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Clonal megakaryocyte dysplasia with normal blood values: a covert, thrombosis-prone, early myeloproliferative neoplasm

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Running title: Clonal megakaryocyte dysplasia with normal blood values

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Abstract

To improve our knowledge on the epidemiological, clinical and pathobiological profile of clonal megakaryocyte dysplasia with normal blood values (CMD-NBV), a *BCR::ABL*-negative myeloproliferative neoplasms (MPNs) clinical variant, we here report a series of 30 consecutive subjects with CMD-NBV. Sixteen subjects were men and the median age was 48 years (IQR, 39-53 years). A situation-driven diagnosis (70% of cases had the diagnosis triggered by an incidental or symptomatic venous or arterial thrombosis), high incidence of thrombotic events (6.5 events x 100 subject-years), and indolent disease (the 10-year CMD-NBV specific survival was 100%) were common. Nineteen subjects had a high BMI at diagnosis and 14 had ≥ 1 Charlson co-morbidities. In 21 the driver variant was *JAK2*^{V617F} with a median variant allele frequency at diagnosis of 8.9% (IQR, 5.4–18.4%). Six of 24 (25%) subjects with data on next generation sequencing (NGS) for myeloid neoplasm-related genes had ≥ 1 pathogenic somatic variant in *ASXL1*, *TET2, DNMT3A or SRSF2*, a frequency in the lower range of values of chronic MPNs. Twelve putative germline, non-pathogenic, missense variants in *ASXL1*, *TET2*, *DNMT3A, RUNX1*, *CUX1*, *ABL1*, *NF1*, *KIT* and *CSF3R* or 5' UTR in *NF1* and 3' UTR in *ASXL1* were detected in 10 of 24 (42%) subjects. These data further support identification of CMD-NBV as a distinct entity.

Introduction

The World Health Organization (WHO) and the International Consensus Conference (ICC) classify BCR::ABL-negative classical myeloproliferative neoplasms (MPNs) into three major types, essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF). PMF is further divided into two distinct subtypes, prefibrotic (pre-MF) and overt myelofibrosis (overt-MF).[1,2] We recently proposed two cognate variants in the MPN domain, named clonal megakaryocyte dysplasia with normal blood values (CMD-NBV),[3] and clonal megakaryocyte dysplasia with isolated thrombocytosis (CMD-IT).[4] Pre-MF, overt-MF, CMD-NBV and CMD-IT share bone marrow (BM) morphological feature of megakaryocyte hyperplasia and dysplasia and were clustered in the new category of myelofibrosis-type megakaryocyte dysplasia (MTMD).[5]

Facing the new classificatory complexity, we conceptualized MTMD as a spectrum of disorders with a distinct phenotype and prognosis.[5-7] This view highlights the interest on the molecular events that drive specific disease presentations and explain their clinical features and laboratory findings.

Among the MTMD variants, CMD-NBV is the rarest and least characterized. CMD-NBV connotes normal hematologic values or minimal abnormalities and is mostly diagnosed in the context of venous or arterial thrombosis. In the cohort of 15 cases, we reported in 2022,[3] 10 had the canonical somatic *JAK2*^{V617F} mutation, while in the remaining cases the driver of clonal expansion was not identified.

With the aim to improve our knowledge on the epidemiological, clinical and pathobiological profile of CMD-NBV, we now report an expanded series of 30 consecutive subjects with CMD-NBV. To delineate subjects' molecular characteristics that could represent disease specific and defining molecular markers, we studied variant topography by next generation sequencing (NGS) technique.

Methods

Subjects characteristics and clinical procedures

In this single-centre retrospective study, subjects with CMD-NBV were identified from the institutional database of the Centre for the Study of Myelofibrosis of the IRCCS Policlinico S. Matteo Foundation in Pavia, Italy (Pavia-CSM-database). The database contains

consecutive individuals registered since 1998 with a diagnosis of MPN and examined at least once. This report consists of 14 cases we published in 2022,[3] and 16 newly referred cases. One previously reported subject was excluded since a missed history of thrombocytosis (platelet count > 450 x10E+9/L) contrasted our adjudicated CMD-NBV diagnostic criteria. All the subjects gave written informed consent approved by the IRCCS Policlinico S. Matteo Foundation Institutional Ethics Committee to be included in the database and to donate samples for genetic and molecular research on their disease.

Diagnosis of CMD-NBV was based on two distinct criteria:[3] 1. BM megakaryocyte hyperplasia and dysplasia consistent with the 2009 WHO diagnostic criteria for pre-MF.[8] 2. Failure to meet the clinical-hematological WHO criteria for PV or ET, and any of the four minor diagnostic criteria for pre-MF, i.e. palpable splenomegaly, anemia, white blood cell (WBC) count ≥11 x 10E+9/L, and increased serum lactate dehydrogenase level (LDH). As a deviation from these criteria, in this report we classified as CMD-NBV also subjects presenting with a palpable splenomegaly (no more than 5 cm from the costal margin) who had concurrent splanchnic vein thrombosis.

