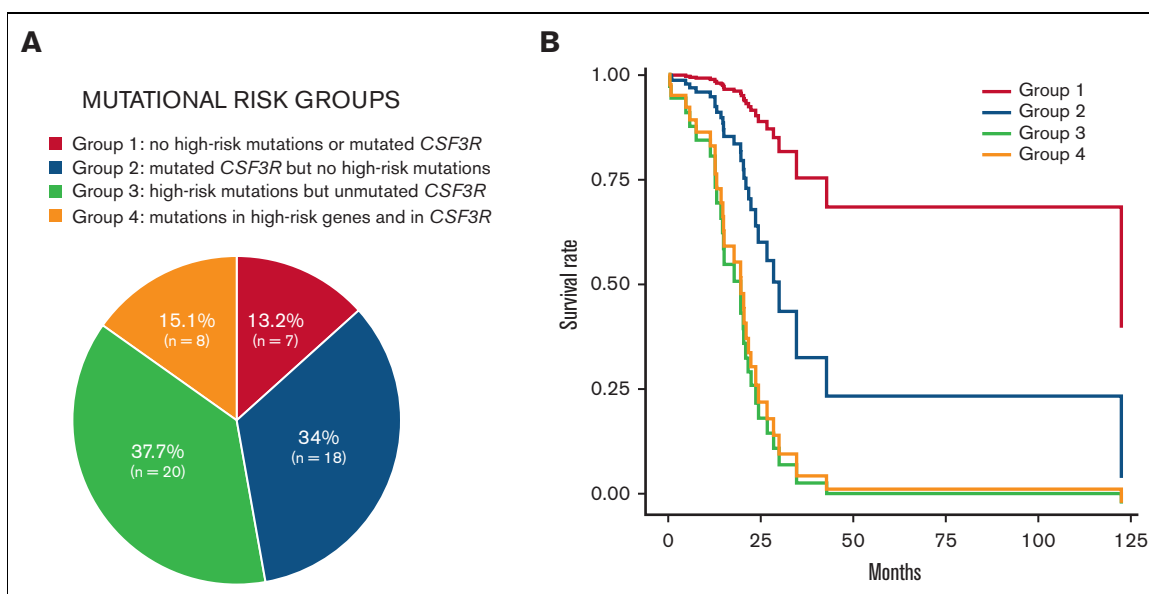


Figure 4. Molecular risk of CNL/aCML. (A) shows the percentage and number of patients in each group and (B) the survival of different groups (curves under a Cox model adjusted by age, sex, and leukocyte count). Group 1 (red, reference group): no high-risk mutations or mutated *CSF3R*; group 2 (blue, HR 4.5, $P = .192$): mutated *CSF3R* but no high-risk mutations; group 3 (green, HR 18.0, $P = .012$): patients with high-risk mutations but unmutated *CSF3R*; group 4 (orange, HR 15.6, $P = .003$): patients mutated *CSF3R* and high-risk mutations. High-risk mutations include *CBL*, *CEBPA*, *EZH2*, *NRAS*, *TET2*, and *U2AF1*.

Table 3. Patients characteristics in 53 patients with aCML or CNL according to mutational risk groups

	Group 1 (n = 7)	Group 2 (n = 18)	Group 3 (n = 20)	Group 4 (n = 8)	P
Gender = % male (n)	71.4% (5)	66.7% (12)	70% (14)	62.5% (5)	.978
Age (mean, range)	69.5 (54.1-87.4)	68.5 (33.0-92.50)	72.2 (45.6-91.4)	76.8 (66.4-84.7)	.474
Median OS (mo)	42.8	34.7	19.6	13.0	<.001
Transformation to AML	0% (0)	33.3% (6)	40.0% (8)	12.5% (1)	.150
Median time to transformation (mo)	NR	16.9	10.7	8.6	.037
HSCT	16.7% (1)	13.3% (2)	17.6 (3)	0% (0)	.732
Cause of death					.345
Progression	100% (1)	100% (7)	64.3% (9)	40% (2)	
Bleeding	0% (0)	0	14.3% (2)	40% (2)	
Other	0% (0)	0	21.4% (3)	20% (1)	
Splenomegaly	57.1% (4)	66.7% (12)	45% (9)	62.5% (5)	.582
Hepatomegaly	16.7% (1)	40% (4)	10% (2)	50% (1)	.194
Cutaneous bleeding	0% (0)	10% (1)	15% (3)	0% (0)	.678
Mucosal bleeding	0% (0)	10% (1)	5% (1)	0% (0)	.826
Hemoglobin (g/L)	72.88	82.74	79.91	106.00	.631
Platelets ($\times 10^9/L$)	332.00	246.72	394.8	253.88	.537
WBCs ($\times 10^9/L$)	27.23	53.24	75.18	93.28	.087
Neutrophils ($\times 10^9/L$)	20.96	48.56	65.06	79.41	.114
Lymphocytes ($\times 10^9/L$)	3.37	3.22	4.74	3.30	.245
Monocytes ($\times 10^9/L$)	1.93	1.63	2.38	1.93	.830
Altered cytogenetics	0% (0)	6.7% (1)	25.0% (4)	0% (0)	.176

Group 1: unmutated *CSF3R* without high-risk mutations. Group 2: mutated *CSF3R* without high-risk mutations. Group 3: unmutated *CSF3R* with high-risk mutations. Group 4: mutated *CSF3R* with high-risk mutations. High-risk mutations include *CBL*, *CEBPA*, *EZH2*, *NRAS*, *TET2*, *U2AF1*. P values (ANOVA, χ^2 , or Fisher exact test) are for the presence of differences between groups. Statistically significant differences are in bold.

