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Myeloproliferative neoplasms: Biology and treatment

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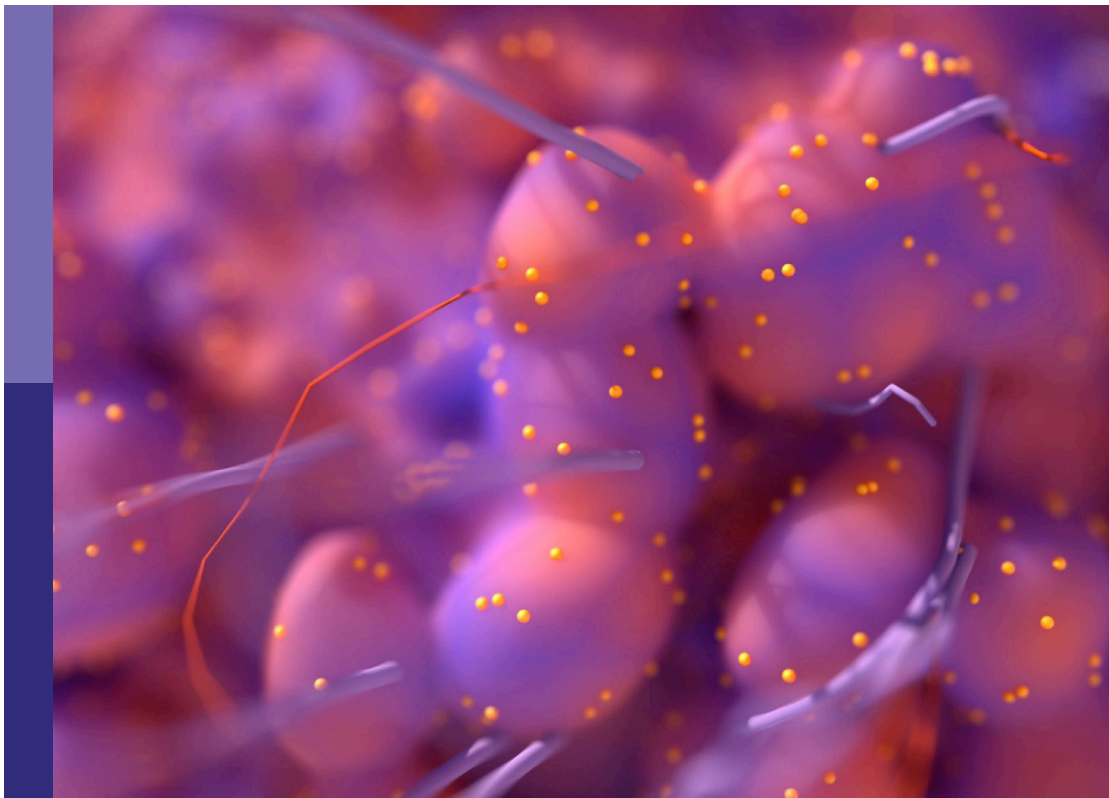

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Myeloproliferative neoplasms: Biology and treatment

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Case report: Double L611S/V617L *JAK2* mutation in a patient with polycythemia vera originally diagnosed with essential thrombocythemia

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Double *JAK2* mutations have rarely been described in myeloproliferative neoplasms (MPNs) and are demonstrated to be associated with the polycythemia vera (PV) phenotype. Here, we first report a case of a PV patient with a de novo double L611S/V617L in cis mutation of *JAK2*. A 40-year-old woman was admitted to the hospital with massive splenomegaly, multiple splenic infarcts, and abdominal pain. She had a 4-year history of erythrocytosis with an antecedent 10-year history of thrombocytosis before coming to our hospital. She was diagnosed with *JAK2* L611S/V617L double-mutant PV after a detailed medical examination in 2017. According to the literature, IFNα therapy can induce clinical, hematological, histopathological, and occasionally molecular remission in individuals with MPNs. Our report demonstrates that combination therapy with ruxolitinib and IFNα can lead to a substantial reduction in *JAK2* L611S/V617L double-mutant allele burden.

KEYWORDS

polycythemia vera, essential thrombocythemia, myeloproliferative neoplasm, *JAK2* L611S/V617L mutation, *CSF1R* mutation, phenotype transformation

Introduction

Somatic *JAK2* mutations are the most common disease-causing events in patients with myeloproliferative neoplasms (MPNs). Double *JAK2* mutations have rarely been described. A *JAK2* L611V missense mutation that presented in cis with V617F has been reported in three adult polycythemia vera (PV) patients, which resulted in the *JAK2* L611V/V617F double mutation (1). The coexistence of *JAK2* L611S and *JAK2* V617F variants was described in one adult PV patient who carried the *JAK2* L611S mutation in cis with the *JAK2* V617F mutation (2). A double L611S/V617F *JAK2* mutation was detected in a pediatric patient with PV (3). These double *JAK2* mutations were associated

with a PV phenotype. Acquisition of additional mutations in hematopoietic stem cells (HSCs), which creates either compound-mutant *JAK2* alleles or a compound-heterozygous state (4), could influence the subclones and affect the disease phenotype in MPNs.

Here, we first report a case of a PV patient with a *de novo JAK2* L611S/V617L double mutation *in cis*. A 4-year history of erythrocytosis with an antecedent 10-year history of thrombocytosis in this case suggests that essential thrombocythemia (ET) and PV are a continuum of one basic disease. Sørensen et al. had reported promising 2-year end-of-study results of the clinical trial investigating combination treatment with ruxolitinib and low-dose pegylated interferon-α2 (PEG-IFNα2), with improved cell counts, reduced bone marrow cellularity and fibrosis, decreased *JAK2* V617F burden, and reduced symptom burden with acceptable toxicity in several patients with PV or myelofibrosis (5). In our case, the combination of ruxolitinib and IFNα could improve treatment efficacy and produced a good outcome.

Case description

In January 2003, a woman suffered her first seizure. The initial hemogram showed a high platelet count (517×10^9/L) and a normal hemoglobin concentration, hematocrit level, and white blood cell count. She had no special medical, family, or psychosocial history. A diagnosis of epilepsy was made, and carbamazepine was prescribed by the doctor at the local hospital as seizure treatment. While the patient was taking carbamazepine for epilepsy from January 2003 to May 2009, her hemogram showed a moderately high platelet count (maximum value: 659×10^9/L). However, she did not have another blood test until November 2010.

In November 2010, her hemogram test showed a much higher platelet count (798×10^9/L) and a slightly elevated hemoglobin concentration (157 g/L), hematocrit level (47.20%), and white blood cell count (11.66×10^9/L). The bone marrow characteristics are the increase and clustering of pleiomorphic megakaryocytes with multi-lobulated nuclei and proliferation of myelopoiesis and erythropoiesis in a marked hypercellular bone marrow. Abdominal ultrasound examination revealed a mildly enlarged spleen (thickness, 4.4 cm; length, 11.8 cm). *BCR-ABL1* fluorescence in situ hybridization (FISH) showed a negative result. The patient was clinically diagnosed with ET without comprehensive genetic analysis for MPNs at the local hospital after exclusion of secondary causes for ET and received intermittent hydroxyurea therapy because of poor treatment co*MPL*iance. During the intermittent hydroxyurea treatment, the patient's hemogram showed fluctuating, moderately high platelet counts ($370–593 \times 10^9$/L).

Due to poor treatment co*MPL*iance, the patient did not receive hydroxyurea therapy from September 2011 to October 2017. A single hemogram test in October 2014 showed a high hemoglobin concentration (172 g/L), hematocrit level (55.5%), platelet count (393×10^9/L), and white blood cell count (10.7×10^9/L).

In November 2017, the patient required emergency medical treatment for abdominal pain. The hemogram showed a high hemoglobin concentration (182 g/L), hematocrit level (57%), platelet count (347×10^9/L), and white blood cell count (12.97×10^9/L). Abdominal MRI revealed giant splenomegaly (thickness, 8.6 cm; length, 23.5 cm), multifocal hemosiderin deposition, and multiple infarcts in the spleen. The patient had a 4-year history of erythrocytosis with an antecedent 10-year history of thrombocytosis before coming to our hospital. Her medical, family, or psychosocial histories were otherwise unremarkable. As such, she was directly transferred from the outpatient clinic to the inpatient department for further medical examination.

After detailed hematologic examination in November 2017, the patient's bone marrow morphological examination revealed hyperplasia of all three lineages with megakaryocyte hyperplasia with micromegakaryocytes. Reticular fiber staining showed mild increase in reticular fibers (grade 0 myelofibrosis). The bone marrow sa*MPL*es were investigated by flow cytometry. Flow cytometric analysis of HLA-DR, CD33, CD34, CD117, CD10, CD56, CD19, CD5, CD7, CD2, CD11b, CD15, CD13, CD16, CD71, CD41, and CD45 revealed no obvious expression disorder. There was no detection of abnormal blasts and the immunophenotypic characteristics analyzed by flow cytometry were normal. Chromosomal analyses of the bone marrow showed a normal karyotype. *BCR-ABL1* fusion gene variants (p190, p210, p230, and rare variants) were not detected by reverse transcription-polymerase chain reaction (RT-PCR). Rearrangements of the *PDGFRA, PDGFRB, or FGFR1* genes were not detected by FISH analysis. There was no detection of a *JAK2* V617F mutation, *JAK2* exon 12 mutation, *MPL* W515L mutation, *MPL* W515K mutation, or a *CALR*eticulin (*CALR*) exon 9 deletion or insertion by PCR with Sanger sequencing. We then performed next-generation sequencing (NGS) for whole exome sequencing (WES) of her bone marrow cells. This revealed *de novo* double mutations in *JAK2*, including the *JAK2* V617L mutation (c.1849G>C; p.V617L), with a variant allele frequency (VAF) of 44.0%, and the *JAK2* L611S mutation (c.1832T>C; p.L611S), with a VAF of 44.7%. She also had a colony-stimulating factor 1 receptor (*CSF1R*) mutation (c.1460C>T; p.A487V; VAF 46.2%). Neither the double L611S/V617L *JAK2* mutation nor other MPN-associated mutations were detected in her buccal mucosal cells by NGS-WES, but the *CSF1R* A487V mutation was detected with a VAF of 46.2%, and thus proven to be germline. The Integrative Genomics Viewer visualization tool showed that both *JAK2* variants were located in the same reads (Figure 1). Cranial MRI revealed ischemic degeneration in the bilateral frontal cortex. Gene mutations associated with epilepsy were not detected.

The patient was diagnosed with *JAK2* L611S/V617L double-mutant PV after a detailed medical examination,

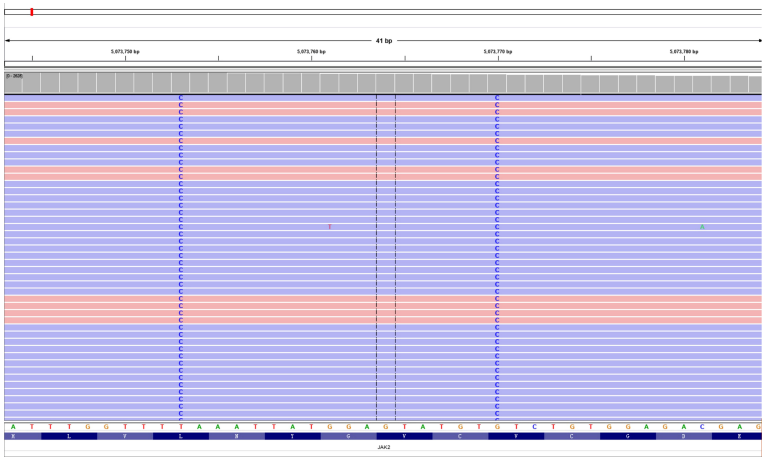

FIGURE 1
Double L611S/V617L *JAK2* mutation. Visualization of the two single-nucleotide variants in the aligned sequencing data using the Integrative Genomics Viewer (IGV) tool.

and she received continuous treatment with ruxolitinib from November 2017 and combination therapy with ruxolitinib and IFNα from January 2021 (Table 1). During the follow-up time from November 2017, the hemoglobin concentration, hematocrit level, platelet count, and white blood cell count returned to normal 10 weeks after treatment initiation. Significant reductions of 45% and 56% in the cross-sectional area of the spleen were achieved 12 weeks (spleen: thickness, 6.3 cm; length, 17.5 cm) and 2 years later (spleen: thickness, 5.7 cm; length, 15.7 cm), respectively. Alleviation of the constitutional symptoms including excessive and disabling fatigue, early satiety, anorexia, night sweats, abdominal pain and discomfort, and cognitive co*MPL*aints had improved her quality of life 6 months later. VAFs of both the *JAK2* L611S and *JAK2* V617L mutations were 44%, 15%, and 11% in November 2017, November 2019, and November 2020, respectively, which suggested that these mutations had occurred in the same clone. The addition of IFNα to ruxolitinib therapy substantially decreased *JAK2* L611S/V617L allele burden. After the patient received the combined treatment of IFNα and ruxolitinib for 6 months, the VAF of the double L611S/V617L *JAK2* mutation was reduced from 11% to 5% (Table 2). During the follow-up period from November 2017 to November 2020 (ruxolitinib alone), ruxolitinib improved the patient's splenomegaly and other symptoms, including early satiety, bloating, portal hypertension, fatigue, and undesired weight loss, which improved her overall quality of life.

TABLE 1 Clinical and biological characteristics of this patient with the double L611S/V617L *JAK2* mutation.

Time	January 2003	January 2003 to May 2009	November 2010	November 2010 to September 2011	October 2011 to October 2017	November 2017	December 2017 to December 2020	January 2021~
Diagnosis	Epilepsy	Epilepsy	ET Epilepsy	ET Epilepsy	ET switching to PV in 2014? Epilepsy	PV Epilepsy	PV Epilepsy	PV Epilepsy
PLT ($\times10^9$/L)	517	659	798	370–593	October 2014: 393	347	Normal	Normal
HGB (g/L)	142	Normal	157	Normal	October 2014: 172	182	Normal	Normal
HCT	40.0%	Normal	47.2%	Normal	October 2014: 55.5%	57.0%	Normal	Normal
Treatment	CBZ	CBZ	HU CBZ	HU CBZ	CBZ	Ruxolitinib CBZ	Ruxolitinib CBZ	IFNα + Ruxolitinib CBZ

WBC, white blood cell; HGB, hemoglobin; HCT, hematocrit; PLT, platelet; ET, essential thrombocythemia; PV, polycythemia vera; HU, hydroxyurea. CBZ, carbamazepine.

TABLE 2 Next-generation sequencing screening result of this patient.

Gene	Mutation	Exon	VAF (2017)	VAF (2019)	VAF (2020)	VAF (2021)
JAK2	NM_004972.3:c.1849G>C(p.V617L)	exon14	44.0%	15.2%	11.0%	5.6%
JAK2	NM_004972.3:c.1832T>C(p.L611S)	exon14	44.7%	15.5%	11.4%	5.4%
CSF1R	NM_005211.3:c.1460C>T(p.A487V)	exon10	46.2%	47.0%	50.1%	49.5%

VAF, variant allele frequency.

Diagnostic assessment

According to the new World Health Organization (WHO) 2016 criteria (6), the patient was clinically diagnosed with ET in November 2010. However, the ET may have existed in January 2003 considering disease evolution and medical history. The data in November 2017 confirmed a diagnosis of PV with a double *JAK2* L611S/V617L mutation according to the WHO 2016 criteria for PV. Massive splenomegaly and multiple splenic infarcts are poor prognostic factors.

Discussion

To our knowledge, this is the first report of a double *JAK2* L611S/V617L mutation in PV. The *JAK2* L611S mutation alone was detected in a child with a germline mutation associated with thrombocytosis (7). The presence of the *JAK2* L611S mutation leads to the constitutive activation of *JAK2*/STAT signaling, even in the absence of erythropoietin receptor expression (8). The *JAK2* V617L mutation was detected in an individual with ET, and germline fibroblast testing confirmed the somatic origin of the mutation (9). *JAK2* V617L and *JAK2* V617I mutations have previously been shown to induce cytokine independence and constitutive downstream signaling in Ba/F3 cells randomly mutated at position 617 of *JAK2* (10).

The double *JAK2* L611V/V617F mutation increases the activation of *JAK2*, AKT, and ERK1/2 (but not of STAT5) and is associated with isolated erythrocytosis (1). We speculate that a similar mechanism could underlie the effects of double *JAK2* L611S/V617L mutation.

The studies by Van Egeren et al. (9) and Williams et al. (11) showed that the latency between the acquisition of the driver gene mutation and the manifestation of MPN was much longer than that was generally assumed. This provides fascinating insights into the early steps in the pathogenesis of MPN, which might raise opportunities for earlier intervention and provide a new paradigm for cancer development.

Studies have revealed that the *JAK2* V617F allele burden, differential signal transducer and activator of transcription activation, order of other somatic mutations, and interindividual genetic variation influence the predisposition to specific MPN subtypes (12). However, the specific role of the double *JAK2* L611S/V617L mutation in our PV patient with a history of ET is unclear.

The presence of different mutations within the same gene often results in different biological properties that make it challenging for us to deeply understand the *de novo* double *JAK2* L611S/V617L *in cis* mutation in PV. This patient experienced a decrease in the *JAK2* L611S/V617L double mutation allele burden under the combination therapy of ruxolitinib and IFNα. The *de novo JAK2* L611S/V617L double mutation *in cis* identified in this case might be an oncogenic mutation or driver mutation, as *JAK2* V617F was discovered in 2005 as the driver mutation of the majority of non-*BCR-ABL1* MPNs (13, 14).

The patient in this study had a germline CSF1R mutation (c.1460C>T; p.A487V). Unlike the reported pathogenic germline CSF1R mutations located within the PTK domain (15), the mutation identified in our patient was located within the immunoglobulin domain. The clinical significance of the CSF1R A487V mutation remains unknown. The dominant transmembrane receptor controlling the differentiation and survival of almost all macrophages regardless of their origins is the colony-stimulating factor 1 receptor. Although the CSF1R A487V germline mutation is known to be nonpathogenic, we speculate that the presence of *JAK2* mutations in HSCs and endothelial cells, bone marrow-derived macrophages, or yolk sac-derived brain-specific macrophages with the CSF1R A487V mutation might cooperatively promote multiple splenic infarcts and ischemic degeneration in the bilateral frontal cortex (probably microthrombosis), which likely triggered her seizures. These seizures might be TIAs provoked by the increased number of more adhesive thrombocytes.

HSC heterogeneity underlies the disparate phenotypes of MPNs harboring the same initiating mutation, and malignant transformation of neoplasms might involve a specific subset of stem cells within a heterogeneous stem cell population. In ET, HSCs with *JAK2* mutations are more sensitive to IFN signaling to megakaryocyte differentiation and show strong megakaryocyte lineage priming (9). Furthermore, hematopoietic stem and progenitor cells (HSPCs) with *JAK2* mutations show a lineage bias towards the megakaryocyte-erythroid fate and contribute to the development of thrombosis (12). IFNα therapy can induce clinical, hematological, histopathological, and occasionally molecular remission in individuals with MPNs. Some results suggested that upon treatment (including long course therapeutic dose of IFNα), the mutant megakaryocyte-primed HSC population

was reduced by promoting apoptosis or quiescence of the mutant cells (12). Clinical evaluation of pegylated IFNα-2a and ruxolitinib in a phase II study of PV and MF showed substantial reductions of *JAK2* V617F allele burden with 41% of patients showing a molecular response along with improved cytosis and fibrosis as well as acceptable toxicity (5). These findings demonstrate that pegylated IFNα adds substantial clone suppression to ruxolitinib therapy and that the combination with ruxolitinib improves tolerability of IFNα therapy. These results suggested that our patient would benefit from sustained combination therapy with ruxolitinib and IFNα.

A recent study confirmed that therapeutic targeting of Y-Box Binding Protein 1 (YBX1)-dependent ERK signaling in combination with *JAK2* inhibition could eradicate cells harboring mutations in *JAK2* (16). The ruxolitinib/nilotinib/prednisone combination showed synergistic inhibitory effects on the JAK/STAT and MAPK signaling pathways in MPN cells (17, 18). Thus, IFNα combined with *JAK2* or MEK inhibition might improve therapeutic efficacy.