For all subjects, the database contained information at diagnosis about sex, age, body mass index (BMI), spleen size (clinical measurement), complete blood count with differential, and serum LDH level. BMI was categorized into underweight (< 18.5 kg/m^2), normal weight (18.5 to < 25 kg/m^2), overweight (25 to < 30 kg/m^2) and obese (≥ 30 kg/m^2).[9] Abnormal blood concentrations were defined as hemoglobin > 153 g/L (female) or > 160 g/L (male); WBC count > 8.8 x 10E+9/L; monocytes > 0.7 x 10E+9/L, and platelets > 390 x 10E+9/L.[10,11] Blood eosinophils percentage > 7% and blood basophils > 1% were defined outside the normal range.[12] In subjects analyzed at diagnosis and from whom we had peripheral blood slides, slides were re-examined for platelet morphology. For the purpose of the current study, platelets with a diameter ≥5 µm were considered macroplatelets.[13]

The reason for initial clinical presentation and diagnosis and all information on concomitant diseases was retrieved from medical records. Charlson Co-morbidity Index (CCI) was calculated as described.[14] For maintaining a person-centric rather than disease-centric perspective, we defined chronic physical multi-morbidity using the chronic physical illnesses (CPI) based on the modified European Health Interview Survey (EHIS)

guidelines.[15] To contextualize co-morbidities in the field of MPNs, we also categorized conditions diagnosed before or concurrent with CMD-NBV as autoimmune, cardiovascular/metabolic, infectious, and other inflammatory or malignant as described.[16]

In all subjects, key pathological BM features were obtained from the pathology report. Thrombosis was defined as any venous or arterial thrombo-embolism excluding superficial vein thrombosis. Thrombotic events that occurred within 2 years prior to the diagnosis of CMD-NBV were defined as MPN-related.

Data on *JAK2*^{V617F}, *MPL* and *CALR* mutations and variant allele frequencies (VAFs) were available at the time of diagnosis. NGS analyses were done on DNA from granulocyte collected at diagnosis or within 12 months after diagnosis and stored in our institutional biobank. Myeloid mutations were analyzed by NGS at the Lab of Molecular Hematology of the IRCCS Policlinico San Matteo Foundation and University of Pavia, Pavia, Italy. Details of library preparation, sequencing, and variant analysis are provided in the *Supplemental Methods*

Statistics

Subject co-variates are reported as median and interquartile range (IQR) for continuous variables. Categorical variables are reported as frequency rates and percentages and analyzed using Chi-square test. Independent group t-test was used to analyze normally distributed continuous variables. The Kurskal-Wallis test was used for non-normally distributed data. Major study endpoints were progression to active disease, blast transformation, death and thrombotic events. Progression to active disease was defined as: (1) disease-associated hemoglobin concentration < 100 g/L; (2) spleen > 10 cm below the left costal margin; (3) platelets < 150 x 10E+9/L; and/or (4) WBC count < 4 x 10E+9/L or >12 x 10E+9/L. To avoid confounding, we censored development of any of these criteria at the start of any disease-modifying intervention or at the diagnosis of a new cancer. Frequency of thrombotic events was expressed as incidence, calculated as numbers of events x 100 subject-years of observation with 95% Confidence Interval (CI). Results were considered statistically significant if P-values were < 0.05. Computations were done with STATISTICA© software **(**Dell Technologies Inc. Round Rock, TX, USA).

Ethics

The research was conducted in accordance with the World Medical Association Declaration of Helsinki. All subjects gave written informed consent approved by the IRCCS Policlinico S. Matteo Foundation Institutional Ethics Committee. The Ethics Committee of the Hospital also approved a written informed consent for patients to donate samples for molecular research (reference number 20110004143 of the 26.9.2011).

Results

The 30 adults that fulfilled our adjudicated criteria for CMD-NBV represent the 2.4% of all subjects registered in the Pavia-CSM-database for the MTMD category. Sixteen are men and median age is 47.5 years (IQR, 39-53).

Diagnosis

In 21 subjects (70%) the diagnosis of CMD-NBV was synchronous with an unexplained symptomatic venous or arterial thrombotic event (n = 15), incidental discovery of portal cavernoma (n = 5) or a diagnosis of post-embolic pulmonary hypertension (n = 1). In 9 other subjects, the diagnosis was driven by the incidental finding of laboratory abnormalities consistent with an MPN (n = 8), or of vertebral bone MRI abnormality interpreted as bone marrow involvement by a myeloid disorder (n = 1; *Supplemental Table 1*).

Co-variates at diagnosis

Subject co-variates at diagnosis are displayed in *Table 1*. With median hematological co-variates values in the normal range, 4, 5 and 6 subjects had hemoglobin, WBC and platelet concentration above the upper range of normal, while 4 had platelet count (n = 3) or WBC concentrations (n = 1) under the lower range of normal. Nine subjects diagnosed with a synchronous splanchnic vein thrombosis had a palpable spleen (no more than 3 cm below the costal margin). Two subjects had increased eosinophils, 5 increased basophils and 5 increased monocytes, yet 12 subjects (40%) had at least one of the above reported abnormalities. Blood smears at diagnosis was available in 20 subjects: macro-platelets were documented in 16 of them (80%). Macro-platelets were a small proportion of platelets in coexistence with normal platelets. Mean platelet volume was greater than 12 fl in one subject. *JAK2*^{V617F} was detected in 21 subjects (70%) with a median VAF of 7.8% (IQR, 5.2–17.9%). No *CALR*, *MPL* or *JAK2* exon 12 mutations were detected in the 9 remaining individuals. Median BMI at diagnosis was 26.1 m^2/kg (IQR, 23.1-28.7). No subject had a

BMI <18.5 m^2/kg, 11 (37%) were normal weighted, 15 (50%) had a BMI between 25 m^2/kg and 30 m^2/kg, and 4 (13%) were obese.