ANOVA, analysis of variance; HSCT, hematopoietic stem cell transplant; NR, not reached.

cohorts challenging. In addition, as has been mentioned above, our study is retrospective, and our survival analysis could have been affected by variable follow-up data collection and changes to patient care during the study period. Nevertheless, our survival results are consistent with those of others.^{2,6}

Finally, because of their rarity and the absence of a common targetable mutation, there is no standard treatment for these entities. In fact, most cases in our series were treated with hydroxyurea as a first-line therapy instead of other drugs such as azacitidine or ruxolitinib that may have disease-modifying potential.^{2,30} Ruxolitinib, a JAK1/2 inhibitor widely used in PMF, has activity against *CSF3R*, which has resulted in clinically significant responses in a subset of patients, although many of these responses have not been sustained.^{12,21,36} The key role of *CSF3R* mutations and the adverse survival of these patients supports a combination study of ruxolitinib and other drugs with proven efficacy in MDSs (now renamed MDS neoplasms in the new WHO classification),²⁸ a disorder which shows clear mutational overlap with CNL/aCML. The advent of new drugs targeting specific genes and pathways such as spliceosome modulators, MEK inhibitors for cases with RAS-RAF-MEK mutations, and others will open the doors to new treatment options and combinations, for which a molecular classification of these diseases is likely to be important.³⁷⁻³⁹ Given their poor prognosis, hematopoietic stem cell transplant must be considered a priority in patients who are candidates for this procedure.

In summary, we analyzed the clinical and molecular data of one of the largest CNL/aCML cohorts to date. Our study provides insights on how similar these diseases are and supports the idea they should be considered as a continuum of the same disease and classified as a subtype of MDS/MPN given their mutational profile. We have also identified molecular prognostic groups and shown the importance of *CSF3R* mutations, which are targetable by ruxolitinib. Further studies and meta-analysis are needed to clarify the value of these mutations and the best treatment for this group of diseases.

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Authorship

Contribution: G.C.-T. designed the study, analyzed and interpreted the data, and wrote the manuscript with the support of J.M.-L., R.A., and N.C.P.C.; M.R. performed central morphological review of bone marrows; all remaining authors contributed patient data and/or

material for analysis; and all authors contributed to and approved the final version of the manuscript.

Conflict-of-interest disclosure: G.C.-T. has received honoraria from Novartis, Incyte, and Astellas. N.C.P.C. has received research support from Novartis, and honoraria from Novartis, Incyte, and Astellas. C.H. has received clinical research funding from Novartis, Constellation, and Bristol Myers Squibb (BMS), and has served on advisory boards and as a speaker for Novartis, BMS-Celgene, CTI BioPharma, Gilead Sciences, Shire, Roche, Janssen, Promedior, Geron, AOP, Galecto, Sierra Oncology, Constellation, and Keros. F.F.-M. has received research support from Incyte and Cty. A.A.-L. participated in advisory boards from BMS-Celgene/AOP and received payment for lectures from Novartis/AOP/BMS-Celgene. C.A. received research funding from Celgene/BMS and Jazz Pharmaceuticals and has served on advisory boards and as a speaker for AbbVie. S.N. has served as speaker for

BMS and Astellas, served on advisory board for BMS, and has received educational meeting sponsorship from Novartis and BMS.

ORCID profiles: G.C.-T., 0000-0002-9570-5542; J.C.M.-Á., 0000-0003-3332-8195; J.C.H.-B., 0000-0002-4289-3113; F.F.-M., 0000-0002-9520-3243; T.G.-M., 0000-0002-3793-4865; M.P.-E., 0000-0001-9943-4404; K.M., 0000-0001-5335-3641; R.S., 0000-0001-6955-2290; B.P., 0000-0001-8198-9663; C.A., 0000-0003-4391-9802; M.L.M., 0000-0002-0685-8441; A.J.M., 0000-0001-8522-1002; R.A., 0000-0002-2699-8353; N.C.P.C., 0000-0001-5481-2555.

Correspondence: Gonzalo Carreño-Tarragona, Hematology Department, I+12, Hospital Universitario 12 de Octubre, Avda. Córdoba, S/N, 28041 Madrid, Spain; email: gonzalo.carreno@salud.madrid.org; and Rosa Ayala, Hematology Department, I+12, Hospital Universitario 12 de Octubre, Avda. Córdoba, S/N, 28041 Madrid, Spain; email: rosam.ayala@salud.madrid.org.

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