Given the lifelong trajectories of MPNs in humans and considering the underlying mechanisms in MPNs, the heterogeneity of *JAK2* mutant HSCs and HSPCs, and clonal expansion and evolution, studies to increase the understanding of MPNs and to improve our management of MPN patients are needed.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by The Third Affiliated Hospital of Sun Yat-Sen University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

X-QL participated in the clinical care, analyzed the data, and was a major contributor in writing the manuscript. J-JL conducted the literature search and revised the manuscript. C-CL conducted the study, provided patient care, analyzed the data, and contributed with final manuscript drafting. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A multicenter, open-label study for efficacy and safety evaluation of anagrelide in patients with treatment-naïve, high-risk essential thrombocythemia as a primary treatment

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As the discussion of first-line anagrelide treatment is ongoing, we aimed to prospectively examine the efficacy and safety of anagrelide in cytoreduction therapy-naïve high risk essential thrombocythemia (ET) patients in Korea. Seventy patients from 12 centers were treated with anagrelide monotherapy for up to 8 weeks, followed up until 24 months. At week 8, 50.0% of the patients were able to achieve platelet < 600 x 10^9/L, and by 12 months, 55/70 (78.6%) patients stayed on anagrelide, and 40.0% patients showed platelet normalization. 14 patients required additional hydroxyurea (HU) for cytoreduction. The median daily dose of needed HU was 500mg (range 250mg – 1500mg). The efficacy was independent of the somatic mutation

status. There were 4 thromboembolic events and 7 bleeding events during the follow-up period. The most common adverse events associated with anagrelide use were headache, followed by palpitation/chest discomfort, edema and generalized weakness/fatigue. 7 patients wished to discontinue anagrelide treatment due to adverse events (3 due to headache; 2 due to edema; 1 due to palpitation and 1 due to skin eruption). All in all, first-line anagrelide treatment showed a favorable response with tolerable safety profiles regardless of somatic mutation status.

KEYWORDS

essential thrombocythemia, high risk, Anagrelide, phase IV clinical trial, myeloproliferative neoplasms

Introduction

Essential thrombocythemia (ET) is a type of myeloproliferative neoplasm characterized by abnormal proliferation of megakaryocytes in the bone marrow leading to elevated platelet counts (1). Since ET patients have an almost normal survival, any survival effect of treatment is very difficult to prove. Thus, the goal of ET treatment is reduction of thrombotic and hemorrhagic complications, and platelet reduction to <400 x10 ^{9}/L. While cytoreductive therapy with or without low-dose aspirin is the mainstay of thrombosis risk reduction, the optimal choice of a therapeutic agent is less clear. Several agents, including hydroxyurea, anagrelide, and interferon are used for this purpose, but there are only a handful of data directly comparing these agents (2–7). As such, there is a gap in preferred therapeutic agents among the continents: based on the ANAHYDRET study which showed non-inferiority of anagrelide to hydroxyurea (5), anagrelide is used as first-line therapy for high-risk ET patients in Korea. On the other hand, due to concerns about leukemogenesis observed in the EXELS study (6, 8, 9) and conflicting results of PT-1 trial data (7), anagrelide remains a second-line therapy in Europe.

Anagrelide is an amidazoquinazolin derivative, originally developed as an anticoagulation drug, which was shown to have a potent platelet reducing effect (10). It is the only platelet-specific cytoreductive drug known, having no inhibitory effects on red or white cell progenitor proliferation. It reduces platelet production by inhibiting megakaryocyte colony development, thus producing a left-shift in megakaryocyte maturation, reducing megakaryocyte size, and maturation (11). As discussion of anagrelide efficacy and safety is ongoing and a consensus has not been reached, we aimed to prospectively examine the efficacy and safety of anagrelide as cytoreduction therapy-naïve high-risk ET patients in Korea.

Methods

Design overview

This was a multi-center, prospective observation study (ClinicalTrials.gov identifier: NCT03232177). The aim of the study was to examine the efficacy and safety of first-line anagrelide treatment in high-risk Korean ET patients. The primary objective was to determine the percentage of patients who had hematologic response, defined as platelet count < 600 x 10 ^{9}/L by week 8 on anagrelide monotherapy. The secondary objectives included (1) platelet normalization rates (platelet < 400 x 10^{9}/L) at week 8 and 12 months per European LeukemiaNet guidelines (12) (2); platelet reduction by more than 50% at week 8 and 12 months; (3) anagrelide safety, tolerability and compliance at 12 months. We also evaluated somatic mutation profiles and anagrelide efficacy as an exploratory objective. The interest in mutation profiling was instigated by 2 recent small studies suggesting that anagrelide may be more effective in *JAK2* positive patients than *CALR* positive patients (13, 14). Specifics target sequencing methods are available in Supplementary Materials.

Study population

Patients older than 18 years with ET diagnosed according to 2016 WHO classification (1) were screened. Those participants with high-risk ET [age older than 60 years or a history of vascular complications (15–17)] and cytoreductive treatment naïve were invited to participate in the study, regardless of mutational status. Patients with underlying medical conditions requiring active interventions, inadequate organ (cardiac,

hepatic, renal, pancreatic) functions, pregnancy, concurrent malignancies, or taking phosphodiesterase III/IV drugs were excluded. The study was conducted according to the Declaration of Helsinki and was approved by the institutional review board (IRB) of each hospital. Informed consent was taken from all patients before participating in any study-related procedure.

Interventions

From week 1 to week 8, anagrelide monotherapy was required. Patients were started on anagrelide 0.5mg twice a day and after 1-week dose escalation was allowed. From week 9, additional agents for cytoreduction were allowed per attending physician's discretion. The maximum anagrelide dose allowed was 10mg/day (2.5mg four times a day). Patients were followed up at week 1, week 4, week 8, 3 months, 6 months, 9 months, 12 months, 18 months and 24 months for lab testing and drug compliance monitoring. The adverse events (AE) were assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.03 (available at: https://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03/CTCAE_4.03_2010-06-14_QuickReference_8.5x11.pdf). The bleeding and thrombotic complications were graded according to International Society on Thrombosis and Hemostasis (ISTH) (18), which are presented in Supplementary Materials in details.

Statistical analysis

Fisher's exact test was used for nominal variables, and Mann-Whitney U test was used for continuous variables. For all statistical analyses of effective variables, two-tailed tests were performed. p-values of <0.05 were considered statistically significant. All data were analyzed using the Statistical Package for the Social Sciences software (IBM® SPSS®Statistics, version 25.0).

Results

Baseline characteristics

Table 1 shows the baseline characteristics of all 70 patients (34 males and 36 females) enrolled. The median age at ET diagnosis was 69 years (range 24 – 90), and time to study enrollment from diagnosis was median 13 days. The majority of the patients harbored *JAK2V617F* mutation (48/69, 68.6%) and there were 13 (18.8%) triple-negative patients. As for the adjunct anticoagulation therapy, 51/70 (72.9%) took aspirin, 14/70 (20.0%) plavix, 2/70 (2.9%) enoxaparin, 2/70 (2.9%) edoxaban and 1/70 (1.4%) dabigatran.

TABLE 1 Baseline characteristics.

Characteristics	Total (N=70)
Sex, male (N, %)	34
Age at study enrollment, years (median, range)	69 (24-90)
>60 years old	50 (71.4)
Diagnosis to study enrollment, days (median, range)	13 (0-792)
Mutation status (N, %)	
JAK2V617F	48/69 (68.6)
CALR	6/69 (8.6)
MPL	6/69 (8.6)
Triple negative	13/69 (18.8)
Previous drug exposure (N, %)	
Hydroxyurea	0 (0)
Anagrelide	0 (0)
Interferon	0 (0)
Baseline laboratory findings (mean, ± SD)	
WBC ($x10^9$/L)	10.4 (0.4)
Hemoglobin (g/dL)	13.8 (0.2)
Platelet ($x10^9$/L)	931.4 (40.0)
Baseline platelet > 1000 x 10^9/L	21 (30.0)
Cardiovascular risk factors (N, %)	
Hypertension	29 (41.4)
Diabetes	11 (15.7)
Dyslipidemia	10 (14.3)
BMI > 25	27 (38.6)
Prior history of thrombosis	13 (18.6)
IPSET-Thrombosis risk stratification	
Low	7 (10.0)
Intermediate	6 (8.6)
High	57 (81.4)

ET, essential thrombocythemia; SD, standard deviation; WBC, white blood cell count; BMI, body mass index; IPSET, International Prognostic Score.

Treatment outcomes

The response was evaluated in the 64 patients who stayed on anagrelide treatment at week 8 (Table 2). Platelet normalization was documented in 20.3% of the patients by week 8, and 40.0% by month 12. Platelet reduction by more than 50% was seen in 18.8% of the patients by week 8, and in 43.6% of the patients by month 12.

As shown in Table 2, there were 4 thromboembolic events (20) (3 major arterial thromboses; 1 minor arterial event) and 7 bleeding events (2 major bleeding events; 5 minor bleeding events) during the follow-up period. There were no venous thrombosis/events during the follow-up. One patient with a history of carotid artery stenosis and hepatitis B suffered from an angina pectoris attack requiring intervention. Of the 3 patients who experienced cerebral infarction, one had the previous history of transient ischemic attack, one had a history of thyroid cancer, and one had alcoholic liver cirrhosis. As for the bleeding events, the patient who had intracranial

TABLE 2 Treatment related outcomes.

Parameters	N, %
Response*	
Platelet < 600 (x10^9/L) at 8 weeks	32/64 (50.0)
Platelet reduction ≥ 50% at 8 weeks	12/64 (18.8)
Platelet normalization (≤ 400 x 10^9/L) at 8 weeks	13/64 (20.3)
Platelet reduction ≥ 50% at 12 months	24/55 (43.6)
Platelet normalization (≤ 400 x 10^9/L) at 12 months	22/55 (40.0)
Additional cytoreductive agents use	
Hydroxyurea	14/69 (20.3)
250mg	3
500mg	8
1000mg	1
1500mg	2
Thromboembolic events, patients**	
Cerebral infarction (major arterial thrombosis)	3
Angina pectoris (minor arterial event)	1
Bleeding events, patients*	
GI bleeding (major bleeding event)	1
Ecchymosis (minor bleeding event)	2
Epistaxis (minor bleeding event)	3
Intracranial hemorrhage (major bleeding event)	1

*Response was calculated from patients stayed on anagrelide treatment regardless of compliance.
**According to predefined criteria (19).

hemorrhage had a history of cerebral infarction and hypertension.

Adverse events

As for tolerability, 64/70 (91.4%) patients remained on anagrelide monotherapy by week 8. Among the 64 patients remaining on anagrelide, all but 1 had taken ≥80% of the prescribed medication. By 12 months, 55/70 (78.6%) patients stayed on anagrelide and 54/55 patients showed good compliance to the drug (Table 3).

Anagrelide treatment had minimal effects on white blood cell counts and hemoglobin level as shown in Figure 1. The median dose of anagrelide required was 2.5mg per day (Figure 2). Fourteen patients required additional hydroxyurea (HU) for cytoreduction. The median dose of needed HU was 500mg (range, 250mg – 1500mg). Fortunately, there were no differences in adverse events profile among those taking additional hydroxyurea versus those who did not.

The most frequent AE associated with anagrelide use was headache, followed by palpitation/chest discomfort, edema and generalized weakness/fatigue. The most common AE ≥ grade 3 was epigastric pain, followed by headache, edema and dyspepsia. 7 patients wished to discontinue anagrelide treatment due to adverse events (3 due to headache; 2 due to edema; 1 due to

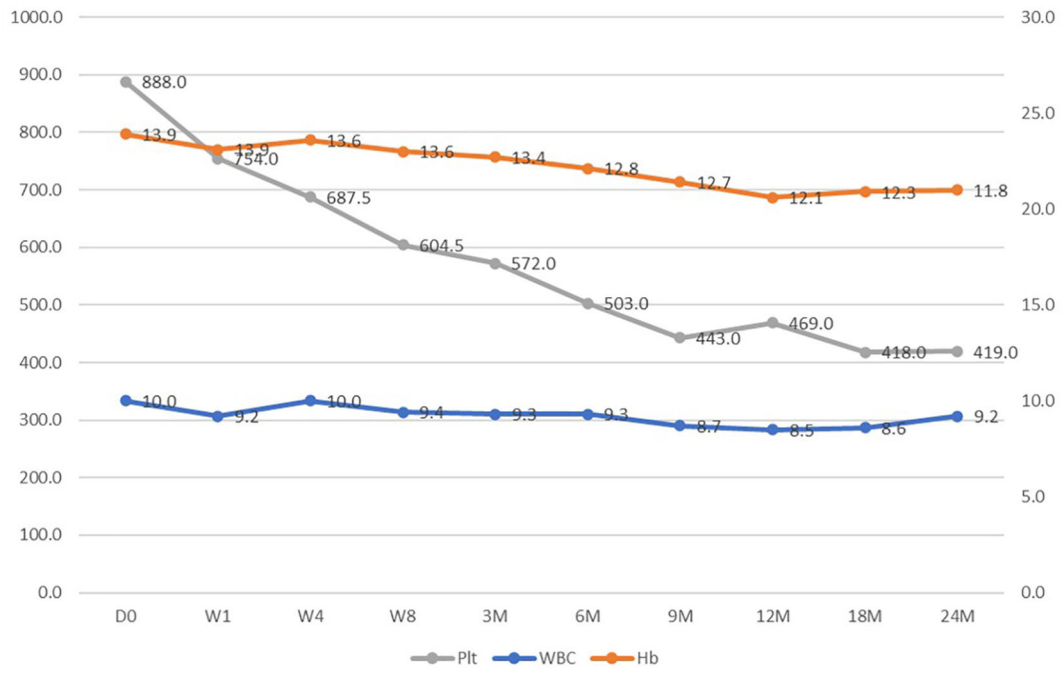


FIGURE 1
Hemogram changes over the treatment course.

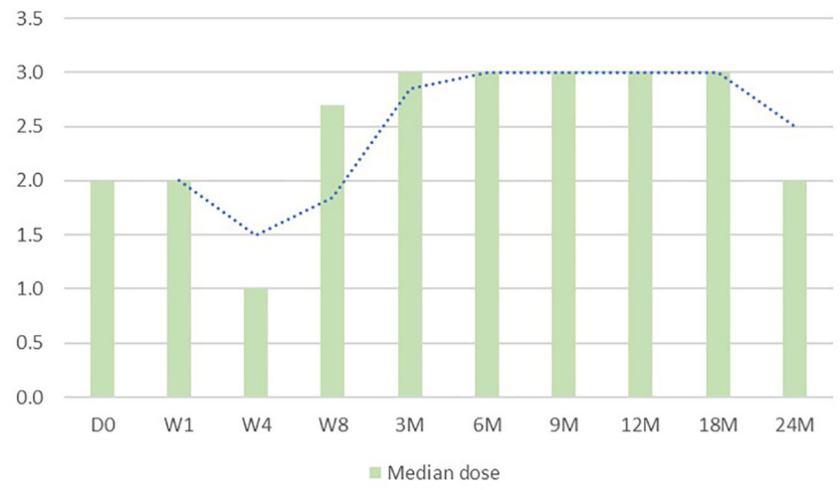


FIGURE 2
Changes in anagrelide dose over the treatment course.

palpitation and 1 due to skin eruption). There were no acute leukemia transformation or myelofibrosis transformation during the follow-up.

Somatic mutational profile

The overall somatic mutational profile is shown in Figure 3. Interestingly, 4 patients showed concurrent *JAK2V617F* mutation and *MPL* mutation. There were no patients harboring *JAK2 exon 12* mutation. Other than the known driver mutations, *ASXL1* was identified in 21/70 (30.0%) patients, followed by *TET2* 10/70 (9.8%) and *KIT* 6/70 (8.6%).

Figure 4 shows the mutation status according to treatment efficacy. The mutation status was not related to anagrelide treatment response. More specifically, among patients meeting primary endpoint of platelet count < 600 x 10^9/L by week 8 74.2% harbored *JAK2V617F* mutation while 65.6% of the non-

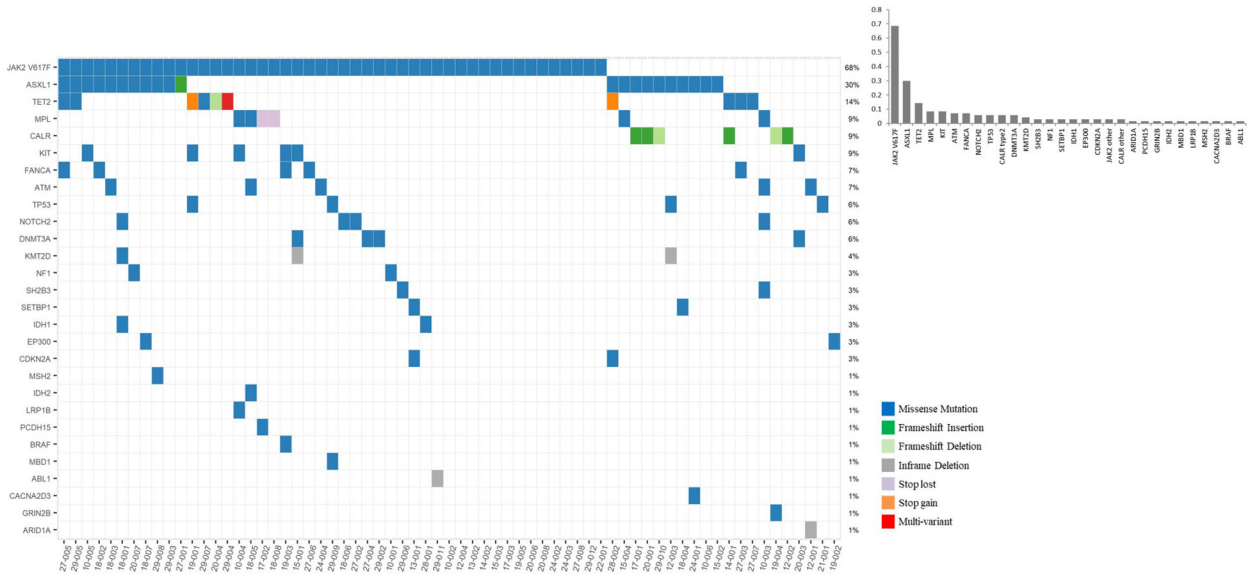
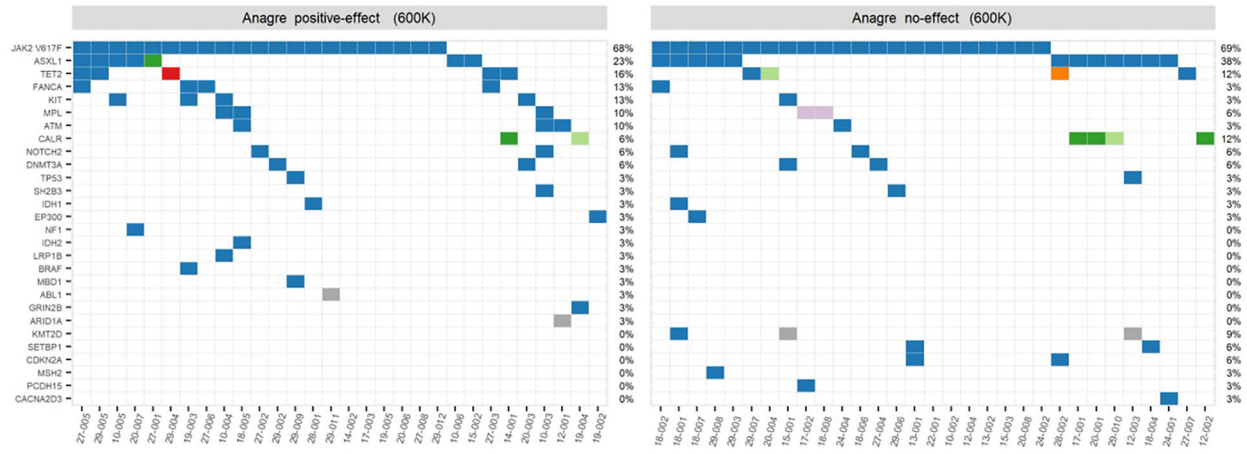


FIGURE 3
Baseline somatic mutation profiles of essential thrombocythemia patients treated with anagrelide.



	Responsive	Non-responsive	P
JAK2 V617F positivity	23/31 (74.2%)	21/32 (65.6%)	0.459
Mutations other than JAK2 V617F	8/31 (25.8%)	10/32 (31.3%)	0.438

FIGURE 4
Somatic mutation landscape according to treatment efficacy, patients reaching platelet level < 600 x 10^9/L versus ≥600 x 10^9/L at week 8.

responders harbored *JAK2V617F* mutation (p=0.459). Non-*JAK2V617F* mutations were noted in 8 responders (25.8%), while 10 (31.3%) non-responders harbored mutations other than *JAK2V617F* (p=0.438).