Co- and multi-morbidities

At the time of our Centre referral, 14 subjects (47%) had one or more comorbidities according to the Charlson co-morbidity criteria (CCI ≥ 1; *Table 2*): 7 had a CCI = 1, 6 a CCI = 2, and 1 a CCI = 3, with a median of 0.8 co-morbidities *per* subject. The most common co-morbidities were TIA/stroke (n = 4), solid neoplasia (n = 4), peripheral vascular disease (n = 3). Multi-morbidity was present in 14 subjects (47%): 8 had one co-occurring morbidity, while 3 had 2, and 3 had 3 co-occurring morbidities. The most frequent CPI was arterial hypertension (n=10; *Supplemental Table 2*). According to the Horvat-defined co-morbidities, 15 subjects had 1 or more co-morbid condition (*Supplement Table 3*). Thirteen subjects had a co-morbidity classified as cardiovascular or metabolic, 9 as inflammatory or autoimmune, and 4 as malignant. Four co-occurring inflammatory/autoimmune diseases were rare diseases: one subject was diagnosed with osteopecilia, a rare benign condensing osteopathy, one had familial sclerosing cholangitis, one Horton arteritis and one dural arteriovenous fistula due to sinus thrombosis (currently defined related to an inflammatory micro-environment).[17]

Bone marrow histology

Results of BM histology are displayed in *Table 3*. Being a necessary criterion for the diagnosis of CMD-NBV, megakaryocyte hyperplasia was a common feature. Age-corrected overall BM cellularity was increased in 1, normal in 9 and decreased in 3. All subjects had ≥1 signs of megakaryocyte dysplasia, including loose megakaryocyte clusters (n = 19), dense megakaryocyte clusters (n = 3), bulbous megakaryocytes (n = 14), or micromegakaryocytes (n = 6). No subject had granulocyte or erythroid lineages dysplasia. BM fibrosis was grade 0 (n = 20) or grade 1 (n = 10). Ten subjects had an increased vascular component and one showed megakaryocytes in the blood vessels. Lymphocyte hyperplasia was present in 20 cases. Absence of lymphocytic clonality was established in all the cases. In 7 subjects an increased number of BM eosinophils and in 3 increased mast cells was reported.

Somatic and germline variants

A panel of 45 genes was sequenced in 24 out of 30 subjects (80%). A total of 10 variants were classified as pathogenic or likely pathogenic somatic variants spread across 4 genes and 6 subjects (25%; 1.7/case mutation burden) (*Table 4; Supplemental Table 4***).** Subject UPN14 had 2 mutations in *ASXL1,* subject UPN23 co-occurring mutations in *DNMT3A, TET2, SRSF2*, while subject UPN29 in *DNMT3A* and *TET2*. The range of variant allele frequency (VAF) at diagnosis was 2.3-41%.

Ten subjects, representing 42% of those tested for NGS, harboured 12 heterozygous variants in *RUNX1*, *CUX1*, *ABL1*, *ASXL1*, *DNMT3A, CSF3R, TET2, NF1,* and *KIT* we defined germline having VAFs within the 45-55% range. Subject UPN10 had co-occurring variations in *CUX1* and *ABL1*. The putative germline gene variations were non-synonymous, missense, single nucleotide changes (n = 10) or 3' UTR (n = 1), 5' UTR (n=1) and were classified by *ClinVar* (https://www.ncbi.nlm.nih.gov/clinvar) as benign (n = 2), benign/likely benign (n = 2), likely benign (n = 2), of uncertain significance (n = 4), with conflicting classification of pathogenicity (n = 2), or were unknown to the *ClinVar* database (n = 2). No subject with a putative germline mutation had a family history of a highly penetrant cancer-predisposing variation.

Thromboses

With a median follow-up of 9.1 years (IQR, 4-14.2 years), 27 subjects (90%) had at least one major thrombotic event from 2 years before diagnosis to last follow-up (*Table 5*). Overall thrombotic events were 38 (mean, 1.3 events x subject) with an incidence of 6.5 events x 100 subject-years (95% CI, 3.4-11.7). Twenty-seven out of 38 (71%) thromboses were vein thrombosis in atypical sites including splanchnic (n = 21), Budd-Chiari syndrome (n = 3), and sinus vein thrombosis (n = 3). Post-diagnosis thrombosis occurred with an incidence of 4.4 events x 100 person-years (95% CI, 2.2-8.8).

Outcomes

Subjects with portal vein thrombosis or Budd-Chiari syndrome were permanently anticoagulated, whilst subjects with peripheral arterial thrombosis or myocardial infarction received anti-platelet therapy. During the follow-up, 13 subjects received hydroxyurea as antithrombotic prophylaxis at a median time from diagnosis of 1.8 months (IQR, 1.2-3.7 months). No subject had a splenectomy or a hematopoietic cell transplant. No subject had blast transformation. Subject UPN14 progressed at 14.2 years after diagnosis towards an active disease consisting in splenomegaly > 10 cm from the costal margin, hemoglobin

concentration 103 g/L, platelet concentration 108 x 10E+9/L, blood immature myeloid cells, blood CD34-positive cells 44 x 10E+6/L, *JAK2*^{V617F} VAF 98% and bone marrow fibrosis grade-3 (previous grade-1). The subject received hydroxyurea and ruxolitinib sequential therapy.

