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

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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 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 (interquartile range [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 body mass index 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 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 myeloproliferative neoplasms. 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 ten 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 (MPN) 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] ten 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-center retrospective study, subjects with CMD-NBV were identified from the institutional database of the Center 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 >450x10^9/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] (i) BM megakaryocyte hyperplasia and dysplasia consistent with the 2009 WHO diagnostic criteria for pre-MF;[8] (ii) 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 ≥11x10^9/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 >8.8x10^9/L; monocytes >0.7x10^9/L, and platelets >390x10^9/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 MPN, 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 (VAF) 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 laboratory 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 *Online Supplemental Appendix*.

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 χ^2 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: (i) disease-associated hemoglobin concentration <100 g/L; (ii) spleen >10 cm below the left costal margin; (iii) platelets

<150x10^9/L; and/or (iv) WBC count <4x10^9/L or >12x10^9/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 nine 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; *Online Supplementary Table S1*).

Co-variates at diagnosis

Subject co-variates at diagnosis are displayed in Table 1. With median hematological co-variates values in the normal range, four, five and six subjects had hemoglobin, WBC and platelet concentration above the upper range of normal, while four 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, five increased basophils and five 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 co-existence 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 nine 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 four (13%) were obese.

Co- and multi-morbidities

At the time of our center referral, 14 subjects (47%) had one or more co-morbidities according to the Charlson co-morbidity criteria (CCI ≥1; Table 2): seven had a CCI =1, six a CCI =2, and one 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%): eight had one co-occurring morbidity, while three had 2, and three had 3 co-occurring morbidities. The most frequent CPI was arterial hypertension (N=10; *Online Supplementary Table S2*). According to the Horvat-defined co-morbidities, 15 subjects had 1 or more co-morbid condition (*Online Supplementary Table S3*). Thirteen subjects had a co-morbidity classified as cardiovascular or metabolic, nine as inflammatory or autoimmune, and four 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 one, normal in nine and decreased in three patients. 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 seven subjects an increased number of BM eosinophils and in three increased mast cells was reported.

Somatic and germline variants

A panel of 45 genes was sequenced in 24 of 30 subjects (80%). A total of ten variants were classified as pathogenic or likely pathogenic somatic variants spread across four

genes and six subjects (25%; 1.7/case mutation burden) (Table 4; *Online Supplementary Table S4*). Subject UPN14 had two mutations in *ASXL1*, subject UPN23 co-occurring mutations in *DNMT3A, TET2, SRSF2*, while subject UPN29 in *DNMT3A* and *TET2*. The range of VAF at diagnosis was 2.3-41%.

Ten subjects, representing 42% of those tested for NGS, harbored 12 heterozygous variants in *RUNX1, CUX1, ABL1, ASXL1, DNMT3A, CSF3R, TET2, NF1*, and *KIT* we defined germline having VAF 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.

Table 1. Baseline co-variates of subjects with clonal megakaryocyte dysplasia with normal blood values (N=30). Data are shown for the whole population and according to sex.

	All subjects N=30	Males N=16	Females N=14	*P*
Demography and anthropometry				
Age, years, 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 x10^9/L, median (IQR)	6.2 (5.7-7.8)	6.1 (5.3-7.8)	6.7 (5.9-8.7)	0.41
WBC ≥8.8x10^9/L, N (%)	5 (17)	3 (19)	2 (14)	-
WBC <4x10^9/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 x10^9, median (IQR)	486 (410-556)	496 (409-548)	463 (411-743)	0.98
Monocytes >700x10^9, N (%)	5 (17)	2 (12)	3 (21)	-
Platelets x10^9/L, median (IQR)	274 (205-371)	209 (192-278)	356 (277-396)	0.003
Platelets >390x10^9/L, N (%)	6 (20)	2 (12)	4 (28)	-
Platelets <150x10^9/L, N (%)	3 (10)	3 (19)	0 (0)	-
Spleen size, cm^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, xULN, 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 x10^6, 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

By dividing subjects according to sex, males had significantly higher hemoglobin concentrations than females. By contrast, males had lower platelet count and lactate dehydrogenase (LDH) plasma concentration than females. BMI: body mass index; IQR: interquartile range; ULN: upper limit of normal; LDH: lactate dehydrogenase; WBC: white blood count.

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 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 $108x10^9$/L, blood immature myeloid cells, blood CD34-positive cells $44x10^6$/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 <$150x10^9$/L at diagnosis and subjects UPN15 also had a WBC <$4x10^9$/L 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 second 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, five of 30

Table 2. Co-morbidities of subjects with clonal megakaryocyte dysplasia with normal blood values according to the Charlson co-morbidity index. Data were obtained at the first referral at our center.