Discussion

In this study, we present the efficacy and safety results of first-line anagrelide treatment for high-risk ET based on prospective clinical data. The importance of our study lies in that 1) this is a rare prospective study using anagrelide as first-line; 2) the efficacy of anagrelide monotherapy was tested, and in relation to genetic background; 3) ethnically homogeneous population with high-risk ET were enrolled. Overall, we report a 50% response rate with anagrelide monotherapy and a 40% complete remission rate at 1 year with 78.6% of the patients staying on the medication with good tolerability.

It is known that approximately 60% of ET patients harbor *JAK2V617F* mutation (21). Likewise, 68.6% (48/69) of our patients showed *JAK2V617F* mutation (Figure 1). Although relatively small in number, along with a previous Japanese study reporting JAK2V617F mutation rate at 64.2% for ET (22), there seem to be no ethnical differences in ET mutational profiles. Interestingly, there were only 6 patients (8.6%) harboring *CALR* mutation. This is probably because our study enrolled only high-risk ET patients defined as older than 60 years old or having a history of vascular complications. It is well-known that *CALR*-mutated ET patients show higher platelet count and lower thrombotic risk compared to *JAK2V617F*-positive ET patients (23).

Interestingly, there were 4 patients who harbored both *JAK2V617F* and *MPL* mutations. *MPL* mutations were noncanonical mutation (*T374A*) in 3 patients, but a canonical mutation in the other one patient (*W515L*). Somatic mutations in *TP53*, an important prognostic factor in MPN, was found in 4 patients (5.8%). Another DNA repair gene, *ATM* mutation was found in 5/69 (7.2%) patients. Longer follow-ups are required to determine the prognostic values of these mutations.

The efficacy of anagrelide, on the other hand, was not determined by mutational status. Half of the enrolled patients achieved platelet level < 600 x 10 ^{9}/L by week 8 with anagrelide monotherapy, irrespective of driver mutation status (Table 2; Figure 4; Supplementary Figure). This is numerically lower than the one reported by Ito et al. (22), who reported response rate of 83%. We believe such discrepancy is due to the difference in study schema and study population. At 12 months, regardless of driver mutation status, 40.0% of the patients achieved platelet normalization and 43.6% of the patients saw more than 50% reduction in platelet level from baseline. Since anagrelide is not a specific targeted agent but rather its platelet-reducing effect is mediated through reduction in pro-platelet formation (6, 8), it is understandable that the mutation status does not affect the drug's efficacy.

The medication was relatively well tolerated: 74.3% of the patients stayed on anagrelide at 24 months with a median dose of 2 mg. Fortunately, no one experienced anemia development with anagrelide treatment. As with previous reports (3, 19, 22),

TABLE 3 Adverse events.

Parameters	N
Stayed on anagrelide treatment	
Baseline	70/70 (100)
Week 1	69/70 (98.6)
Week 4	66/70 (94.3)
Week 8	64/70 (91.4)
6 months	59/70 (84.3)
12 months	55/70 (78.6)
24 months	52/70 (74.3)
Medication compliance*	
Week 1	65/69 (94.2)
Week 4	63/66 (95.5)
Week 8	63/64 (98.4)
12 months	54/55 (98.1)
Reasons for anagrelide discontinuation	
Adverse events	7/17 (41.2)
Lost to follow-up	3/17 (17.6)
Consent withdrawal	7/17 (41.2)
Symptoms related to anagrelide, any (grade ≥3), events	
Headache	26 (4)
Palpitation/chest discomfort	19 (2)
Edem	17 (4)
Generalized weakness/fatigue	17 (1)
Dyspepsia	12(4)
Itching/pruritis	10(3)
Diarrhea/loose stool	10 (2)
Dizziness/light headedness	10 (0)
Epigastric pain	8 (5)
Dyspnea	7 (1)
Abdominal pain	3 (0)
Skin eruption/rash	5 (1)
Bone pain	5 (1)
Anorexia	5 (2)
Constipation	4 (1)
Neuropathy/tingling sensation	4 (2)
Nausea	3 (1)
Uncontrolled blood pressure	3 (1)
Myalgia	3 (0)
Liver function test elevation	2 (1)
Creatinine elevation	2 (2)
Insomnia	1 (1)
Serum glucose elevation	1 (0)

*Taken ≥80% of the prescribed drugs.

the 2 most common adverse events were headaches and palpitations. In our study, 3 patients decided to discontinue anagrelide due to headache and 1 due to palpitation. Overall, there were only 4 thromboembolic events and 7 bleeding events during the 24-month follow-up period. Numerically, these are fewer compared to previous studies, which reported 11.5% (14/122) major events and 36.9% (45/122) minor events (5). Interpretation of such discrepancy warrants caution: traditionally Asians have been associated with lower incidence of thromboembolism (19, 24, 25) but recently it was reported that Korean patients have similar frequency of thrombosis compared to Western patients (26, 27). In fact, one of the major limitations of our study is that we are not able to determine if anagrelide can significantly decrease thrombosis or bleeding risk because this was a single-arm study without a control arm and because only 4 thrombotic events occurred during a follow-up time of 24 months. Since the fundamental goal of treatment in high-risk ET is a reduction of thrombosis and hemorrhage through platelet reduction to preferably normal range, we aim to investigate further if platelet response correlates with vascular events through a longer follow-up. Lastly, also due to short follow-up period, we were not able to document secondary transformation of ET. This, too, warrants a longer follow-up study.

In conclusion, our study supports the use of anagrelide as a first-line cytoreductive agent in high-risk ET. Longer and more detailed follow-up on thrombotic and bleeding complications should ensue.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA853096.

Ethics statement

The studies involving human participants were reviewed and approved by Seoul National University Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Contribution: D-YS and S-SY created the concept and design; all authors provided the study materials or patients; JB and D-YS collected and assembled the data; and all authors contributed to the writing and the final approval of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.989984/full#supplementary-material

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An alternative dosing strategy for ropeginterferon alfa-2b may help improve outcomes in myeloproliferative neoplasms: An overview of previous and ongoing studies with perspectives on the future

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Ropeginterferon alfa-2b is a novel, long-acting mono-pegylated proline-IFN-alpha-2b approved for treatment of polycythemia vera in adults, regardless of thrombotic risk level or treatment history. Clinical trial data indicate the dose and titration of ropeginterferon alfa-2b is safe and effective. However, additional studies may provide rationale for an amended, higher initial dosage and rapid titration. This article is an overview of current and upcoming studies of ropeginterferon alfa-2b in myeloproliferative neoplasms that support the exploration of an amended dosing scheme in order to optimize patient tolerability and efficacy outcomes.

KEYWORDS

ropeginterferon alfa-2b, myeloproliferative neoplasm, polycythemia vera, alternative dosing strategy, clinical study, interferon, pegylated interferon

Introduction

Polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF) including pre-fibrotic/early primary myelofibrosis (PMF) are classical Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs) characterized by the uncontrolled clonal proliferation of hematopoietic stem or progenitor cells due to driver mutations of genes including *JAK2*, *CALR*, or *MPL* (1, 2). Thrombohemorrhagic complications and leukemic transformations are part of the natural history of MPNs (3, 4). Most PV patients harbor a *JAK2* mutation, the vast majority being a point mutation on exon 14 (V617F) (4, 5). PV is considered an inflammatory neoplasm (4, 6) characterized by clonal erythrocytosis, often

accompanied by thrombocytosis and leukocytosis, leading to a high risk of thromboembolic events (7).

Interferon (IFN) alfa-based therapies demonstrate preferential activity against neoplastic hematopoietic stem or progenitor cells and elicit complete and durable hematologic remission and molecular response, reduce PV progressive events, and shows improvement of patient myelofibrosis-free and overall survival (8–13). Ropeginterferon alfa-2b is a novel mono-pegylated IFN with pharmacokinetic properties allowing dosing once every 2 to 4 weeks (14–18). It was approved by the US Food and Drug Administration (FDA) in November 2021 and the European Medicines Agency (EMA) in February 2019 for adults with PV and is the first and only IFN approved for PV treatment (19, 20). The NCCN Guidelines place ropeginterferon alfa-2b as a treatment option for low (Category 2B) and high-risk PV patients (Category 2A) (2). The European LeukemiaNet (ELN) recommends ropeginterferon alfa-2b and pegylated IFN alfa-2a as a therapeutic option for treatment naive patients with low-risk PV requiring cytoreductive therapy (21).

The safety and efficacy of ropeginterferon alfa-2b was assessed in the pivotal phase 1/2 PEGINVERA, phase 3 PROUD-PV, and its extension CONTINUATION-PV studies (11, 12, 15, 22). Doses in these studies ranged from 50 micrograms (mcg) to 540 mcg every two weeks, leading to the recommended starting dose of 100 mcg [50 mcg for patients receiving hydroxyurea (HU)], and increasing 50 mcg every two weeks to a maximum of 500 mcg, until hematological parameters are stabilized (hematocrit <45%, platelets <400 x 10^9/L, WBC <10 x 10^9/L) (14). The dosing interval may increase to 4 weeks upon achieving hematological stability for at least one year on a stable dose (14). Since these studies were reported, additional clinical investigation into alternative dosing strategies suggest a potential role for rapid titration and higher starting doses of ropeginterferon alfa-2b.

The safety of ropeginterferon alfa-2b was also evaluated in patients with chronic hepatitis B or hepatitis C (genotypes 1 and 2) at doses ranging from 270 to 450 mcg every 2 weeks as monotherapy or in combination with ribavirin (23–27), or in COVID-19 patients in combination with standard of care at 250 mcg (28). Most reported adverse events (AEs) during ropeginterferon alfa-2b treatment were mild or moderate and toxicities ≥grade 3 were uncommon. These results suggest tolerability and safety of higher starting doses of ropeginterferon alfa-2b.

In this article, we review the clinical development of ropeginterferon alfa-2b in PV and current research extending into other related MPNs in order to closely examine a critical connection between an amended dosing schema and key disease outcome measures such as a reduction in thromboembolic risk, complete hematologic response, and reduction in the driver mutation variant allele frequency (VAF), while maintaining adequate safety and tolerability.

Polycythemia vera

Clinical trial experience

The PEGINVERA study was a phase 1/2 multicenter study conducted to evaluate the dosing, tolerability, and efficacy of ropeginterferon alfa-2b in PV patients (15). A diverse PV patient population was enrolled including newly diagnosed, those pretreated with HU, and those receiving HU, who were at low- or high-risk for thromboembolic events. Eight subcutaneously administered dose levels (50, 100, 150, 225, 300, 360, 450, and 540 mcg) given every 2 weeks were explored. The Phase 1 portion of the study aimed to identify the maximum tolerated dose (MTD) of ropeginterferon alfa-2b in 25 patients using a 3 + 3 dose-escalation method. No dose-limiting toxicities (DLTs) were observed and the MTD was determined to be the highest dose level that was evaluated, or 540 mcg. The Phase 2 portion of the PEGINVERA study was designed to assess efficacy in the form of complete response (CR) including hematologic remission according to modified ELN criteria (Table 1) and molecular response (MR) in 26 patients with dose titrations based on disease response and tolerability. After a median time of 5 years of treatment, results from this study showed that 27 of 42 patients (64.3%) attained a complete hematologic response (CHR) from the efficacy analysis set. It required a median time of 34 weeks of treatment for patients to achieve a CHR (22). For *JAK2*V617F VAF, 12/42 patients (28.6%) achieved a complete molecular response (CMR) as the best observed response. A median time of 82 weeks was required for patients to achieve a CMR and 34 weeks to achieve any MR. The study demonstrated that ropeginterferon alfa-2b treatment for up to 7 years was efficacious and well-tolerated (22).

The phase 3 PROUD-PV study (N=254) enrolled early-stage patients diagnosed with PV who were either HU naïve or those who had been treated with HU for <3 years (11, 12). Patients underwent stratified randomization by age, history of thrombosis, and prior use of HU, and then randomized to ropeginterferon alfa-2b or HU group. The primary endpoint was disease response rate after 12 months of therapy. Dose initiation and titration were similar to the PEGINVERA Phase 2 trial. Ropeginterferon alfa-2b treatment was started at a dose of 100 mcg or 50 mcg for patients receiving HU and the dose increased by 50 mcg every 2 weeks for the maximum dose of 500 mcg. It was estimated that the mean efficacious dose was reached after approximately 16.2 weeks (95% CI 14·8–17·6) for ropeginterferon alfa-2b and 11.4 weeks (95% CI 10·2–12·6) for HU (11). At 12 months, the PROUD-PV study failed to demonstrate superiority regarding the primary endpoint (ropeginterferon alfa-2b 21% vs. HU 28%) and secondary endpoints of CHR (ropeginterferon alfa-2b 43% vs. HU 46%) and MR (ropeginterferon alfa-2b 34% vs. HU 42%) (11). It is unknown if the difference in time to reach an effective dose or an optimal maximum dose plateau contributed to the observed outcomes.

The extension study, CONTINUATION-PV, recruited 171 patients who completed the PROUD-PV study (95 in the ropeginterferon alfa-2b group vs. 76 in control) (11). While the control group was allowed to change treatment from HU to best available treatment (BAT), 64/66 patients (97%) remained on HU treatment. The dose for ropeginterferon alfa-2b used in PROUD-PV was continued at the discretion of the investigator and administered every 2, 3, or 4 weeks. Ropeginterferon alfa-2b group showed a continuous trend of improved outcomes. At 36 months, ropeginterferon alfa-2b treatment led to CHR in 67 of 95 patients (71%) compared to 38 of 74 (51%) in the HU/BAT group (p=0·012). The ropeginterferon alfa-2b group showed a superior rate in CHR with improved disease burden than the HU/BAT group (53% versus 38%; p=0·044) (11). A consistent reduction of median *JAK2*V617F

TABLE 1 Measured response definitions for the clinical studies of ropeginterferon alfa-2b for the PV treatment.

	Complete response (CR)	Partial response (PR)	Complete Molecular Response (CMR)	Partial Molecular Response
PEGINVERA	hematocrit < 45% without phlebotomy in the past 2 months, platelet count ≤ 400 x 10^9/L, white blood cell count (WBC) ≤ 10 x 10^9/L, normal spleen size on ultrasound imaging, and the absence of thromboembolic events[a]	Defined as either hematocrit < 45% without phlebotomy but with persistent splenomegaly or platelets >400x10^9/Lor reduction of phlebotomy requirements by at least 50%	Reduction of JAK2 allelic burden from DNA samples to undetectable levels.	Defined as 1) a reduction of ≥50% from baseline value in patients with <50% mutant allele burden at baseline, OR 2) reduction of ≥25% in patients with >50% mutant allele burden at baseline.
PROUD-PV and CONTINUATION-PV	1) CHR[b] with normal spleen size 2) CHR with improved disease burden (i.e., splenomegaly, microvascular disturbances, pruritus, and headache) 3) CHR[b]	Not Reported	Reduction of any molecular abnormality to undetectable levels	Reduction in the JAK2 V617F allele burden of at least 50% from baseline (if the baseline value is <50%) and a reduction of at least 25% from baseline (if the baseline level is at least 50%)
Phase 2 Japan	CHR: hematocrit < 45%, WBC count ≤ 10 x10^9/L, and platelet count ≤ 400 x10^9/L; no phlebotomy in the previous 12 weeks			
Ongoing Studies				
Phase 2 China	CHR: hematocrit <45% without phlebotomy or erythrocyte apheresis in the preceding 12 weeks, platelet ≤400×10^9/L, leukocyte ≤10×10^9/L			
IIT Korea	CHR (hematocrit <45% without phlebotomy in the previous 12 weeks, platelet ≤400×10^9/L, leukocyte ≤10×10^9/L) and MR			

CHR, complete hematologic response, IIT, investigator-initiated trial.
[a]CR for PEGINVERA was defined according to the modified ELN (European Leukemia Net) criteria.
[b]CHR defined as hematocrit < 45% without phlebotomy in the past 3 months, platelet count ≤ 400 x 10^9/L, WBC ≤ 10 x 10^9/L.

allele burden was observed at every timepoint starting from screening (38%) to month 60 (8%) (12).

A multicenter, 52-week single-arm study evaluated the safety and efficacy of ropeginterferon alfa-2b in 29 Japanese patients diagnosed with PV (29). This trial followed the same titration regimen as the PROUD-PV study. The primary outcome was a durable CHR at 9 and 12 months and it was achieved in 8/29 patients (28%). At 12 months, 51% of patients achieved CHR, similar to 43% at 12 months reported in PROUD-PV. One serious treatment emergent AE (TEAE) of gastroenteritis was reported but was not deemed treatment-related. All patients experienced at least one TEAE and none were grade ≥3 in severity. An extension of the study continues (NCT04655092) (30) and its results may confirm the long-term effect of ropeginterferon alfa-2b observed in the PROUD/CONTINUATION-PV study.

A phase 2b, multicenter, open-label, parallel-group, randomized study evaluated the safety and efficacy of ropeginterferon alfa-2b at a fixed dose of 100 mcg every 2 weeks with phlebotomy and aspirin compared to phlebotomy and aspirin alone in 127 patients with low-risk PV (31). The primary endpoint was the maintenance of median hematocrit <45% during a 12-month period without disease progression. Following treatment, 84% of patients in the experimental group achieved the primary end point versus 60% in the control group (CI: 7-41%; p=0.0075). No disease progression was noted in the ropeginterferon alfa-2b group compared to 8% in the control. There was no statistically significant difference between the groups in frequency of AEs of grade 3/4 observed.

Ongoing investigational trial experience

Based on the hematological outcomes observed in the clinical trials for ropeginterferon alfa-2b, several international investigational studies were designed to further understand the relationship between short and long-term outcome measures and safety with an amended, and potentially optimized dosing regimen in PV patients.

An ongoing, phase 2, single-arm study in 49 Chinese PV patients who are resistant or intolerant to HU is evaluating the safety and efficacy of ropeginterferon alfa-2b when administered in a 250-350-500 mcg dosing titration regimen (32, 33). In this dosing scheme, the starting dose is 250 mcg, followed by 350 mcg at Week 2, and a target dose of 500 mcg at Week 4. Interim results reveal a 52% CHR rate at Week 24, comparable to 43% at Week 52 observed in PROUD-PV. *JAK2*V617F VAF decreased over time with two patients achieving a level <3%. TEAEs were reported in >95% of patients with possible treatment-related Grade ≥ 3 AEs in five patients (10.2%). This suggests that the more aggressive dosing schema results in a more rapid time to CHR.

A single-arm open-label, multicenter study in South Korea is currently evaluating the safety, efficacy, and tolerability of 250-350-500 mcg ropeginterferon alfa-2b dosing in PV patients who are either HU naïve or previously treated (34). Only 4.4% of patients at an interim data-cut required dose reductions during dose escalation, indicating good tolerability of this dosing approach. No treatment-related serious AEs were reported, and the majority of the AEs were grade 1 or 2. At the interim data-cut, 45, 20, and 6 patients were evaluable at 3, 6, and 9 months, respectively. The mean hematocrit, platelet, and WBC counts steadily decreased from baseline to Month 6 and 9. The *JAK2*V617F VAF showed a trend of rapid decrease with treatment from 59.7% at baseline to 42.4% at Month 3, and 37.1% at Month 6. The data suggests that ropeginterferon alfa-2b therapy with the 250-350-500 mcg dosing regimen induced hematological and molecular responses and was well-tolerated in Korean patients with PV (34).

ECLIPSE-PV (NCT05481151) is a phase 3b study to assess the efficacy, safety, and tolerability of ropeginterferon alfa-2b in North American adult PV patients utilizing the 250-350-500 mcg regimen

(35, 36). It is hypothesized that the response rates of PROUD/CONTINUATION-PV will be observed in a shorter period of time (36).

Clinical experience of ropeginterferon alfa-2b in other MPNs

Decades of clinical research conducted with IFN alfa support its role as a treatment option across MPNs (37–40). While ropeginterferon alfa-2b is not currently approved to treat ET or MF, the existing IFN alfa data support further clinical evaluation of ropeginterferon alfa-2b for other MPN diagnoses (Table 2).

The ongoing global phase 3, multicenter, randomized, controlled SURPASS-ET trial (NCT04285086) (41), and the single-arm EXCEED-ET trial (NCT05482971) in US and Canada (47), are currently evaluating the 250-350-500 mcg dosing scheme. Previous exploratory clinical research and meta-analysis already indicated that interferon therapy could be a safe and effective treatment for ET (42). This Phase 3 trial aims to provide pivotal data to support the approval of ropeginterferon alfa-2b for the treatment of ET.