Subjects UPN7, UPN15, and UPN21 had a platelet concentration < 150 x10E+9 at diagnosis and subjects UPN15 also had a WBC < 4 x10E+9 at diagnosis. These abnormalities recovered without intervention. No subject other than UPN14 had a >10% increase in *JAK2*^{V617F} VAF.

Twenty-eight subjects had a 2nd BM biopsy. Three of 19 subjects with grade-0 BM fibrosis at diagnosis progressed to grade 1, and 2 of 9 with BM fibrosis grade-1 progressed to ≥ grade 2. There were no concurrent changes in blood cell concentrations save in subject UPN14. The 10-year CMD-NBV-specific survival was 100%.

Subject UPN3 developed intra-ductal breast cancer 10 months after diagnosis. Subject UPN23 developed breast cancer 2 years after diagnosis. Subject UPN29 developed lung carcinoma 4 years after diagnosis and she died 6 months thereafter. Subject UPN30 developed small lymphocytic lymphoma 8 years after diagnosis followed by lung adenocarcinoma 10 years after diagnosis. Subject UPN14 was diagnosed with monoclonal gammopathy of uncertain significance 10 years after diagnosis. In summary, 5 out of 30 subjects (17%) developed 6 primary second malignant or premalignant diseases, giving a post-diagnosis incidence of 6 events x 100 subject-years (95% CI, 2.4-12.5). Subject UPN19 died 12 years after diagnosis for liver sequelae of Budd-Chiari syndrome, and subject UPN30 died 11.2 years after diagnosis for lung adenocarcinoma. The 20-year survival was 78% (95% CI, 53-99%) from diagnosis.

Discussion

Our analysis of 30 consecutive subjects CMD-NBV highlights the uniqueness of the clinical characteristics we delineated in the original description of this form of MPN,[3] and allowed us to derive new insights on its bio-pathology. One distinct clinical hallmark of CMD-NBV is the situation-based diagnosis: in this cohort, 70% of cases had the diagnosis made whilst investigating a possible MPN triggered by an incidental or symptomatic venous or arterial thrombosis. Another clinical feature is the markedly elevated risk of thrombosis, especially

splanchnic vein thrombosis, with an incidence of a major thrombotic event of 6.5 events x 100 subject-years. Third, at a median follow-up of 9.1 years, all but one subjects remained asymptomatic with no change in hematological values, despite coincidental increase of BM fibrosis.

The situation-based diagnosis and the indolent disease phenotype challenge the knowledge of a trustworthy epidemiology of CMD-NBV. The median age at diagnosis of the cohort was 45 years old with a range from 20 to 75 years. However, the age at diagnosis mostly reflects the age of incidental thrombosis. Moreover, the 3 percent incidence of the variant in our database arguably does not portrait its prevalence since screening for an occult MPN in subjects with thrombosis is case-specific. In particular, it is common in splanchnic vein thrombosis,[18] but uncertain in unexplained peripheral vein or arterial thrombosis, and uncommon in older subjects.[19-21.]

If normal blood values we entered into the definition of the CMD-NBV variant resulted coherent with the values of blood parameters of the cohort, the morphological picture of peripheral blood does not. In fact, in more than 40% of cases blood eosinophilia, basophilia or monocytosis at diagnosis, and in 90% of cases a small population of macro-thrombocytes was documented. We interpreted these signs as an expression of the early CMD-NBV malignancy.

Aligning with the literature suggesting that individuals with MPNs generally have poorer health compared with the normal population, here we documented that the median value of BMI at diagnosis (26.2 kg/m^2) felt in the category of overweight, and was higher than that reported in Italian cases with PV (24.2 kg/m^2),[22] or PMF candidate to ruxolitinib (23.9 kg/m^2),[23] or allogeneic HSCT (24.9 kg/m^2).[24] Moreover, 14% of cases were obese. This result suggests a possible mechanistic relation between obesity and myeloproliferation applies in CMD-NBV, as has been documented in other pre-cancers and cancers.[25]

We also documented co-morbidities were common in subjects with CMD-NBV. By considering the Charlson's co-morbidity index, 48% of subjects had one or more comorbid condition at diagnosis, mirroring the results of Italian PV and PMF cases in whom 40% and 51% of subjects, respectively, had at least one Charlson's comorbidity.[23,24] European Health Interview Survey (EHIS) multimorbidity analysis showed that 48% of individuals

with CMD-NBV have one or more comorbidity, a rate higher than the 26.2 % reported in an European control population.[26] Finally, by using the Horvat classification of comorbidity, cardiovascular and autoimmune comorbidities resulted to dominate our population of subjects. These findings highlight the importance of host and environmental risk factors in CMD-NBV. Moreover, the co-occurrence of rare diseases, like Horton arteritis, familial sclerosing cholangitis, osteopecilia and dural artero-venous fistula, suggest etiological heterogeneity of CMD-NBV.