Co-morbidities, N (%)	N of subjects (%) Total N=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 clonal megakaryocyte dysplasia with normal blood values 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 of subjects (%)**		
Megakaryocyte clusters Loose Dense	22 (73) 19 (63) 3 (10)		
Megakaryocyte nuclei Hyper-lobulated Bulbous	 2 (7) 14 (47)		
Small megakaryocytes	6 (20)		
Fibrosis Grade 0 Grade 1 Grade 2 Grade 3	 20 (67) 10 (33) 0 0		

subjects (17%) developed six primary second malignant or premalignant diseases, giving a post-diagnosis incidence of six 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

Table 4. Genetic and molecular profile of the 30 subjects diagnosed with clonal megakaryocyte dysplasia with normal blood values. Data were obtained at diagnosis.

Case #	Sex/age, years	Driver mutation (VAF%)	NGS-somatic variants (VAF%)	Cytogenetics
UPN1	M/25	*JAK2*^{V617F} (21)	Neg	-
UPN2	M/52	*JAK2*^{V617F} (10)	ND	XY
UPN3	F/49	*JAK2*^{V617F} (5)	Neg	-
UPN4	M/57	*JAK2*^{V617F} (5.2)	Neg	-
UPN5	F/44	*JAK2*^{V617F} (7.7)	Neg	-
UPN6	M/23	TN	ND	-
UPN7	M/49	*JAK2*^{V617F} (5.9)	Neg	-
UPN8	M/49	TN	Neg	XY
UPN9	F/38	*JAK2*^{V617F} (ND)	*TET2* (c.4585C>T) (p.Gln1529*) (2.7)	XX
UPN10	M/44	TN	Neg	-
UPN11	F/42	*JAK2*^{V617F} (33)	Neg	XX
UPN12	F/32	TN	Neg	XX
UPN13	M/52	*JAK2*^{V617F} (16)	ND	-
UPN14	F/46	*JAK2*^{V617F} (ND)	*ASXL1* (c.2077C>T) (p.Arg693*) (3.8) *ASXL1* (c.1900_1922 del) (p.Glu635fs) (41)	-
UPN15	M/49	*JAK2*^{V617F} (0.65)	*TET2* (c.4045-1G>A) (null) (3.9)	-
UPN16	F/37	TN	Neg	-
UPN17	M/71	TN	Neg	-
UPN18	F/46	*JAK2*^{V617F} (19)	Neg	-
UPN19	F/54	*JAK2*^{V617F} (ND)	ND	XX
UPN20	M/29	*JAK2*^{V617F} (7.8)	Neg	XY
UPN21	M/52	*JAK2*^{V617F} (0.19)	Neg	-
UPN22	F/53	TN	Neg	-
UPN23	F/70	*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	*JAK2*^{V617F} (5)	Neg	-
UPN25	M/20	TN	Neg	-
UPN26	M/49	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: variant allele frequency; M: male; F: female; Neg: negative; TN: triple negative; ND: not determined; NGS: next-generation sequencing.

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 subject 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% 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 MPN 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 co-morbid 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 co-morbidity.[23,24] European Health Interview Survey (EHIS) multimorbidity analysis showed that 48% of individuals with CMD-NBV have one or more co-morbidity, a rate higher than the 26.2 % reported in an European control population.[26] Finally, by using the Horvat classification of co-morbidity, cardiovascular and autoimmune co-morbidities 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.

Table 5. Major thrombotic events occurring in 30 subjects diagnosed with clonal megakaryocyte dysplasia with normal blood values, considering a time frame of 2 years before diagnosis up to the last follow-up.

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

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 four 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 strate-

gies 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.

Disclosures

RPG is a consultant to BeiGene Ltd., Fusion Pharma LLC, LaJolla NanoMedical Inc., Mingsight Parmaceuticals Inc., Kite Pharma and CStone Pharmaceuticals; is an advisor to Antegene Biotech LLC; is the medical director of FFF Enterprises Inc.; is a partner in AZACA Inc.; is on the board of directors of RakFond Foundation for Cancer Research Support; and is on the scientific advisory board of StemRad Ltd. All other authors have no conflicts of interest to disclose.

Contributions

GB designed the study, analyzed the data, and wrote the first version of the manuscript. VR and RPG contributed to writing the manuscript. AI, CT, MCF, AR and VDS enrolled patients. MM, RC, AC and CA led the database sample collection and clinical characterization efforts. TB, AR and LM revised the typescript and discussed the results. PC performed driver mutations genotyping for the dataset. LP and AG reviewed the bone marrow biopsies of patients diagnosed in the Hospital of Bergamo and Crema, respectively. AG, MG and MB performed DNA NGS analysis. AC helped with the statistical analysis. All authors have read and agreed to the published version of the manuscript.

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Data-sharing statement

All data generated or analyzed during this study are included in this article. Further enquieries can be directed to the corresponding author.

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