A phase 2 study evaluated the efficacy of ropeginterferon alfa-2b in 25 patients with pre-fibrotic PMF (43). Ropeginterferon alfa-2b was administered at doses between 50 to 200 mcg every 4 weeks during the 24-month treatment period in the treatment-naïve group. Clinical improvements were observed, and no patient showed disease progression at 2 years. Two patients withdrew from the study one year after starting ropeginterferon alfa-2b treatment due to psychological related AEs (43). A second study recruited 8 MF patients (2 early and 6 intermediate/high-risk) who received 50 mcg with dose titration to 300 mcg every two weeks (44). Preliminary clinical improvement regarding spleen size and symptom scores was observed. One patient discontinued therapy due to dizziness and atrial fibrillation (44).

An ongoing, phase 2 investigator-initiated trial assessing the efficacy and safety of the 250-350-500 mcg dosing titration of ropeginterferon alfa-2b in early MF (NCT04988815), includes 56 patients with pre-fibrotic/early MF, overt PMF, post-PV MF or post-ET MF, and low/intermediate - 1 risk category according to dynamic international prognostic scoring system (DIPSS) (46). Interim results indicate 71% of treated patients at Week 12 and 67% at Week 24 achieved clinico-hematologic complete response. Of 39 patients harboring *JAK2* V617F, 36 reported reductions in VAF at 24 weeks with a reduction >50% in three treated patients and undetectable levels in one patient. No progression to overt MF or blast phase disease was observed. No treatment discontinuation, new safety signals, or deaths were reported thus far. The treatment options for patients with early MF including pre-fibrotic PMF have been very limited. The data from this study suggest that ropeginterferon alfa-2b potentially provides an effective treatment option for patients with early MF.

Further, a compassionate use program (CUP) recruited HU and/or anagrelide-resistant or intolerant MPN patients and administered ropeginterferon alfa-2b mostly using the 250-350-500 mcg dosing regimen in Taiwan (45). Published data from an initial cohort of nine patients show tolerability and substantial efficacy. Additional data in 20 MPN patients (14 PV, 4 ET, 1 post-ET MF, 1 pre-fibrotic PMF) further demonstrate tolerability and efficacy (45). At Week 52, eight of 11 response-evaluable PV patients (72.7%) achieved CHR and two of three ET patients (66.7%) achieved CHR. The median time to CHR was 27 and 24 weeks for PV and ET, respectively.

Overall, ropeginterferon alfa-2b treatment of MPNs at the 250-350-500 mcg dosing regimen appears to lead to notable and rapidly occurring clinical efficacy or activities with tolerability. The drug-related adverse events were generally well-tolerated and manageable, indicating a favorable benefit-risk profile.

Discussion

The response outcomes observed in multiple clinical trials of ropeginterferon alfa-2b confirm clinical benefits in MPN patients. Emerging data utilizing the 250-350-500 mcg regimen suggest that a higher initial dose with faster dose titration may lead to earlier complete hematological remission. Although a direct comparison is not possible in the absence of head-to-head data, treatment with this regimen in PV for 6 months led to CHR rates comparable to those observed at 12 months in the studies utilizing the low starting dose and slower titration schema.

Support for a higher starting dose of ropeginterferon alfa-2b comes from the outcomes noted in the PROUD-PV and the study by Edahiro et al. compared to the outcomes from the emerging data of the 250-350-500 mcg ropeginterferon alfa-2b titration regimen from several recent sources including a CUP in Taiwan (46, 48), a Phase 2 study in China (32, 33), an IIT study in Korea (34). For example, the study by Edahiro et al. followed the slow titration regimen, whereas the 14 patients with HU and/or anagrelide resistance or intolerance enrolled in the CUP study followed the 250-350-500 mcg titration regimen, and the median time to response was 52 weeks vs 27 weeks. At 52 weeks, patients from Edahiro et al. study had a CHR rate of 51% compared to 73% in the CUP study. Interim results from the Chinese Phase II study in HU-resistant or intolerant PV patients showed a CHR rate of 52% at 24 weeks (32). The CHR rate at 24 weeks (6 months) was even notably higher than the rate of 43% observed at 12 months in the PROUD-PV study. The data from the Korean IIT study in HU naïve or pre-treated PV patients also indicate higher hematologic and molecular responses at 6 months (34).

Indeed, the risk of a thromboembolic event is highest immediately before or after establishing the diagnosis of PV (3, 49–51). A potential risk of thrombosis may not be adequately addressed by the low starting dose and slow titration regimen as time to hematologic response is delayed. Furthermore, it is widely known that high *JAK2*V617F VAF poses greater risk of myelofibrotic transformation (52–54), and that high *JAK2*V617F VAF is associated with high leukocyte count (55, 56) which has also been implicated as an independent risk factor for thromboembolic events (57, 58). Results from ongoing clinical studies utilizing the 250-250-500 dosing regimen will provide key insights into the correlation between dosage and outcome response and rates of thromboembolic complications. Thromboembolic complications have not been reported during the intra-patient dose escalations with the 250-250-500 dosing regimen from the existing data. Given that it has only three step dose escalations with a higher starting dose, it is reasonable to believe that the dosing regimen might potentially minimize the risk of thrombosis and hemorrhage associated with an under-dosing during dose titrations. Although a thromboembolic risk associated

TABLE 2 Overview of clinical studies with ropeginterferon alfa-2b.

First author [Reference]	Publication type	Year	Study name	Discipline	Disease	Phase	Dosing regimen	Maximum dose (mcg)
Gisslinger (15)	Original article	2015	PEGINVERA	Hematology/ Oncology	Polycythemia vera (PV)	I/II	Starting dose at 100 mcg or 50 mcg (under HU treatment). Increase 50 mcg every 2 weeks	540
Gisslinger (11)	Original article	2020	PROUD/ CONTI-PV	Hematology/ Oncology	PV	III	As above	500
Kiladjian (12)	Letter to editor	2022	PROUD/ CONTI-PV	Hematology/ Oncology	PV	III	As above	500
Edahiro (29)	Original article	2022	NCT04182100	Hematology/ Oncology	PV	II	As above	500
Jin (32)	Conference abstract	2022	NCT05485948	Hematology/ Oncology	PV	II	250 mcg as the starting dose, 350mcg at Week 2, then 500 mcg at week 4	500
Lee (34)	Conference abstract	2022	N/A	Hematology/ Oncology	PV	II	As above	500
Barbui (31)	Original article	2021	Low-PV	Hematology/ Oncology	PV	II	Fixed dose at 100 mcg	100
Verstovsek (41)	Review	2022	Surpass-ET	Hematology/ Oncology	ET	III	250 mcg as the starting dose, 350mcg at Week 2, then 500 mcg at week 4	500
Gisslinger (42)	Conference abstract	2018	N/A	Hematology/ Oncology	Myelofibrosis (MF) (prefibrotic/ early)	II	50 to 200 mcg every 4 weeks	200
Palmer (43)	Conference abstract	2018	NCT02370329	Hematology/ Oncology	MF	II	50 mcg with dose titration to 300 mcg every 2 weeks	300
Gill (44)	Conference abstract	2022	NCT04988815	Hematology/ Oncology	MF (prefibrotic/ early)	II	250 mcg as the starting dose, 350mcg at Week 2, then 500 mcg at week 4	500
Chen (45)	Conference abstract	2019	CUP	Hematology/ Oncology	PV, ET, and MF	N/A	250 mcg as the starting dose, 350mcg at Week 2, then 500 mcg at week 4	500
Huang (46)	Original article	2020	CUP	Hematology/ Oncology	PV, ET, and MF	N/A	250 mcg as the starting dose, 350mcg at Week 2, then 500 mcg at week 4	500
Huang (23)	Original article	2020	N/A	Hepatology	Hepatitis B	I/II	Fixed dose at 350 or 450 mcg	450
Hsu (24)	Original article	2021	N/A	Hepatology	Hepatitis C	II	Fixed dose at 270, 360 or 450 mcg	450
Lin (25)	Original article	2021	NCT01587586	Hepatology	Hepatitis C	II	Fixed dose at 180, 270 or 450 mcg	450
Chen (28)	Original article	2022	CUP	Infectious disease	Coronavirus disease 2019	N/A	Single dose at 250 mcg	250
Huang (18)	Original article	2021	N/A	Pharmacokinetics	Healthy participants	I	Single dose at 90, 180, and 270 mcg	270
Huang (17)	Original article	2022	NCT05129644	Pharmacokinetics	Healthy participants	I	Single doses ranging from 24 to 270 mcg	270
Miyachi (16)	Original article	2021	NCT03546465	Pharmacokinetics	Healthy participants	I	Single dose at 100, 200, or 300 mcg	300

CUP, compassionate use program; N/A, not applicable; NR, not reported.

with an under-dosing during the slow dose titrations in PV patients could potentially be managed with phlebotomies, the 250-350-500 dosing regimen of ropeginterferon alfa-2b provides a treatment option of leveraging the risk with rapid induction of hematologic remission and molecular response associated with VAF decreases of drive mutations such as *JAK2*V617F in broader MPN patients. Therefore, the importance of an optimal dosing and titration schedule for ropeginterferon alfa-2b may need to be further explored to better understand its potential impact on critical efficacy and tolerability outcomes in PV or other MPNs.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

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The multidisciplinary approach to eosinophilia

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Eosinophilic granulocytes are normally present in low numbers in the bloodstream. Patients with an increased number of eosinophilic granulocytes in the differential count (eosinophilia) are common and can pose a clinical challenge because conditions with eosinophilia occur in all medical specialties. The diagnostic approach must be guided by a thorough medical history, supported by specific tests to guide individualized treatment. Neoplastic (primary) eosinophilia is identified by one of several unique acquired genetic causes. In contrast, reactive (secondary) eosinophilia is associated with a cytokine stimulus in a specific disease, while idiopathic eosinophilia is a diagnosis by exclusion. Rational treatment is disease-directed in secondary cases and has paved the way for targeted treatment against the driver in primary eosinophilia, whereas idiopathic cases are treated as needed by principles in eosinophilia originating from clonal drivers. The vast majority of patients are diagnosed with secondary eosinophilia and are managed by the relevant specialty—e.g., rheumatology, allergy, dermatology, gastroenterology, pulmonary medicine, hematology, or infectious disease. The overlap in symptoms and the risk of irreversible organ involvement in eosinophilia, irrespective of the cause, warrants that patients without a diagnostic clarification or who do not respond to adequate treatment should be referred to a multidisciplinary function anchored in a

hematology department for evaluation. This review presents the pathophysiology, manifestations, differential diagnosis, diagnostic workup, and management of (adult) patients with eosinophilia. The purpose is to place eosinophilia in a clinical context, and therefore justify and inspire the establishment of a multidisciplinary team of experts from diagnostic and clinical specialties at the regional level to support the second opinion. The target patient population requires highly specialized laboratory analysis and therapy and occasionally has severe eosinophil-induced organ dysfunction. An added value of a centralized, clinical function is to serve as a platform for education and research to further improve the management of patients with eosinophilia. Primary and idiopathic eosinophilia are key topics in the review, which also address current research and discusses outstanding issues in the field.

KEYWORDS

clinical, diagnosis, eosinophilia, multidisciplinary, review, trial, treatment

1 Introduction

The differential count of white blood cells is a simple analysis to obtain diagnostic information, and deviations in the number of leukocytes reflect perturbed homeostasis. Leukocytosis and leukopenia, associated with neutrophilic granulocytes or lymphocytes, are important clues when evaluating a patient, indicating, for example, a feedback control request for immunocompetent cells to fight infections or a derailed leukopoiesis with or without maturation (1, 2). Eosinophilic granulocytes (eosinophils) are normally among the least abundant circulating white blood cells ($<0.5 \times 10^9$/L). Unlike common leukocytes, a reduced number of eosinophils is not captured by the differential count and is normally not clinically paid attention to. In contrast to this, the observation of an increase in eosinophils in a differential count of blood or other samples can be a key piece of information that should be contextualized in advance in the individual (adult) patient. However, a structured approach is required to guide diagnostic and therapeutic decisions clinically, and the task is to isolate the impact of an increased eosinophil count (eosinophilia) from all other etiologic factors in the overall assessment (3–8).

Chronic myeloproliferative neoplasms (MPNs) include several distinct disorders, representing an autonomous turnover of one or more of the cells, circulating in the blood. MPNs include, according to the current WHO classification, breakpoint cluster region—Abelson1 (*BCR-ABL1*)-positive chronic myeloid leukemia (CML) and the *BCR-ABL1*-negative neoplasms, many of which are considered to be inflammatory conditions driving a clonal evolution in a biological continuum involving variable mutations and genetic structural aberrations (9–14). A separate category in the WHO classification is the myeloid/lymphoid neoplasms with eosinophilia and tyrosine kinase fusion genes (MLN-TK), which are usually prominent features at diagnosis (9).

Primary eosinophilia is rare and reflects clonal hematopoiesis in which the production of eosinophils is driven by a genetic or intrinsic cause. Causes of secondary eosinophilia are common and very different in nature and are characterized as being reactive to factors with an extrinsic impact on the eosinopoiesis. Secondary or reactive eosinophilia is driven, in particular, by the cytokine interleukin (IL)-5, produced by activated T lymphocytes (15, 16). This scenario of immunological crosstalk is associated with autoimmune, infectious, and inflammatory diseases; malignancy; and allergy, including iatrogenic, drug-induced adverse reactions (3–8). When no congenital, clonal, or reactive cause can be demonstrated, patients with persistent eosinophil counts of at least 1.5×10^9/L are categorized as idiopathic hypereosinophilic. This group can be subdivided into patients with no manifestations of eosinophilia [iHE, or hypereosinophilia of undetermined significance (iHE_{US})] or idiopathic hypereosinophilic syndrome (iHES), when organ involvement due to eosinophils is present (3–8, 17–20). However, the overlap of symptoms, regardless of whether the cause of the disease is primary, secondary, or idiopathic eosinophilia—or a combination of them—is considerable, and characterizes the patient with an increased eosinophil count in blood as a clinical challenge.

Notwithstanding the cause, the presence of an increased number of circulating eosinophils in the blood may be associated with inappropriate organ involvement. In most cases, patients with secondary eosinophilia are treated successfully by the general practitioner (GP) or at departments specialized in the management of individual manifestations. However, it can be difficult to prove whether the patient has primary eosinophilia, secondary eosinophilia, or iHES—or whether the symptoms and cause of the eosinophilia can be attributed to an atypical presentation or represent more than one etiology. An insufficient response to symptoms, and unexplained persistent or recurrent eosinophilia despite adequate treatment may be a reason for a

thorough reassessment. This review describes eosinophilia in a clinical context, particularly how a dedicated function with a multidisciplinary team is one way to provide a rational approach due to the complexity of demonstrating differential diagnosis and options for targeted treatment. Current unresolved issues in the management of eosinophilia are discussed.

2 Review of the eosinophil granulocyte in health and disease

2.1 The eosinophil granulocyte and pathophysiology

Eosinophils originate from a myeloid cluster of differentiation (CD) CD34+ precursors in the bone marrow and are part of the innate immune system (16, 21). Maturation takes a week on average and is influenced by a granulocyte-macrophage colony-stimulating factor (GM-CSF), IL3, and IL-5 and is driven by activation of transcription factor networks including PU.1, CCAAT/enhancer-binding protein (C/EBP), and GATA-binding protein 1 (GATA-1). All of the above are involved, but IL-5 and GATA-1 have key roles in eosinopoiesis and turnover; IL-5 is involved in egress from the bone marrow microenvironment into the bloodstream, promoting activation and survival and preventing apoptosis; and GATA-1 is a vital regulator of cell maturation (22–24).

The course of eosinophils subsequent to bone marrow release starts with circulation in the bloodstream for 8–18 h and terminates in peripheral organs for up to 12–14 days or longer (25, 26). At some point during circulation in the bloodstream, the eosinophil migrates through the lining of blood vessels and enters one of the numerous organs, possibly attracted by chemokines (27). Being mobile cells, they are distributed to the liver, lungs, skin, heart, reticuloendothelial system, glands, and digestive tract, but not to the esophagus, which is normally devoid of eosinophils. The cells remain in the organs as tissue-resident cells under homeostatic conditions. During this time, the cell may proliferate under inflammatory conditions and undergo terminal apoptosis (28, 29).

Eosinophils in the bloodstream or tissues are large, spherical cells, 12–17 μm in size with a bi-lobed nucleus, without a nucleolus, and exhibiting numerous coarse, rounded, and red-purple granules in the cytoplasm by routine staining (16, 24, 28). Figure 1 shows peripheral blood smears, illustrating mature eosinophil granulocytes from a patient with mild eosinophilia. Eosinophils can be compared morphologically to neutrophil granulocytes, lymphocytes, and platelets. Typically, less than one eosinophil granulocyte will be recognized in a field using light microscopy. Detection of more than one eosinophil in a 400× light microscopy field examination is an indication of eosinophilia, normally representing 1%–4% of all white blood cells (1, 2).

The cell is easily identified by light microscopy in a blood smear by the appearance of the nucleus and coarse granules, which distinguish the appearance of eosinophils from other leukocytes (Figure 1). The cytoplasm contains not only the abundant and phenotypically characteristic-specific (also named secondary) granules, but also other organelles (not readily visible using light-microscopy) such as the smaller, azurophilic, and fewer primary granules. Both granules are lysosomes, storage sites for agents involved in tissue damage and inflammation. The secondary granules contain chemokines, growth factors, cytokines, and proteolytic enzymes such as the major basic protein, eosinophil peroxidase, eosinophil-derived neurotoxin, and eosinophil cationic protein (15, 23, 24, 26, 30). A predominant protein in eosinophils is galectin-10, which is now identified as a component of the peripheral cytoplasm. Upon secretion, it precipitates in tissues and body fluids as the Charcot–Leyden crystal protein, a lysophospholipase indicative of eosinophil granulocyte activity (31). The various proteases released from the specific granules contribute to the antimicrobial effect of the phagocytosing eosinophil granulocyte but may, at the same time, cause epithelial cell damage, and cytotoxicity, and contribute to fibrosis. The release of substances into tissues is damaging to microorganisms or bystander cells, causing organ damage. The concentration of proteases in body fluids may serve as a biomarker of inflammation involving eosinophils, reflecting a potential effect of circulating granule components (15, 28, 32).

Eosinophil granulocytes interact *via* their arsenal of surface-bound receptors (e.g., IgE, histamine, chemokine, cytokine, and adhesion) and the ability to secrete various proteins and other substances as mentioned above, that characterize both circulating and resident granulocytes (15, 23, 24, 33, 34). The dynamics are normally reflected in their ability to respond to infectious and inflammatory stimuli by increased numbers, eosinophilia in the blood and affected tissues, and activation. The release of preformed or stimulus-dependent chemokines, interleukins, leukotrienes, growth factors, and proteins behaves like a cascade, accompanied by a respiratory burst. The generation of reactive oxygen species upon assembly of the components of the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex in the plasma membrane is higher per cell in eosinophils than in neutrophils (35, 36). Although the number of neutrophils is usually about 10 times higher, the increase in eosinophils in response to inflammatory or infectious stimuli contributes to the potential toxicity in the process, including harmful effects on bystander cells, through the release of granule content and reactive oxygen species. Like neutrophils, eosinophils are phagocytes that contribute to the direct control of helminth infections, and both granulocytes can form extracellular traps. This complex network of granule components and DNA is the ultimate contribution of the dying cell participating in parasitic infection and inflammation (15, 34, 37).