A major aim of our report was to investigate the molecular profile of CMD-NBV subjects. We found most subjects with CMD-NBV had *JAK2*^{V617F}. What makes CMD-NBV unique is the low *JAK2*^{V617F} VAF at diagnosis (median value, 7.8%) and no *CALR* and *MPL* variants. Twenty-five percent of subjects had one or more additional non-driver somatic mutations, a common feature of people with a chronic phase MPN.[27-29] Although limited by the low number of cases, this proportion appears to be in the low range of values.[29]

A high proportion (42%) of subjects had variants in genes involved in hematopoiesis and leukemia which we interpreted as germline. These variants overlap somatic variants in *ASXL1*, *TET2*, *DNMT3A*. None of these putative germline variants is reported as high-penetrance cancer predisposing. However, the *RUNX1* (c.167C>T) variant, classified now as benign, may be up-graded to higher level of pathogenicity considering additional segregation data reporting two families where the germline variant was associated with thrombocytopenia and with evolution to a myelodysplastic syndrome (MDS).[30,31] Germline *CSF3R* (c.2422G>A) is reported in MDS and MDS/MPN.[32-35] Germline *ASXL1* (c.3306G>T) is reported in 4 out of 62 children with chronic myeloid leukemia,[36] germline *DNMT3A* (c.1502A>G) is reported in a child with acute myeloid leukemia.[37] Finally, 3'UTR *ASXL1* (c.*87A>G) variant is associated with a low blood basophils concentration and with lower eosinophils and monocytes concentration.[38]

In conclusion, this expanded cohort of subjects with CMD-NBV highlights the clinical variant presents as a covert, thrombosis-prone, early MPN. The characterization of somatic mutation profiles fosters the development of strategies for early interception and intervention. The hypothesis of high incidence of predisposing germline variants in myeloid genes drives the future research with the perspective to investigate the heritability of the identified germline variants.

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Table 1. Baseline co-variates of subjects with CMD-NBV (n = 30). Data are shown for the whole population and according to sex

	All subjects (n=30)	**Males (n=16)**	**Females (n=14)**	**p-value**
Demography and anthropometry				
Age, yrs, median (IQR)	47.5 (39-53)	49 (30-52)	46 (41-54)	0.53
Sex male, n (%)	16 (53)			
BMI, kg/m^2, median (IQR)	26.1 (23.1-28.7)	25.9 (23.6-28.2)	26.4 (21.7-28.7)	0.91
BMI, kg/m^2, ≥ 30, n (%)	4 (13)	1 (6)	3 (21)	
Clinical-hematological co-variates				
Hemoglobin, g/L, median (IQR)	137 (128-150)	149 (140-155)	133 (127-135)	0.003
Mean cell volume, fl, median (IQR)	87.3 (81.2-89.4)	86.7 (80.8-88.5)	88 (85-89.9)	0.61
WBC x 10E+9/L, median (IQR)	6.2 (5.7-7.8)	6.1 (5.3-7.8)	6.7 (5.9-8.7)	0.41
WBC ≥ 8.8 x 10E+9/L, n (%)	5 (17)	3 (19)	2 (14)	
WBC <4 x 10E+9/L, n(%)	1 (3)	1 (6)	0 (0)	
Eosinophils percent, median (IQR)	3.2 (2-4.9)	3.1 (2-3.9)	3.4 (2.7-6.3)	0.075
Eosinophils percent >7, n (%)	2 (7)	0	2 (14)	
Basophils percent, median (IQR)	0.7 (0.3-1)	0.5 (0.2-1)	0.9 (0.5-1.1)	0.22
Basophils percent >1, n (%)	5 (17)	1 (6)	4 (28)	
Monocytes x 10E+9, median (IQR)	486 (410-556)	496 (409-548)	463 (411-743)	0.98

Monocytes > 700 x 10E+9, n (%)	5 (17)	2 (12)	3 (21)	
Platelets x10E+9/L, median (IQR)	274 (205-371)	209 (192-278)	356 (277-396)	0.003
Platelets > 390 x 10E+9/L, n (%)	6 (20)	2 (12)	4 (28)	
Platelets < 150 x 10E+9/L, n (%)	3 (10)	3 (19)	0 (0)	
Spleen size, cm E+2, median (IQR)	90 (90-110)	90 (90-120)	90 (90-90)	0.36
Spleen size > 90 cm^2, n (%)	9 (30)	6 (37)	3 (21)	
Plasma LDH, x ULN, median (IQR)	0.86 (0.78-1.00)	0.78 (0.66-0.90)	0.93 (0.83-1.17)	0.007
Serum cholesterol, mg/dL, median (IQR)	190 (146-217)	178 (133-200)	194 (179-218)	0.21
Blood CD34-positive cells x 10E+6, median (IQR)	2.37 (1.60-4.31)	2.19 (1.36-4.72)	2.56 (1.63-3.39)	0.85
Molecular co-variates				
JAK2^{V617F}, n (%)	21 (70)	10 (62.5)	11 (78.6)	0.33
JAK2^{V617F} allele frequency, median (IQR)	7.8 (5.2-17.9)	5.9 (3.7-10)	14.2 (6.8-19.5)	0.11
CALR mutation, n (%)	0	0	0	
MPL mutation, n (%)	0	0	0	
Triple negative, n (%)	9 (30)	6 (37.5)	3 (21.4)	0.33

BMI: Body mass index; IQR: Interquartile range. ULN: upper limit of normal.

By dividing subjects according to sex, males had significantly higher hemoglobin concentrations than had females. By contrast, males had lower platelet count and LDH plasma concentration than females

Table 2. Co-morbidities of subjects with CMD-NBV according to the Charlson co-morbidity index. Data were obtained at the first referral at our Center

Co-morbidities, n (%)	**Subjects number (%)** **Total = 30**
Acute myocardial infarction	2 (7)
Solid neoplasia - Localized - Metastatic	4 (13) 2 (7) 2 (7)
Diabetes mellitus - Uncomplicated - Complicated	1 (3) 1 (3) 0
Transient ischemic attack/stroke	4 (13)
Chronic obstructive pulmonary disease	1 (3)
Peptic ulcer disease	0
Peripheral vascular disease	3 (10)
Liver disease - Mild - Moderate-severe	2 (7) 1 (3) 1 (3)
Connective tissue disease	1 (3)
Congestive heart failure	1 (3)
Chronic cognitive deficit	0
Hemiplegia	0
Lymphoma	1 (3)
Leukemia	0
Acquired immune deficiency syndrome	0

Table 3. Detailed analysis of the bone marrow features of 30 subjects with CMD-NBV at diagnosis

Quantitative variables			
	Reduced n (%)	Normal n (%)	Increased n (%)
Cellularity	3 (10)	9 (30)	18 (60)
Erythropoiesis	2 (7)	11 (37)	17 (57)
Granulopoiesis	2 (7)	12 (40)	16 (53)
Megakaryopoiesis	0	0	30 (100)
Qualitative variables			
	Present, n subjects (%)		
Megakaryocyte clusters	22 (73)		
- Loose	19 (63)		
- Dense	3 (10)		
Megakaryocyte nuclei			
- Hyper-lobulated	2 (7)		
- Bulbous	14 (47)		
Small megakaryocytes	6 (20)		
Fibrosis			
- Grade 0	20 (67)		
- Grade 1	10 (33)		
- Grade 2	0		
- Grade 3	0		

Table 4. Genetic and molecular profile of the 30 subjects diagnosed with CMD-NBV. Data were obtained at diagnosis

Case #	Sex/age	Driver mutation (VAF%)	NGS-somatic variants (VAF%)	Cytogenetics
UPN1	M/25 yrs	*JAK2*^{V617F} (21)	Neg	
UPN2	M/52 yrs	*JAK2*^{V617F} (10)	ND	XY
UPN3	F/49 yrs	*JAK2*^{V617F} (5)	Neg	
UPN4	M/57 yrs	*JAK2*^{V617F} (5.2)	Neg	
UPN5	F/44 yrs	*JAK2*^{V617F} (7.7)	Neg	
UPN6	M/23 yrs	TN	ND	
UPN7	M/49 yrs	*JAK2*^{V617F} (5.9)	Neg	
UPN8	M/49 yrs	TN	Neg	XY
UPN9	F/38 yrs	*JAK2*^{V617F} (ND)	*TET2* (c.4585C>T) (p.Gln1529*) (2.7)	XX
UPN10	M/44 yrs	TN	Neg	
UPN11	F/42 yrs	*JAK2*^{V617F} (33)	Neg	XX
UPN12	F/32 yrs	TN	Neg	XX
UPN13	M/52 yrs	*JAK2*^{V617F} (16)	ND	
UPN14	F/46 yrs	*JAK2*^{V617F} (ND)	*ASXL1* (c.2077C>T) (p.Arg693*) (3.8) *ASXL1* (c.1900_1922 del) (p.Glu635fs) (41)	
UPN15	M/49 yrs	*JAK2*^{V617F} (0.65)	*TET2* (c.4045-1G>A) (null) (3.9)	
UPN16	F/37 yrs	TN	Neg	
UPN17	M/71 yrs	TN	Neg	
UPN18	F/46 yrs	*JAK2*^{V617F} (19)	Neg	
UPN19	F/54 yrs	*JAK2*^{V617F} (ND)	ND	XX
UPN20	M/29 yrs	*JAK2*^{V617F} (7.8)	Neg	XY
UPN21	M/52 yrs	*JAK2*^{V617F} (0.19)	Neg	
UPN22	F/53 yrs	TN	Neg	
UPN23	F/70 yrs	*JAK2*^{V617F} (5)	*DNMT3A* (c.2320G>T) (p.Glu774*) (8) *TET2* (c.4791del) (p.Tyr1598Ilefs*12) (4) *SRSF2* (c.161C>T) (p.Ser54Phe) (3)	XX
UPN24	F/43 yrs	*JAK2*^{V617F} (5)	Neg	
UPN25	M/20 yrs	TN	Neg	
UPN26	M/49 yrs	TN	ND	

UPN27	F/70	*JAK2*^{V617F} (17.9)	*DNMT3A* (c.1656 delC) (p.Asn552fs) (2.5)	
UPN28	M/39	*JAK2*^{V617F} (3.7)	Neg	
UPN29	F/55	*JAK2*^{V617F} (12)	*DNMT3A* (c.1490G>A) (p.Cys4977Tyr) (2.8) *TET2* (c.4393C>T) (p.Arg1465*) (2.3)	
UPN30	M/75	*JAK2*^{V617F} (ND)	ND	Trisomy 9/del Y

VAF: variation allele frequency; TN: triple negative; ND: not determined; NGS: next generation sequencing

Table 5. Major thrombotic events occurring in 30 subjects diagnosed with CMD-NBV, considering a time frame of two years before diagnosis up to the last follow-up