The phenotype of mature eosinophil granulocytes and precursor cells can be separated by the CD11b/CD62L expression, accompanied by upregulation and co-expression of various surface markers. These include C-C motif chemokine receptor 3 (CCR3, CD193), IL-5 receptor alpha (CD125), and sialic acid-binding Ig-like lectin 8 (siglec-8), a member of the CD33-related siglec subfamily, all of which are highly expressed on the surface of eosinophil granulocytes in blood and bone marrow (23, 28, 38, 39). Flow cytometry to identify eosinophils may be routinely performed, including a panel of monoclonal antibodies to detect

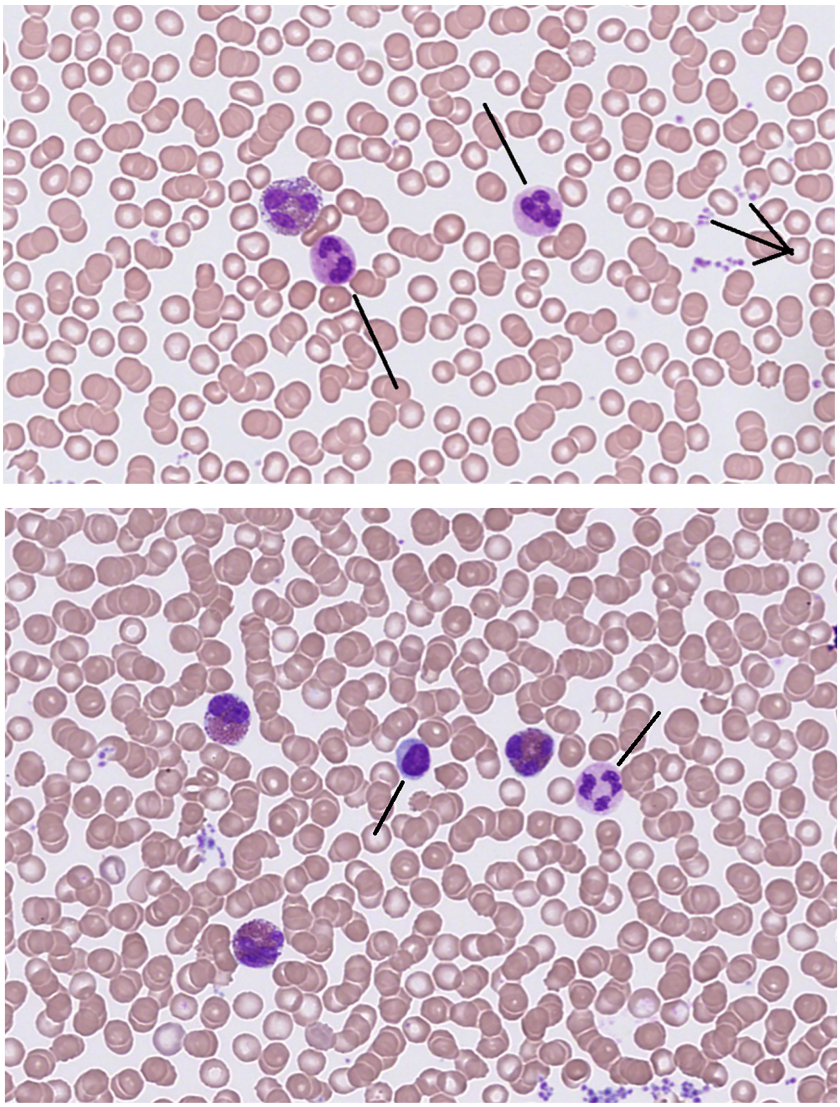

FIGURE 1
Eosinophil granulocytes in peripheral blood smear. Giemsa stain, 400×. Upper panel illustrates one eosinophil, neutrophil granulocytes (/), and aggregates of platelets (>). Lower panel shows eosinophil granulocytes, a mononuclear cell (/ lymphocyte), a polynuclear granulocyte (/ neutrophil), and thrombocytes (not marked).

myeloid proliferation according to Euroflow (40). However, flow cytometry may not be relevant if the eosinophil count is normal.

Being classified as an MPN, iHES carries the risk for vascular events both before and after diagnosis. The manifestations are almost always thrombotic, in both arterial and venous locations, and may be more frequently associated with clonal, primary eosinophilia. The thrombogenic potential of eosinophilia may be manifested in addition to other inherited or acquired risk factors for vascular events. The risk of bleeding may be minimal because severe thrombocytopenia or another-acquired hemorrhagic diathesis is very unusual in iHES. Retrospective analyses of HES cohorts report that 21%–24% of patients have experienced at least one event before diagnosis (41, 42). One study has linked increased expression of tissue factor (factor III or CD142), which is the initiator of thrombin generation, in eosinophil granulocytes examined in patients with iHES and secondary hypereosinophilia (43). In addition, eosinophils interact with platelets to promote atherosclerosis and thrombosis (44).

2.2 Epidemiology and definitions

An increase in the number of eosinophils is common and can be arbitrarily divided into three levels: mild (0.5–1.5 × 10^9/L), moderate (≥1.5 × 10^9/L), and severe (>5 × 10^9/L) eosinophilia. The term hypereosinophilia may be used to characterize all cases of moderate or severe eosinophilia (3–6).

The number of eosinophil granulocytes in the blood is routinely measured by automated machine analysis as part of the differential count. Minor variations in the normal threshold, defined as 0.4–

0.5×10^9/L blood, may be observed between different machines and laboratories, but a manual count of (mature) eosinophils in the blood is rarely if ever, needed (45). An association within the normal range of blood eosinophil counts in adults has been reported to be correlated with several demographic factors including age, biological sex, race, BMI, and smoking status (46, 47). A diurnal variation in eosinophil count in healthy individuals has been reported to be higher at night and lower in the morning (48). Despite these variations in normal subjects, no specific recommendation has been made or deemed relevant for the interpretation of cell counts in patients with eosinophilia.

The incidence of eosinophilia varies worldwide. Although due to a plethora of reactive causes, it is more likely due to infection in tropical areas and inflammation in industrialized regions. A hospital incidence of over 10% has been reported in South Korea (49) and India (50). The incidence of eosinophilia in subjects having a blood sample taken over 10 years in the primary sector of a Western capital city was reported to be 4% in adults, reflecting that eosinophilia is a common problem to be contextualized clinically (51). In contrast, the incidence of eosinophilia in a large Canadian island district was 0.1% (52). The elevated cell count is transient in almost all patients with secondary eosinophilia, due to the impact of treatment or the self-limiting nature of the reaction, and the prevalence of eosinophilia remains low.

The definition of HES was introduced in 1968 and required that patients presented moderate or severe blood eosinophilia of unknown origin for more than 6 months, and for it to be responsible for organ damage (53, 54). The term in its original meaning is no longer applicable due to the options for treatment, the risk of irreversible symptoms, and the improvements in the diagnostic tools. Today, according to the agreed-upon definitions, HES reflects a heterogeneous group of disorders, presenting with persistent peripheral blood eosinophilia $\geq 1.5 \times 10^9$/L on two occasions, the absence of a secondary cause of eosinophilia, and evidence of eosinophil-associated end-organ damage, justified by excessive tissue eosinophilia (55, 56).

The incidence has been reported to be 0.036/100,000 for HES in the USA (57) and 0.018/100,000 specifically diagnosed with the most prevalent primary eosinophilia, a factor interacting with PAPOLA and CPSF1-platelet-derived growth factor receptor alpha (*FIP1L1-PDGFRA*)-positive neoplasms, in France (58). The reports underline the rarity of primary eosinophilia, and more precise estimates may be difficult to collect, although the WHO ICD system provides diagnostic registry codes for different subclasses of hypereosinophilia. The *FIP1L1-PDGFRA* primary myeloid neoplasm is more prevalent in male adults (58), but otherwise, iHES and clonal primary eosinophilia are overall not gender-specific and can be diagnosed at all ages. No valid data are available on the incidence in pediatric patients (59, 60).

Eosinophilia can be classified by diagnostic tests into congenital (familial) causes as primary (intrinsic, clonal) or secondary (extrinsic, reactive) (20, 55). The diagnosis by exclusion of iHES (with symptoms due to eosinophilia) may also be clinically sub-characterized as either a myeloid (mHES) or a lymphoid (lHES) phenotype (61, 62). The lymphoid HES subtype is driven by $CD3^-CD4^+$ IL-5, producing T cells and thus secondary, non-neoplastic eosinophilia that is glucocorticoid sensitive and often associated with, e.g., angioedema, skin lesions, pruritus, and fasciitis (63). Previously, concomitant manifestations such as cardiac involvement, hepatosplenomegaly, anemia, variable leuko- and thrombocyte counts, and steroid resistance were used to phenotypically characterize a myeloid HES (61, 62, 64), similar to other MPNs. Specific diagnostic tests are required to identify patients according to the updated criteria. The revisions of the WHO classifications of malignant eosinophilic disorders by molecular diagnostic markers since 2008 have established the cluster of MLN-TK identified by specific tyrosine kinase rearrangements (3–9).

2.3 Symptoms of eosinophilia

The presentation of patients with eosinophilia varies considerably, in terms of symptoms and severity. Over days to months, most patients may gradually experience a worsening of symptoms related to the cause of eosinophilia, whereas symptom flares are characteristic of iHES. The presence of an increased eosinophil count in the blood may have been indolent for years, or manifestations may be due to a recent onset involving one or more organs simultaneously. The clinical context at presentation in patients with an increased blood eosinophil count is not related to the classification of primary, secondary, or iHES, because the symptoms due to eosinophilia may mimic or be involved in the diseases listed in Supplementary Table 1 concerning organ manifestations (65–86) and Supplementary Table 2 concerning parasitic causes (87, 88). An exception is iHE/iHE$_{US}$, which by definition is asymptomatic eosinophilia (4–7, 20, 55).

The considerable overlap in patients presenting with eosinophilia as a clinical clue in differential diagnostics reflects how complex the correct diagnosis may be. Nevertheless, common pathologic conditions not associated with eosinophilia may manifest with similar symptoms. The recurrent question is to decide whether eosinophilia is an independent causative factor or whether the presence of eosinophilia is an additional causative factor to disorders that may have been present before the eosinophilia was noticed. Symptoms due to eosinophilia may thus be manifest in all organ systems in addition to pre-existing conditions, masked as a worsening (Supplementary Table 1).

B-symptoms, including weight loss, low-grade fever, and night sweats may occur in all patients with eosinophilia, whether primary, secondary, or iHES. In the individual patient, they may be attributed to cytokine signaling induced by eosinophils or other immunocompetent cells as part of a malignant, infectious, and inflammatory secondary cause (Supplementary Tables 1, 2).

Reports on cohorts with eosinophilia have been published describing manifestations in iHES (65) or cross-sectional symptom registries (66, 67). Studies from Western institutions cannot be compared due to methodological differences, but the institutional reports reflect the diversity of symptoms ascribed to primary or secondary eosinophilia (Supplementary Tables 1, 2). The most common organ involvement in patients with unexplained hypereosinophilia referred for examination involves dermatologic,

respiratory, and gastrointestinal symptoms in approximately 45%, 35%, and 25%, respectively (65, 66). Registration of causes of unclassified eosinophilia identifies infection, allergy, and non-hematologic malignancy as common causes in different parts of the world (49, 50, 52, 61, 66, 67). These data support a multidisciplinary approach to patients who cannot be classified in a straightforward manner according to diagnostic guidelines or who do not respond adequately to proper treatment (Supplementary Tables 1, 2).

2.4 Diseases associated with eosinophilia

2.4.1 Diagnostic entities

Figure 2 depicts the clinical spectrum, contemplating diagnostic entities that approach a patient with eosinophilia in the causative context, including manifestations of organ involvement that may have overlapping presentations (Supplementary Tables 1, 2) (65–88). The threshold is chosen to align with the definition of iHE/iHE$_{US}$ or iHES. Still, it applies to all cases with mild eosinophilia and patients without symptoms or with symptoms related to diagnoses other than eosinophilia. It may be important to examine in the same way patients with an increased or fluctuating eosinophil count observed over weeks to months or even years.

The descriptive manner in which eosinophilia is elucidated by pathophysiologic drivers can contribute to structuring the clinical approach and qualify diagnostic testing in a patient with newly diagnosed eosinophilia or for a second opinion at a later stage in a multidisciplinary forum. The information provided in Figure 2 is not exhaustive, as it is not possible to present a complete list. Figure 2 includes major groups of hematologic diagnoses as defined by the current WHO classification (9, 89). iHE and iHES are listed as clonal hematologic disorders, assuming that most cases, which at the moment are diagnosed by exclusion, may harbor a clonal driver in the eosinopoiesis (90). The algorithm indicates when eosinophils are part of the clonal disease, as described, for example, in some acute myeloid leukemias (AML) with recurrent genetic abnormalities, such as core-binding factor AML with t(8;21) or inv (16), including immature eosinophilia (91, 92). The differential diagnosis may include MLN-TK. Eosinophilia appears to be reactive and induced by cytokine signaling (IL-3) in B-acute lymphoblastic leukemia (B-ALL) with t(5;14) and with mature eosinophils circulating in the blood (93). Characterization of secondary eosinophilia can be hampered by difficulties in quantifying specific cytokine stimuli. These analyses are not done routinely at most institutions and therefore require scientific projects to accumulate data and describe the involvement of genetics as well as inflammation, e.g., in histiocytosis.

Eosinophilia is always present in iHE/iHE$_{US}$ and iHES and is part of the diagnostic criterion. Eosinophilia is present in almost all cases of MLN-TK, caused by fusion genes involving a receptor [PDGFRA or B, fibroblast growth factor receptor (FGFR), fms like

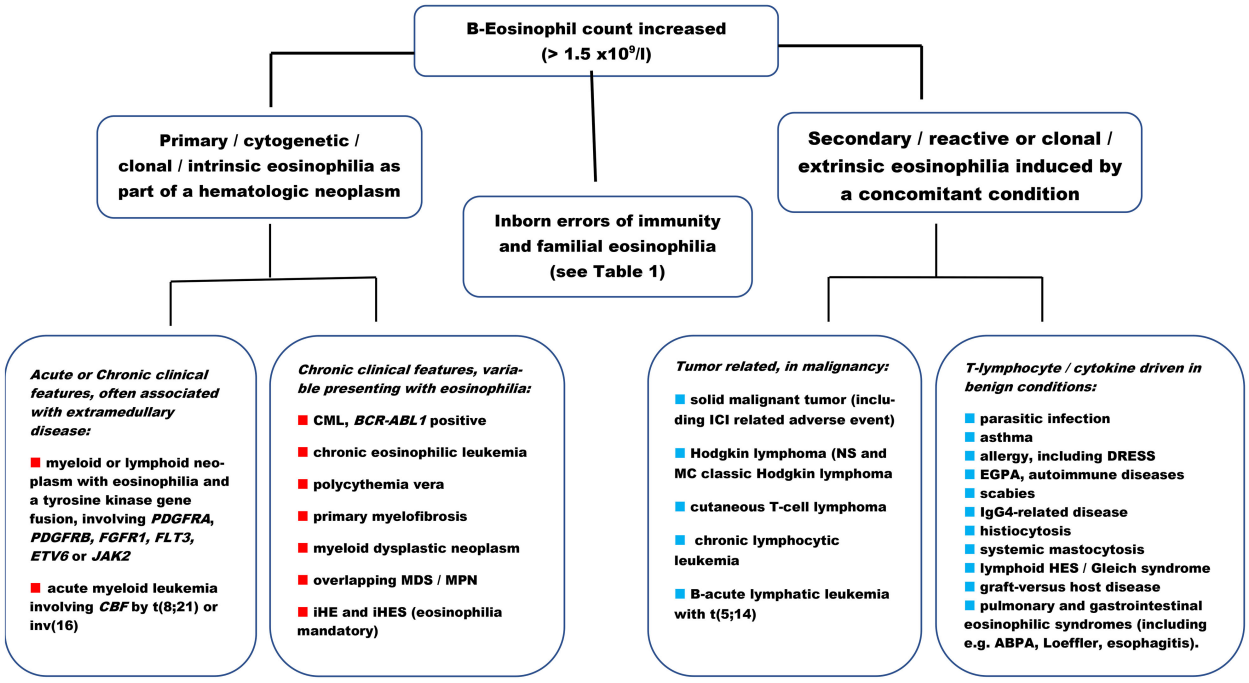

FIGURE 2
Pathophysiological algorithm and examples of diagnostic entities in patients with (moderate) eosinophilia. The diagram is not exhaustive. ABPA, allergic bronchopulmonary aspergillosis; BCR-ABL, breakpoint cluster region—abelson1; CBF, core binding factor; CML, chronic myeloid leukemia (t9;22) positive; DRESS, drug reaction eosinophilia systemic symptoms; EGPA, eosinophil granulomatous polyangiitis; ETV6, ETS variant transcription factor 6; FGFR1, fibroblast growth factor receptor 1; FLT3, Fms-related tyrosine kinase 3; HES, hypereosinophilic syndrome; ICI, immune checkpoint inhibitor; Ig, immunoglobulin; iHE, idiopathic hypereosinophilia; iHES, idiopathic hypereosinophilic syndrome; inv, inversion; JAK2, Janus kinase 2; MC, mixed cellularity; MDS, myelodysplastic neoplasm; MPN, myeloproliferative neoplasm; NS, nodular sclerosis; PDGFRA/B, platelet-derived growth factor A or B; t, translocation.

tyrosine kinase 3 (FLT3)], a transcription factor (ETV-6), or a non-receptor kinase (Janus kinase 2, JAK2). Eosinophilia is mandatory at diagnosis in chronic eosinophilic leukemia (CEL) (Figure 2) (6–9, 20, 58). Most patients with CML (in the chronic phase) present with mild or moderate absolute eosinophilia as part of the *BCR-ABL1* oncogene-driven leukocytosis and may rarely present as eosinophilia (94). Eosinophilia is variable, and mild, if present, in Philadelphia-negative MPNs such as polycythemia vera (PV) or primary myelofibrosis (PMF), and essential thrombocytosis (ET) but is not part of the diagnostic criteria (9, 19, 95). Overlapping MPN/myelodysplastic neoplasms (MPN/MDS) and myeloid dysplastic neoplasms are rarely associated with eosinophilia, but eosinophilia may be observed as part of the perturbed hematopoiesis (9, 17, 95).

Figure 2 may serve as a catalog and inspiration for relevant diagnostic groups: autoimmune, infectious, clonal, etc., in the initial approach, emphasizing that the list is not exhaustive and that secondary, reactive causes are the most common (3–7, 20, 55, 64, 95) (Supplementary Tables 1, 2). The listed examples of benign disorders may (all) be associated with T-lymphocyte/cytokine-driven mature eosinophilia in the blood (or tissues, e.g., skin, lung, or gastrointestinal tract) and include common or very rare diagnoses such as asthma exacerbation (96), sarcoidosis (97), rheumatoid arthritis (98), and atopy (99) or IgG4-related diseases (IgG4-RD) (100). Eosinophilia may be part of the diagnostic criteria, e.g., a B-eosinophil count of at least 1×10^9/L (or evidence of extravascular eosinophilic predominant inflammation on biopsy) in eosinophilic granulomatosis with polyangiitis (EGPA, formerly Churg–Strauss syndrome) (75). Similarly, a significant number of eosinophils in the sputum, airway, or blood is required to diagnose eosinophilic asthma (73).

The eosinophil count is frequently increased in parasitic infections (Supplementary Table 2) (87, 88, 101), allergic bronchopulmonary aspergillosis (ABPA) (88, 102), or scabies (88, 103). Eosinophilia is a predominant clinical component in lHES, characterized by marked overproduction of eosinophil factor(s) by dysregulated CD3-CD4+ T cells, which are clonal in most cases (63), and in episodic angioedema with eosinophilia (Gleich syndrome), which is a multilineage cell cycle disorder (104). The entities Eosinophilic Pulmonary Disease (EPD) (71–73) and Eosinophilic Gastrointestinal Diseases (EGID) (78) can be considered working diagnoses, including chronic, immune-mediated disorders with a multifactorial etiology and characterized by an increase in eosinophil-predominant tissue inflammation on biopsy. Organ-specific entities include several specific diagnoses such as eosinophilic esophagitis and Loeffler's syndrome, which are often accompanied by blood eosinophilia. In Loeffler's syndrome, the eosinophilia is transient, accompanied by fluctuating, mild to severe respiratory symptoms with fever, and interstitial, migratory pulmonary infiltrates. The shading represents an accumulation of eosinophils, most often in response to parasitic infection (69–73, 78, 105) (Supplementary Table 1). Finally, the information provided in Figure 2 may emphasize as clinically important that a patient may have eosinophilia for more than one reason, requiring separate treatments for proper care.

2.4.2 Eosinophilic granulomatosis with polyangiitis

Eosinophilic granulomatosis with polyangiitis (EPGA) (Churg–Strauss) is a rare and potentially life-threatening systemic vasculitis. For all practical purposes, EPGA always develops in patients with pre-existing asthma (74).

The disease is characterized by predominantly small-vessel vasculitis and extravascular necrotizing granulomas associated with eosinophilic inflammation. Most patients have moderate to high blood eosinophil counts. A blood eosinophil count $\geq 1 \times 10^9$/L and/or extravascular eosinophilic predominant inflammation on biopsy of affected tissue may support the current diagnostic criteria (75). It has been consistently found that 30%–40% of affected patients have antineutrophil cytoplasmic antibodies (ANCA), which can pose a diagnostic challenge in relation to the more common systemic vasculitis, although these are also rare in a tertiary rheumatology outpatient clinic (Supplementary Table 1 and Figure 2).

The involvement of the lungs is the most common organ affected together with maxillary sinusitis (allergic rhinitis and/or sinus polyposis). Other major manifestations in EGPA involve the skin, peripheral nerves and kidney, as palpable purpura, mononeuritis multiplex and glomerulonephritis, respectively. Less commonly, the heart is involved with congestive heart disease symptoms and subendocardial fibrosis, and the gastrointestinal tract and the eye are affected (74, 75). Therapy includes corticosteroids (CS) and immunosuppressive agents, which overlap with the treatment of iHES.

2.4.3 Histiocytosis and IgG4-related disease

Histiocytoses are very rare diseases. The prediagnostic phase is often long. It is uncommon for histiocytosis alone to present with peripheral eosinophilia, but it does occur, particularly when associated with another myeloid neoplasm. A retrospective study showed that 10% of adults with non-Langerhans cell histiocytosis have a concomitant MPN (106). Therefore, patients with histiocytosis discovered during the evaluation for eosinophilia should be offered bone marrow examination and testing for recurrently mutated myeloid genes. Hodgkinoid histiocytosis—a very rare entity—presents with eosinophilia and may mimic lymphoma (107).

In contrast to peripheral eosinophilia, tissue eosinophilia is common in histiocytosis, particularly Langerhans cell histiocytosis (formerly called eosinophilic granuloma), Erdheim–Chester disease, and ALK-positive histiocytosis (9, 95, 108–110).

In the setting of eosinophilia, the histiocytoses are most relevant as differential diagnoses to IgG4-RD, as the organ manifestations of Langerhans cell histiocytosis and Erdheim–Chester disease may resemble IgG4-RD (100, 108, 109). The distribution of lesions revealed by PET-CT scans may help to differentiate the diseases: bone involvement favors histiocytosis over IgG4-RD. Moreover, observation by imaging of perinephric changes is indicative, and demonstration of flasklike deformation in the distal femur due to meta-diaphyseal osteosclerosis, is pathognomonic for Erdheim-Chester disease. The finding of mutated v-Raf murine sarcoma

viral oncogene homolog B (*BRAF*), rat sarcoma (*RAS*), or mitogen-activated protein kinase (*MAPK*) pathway genes supports a histiocytic diagnosis. Rosai–Dorfman disease is another histiocytosis that can be mistaken for IgG4-RD, as IgG4+ cells are often prominent in this condition (100).

It is currently unclear whether the eosinophilic infiltrate in histiocytic diseases is due to the disease itself or a phenomenon secondary to the inflammatory microenvironment.

IgG4-RD is an important differential diagnosis in hypereosinophilia (100). Of 100 patients with eosinophilia evaluated at a tertiary center, 9 had IgG4-RD (66). Presenting features of IgG4-RD are variable but include eosinophilia, allergy and nasal polyposis, salivary gland involvement, lymphadenopathy, sclerosing cholangitis, autoimmune pancreatitis, retroperitoneal fibrosis, and glomerulonephritis. Of note, only approximately 50% of Caucasians with IgG4-RD have elevated IgG4 levels in peripheral blood (100). Moreover, the organ manifestations of the other histiocytic diseases, Langerhans cell histiocytosis and particularly Erdheim–Chester disease may resemble IgG4-RD. PET-CT scans including the extremities to below the knee and evaluation for diabetes insipidus may help to discern between the disorders (108, 109). Kimura's disease is a rare entity that primarily affects young to middle-aged Asians, typically causing cervical lymphadenopathy, peripheral eosinophilia, and increased IgE levels; it may resemble IgG4-RD and Rosai–Dorfman disease (111). Interestingly, Kimura's disease has been reported to respond to an anti-Il5 monoclonal antibody (mepolizumab) (112).

2.4.4 Eosinophilia in malignancies

Blood eosinophilia and/or infiltration of eosinophils in the tumor tissue is encountered in patients diagnosed with common solid tumors, and the presence of eosinophilia in blood or infiltrating solid tumors is not consistent in any neoplasm (113). A potential beneficial role may be explained by the secretion of various enzymes and cytokines by eosinophils that influence tumor immunity and reduce tumor progression (114). Severe blood eosinophilia during symptom development may be a diagnostic clue for malignancy and therefore guide the diagnostic process (115, 116). Results indicate that blood eosinophilia may be a positive prognostic factor in some malignant solid tumors, when present (113, 117), whereas the presence of blood eosinophilia after surgical resection may indicate an unfavorable prognosis, relapse, or rapid disease progression (118). The inconsistent observation of blood or tissue eosinophilia in malignant tumor entities and the lack of robust, prospective data indicate that blood eosinophilia may be used cautiously as a simple biomarker in some oncologic patients, similar to a leukemoid reaction. Nonetheless, the numerous reports and studies of eosinophilia in solid tumors add to the possible functions of eosinophil granulocytes in this developing field related to the tumor microenvironment, which awaits further clarification (119, 120).

A unique feature has emerged with the introduction of immune checkpoint inhibitors (ICIs), approved for the treatment of several cancers. Mild to moderate, though rarely severe. blood eosinophilia may be observed in less than 5% of patients, a few weeks to many months after treatment initiation. Eosinophilia may be asymptomatic, and therapy with the ICI may proceed in a small proportion of patients (121, 122). Decisions on treatment strategy are based on the individual patient's response to targeted treatment, the severity of organ damage, and measures to control the symptoms, including CS treatment. Close monitoring of eosinophil counts, manifestations due to eosinophil activity, and response to the ICI is appropriate. Co-administration of CS to lower the eosinophil count may be acceptable, while a differential diagnosis must be excluded (Figure 2). Blood eosinophilia may persist after discontinuation of ICI therapy, which poses additional concerns regarding monitoring and treatment strategies for eosinophilia and malignant diseases. ICI-induced eosinophilia may have a favorable prognostic impact (122, 123). In a significant percentage of patients with ICI-induced eosinophilia, treatment must be discontinued due to eosinophil-associated organ damage, such as heart, skin, and colon (124) (Supplementary Table 1).

3 Diagnostic workup in patients with eosinophilia

The clinical challenge of identifying a diagnosis in the individual patient with eosinophilia warrants a detailed medical history and examination, in addition to the results of routine blood tests. The circumstances mirror those of patients with MPN, when serial measurements of blood cell counts show increased numbers over time, and specific analyses are often needed for clarification.

Quantification of differential white blood cell counts may be more commonly performed in specialties that treat with immunosuppressants or chemotherapy, in the interest of neutrophil granulocytes and lymphocytes. These treatments may, however, reduce the absolute number of eosinophils or be associated with fluctuating numbers, perhaps masking a concurrent condition related to eosinophilia (Figure 2). Observation of eosinophils in such patients at the end of cycles or in treatment-free periods may be clinically informative, providing clues for extended follow-up, e.g., in a multidisciplinary approach.

Once the presence of a repeatedly elevated eosinophil count has been confirmed, the diagnostic workup can be viewed as a stepwise process as follows:

1. exclusion of secondary causes;
2. evaluation of primary causes; and
3. diagnosis by exclusion of idiopathic hypereosinophilia.

Secondary causes are overall much more common than primary eosinophilia or iHES. A wide variety of diseases may be associated with eosinophilia, and thus a thorough medical history and clinical examination are essential to identify the causes of (secondary) eosinophilia. Relevant information includes familial predisposition, concomitant disorders, previous malignancies, medications, travel, migration and exposures, medications, and the risk of drug reactions (Supplementary Tables 1, 2; Figure 2) (65–88, 101–103, 125, 126). Since all organs may be involved in patients with eosinophilia, it is essential to ask about symptoms and

observe for findings, that may not be mentioned or readily identified but may be (highly) relevant in the clinical context.

If no obvious causes of secondary eosinophilia are found, the next step is to evaluate for primary causes. This evaluation includes the following:

a. complete blood cell count;
b. bone marrow biopsy, aspirate, and blood for morphologic studies; and
c. karyotype, molecular analysis, flow cytometry, or fluorescence *in situ* hybridization (FISH) to determine clonality.

Blood counts and morphology reveal the severity of eosinophilia and abnormalities in other blood cells that may point to an underlying hematologic disease/clonal eosinophilia. Abnormalities in the morphology of eosinophils have been described in HES and CEL, but they may also be seen in reactive conditions. Bone marrow biopsy including morphology, immunophenotyping, cytogenetics, and molecular analysis may reveal an underlying hematologic disease/clonal eosinophilia. In the case of eosinophilia, FISH/cytogenetics and molecular analysis (on bone marrow aspirate or peripheral blood cells) should specifically look for *PDGFRA, PDGFRB, FGFR1, FLT3, ETV6*, and *JAK2* gene rearrangements (Figure 3).

The diagnosis of iHE or iHES is established when the diagnostic workup for primary and secondary eosinophilia is inconclusive.

Figure 3 presents a flow diagram indicating primary, secondary, and idiopathic eosinophilia (3–7, 17–20, 61). The diagram is also applicable in patients with minimal eosinophilia but is aligned with iHES. The blood cell count may be repeated once or twice, if not retrospectively available over weeks and months, to ascertain a baseline value. The cell count may fluctuate and show a (significant) increase from day to day, which may be associated with worsening symptoms and the need for immediate treatment.

Examination of specific organs with biopsy or specialized analysis like magnetic resonance imaging (MRI), echocardiography, positron emission tomography (PET) scan, or organ function test (especially lung and heart) may be a part of the baseline workup for both secondary and primary causes, iHES, and iHE.

Routinely, patients with eosinophilia can be attended to in the outpatient clinic (67), but the urgency in symptom development, risk in procedures, and access to diagnostic procedures, or onset of treatment, e.g., with CS, may require hospitalization and parallel examination of primary and secondary causes. Access to specialized exams (MRI, PET echocardiography, organ function tests, etc.) may be limited to daytime or weekdays or may not be available at the current institution, which may lead to referral of patients with eosinophilia and acute manifestations to other institutions specialized in this condition.

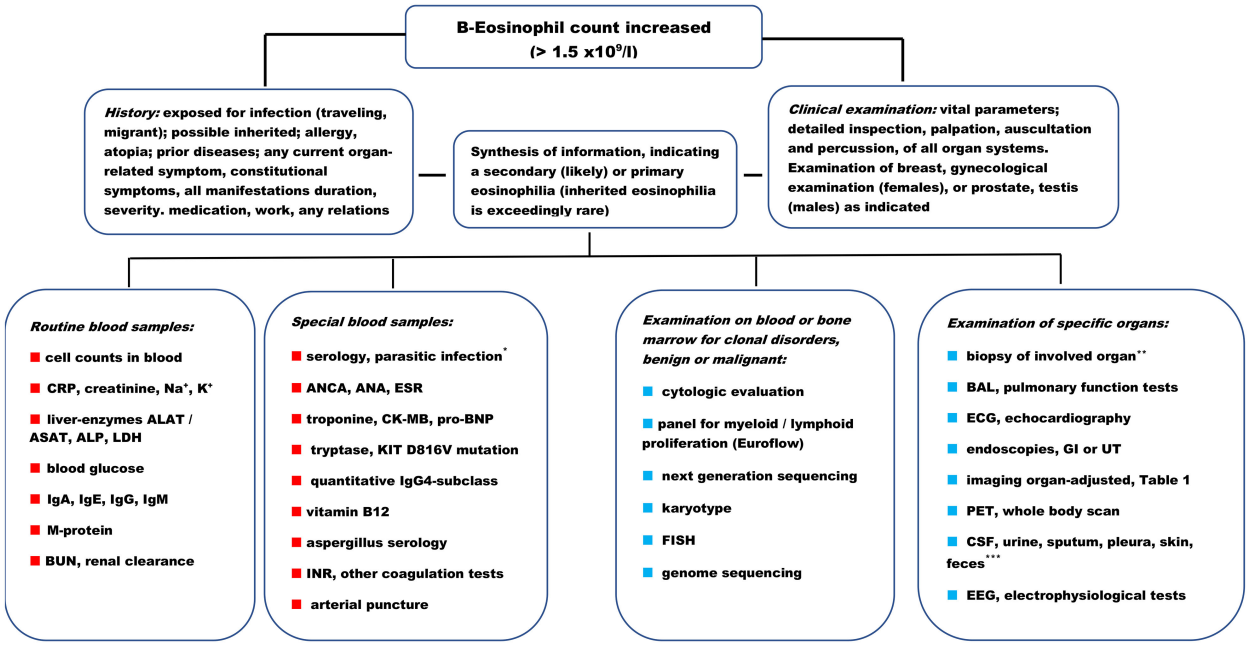

FIGURE 3
Diagnostic workup in patients with (moderate) eosinophilia. ALAT/ASAT, alanine aminotransferase/aspartate aminotransferase; ALP, alkaline phosphatase; ANA, antinuclear antibodies; ANCA, anti-neutrophil cytoplasmic antibodies; BAL, bronchoalveolar lavage; BUN, blood urea nitrogen; CK-MB, creatine kinase—myocardial band; CRP, C-reactive protein; CSF, cerebrospinal fluid; ECG, electrocardiogram; EEG, electroencephalogram; ESR, erythrocyte sedimentation rate; FISH, fluorescence *in situ* hybridization; GI, gastrointestinal tract; Ig, immunoglobulin; INR, international normalized ratio; K+, potassium; LDH, lactate dehydrogenase; M-protein, monoclonal protein; Na+, sodium; PET, positron emission tomography; pro-BNP, pro-b-type natriuretic peptide; UT, urinary tract. ⋆ See information provided in Supplementary Table 2. ⋆⋆ Skin, lung, lymph node, nasal polyp, liver, mucosa (GI, UT), muscle, myocardial, kidney, and brain. ⋆⋆⋆ For microscopy, culture, and other diagnostic tests (e.g., Mantoux in the skin). Proposals for blood samples and other tests to be adapted to pre-planned procedures at the department (e.g., routine laboratory packages). Proposals are not prioritized but must be selected and customized to the individual patient.

Documentation of eosinophilic infiltration in tissues, such as skin, heart, kidney, lung, lymph nodes, and bone marrow, should be performed to demonstrate the association with iHES and primary eosinophilia or to help establish the diagnosis of a secondary course. However, an issue may be to commence cytoreductive treatment within hours of having established or recognized the potential association of eosinophilia and critical symptoms, to stabilize the patient and mitigate worsening. Response to treatment in an acute setting may be rapid—from hours to a few days. Therefore, it is valuable for the diagnostic workup if blood samples (and skin biopsy if relevant and bone marrow if possible) are obtained and kept for microscopy, flow cytometry, and clonal mutation analysis before treatment is initiated. If transfer to a tertiary center for eosinophilia is planned, a decision based on a conference with the center, or the regional hematology department is relevant to decide whether blood and bone marrow samples can be obtained for analysis before treatment starts. More invasive procedures or biopsies may be challenging to perform before initiation of treatment or transferal. Performing analysis in responding patients when the patient has a very low blood eosinophil count is unlikely to be similarly informative (Figure 3). The diagnostic process may be delayed, and the analysis may need to be repeated as the CS is tapered and the eosinophil count increases.

In conclusion, the diagnostic workup should be customized to the individual patient, based on organ manifestations (Supplementary Tables 1, 2) and interpretation of the pathophysiologic driver (Figure 2), and translated to conduct the relevant examinations (Figure 3).

In addition to the diagnostic workup, in cases of primary eosinophilia, iHE, and iHES, it may be recommended to register baseline cardiac and pulmonary function tests, notwithstanding any manifestations, but because vital organs are often involved in primary eosinophilia (3–7, 20, 41, 42, 53, 54, 58, 65–67).

4 Treatment of patients with eosinophilia

The first-line treatment in acute circumstances due to eosinophilia is CS, orally or intravenously at high doses (maximum 1 mg/kg prednisolone or equipotent Solu-Medrol), once a day. This is effective in approximately four out of five patients, with a gradual, often cytolytic reduction in the eosinophil count and typically an associated symptom relief. This improvement may be supported by other treatments, such as those for organ failure, infection, or a specific diagnosis, as indicated (Supplementary Tables 1, 2; Figure 2). The effect of CS on eosinophilia is rapid in CS-sensitive patients. CS can reduce the survival and function of eosinophils, block autocrine cytokine signaling, and impact the production of eosinopoietic factors derived from T lymphocytes or other immunoregulatory cells (127–130). It is not possible to predict whether a patient will respond to CS treatment (the first time CS is administered for eosinophilia) and benefit from the often-rapid effect. In patients who do not respond (sufficiently) to CS, this feature may support the interpretation of a myeloid, clonal genesis, driving the eosinophilia and overriding cytokine stimulation of eosinopoiesis, derived from T lymphocytes or macrophages. Patients with the *FIP1L1-PDGFRA* myeloid neoplasm with a tyrosine kinase fusion (Figure 2) are usually not sensitive to CS (58). The pattern of responsiveness to CS is often characteristic in the individual patient at the time of relapse of primary eosinophilia.

Upon diagnosis of secondary eosinophilia, treatment is initiated according to guidelines, perhaps supported by institutional recommendations, all aimed at reducing or eliminating symptoms and improving quality of life. Treatment may be directed at infectious diseases, inflammatory diseases, including autoimmune diseases, or malignancies accompanied by eosinophilia (Figure 2). Numerous medications are available for the management of this large patient population, which includes almost all patients with eosinophilia, and include antimicrobials, immune suppressants, anti-inflammatory drugs, or chemotherapy. Medical treatment is administered in different ways: orally, parenterally, applied on the skin, inhaled, or in other ways that target specific compartments (e.g., intrathecally), or may involve radiotherapy. CS is an example of an agent used systemically in EGPA, while it is routinely used by inhalation in asthma or ABPA. In addition, drugs supporting organ function may be needed, whether the cause is ascribed to eosinophilia or other factors. The list reflects the standard of care for all internal medicine diagnoses.

If the patient does not improve with treatment, or if (isolated) eosinophilia persists, then a follow-up assessment may be needed to exclude a concomitant disease, representing primary eosinophilia, (other) secondary eosinophilia, or very rare inborn errors of immunity or familial eosinophilia (Supplementary Tables 1, 2; Figure 2). Mild (perhaps moderate) eosinophilia may be acceptable, reflecting the number of eosinophils as a potential biomarker in patients with non-malignant disease who are otherwise, responding satisfactorily and are being monitored regularly according to standard of care (131, 132). However, an increased eosinophil count in patients with solid tumors (118) and Hodgkin's lymphoma (133) should be followed, reflecting incomplete remission or relapse as secondary eosinophilia (Figure 2).

5 Primary and idiopathic eosinophilia

5.1 Specific diagnoses

The diagnostic entity "myeloid/lymphoid neoplasms with eosinophilia and tyrosine kinase gene fusions" (MLN-TK) was introduced in the 2008 WHO classification (134). The dissection of the genetic aberrations has contributed to identifying and including more patients in this clinically heterogeneous group and added information relevant to prognosis and treatment (9, 95). The neoplasms in this group are driven by constitutively active domains in tyrosine kinase fusion genes and may carry additional mutations in other genes that promote an increase in the number and survival of malignant cells (19, 135–137). Consequently, the pathophysiology of the disease and the clinical presentation of the patients with MLN-TK are very heterogeneous, emphasizing the importance of establishing a correct diagnosis. The application of cytogenetic and molecular

analysis can be guided by the history (acute or chronic), examination findings (lymphadenopathy and splenomegaly), and laboratory tests (tryptase, blasts) (Figure 3) (138).

All the specific diagnoses included in the MLN-TK must be *BCR-ABL1* negative and instead arise due to rearrangements and abnormal gene products in *PDGFRA*, *PDGFRB, FGFR1*, *FLT3, ETV6*, or *JAK2* (9, 95, 135–137). The origin of fusion genes involves cryptic deletions or translocations of chromosomal regions during mitosis in hematopoietic stem cells, which result in dysregulated intracellular signaling and the development of distinct AML, MPN, MDS, overlapping forms, mixed phenotype acute leukemia, and T- or B-ALL or lymphoma.

Figure 4 provides examples of the clinical presentation and management of MLN-TK, representing the diversity of disease in primary, neoplastic (intrinsic) eosinophilia. The figure is not exhaustive, as the individual patient may present with variable symptoms from different organ systems (Supplementary Table 1). The partner combinations of the fusion genes in MLN-TK influence the clinical presentation, and a more—in this context—common partner and the associated phenotype is presented as an example in each of the six categories (Figure 4). Specific features that may be observed in blood samples, are indicated, but are not universally observed and may be influenced by variations in disease dynamics, patient comorbidity, and latency or initiation of treatment before referral for examination, e.g., anemia, thrombocytopenia, and related symptoms. In principle, all organs may be involved in MLN-TK (Supplementary Table 1), and the manifestations and clinical course from the presentation may be similar to those of acute or chronic leukemia (Figure 4).