	Number of events
Overall thrombotic events, n	38
Arterial thrombosis, n (%)	8 (21)
- In 2 years before diagnosis, n	5
- At diagnosis, n	2
- After diagnosis, n	1
Deep vein thrombosis in typical sites, n (%)	3 (8)
- In 2 years before diagnosis, n	3
- At diagnosis, n	0
- After diagnosis, n	0
Venous thrombosis in atypical sites, n (%)	27 (71)
- In 2 years before diagnosis, n	6
- At diagnosis, n	16
- After diagnosis, n	5

Supplemental material

Clonal megakaryocyte dysplasia with normal blood values: a covert, thrombosis-prone, early myeloproliferative neoplasm

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Supplemental Methods

Genomic profiling

Screening for myeloid cancer associated mutations was performed using oncoReveal Myeloid Panel (Pillar Biosciences, MA, USA) an amplicon-based library preparation chemistry that interrogates genes recurrently mutated in myeloid neoplasms. We focused on a core panel of 45 genes (ANKRD26 (exon 1), ASXL1 (exons 12-13), BCOR (exons 2-15), BCORL1 (exons 1-12), CALR (chr 19: g.13054521-13054710), CBL (exons 1-3, 5, 8-10, 12-13, 16), CEBPA (exon 1), CSF3R (exons 14-16), CUX1 (exons 1-5, 9-10, 12, 17, 20-21, 24), DDX41 (exons 1, 3, 5-6, 8, 10-11, 14-15), DNMT3A (exons 1-23), ETNK1 (exon 3), ETV6 (exons 1-8), EZH2 (exons 2-20), FLT3 (exons 14, 20), GATA2 (exons 3-7), GNAS (exons 8-9), HRAS (exons 2-3), IDH1 (exon 4), IDH2 (exons 4, 6), JAK2 (exons 12-15), KIT (exons 2, 8-11, 13-15, 17-18), KMT2A (exons 1-11, 27, 31, 35), KRAS (exons 2-4), MPL (exon 10), NF1 (exons 1-4, 6, 12, 30, 37-39, 41, 45, 49, 52, 58), NPM1 (exon 11), NRAS (exons 2-4), PDGFRA (exons 12, 14-15, 18), PHF6 (exons 2-10), PIGA (exons 2-6), PPM1D (exon 6), PTEN (exons 5, 7), PTPN11 (exons 3, 13), RAD21 (exons 2-14), RUNX1 (exons 2-9), SETBP1 (exon 4), SF3B1 (exons 13-16), SRSF2 (exon 1), STAG2 (exons 3-35), TET2 (exons 3-11), TP53 (exons 2-11), U2AF1 (exons 1-3, 5-6, 8), WT1 (exons 1-10), ZRSR2 (exons 1-11)). Briefly, pairs of DNA oligos targeting each region of interest were used in the first round of gene-specific PCR and the products subsequently purified via size selection. After purification, a second round of PCR adds index adaptors and P5 & P7 sequences to each library for sample tracking and sequencing. The resulting libraries were further purified and 2 x 250 bp paired-end sequenced on an Illumina MiSeq-system platform.

NGS Data Analysis

The analysis of NGS data performed all the typical steps of secondary analysis from reads mapping and refinement [1, 2] to the identification of single nucleotide variants (SNVs) and insertions/deletions (Indels). The variant calling step integrated four variant calling algorithms [3, 4, 5, 6] since such approach allows identifying variants with higher sensitivity and accuracy than using any single algorithm [7, 8]. Detected variants were annotated with information about their effects on the structure or function of proteins, their frequency in populations (e.g. dbSNP, ESP6500, ExAC, gnomAD) or their presence in cancer databases (e.g. COSMIC, cBioPortal for Cancer Genomics, ClinVar). Synonymous

variants, variants located outside protein coding regions and variants with VAF <2% were filtered. The remaining variants, were tagged using different criteria based on information retrieved from literature, sequence conservation and in silico prediction effect. Classification of germline variants was performed using Franklin (https://franklin.genoox.com) a curation tools based on the American College of Medical Genetics (ACMG) guidelines.

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Supplemental Table 1. Circumstance of CMD-NBV diagnosis.

		Precipitating event	**Diagnostic strategy**
UPN1	M/25	Idiopathic mesenteric, portal, splenic vein thrombosis	Screen for MPN
UPN2	M/52	Portal cavernoma, oesophageal varices, splenomegaly	Screen for MPN
UPN3	F/49	Unexplained popliteal arterial thrombosis	Screen for MPN
UPN4	M/57	Idiopathic sagittal cerebral sinus venous thrombosis	Screen for MPN
UPN5	F/44	Recurrent idiopathic mesenteric and portal vein thromboses	Screen for MPN
UPN6	M/23	Hemoglobin = 16 g/dL, familial sclerosing cholangitis	Evaluate polycythemia
UPN7	M/49	Idiopathic portal and spleen vein thromboses	Screen for MPN
UPN8	M/49	Popliteal arterial portal vein thromboses	Screen for MPN
UPN9	F/38	Idiopathic portal vein thrombosis	Screen for MPN
UPN10	M/44	Vertebral bone alteration	Screen for MPN
UPN11	F/42	Portal cavernoma, splenomegaly	Screen for MPN