Eosinophilia is a hallmark of patients with MLN-TK that develops *de novo* but may be absent in a minority of patients. The increase in blood eosinophils may be mild to severe, and variable within the same MLN-TK entity. This feature reflects the complexity of the perturbed cell biology and the importance of identifying the partner gene through different analyses for clonality to confirm the correct diagnosis and possibly initiate a targeted therapy (Figures 3, 4). The number of potential partners is different for each fusion gene, but one gene can be involved in different fusion gene relations, and the number of genes and related pathways involved is increasing, with at least 72 fusion gene

FIGURE 4
Myeloid/lymphoid neoplasms with eosinophilia and tyrosine kinase fusions (MLN-TK). One disease with a specific rearrangement occurring in the MLN-TK is described briefly. ABL1, abelson1; ALL, acute lymphocytic leukemia/lymphoblastic lymphoma; AML, acute myeloid leukemia; BM, bone marrow; CEL, chronic eosinophilic leukemia; CML, chronic myeloid leukemia; CMML, chronic myelomonocytic leukemia; MPN, myeloproliferative neoplasm; CS, corticosteroid; del, deletion; EMD, extramedullary disease; ETV6, ETS variant transcription factor 6; FGFR1, fibroblast growth factor receptor 1; FIP1L1, Factor interacting with PAPOLA and CPSF1; FISH, fluorescence *in situ* hybridization; FLT3, fms-related receptor tyrosine kinase 3; HSCT hematopoietic stem cell transplantation; incl, including; JAK2, Janus kinase 2; LDH, lactate dehydrogenase; M, male; MDS, myelodysplastic neoplasm; MPAL, mixed-phenotype acute leukemia; PCM1, Pericentriolar material 1; PDGFRA/B, platelet-derived growth factor A or B; RT-PCR, reverse transcription polymerase chain reaction; t, translocation; TKI, tyrosine kinase inhibitor, here: imatinib, dasatinib, and ponatinib; WBC, white blood cell count; ZMYM2, Zinc Finger MYM-Type Containing 2; *eosinophilia, mild–severe, almost always present.

combinations overall in MLN-TK (9, 19, 95, 135–138). A challenge in the diagnostic process is how to combine panels and methodologies with varying sensitivity and specificity in bone marrow samples (Figures 3, 4). The partner in a rearrangement may not always be identified at diagnosis. Tissue samples may also be taken from enlarged lymph nodes, skin, or organs, and samples may be stored in the freezer for additional analysis if needed later.

Eosinophilia is the dominant feature of blood cell analysis in CEL and the most frequent *PDGFRA*-associated MLN-TK (9, 58, 139). The entity CEL is characterized by persistent hypereosinophilia for at least 4 weeks, organ involvement due to eosinophilia, and evidence of both clonality and abnormal bone marrow morphology (e.g., erythroid or megakaryocytic dysplasia), but does not require increased blasts (≥2% in peripheral blood or 5%–19% in bone marrow). The former CEL "not otherwise specified (NOS)," has, by this revised description, been left out of the fifth WHO classification (9, 95, 134). The result of clonal analysis in patients with CEL must exclude the other MLN-TKs specifically as primary eosinophilia, and acute or chronic MPN in general, defined by cytogenetic or mutational criteria in the WHO classification (9, 95). The information provided by cytogenetic, mutational, and cytologic analysis justifies that CEL represents an independent MPN (Figures 2, 4), which often presents with organ involvement due to eosinophilia, and may share clinical features with *PDGFRA-FIPL1* MLN-TK. CEL is different from iHE or iHES, both benign conditions, and is defined by persistent hypereosinophilia in the absence of a clonal or reactive cause, by clonality, and abnormal bone marrow morphology (140). Mutational analysis for a single or concurrent clonal driver in patients with CEL has been reported in a variety of genes involved in myeloproliferation, such as *JAK2*, additional sex combs like 1 (*ASXL1*), chaperonin containing TCP1 subunit 6B (*CCT6B*), tet methylcytosine dioxygenase 2 (*TET2*), enhancer of zeste homolog 2 (*EZH2*), Casitas B-lineage lymphoma (*CBL*), or signal transducer and activator of transcription (*STAT*). The clinical phenotype may therefore be differentially associated with different genotypes (141–144). Next-generation sequencing (NGS) or polymerase chain reaction (PCR) analysis can be performed in cases without clonality by other tests to examine the differential diagnosis of CEL and iHE_{US} or iHES, which may be therapeutically important, demonstrating a potential target (Figures 2–4).

Eosinophilia may be observed in a minority of patients with MPNs such asPV, PMF, or ET. However, it may be difficult to disentangle eosinophilia as part of the neoplastic process in these cases and, alternatively, it is appropriate to consider eosinophilia in these patients to be secondary, reactive to another condition. The diagnosis of an infectious or inflammatory condition in a patient with classic Philadelphia-negative MPN is imperative for optimal management (Supplementary Tables 1, 2; Figures 2, 3).

5.2 Treatment of primary and idiopathic eosinophilia

The approach may be wait-and-watch or may include any symptomatic treatment pending diagnostic clarification, such as antibiotics, xanthine oxidase inhibition, and fluid replacement, as indicated by the clinical circumstances since the dynamics and manifestations in the conditions are variable (Supplementary Table 1; Figures 2, 4). Unless diagnostic information is available at an early stage, e.g., by flow cytometry in AML, CS may be part of a first-line treatment to reduce symptoms and the blood eosinophil count. The final treatment strategy is decided by shared decision-making with the patient, based on full information and a complete diagnostic analysis (Figure 3). Participation in a clinical trial is recommended for all patients with primary eosinophilia and iHES, if possible. No treatment is indicated for eosinophilia in iHES.

Imatinib is authorized by the FDA and EMA for the treatment of advanced iHES and MLN-TK with *FIP1L1-PDGFRA* rearrangement, and mepolizumab is approved as an add-on treatment for adult patients with inadequately controlled iHES without an identifiable non-hematologic secondary cause. The administration of a tyrosine kinase inhibitor (TKI) or anti-IL5 monoclonal antibody (anti-IL5 mAB) is rational given the pathophysiology of iHES and MLN-TK with *FIP1L1-PDGFRA*, which involves the potential stimulation of eosinophils by IL-5 (23–27, 145, 146) and a highly sensitive constitutively active tyrosine kinase (58, 139, 147), respectively.

The treatment strategy in all patients other than MLN-TK with *FIP1L1-PDGFRA* in the first line is therefore based on established use and extrapolation, and a highly individual assessment. This includes information on the diagnosis of CEL, type of MNL-TK or iHES, symptoms, comorbidity, and patient preferences. Adverse events or a planned pregnancy may impact the decision during treatment (3–7, 20, 61, 62, 64, 65). Treatment of primary eosinophilia and iHES includes one or more drugs to reduce and preferably normalize the eosinophil count and any additional medications indicated to mitigate symptoms caused by eosinophilia and organ symptoms. Supportive treatment is highly individualized and may involve all internal medicine specialties (Supplementary Table 1).

Figure 4; Table 1 present the treatment options in MNL-TK, CEL, and iHES. Given the pathophysiologic mechanisms in MLN-TK, information on the clonal nature may indicate the use of TKI, chemotherapy, high-dose cytotoxic regimens, and stem cell support, including allogeneic hematopoietic stem cell transplantation (HSCT), with variable outcomes. Treatment of *FIP1L1-PDGFRA* MLN-TK shows a very high rate of durable complete remissions to low-dose imatinib, including undetectable residual disease by FISH or quantitative reverse transcriptase (RT) PCR. The potential cure of the patient is reflected by the discontinuation of imatinib in a small group of *FIP1L1-PDGFRA* MLN-TK patients (58). In contrast, the diversity of the diagnoses is reflected by indications for AML or ALL regimens in other patients of the same entity (Figure 4).

The treatment of CEL and iHES reflects the possible lymphoid or myeloid nature since the specific mechanism of the disease is not known in iHES and may not be translated from a clonal finding to a therapeutic agent in CEL (Table 2). There are no randomized clinical trials (RCTs) or robust data to support international agreement on the first or subsequent lines of treatment, after CS,

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TABLE 1 Treatment options in chronic eosinophilic leukemia and idiopathic hypereosinophilic syndrome.

Drug	Mode-of-action in eosinophilia	Dosage (adults)	Administration	Potential adverse events	Precautions/Comments
Prednisolone (solu-medrol)	Induce lymphocyte apoptosis, reduce IL-5 production, and may induce apoptosis in eosinophil granulocytes	Starting dose: 0.5–1 mg/kg, tapering over weeks–months (40–100) daily	Oral (IV) guided by clinical circumstances Tablet (or injection)	Osteoporosis, diabetes mellitus, anti-pyretic, risk for infections, increase in serum cholesterol and triglycerides, hypertension, reduced wound healing, impaired skin integrity, mental disturbance	Check blood-glucose and lipids, institute osteoporosis prophylaxis and BMD monitoring if other risk factors and/or repeated courses or maintenance treatment with CS is administered. Slow tapering in weeks to avoid adrenal insufficiency after long-term treatment. Patients may carry an information card
Interferon-alpha 2a	Receptor binding and immunomodulation, acting on lymphoid and myeloid cells	62.5–180 µg 7–10 (sometimes 14) day interval	SC (preceded by paracetamol). Pre-filled syringes	Mental and physical fatigue, gastrointestinal myalgia and arthralgia, anemia, thrombopenia, and/or leukopenia, hepatotoxicity, and thyroid dysfunction	Recommend gradually increasing dosage over weeks, according to vial content, to increase tolerance. Acceptable in pregnancy. To be self-administered by the patient (or caretaker)
Hydroxyurea	Cytoreductive, phase-related inhibition of DNA synthesis	500–1,500 mg once daily (rarely less, not recommend a higher dose)	Oral Tablet or capsule, one dosage size	Pan-cytopenia, an increase in MCV, oral mucositis, alopecia, skin ulcers, melanoma, and possibly carcinogenic	Avoid exposure to sunlight. Later-line treatment in patients <60–65 years due to long-term risk. Consider sperm deposit. Contraindicated in pregnancy, lactation
Mycophenolat mofetil	Inosine-5'-monophosphate dehydrogenase blocking agent, inhibiting T- and B-lymphocyte proliferation and function, immune suppressive	500–1,500 mg in one or two dosages daily	Oral Tablet	Abdominal pain, anemia, thrombo- and/or leuko/lymphopenia, infection, hepatotoxicity, alopecia	Recommend increasing dosage gradually and await an effect in at least 3 months. Contraindicated in pregnancy, lactation
Azatioprin	Antagonist of purine metabolism, immune suppressive	50–150 mg once daily	Oral Tablet, variable dose	Anemia, thrombo- and/or leuko/lymphopenia, hepatotoxicity, infection	May be used in pregnancy, but not while lactating. Interactions
Cyclosporine A	Immune suppressive, inhibition of the production of cytokines, e.g., IL-2, involved in T-lymphocyte activation	100–200 mg once daily (low dosage)	Oral Capsule or mixture	Tremor and cramps, hypertension, renal insufficiency (but less than observed in organ-transplants)	Contraindicated in renal insufficiency. Monitoring P-concentrations should not be needed
Methotrexate	Immune suppressive, inhibits purine and pyrimidine synthesis, reducing T-lymphocyte cytokine signaling	5–20 mg once weekly	Oral Tablet, variable dose	Anemia, thrombo- or leuko/lymphopenia, infection (including HZV re-activation), stomatitis, gastrointestinal, and pneumonitis	Contraindicated in pregnancy and lactation. Regular monitoring of blood cell-counts. HZV vaccination?
Mepolizumab	Anti-IL5 monoclonal antibody	300 mg every 4 weeks	SC	Abdominal and back pain, eczema, and allergic reactions.	Risk for parasitic infections (Supplementary Table 2)
Imatinib	Tyrosine kinase inhibitor	100–400 mg daily	Oral Tablet, variable dose	Muscle cramps and stiffness, abdominal pain, periorbital or extremity edema, anemia, thrombo- and/or leukopenia, alopecia	Dose relation in adverse events. Contraindicated in pregnancy and lactation

BMD, bone mineral density; HZV, herpes zoster virus; IL-2, interleukin 2; IL-5, interleukin 5; IV, intravenous; SC, subcutaneous.

and the decision will depend on the evaluation of the individual patient and treatment options.

CS is the drug of choice when immediate treatment is indicated in CEL and iHES, until clarification. Maintenance treatment, if CS is effective, should include steroid-sparing agents, due to the chronic nature or repeated need for CS, while tapering CS. Lack of CS sensitivity may indicate a "myeloid" phenotype, supporting, for example, interferon-alpha or hydroxyurea as a non-targeting treatment as the next line of treatment. Access to serum IL-5 or other cytokine levels may guide treatment with an

TABLE 2 Ongoing clinical trials in patients with primary eosinophilia.

Drug (NCT number)	Title	Start date–estimated completion date	Estimated enrollment (age at inclusion)	Phase (Design)	Comparator	Primary outcome (route of administration)	Locations (number of sites)
Benralizumab (anti-IL-5r mAb) (NCT04191304)	A phase 3 study to evaluate the efficacy and safety of benralizumab in patients with hypereosinophilic syndrome (HES) (NATRON)	22 July 2020–4 November 2024	120 participants (≥12 years)	Phase 3 (Double-Blind)	Placebo	Time to first HES worsening/flares (SC)	United States, Austria, Belgium, Denmark, France, Germany, Israel, Italy, Japan, the Netherlands, Poland, Switzerland (46)
Depemokimab (anti-IL-5 mAb) (NCT05334368)	Depemokimab in participants with hypereosinophilic syndrome, efficacy, and safety trial (DESTINY)	6 September 2022–30 May 2025	120 participants (≥18 years)	Phase 3 (Double-blind)	Placebo	Frequency of HES flares (SC)	United States, China, Japan, Republic of Korea, Spain (12)
Imatinib (TKI) Ruxolitinib (JAK inhibitor) (NCT00044304)	Tyrosine kinase inhibition to treat myeloid hypereosinophilic syndrome	26 September 2002–1 January 2026	60 participants (>2 years of age for imatinib, and ≥18 years of age for ruxolitinib)	Phase 2 (Open-label)	None	Peripheral blood absolute eosinophil count (oral)	United States (1).
Ruxolitinib (JAK inhibitor) (NCT03801434)	Ruxolitinib in treating patients with hypereosinophilic syndrome or primary eosinophilic disorders	15 November 2019–21 October 2023	25 participants (≥18 years)	Phase 2 (Open-label)	None	Overall response rate (oral)	United States (4)
Mepolizumab (anti-IL-5 mAb) (NCT04965636)	Study in pediatrics with hypereosinophilic syndrome (SPHERE)	14 July 2022–13 September 2023	25 participants (6–17 years)	Phase 3 (Open-label)	None	Number of HES flares experienced by participants per year (SC)	United States, Argentina, Spain (9)
Benralizumab (anti-IL-5r mAb) (NCT02130882)	Study to evaluate safety and efficacy of benralizumab in subjects with hypereosinophilic syndrome (HESIL5R)	19 May 2014–31 December 2023	20 participants (≥18–75 years)	Phase 2 + 3 (Double-blind)	Placebo	Number of participants with a 50% reduction in peripheral blood eosinophilia at week 12 (SC)	United States (1)

anti-IL-5, anti-interleukin 5; anti-IL-5r, anti-interleukin 5 receptor; HES, hypereosinophilic syndrome; JAK, Janus kinase; mAB, monoclonal antibody; SC, subcutaneous injection; TKI, tyrosine kinase inhibitor. Database search of clin.trial.gov March 2023.

immunosuppressive drug. However, validated and robust prospective results are not available, and pretreatment with CS may impact the analysis (145). The decision to introduce methotrexate, azathioprine, cyclosporine, or mycophenolate, instead of, for example, interferon-alpha or hydroxyurea, remains a clinical one (Table 1). The administration of these drugs is supported by case reports, small studies, retrospective analyses, and established use extrapolating from experience in lymphoproliferative and myeloproliferative diseases (63, 104, 148–151) (Table 2).

The lowest dose of CS or any agent should be used, and combination treatment may be administered, as in selected cases with PV or PMF, to reduce adverse events and maintain optimal efficacy. Efficacy may last from weeks to 2 - 3 months, preferably introducing only one drug at a time. Prophylaxis against osteoporosis should be initiated at an early stage in CS-sensitive patients with risk factors for bone density reduction, as CS may be used for months and repeatedly. Prophylactic antimicrobials for *Pseudomonas* infection may be contemplated in selected patients with chronic lung disease and long-term therapy with immunosuppressive agents (Table 1).

Imatinib is approved for advanced iHES, which is a heterogeneous group clinically identified with persistent symptoms due to eosinophilia, and unresponsive to administered lines of treatment. Imatinib may be effective in CEL or iHES because fusion genes, not detected by analysis, may be sensitive to TKI (152). A standard dose may be started and tapered if effective to the lowest effective dosage. Response to any treatment in CEL or iHES is variable (Table 1).

There are no internationally agreed-upon definitions of response to treatment in primary eosinophilia or iHES. Reduction

in symptoms, flares, eosinophil count (complete response requires durable normalization), and any biomarker, particularly the clonal marker, may reflect a satisfactory effect.

Irrespective of the choice of treatment in CEL or iHES, drug discontinuation may be planned after months to years of treatment. It may be reasonable to taper the dosage, considering the drug and being aware that relapse of symptoms, typically manifested at the time of diagnosis is a risk. Patients with rare diagnoses such as primary eosinophilia or iHES should continue to be monitored and treated in specialized departments.

6 Ongoing research in primary eosinophilia

Several clinical trials have been completed or are ongoing with anti-IL5 biologicals in patients with secondary eosinophilia, such as eosinophilic asthma (153), eosinophilic esophagitis (154), and EGPA (155). The results of two RCTs have been published (145, 146) and several clinical studies are ongoing in targeted therapies regarding iHES and clonal eosinophilia. The rarity of these patients is a concern in the planning of clinical studies, and most centers may only be able to enroll one or two patients in a trial. This is a burden for the individual (hematology) department and may be a reason to decline participation. Referral to centers may be a way to mitigate this challenge, but the administrative work and bureaucracy are a challenge for the conduct of clinical studies, which may impact research activity in primary and idiopathic eosinophilia (156).

Table 2 provides information on six trials currently ongoing in Western countries, some of which are still recruiting. The table reflects the challenge due to the rarity of the diseases to conduct active comparator RCTs, instead of "physician's choice," which, in primary eosinophilia and iHES, may be very heterogeneous (Table 1 and Figure 4). This may impact the interpretation of the outcome and the option for blinding the study. Alternatively, blinding of the randomized trial is possible and valuable in placebo-controlled designs, which isolate the effect of the experimental treatment even as an add-on to a standard of care, e.g., CS and hydroxyurea. Inclusion criteria are defined across all ages, reflecting the age distribution of patients with this diagnostic entity. The trials in pediatric patients are of particular interest in order to mitigate any long-term effects of alternative immunosuppressive, immunomodulating, or cytoreductive treatments. It is also an added value for adult patients with iHES or primary eosinophilia to offer biological therapy to achieve disease control by targeted agents with acceptable safety profiles, possibly lifelong, as in all MPNs.

The primary outcomes of the trials are listed and reflect the interest in restricting eosinophilia (by blood cell count) or symptoms (by the rate of response or flares), which are rational and objective endpoints and relevant to patients to reflect a treatment benefit. In this context, the information presented in Table 2 shows that the administration of all agents in the current trials is either orally or subcutaneously, which is beneficial for chronic treatments and allows home administration. If possible, novel drugs in clinical trials for primary eosinophilia or iHES administered intravenously should be avoided in order to reduce patients' time consumption/travel and stay at the hospital during treatment, and to reduce the use of resources at the department. This pattern, reflected in Table 2, is endorsed.

Dexpramipexole is an orally bioavailable synthetic aminobenzothiazole that has demonstrated encouraging results when treating HES with this potentially steroid-sparing agent (157, 158). One trial was started in 2014, but the status of the open-label, phase 2 study to evaluate the safety and efficacy of dexpramipexole (KNS-760704) in adults with iHES is unknown (NCT02101138).

Clinical studies of a second-generation TKI targeting *BCR-ABL1*, like dasatinib and nilotinib in the second line in patients with *FIP1L1-PDGFRA* MLN-TK, failing imatinib, or a *JAK2*-targeting TKI, like ruxolitinib, have possibly met the challenges of conducting clinical studies, even single-arm trials, in rare populations. One consequence may be the off-label use of these drugs, but it is pivotal to accumulate data on their efficacy and safety in patients with MLN-TK. This may be supported by centralized treatment of the patients or reporting to international registries.