UPN12	F/32	Portal vein thrombosis	Screen for MPN
UPN13	M/52	Splenomegaly	Evaluate splenomegaly
UPN14	F/46	Portal cavernoma, splenomegaly	Screen for MPN
UPN15	M/49	Renal vein thrombosis	Screen for MPN
UPN16	F/37	Platelets = 343 x10E+9/L	Evaluate thrombocytosis
UPN17	M/71	Splenomegaly	Evaluate splenomegaly
UPN18	F/46	Portal, mesenteric vein thromboses	Screen for MPN
UPN19	F/54	Portal vein thrombosis, Budd-Chiari syndrome	Screen for MPN
UPN20	M/29	Platelets = 343 x 10E+9/L	Evaluate thrombocytosis
UPN21	M/52	Idiopathic mesenteric vein thrombosis	Screen for MPN
UPN22	F/53	Eosinophilia, WBC = 10 x E+9/L)	Evaluate eosinophilia
UPN23	F/70	Hematocrit = 47,6%	Evaluate polycythaemia
UPN24	F/43	Idiopathic spleen vein thrombosis	Screen for MPN
UPN25	M/20	Idiopathic portal vein thrombosis, Budd-Chiari syndrome	Screen for MPN
UPN26	M/49	Portal cavernoma	Screen for MPN
UPN27	F/70	Pulmonary post-embolic hypertension	Screen for MPN
UPN28	M/39	Portal vein thrombosis	Screen for MPN
UPN29	F/55a	Portal cavernoma	Screen for MPN

UPN30	M/75	Splenomegaly	Evaluate splenomegaly

Supplemental Table 2. European Health Interview Survey (EHIS)-defined co-morbidities present in CMD-NBV subjects at the first referral at our Center

Co-morbidity present	**Subjects number (%)** **Total =30**
Asthma (allergic asthma included)	0
Chronic bromchitis, chronic obstructive pulmonary disease, emphysema	1 (3)
Myocardial infarction (heart attack) or chronic consequences of myocardial infarction	2 (7)
Coronary heart disease or angina pectoris	2 (7)
High blood pressure (hypertension)	10 (33)
Stroke (cerebral haemorrhage, cerebral thrombosis) or chronic consequences of stroke	3 (10)
Arthrosis (arthritis excluded)	0
Low back disorder or other chronic back defect	0
Neck disorder or other chronic neck defect	0
Diabetes	1 (3)
Allergy, such as rhinitis, hay fever, eye inflammation, dermatitis, food allergy or other allergy (allergic asthma excluded)	1 (3)
Cirrhosis of the liver	0
Urinary incontinence, problems in controlling the bladder	0
Kidney problems	1 (3)
Depression	1 (3)

Note: The 15 illnesses diagnosed before or in conjunction with CMD-NBV included hypertension, coronary heart disease and angina pectoris, myocardial infarction, stroke, diabetes, asthma, chronic bronchitis, chronic obstructive pulmonary disease and emphysema, urinary incontinence, kidney problems, spinal and back pain, neck spine pain, allergies, cirrhosis of the liver, arthrosis.

Supplemental Table 3. Horvat-defined comorbidities present in subjects with CMD-NBV. Data obtained at the first referral at our Center

	Subjects number (%) Total=30
Cardiovascular/metabolic	12 (40)
Autoimmune	10 (33)
Inflammatory	0
Malignancy	4 (13)

Supplemental Table 4. Putative germline variations in subjects with CMD-NBV

Patient ID	Gene	Nucleotide change	Aminoacid change	Transcript ID	Consequence	VAF (%)	ExAC Frequency	ClinVar Status	Franklin Status
UPN4	*ASXL1*	c.3306G>T	p.Glu1102Asp	NM_015338	missense	52	0.01	benign	polymorphism
UPN5	*DNMT3A*	c.1502A>G	p.Asn501Serqì	NM_022552	missense	42	0.0003	likely benign	likely benign
UPN8	*RUNX1*	c.167C>T	p.Leu56Ser	NM_001754.5	missense	51	0.01629	benign	benign
UPN10	CUX1	c.2371G>A	p.Ala791Thr	NM_181552R	missense	47	0.000017	NR	uncertain signficance
UPN10	*ABL1*	c.589G>A	p.Glu197Lys	NM_005157.6	missense	54	0.00027	uncertain significante/benign	uncertain signficance
UPN12	*CSF3R*	c.2422G>A	p.Glu808Lys	NM_000760.4	missense	50	0.00622	benign/likely benign	benign
UPN18	*NF1*	c.-22G>C	Null	NM_001042492	5'UTR variant	35	0.00358	benign/likely benign	benign
UPN20	*ASXL1*	c.*87A>G	Null	NM_015338	3'UTR variant	49	NR	NR	benign
UPN25	*TET2*	c.1018A>G	p.Ile340Val	NM_0011127208	missense	51	0.000025	NR	likely benign
UPN27	*NF1*	c.6790A>T	p.Ile2264Leu	NM_001042492	missense	50.6	0.000025	uncertain significante/likely benign	likely benign
UPN28	*TET2*	c.521C>A	p.Pro174His	NM_001127208	missense	44.6	0.0019	uncertain significance/likely benign	likely benign
UPN29	*KIT*	*c.101C>T*	p.Pro34Leu	NM_000222	missense	52	0.00006	Uncertain	uncertain significance

Patient ID	Gene	Nucleotide change	Aminoacid change	Transcript ID	Consequence	VAF (%)	ExAC Frequency	ClinVar Status	Franklin Status
								significance/likely benign	

Abbreviations: VAF, variant allele frequency; NR, not reported.