7 Quality of life in primary and idiopathic eosinophilia

Patient-reported outcome (PRO) measures are today recognized as key endpoints in all clinical trials within hematological disorders (159). For *BCR-ABL1*-negative MPN, disease-specific tools for quality of life (QoL) assessment have been developed, e.g., the Myeloproliferative Neoplasm (MPN) Symptom Assessment Form Total Symptom Score (MPN-SAF-TSS) in ET, PV, and PMF (160), and in myelofibrosis specifically (161). The symptom burden among patients with MPN is highly correlated with QoL (162).

However, no specific tool to capture PRO in iHES, MLN-TK, or CEL has been developed and validated, and the MPN-SAF-TSS did not when established, include patients with these rarer acute and chronic myeloid conditions. Currently, general questionnaires like the European Organization for the Research and Treatment of Cancer Quality of Life Questionnaire (EORTC QLQ-C30) (163, 164), the simpler EuroQol 5-dimensional questionnaire (EQ-5D-5L) (165), or the Patient Reported Outcome Measurement Information System (PROMIS) Short Form (SF) (166) are implemented and recommended in the monitoring of patients and clinical trials in oncology (166, 167). The EORTC-QLQ-C30 in particular has the advantage of being available in more than 100 languages and having electronic records, which may be useful in clinical trials. These tools may be feasible for capturing relevant subjective issues in patients with iHES or primary eosinophilia. The data on QoL are registered as secondary or exploratory endpoints and may therefore be of limited value in single-arm studies or otherwise unblinded trials due to the risk of bias. The multi-faceted manifestations of the target population (Supplementary Table 1)

pose a challenge when trying to develop one homogeneous questionnaire in clinical trials of idiopathic and primary eosinophilia (Figures 2, 4). The placebo-controlled, blinded RCT of mepolizumab in iHES included QoL registered by SF12 (145). The survey demonstrated that baseline parameters were similar in the experimental and control groups and that QoL did not deteriorate during the study period. QoL measures are included in many ongoing trials (Table 2). Robust studies of QoL in primary eosinophilia, iHE, and iHES outside a clinical trial have not been done.

Tools like the Patient Global Impression of Change Item (PGIC) or the Patient Global Impression of Severity Item (PGIS) may be acceptable in clinical studies and for population studies due to the lack of more specific measures (168, 169). The Physician Global Assessment (PGA) has been used in several clinical settings as a disease activity instrument on a visual analog scale and was originally developed for systemic lupus erythematosus (170). However, PGA may not be adequate from an overall perspective in iHES or clonal eosinophilia because, although some symptoms may be objective, several are not (Supplementary Table 1), and the PGA may also be biased, influenced by the physician's experience and circumstances of evaluation.

Thus, today, information regarding QoL in iHES and clonal eosinophilia outside clinical trials should be captured and monitored by detailed interviews with the individual patient due to the known plethora of symptoms in the population. This is a time-consuming challenge in the clinic, and electronic tools for comparing symptoms and variables, e.g., cell counts in blood, may be an important tool to improve the inclusion of this vital information when monitoring patients in the future (171).

8 Multidisciplinary team in eosinophilia

8.1 Description of the collaboration

A multidisciplinary team (MDT) in a hospital is a function specifically suited to cases with complex care needs due to the variable symptoms, diagnostic challenges, and individualized treatment. MDTs are appropriate for rare diseases because expertise is required to provide optimal care and coordinating work in professional MDTs also promotes improvement in patient management. This has been well described in the management of patients with benign or malignant surgical and medical disorders and in research (172–177). MDTs may also be called tertiary centers.

Establishing an MDT has to take into account the different facilities and disciplines at hand, and the effort necessary in an MDT requires a number of clinical and diagnostic facilities to join the collaboration. MDTs are most often organized in regional or university hospitals with centralized functions in several specialties, and thus with extensive and recurrent interaction with local/regional hospitals based on collaborating policies. This development is in line with the centralization of certain functions, to aim for and maintain the highest degree of competence. MDTs and centralized functions reflect the same interest but differ in—at least—the fact that centralized functions are often allocated to one department based on daily routines, whereas MDTs are only operational when several diagnostic and clinical departments have established and agreed to a manner in which to collaborate, as a local policy. The centralized function is expected to run 24/7, whereas MDTs typically have planned meetings at weekly intervals.

A tertiary center for eosinophilia is a specialized function serving a large population since the number of patients with a primary or idiopathic cause in need of specialized treatment at the department of hematology and the incidence of complex secondary cases is low. Inborn errors of immunity and familial cases are exceedingly rare (Figure 2). The population of Denmark is 5.85 million (in 2021), and two MDTs were allocated by government decision in 2017 at two university hospitals, each in a different part of the country. One is in the capital region (at Rigshospitalet, in Eastern Denmark) and the other is in the Southern Denmark region (at Odense University Hospital). Both MDTs are open to referrals from all five regions of the country, primarily for adult patients. Individual patients with eosinophilia can decide which MDT is more convenient for them to attend, but the contact with local/regional departments can be continuous due to the collaboration between departments at different institutions. Similarly, tertiary centers for other rare diagnoses have been established at Danish university hospitals. The Center for Eosinophilia is physically located in the Department of Hematology and collaborates with the six other hematology departments in Denmark, which are routinely involved in the management of patients following an initial referral at a regional level. The Department of Hematology may be appropriate to host the MDT due to the special differential diagnosis, including idiopathic and primary eosinophilia, and also including secondary cases of histiocytosis, malignant lymphoma, and leukemia, whereas the majority of secondary eosinophilia cases are optimally treated at other specialized departments (Supplementary Tables 1, 2; Figures 2–4).

8.2 Elements and benefits of an eosinophilia MDT

Figure 5 shows the principles of patient flow and functions in an eosinophilia MDT. It is important that GPs are also aware of the tertiary centers, e.g., through the dissemination of knowledge at regional meetings and in national medical journals (67, 178) (level 1) and that the specialized departments at the MDT hospital all serve as a filter for a second opinion in the MDT (level 2). Furthermore, GPs should be aware that there are no formal criteria for listing a case for an upcoming conference, which is planned regularly every 4–6 weeks. The MDT program can be planned 6 months in advance, each meeting lasting 60–75 min. The upcoming conference is announced *via* a mailing list to the colleagues in the MDT network and is sent out in advance with anonymized information on usually three to five patients. More detailed information can be retrieved in the secure areas of the hospital's electronic filing system, which can be shared with all other hospitals in the region. The email information includes a

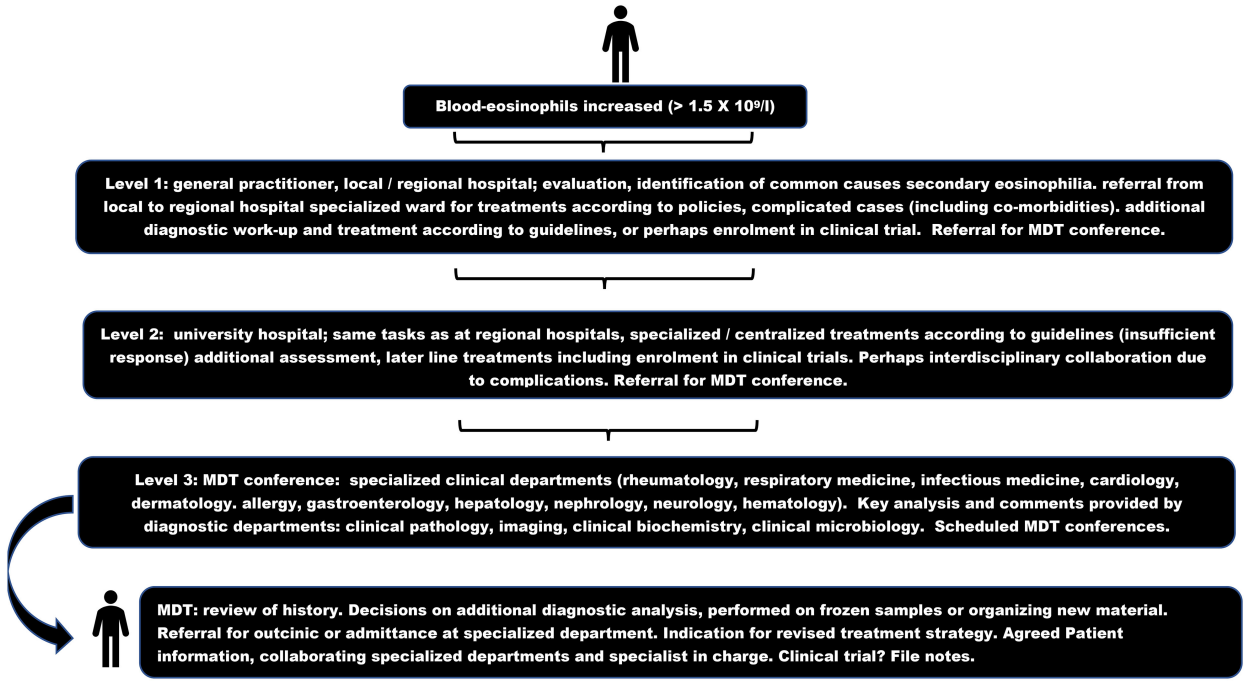

FIGURE 5
Multidisciplinary team flowchart in eosinophilia. MDT multidisciplinary team.

video link for virtual participation in the MDT, which has been a positive outcome of the COVID pandemic (179). In this way, videoconferencing facilitates participation. The two centers for eosinophilia in Denmark organize conferences together four times a year to disseminate knowledge. This activity is made possible due to the use of virtual rooms. In principle, these conferences are open to all interested colleagues (nationwide) and participation is optional. The schedule for the MDT can include fixed days or be a rolling sequence to accommodate the numerous other activities on the ward, in the outpatient clinic, and at other conferences.

A major benefit of the organization of an MDT in eosinophilia is that it allows for direct contact with colleagues participating in the MDT during normal working hours. Colleagues can be contacted *via* an MDT telephone list and are more familiar with the task, and thus are more likely to be able to provide a qualified opinion directly than can be expected from both junior and senior doctors who are generally on-call. Naturally, cases can be discussed in a department within the MDT network, between on-call colleagues and colleagues participating in an MDT, if this is more feasible. It is our experience that an option for personal contact, through telephone lists for the MDT, is one of the aspects facilitating the process when, for various reasons (clinical circumstances, time, and presence) a discussion cannot await the next scheduled MDT to make decisions.

Figure 5 illustrates that most patients are referred from highly specialized departments at the MDT hospital or from collaborating hematology departments (67). The individual patient is presented by the physician most familiar with the case (most often the treating specialist). The case presentation may be in PowerPoint format, which can support the educational dimension of MDT conferences. Patients can be discussed in the MDT either during admission to the hospital or during follow-up in the outpatient clinic, reflecting the wide range of symptoms and severity in the target population (Supplementary Tables 1, 2; Figures 2–4). The purpose of discussing an eosinophilia case in the MDT is to agree on a second opinion, including the need for additional diagnostic tests, e.g., due to persistent eosinophilia and symptoms despite relevant treatment for secondary eosinophilia, and also to establish diagnoses of iHE and iHES *per exclusionem*. The next step is a treatment decision, according to the working diagnosis, to be implemented at the appropriate specialized department, either in the outpatient clinic or during admission to the ward. The scheduled conferences, both in the individual center and in the two national centers, must always include a plan for informing the patient (usually by phone) and any department or function involved (either by verbal information or by referral in writing). All decisions should be recorded as a conference note, and the next MDT will provide the opportunity for follow-up (Figure 5).

9 Discussion

A patient-centered function is fit for purpose due to the complex and challenging manifestations, diagnostics, and options for targeted or revised therapeutic strategy in a small proportion of adult patients with eosinophilia. This is the first presentation of the rationale for the MDT in eosinophilia (Supplementary Tables 1, 2; Table 1 and Figures 2–4). The MDT function is a well-established tool across medical disciplines (172–177), and MDTs have been

implemented in departments treating eosinophilic esophagitis, possibly improving patient outcomes (180, 181), and have also been considered in Loeffler's endocarditis (182). However, one difference between the MDT for patients with eosinophilia and other scheduled MDTs is that the MDT for eosinophilia typically starts by challenging and discussing the working diagnosis (Figure 5), whereas the diagnosis has been established unquestionably in (almost all referred patients) MDTs. The number of clinical and diagnostic departments in an eosinophilia MDT is high, requiring input from all medical specialties and diagnostic expertise (Figure 5). MDTs for established diagnoses in one specialty include additional expertise in specific fields, such as radiotherapists, physiotherapists, specially trained nurses, and nutritionists.

The MDT conferences in eosinophilia, if executed virtually, require an optimal technology for sound and image quality (179), and the sharing of specific parts of the patient's files among the MDT participants. These may include data from blood samples, medical history and clinical findings, CT-PET-MRI scans and descriptions, and light microscopy images of tissue samples. It is very important to prioritize the patient information and decide on the physician in charge of the next steps, and the procedures. Activities must take precautions to avoid disclosure of personal data when disseminating information by mail or virtual systems.

The MDT forum can facilitate educational activities, sharing of experiences at all levels of medical training and specialization. Moreover, the collaboration between the two MDTs in Denmark supports the possibility of participating in international clinical trials, e.g., in iHES (Table 2), because the potential number of patients to be included is higher than in the individual centers. The accumulation and interest of patients with eosinophilia in MDTs facilitate research, e.g., cohort studies in chronic lymphocytic leukemia demonstrating the impact of eosinophilia at diagnosis (183) and a retrospective study advocating PCR analysis for T-cell receptor status in the diagnostic workup (Figures 2, 3), to be performed only if clonality has been demonstrated by flow analysis of lymphocyte subpopulations in blood or bone marrow samples (67).

The differential count in blood and readily identifiable eosinophil granulocytes in blood smear (Figure 1) are a simple source of information to guide toward the broad spectrum of conditions associated with eosinophilia, as opposed to conditions characterized by isolated neutrophilia, monocytosis, or lymphocytosis (1, 2). While isolated blood eosinophilia is characteristic of iHE, concomitant aberrations in other cell lines indicate other hematologic conditions, including iHES and MPNs, in addition to eosinophilia due to secondary causes (Figure 2). The biology of eosinophil granulocytes is well described (21–44), as is their role in various pathophysiologic states (Supplementary Tables 1, 2; Figure 2). The narration of eosinophil functions expanded from "a phagocyte" in innate immunity to a potential key player in the orchestration of homeostasis, normal development, and remodeling (15, 26, 28, 38). However, results from genetic knock-out models in animals, and more recently the possibility to observe any impact of treatments that deplete circulating eosinophils by IL-5 and IL-5 receptor-targeting treatments in functional knock-out humans, challenge the notion of the precise role of eosinophils in health (184–187). More robust results in patients treated with anti-IL5 biologicals or other targeted treatments, in primary or secondary eosinophilia, lowering the eosinophil count durably and maximally as well as research in this field is warranted.

Eosinophils are distributed after extravasation in numerous tissues, but it remains to be understood in more detail what triggers eosinophils to cause various end-organ manifestations in the individual patient (Supplementary Table 1). The distribution of eosinophils in human tissues is normally organ dependent and varies considerably. The distribution may be explained by tissue-specific chemokines or reflect the existence of eosinophil subpopulations *in vivo* (26, 29, 188, 189). The impact of cell subpopulations in primary or idiopathic eosinophilia has not been studied but may contribute to explaining the manifestations in different organs (65).

Current isotope scintigraphy is not readily applicable to eosinophils, in contrast to the information obtained from tracing neutrophils with a clinical benefit in diagnosis and treatment (190). An eosinophil-specific tracing technique could be a major step forward in the diagnosis and management of patients with eosinophilia, replacing or supporting invasive procedures. Perhaps monoclonal antibodies targeting epitopes, preferentially present on eosinophils, such as the IL5-receptor (Table 2) or siglec-8, could be labeled with an isotope serving as a tracer. However, cell death upon binding to the eosinophil plasma membrane would (most likely) be rapidly induced (191, 192). It may be considered whether antibody-functionalized magnetic particle imaging tracers could be a technique applicable to eosinophil granulocytes (193). Currently, imaging techniques indicating inflammation, like PET or gadolinium MRI and biopsies, remain part of the gold standard to demonstrate the involvement of eosinophils in damage.

The natural history of iHE and iHES, including triggering events for progression, is not well understood. Data on the population are available in retrospective, descriptive studies, which indicate the presence of severe anemia, hepatosplenomegaly, older age, and cardiac involvement in a scoring system to be prognostic. Patients with a low-risk score have a 5-year survival of over 95%, while a high-risk score indicates a 5-year survival of 62% (20, 194). Prospective MDT-orchestrated studies and multicenter studies can be a tool to accumulate data, as in the ongoing French COHESION study started in 2019 (NCT04018118). Similarly, the U.S. longitudinal study of familial hypereosinophilia started in 2005 (NCT00091871), may clarify details of the natural history of these rare disorders.

The number of eosinophils in the blood is not itself well-correlated with manifestations in patients with iHES (194, 195). Data have been presented that end-organ damage, to a variable extent in heart, lung, skin, and other tissues, may be explained by extravasation of eosinophils during the process, which affects the eosinophil count, and the risk of symptoms due to eosinophilia does not increase proportionately with counts higher than 1×10^9/L. The clinical implication is that a normal level or even a low number of eosinophilic granulocytes in circulation is not a safeguard for

excluding the risk for eosinophilic involvement in symptomatic patients (196, 197). The clinical implication is that the number of eosinophils in the blood may not in itself be a risk factor in hypereosinophilia, and the clinical assessment and indication for treatment in asymptomatic patients require careful patient information to react to symptoms. The clinical explanation for a correlation between symptoms and eosinophil count, when treatment is started, may therefore reflect the effect of lowering an increased eosinophil count and the clearance of eosinophils in the affected tissues.

Finally, the prognostic information of the blood eosinophil count on admission to the emergency department has been studied in a large, unselected, adult population. Moderate or severe eosinophilia is a risk factor for a significantly increased 3-month mortality, in patients older than 70 years with hematologic disease and an elevated C-reactive protein (198). The results emphasize that awareness of the eosinophil count is a diagnostic clue in the management of hypereosinophilic patients to reduce diagnostic delay, especially of the underlying malignancy (8, 198) (Figure 2).

The iHE_{US} and iHES, respectively, may be examined in more detail at diagnosis to improve the classification. Whole exome sequencing and genome-wide methylation analysis identifying novel disease-associated mutations and methylation patterns, may be appropriate tools to convert the idiopathic status into identifiable mutation-driven entities (90, 141–144). A proportion of cases may thus be contextualized within the MLN-TK or CEL groups, and perhaps be of therapeutic relevance. Casuistic reports of MPN or MDS presenting as iHES, reflecting a diagnostic overlap may not represent a pathophysiologic continuum (199). Rather, the reports reflect the complexity of establishing a strict classification in the chronic myeloid neoplasms with eosinophilia.

The introduction of IL-5 targeting treatment in neoplastic or idiopathic hypereosinophilia mirrors the increasing interest in recent years in mABs targeting the IL-5 pathway for the treatment of hypereosinophilic conditions such as severe asthma and EGPA (33, 153, 200). Table 2 shows that trials are ongoing in two mABs against free IL-5 with mepolizumab or a long-acting formulation (depemokimab), both of which neutralize IL-5 and prevent receptor binding. One study involves an mAB targeting IL-5r (benralizumab). Preliminary, encouraging results from the trial (NCT02130882) on the efficacy and safety of the concept of receptor binding-induced antibody-dependent cellular cytotoxicity of eosinophils in iHES have been presented (201). Reslizumab, a different mAB against free IL-5, may be a candidate for the treatment of iHES (191). Therapeutic trials in the pipeline to support the ongoing studies in MLN-TK, CEL, and iHES (Table 2), may include TKIs targeting FRGFR, FLT3, or STAT pathways, anti-IgE mABs, perhaps anti-CD52 mABs, and biologicals interfering with IL signaling, all of which have been studied in other diseases (202, 203). All agents may be CS-sparing but are also associated with adverse events that must be included in the benefit–risk assessment to induce longer remissions and disease control in chronic conditions. siglec-8 may be a target for treating iHES and primary eosinophilic conditions in RCTs (92, 204, 205). Still, evidence-based treatment algorithms are missing on eosinophil-directed agents, as are additional guidelines for thrombotic prophylaxis in the target population. Current guidelines in eosinophilia are excellent, describing agents for individualized treatment while taking age, comorbidity, manifestations, and information on cytogenetic and mutational drivers into account (3–7, 17, 18, 20, 58, 61, 136, 137, 145–152) (Table 1; Figure 4).

QoL is reduced in symptomatic patients with neoplastic or idiopathic eosinophilia and may also be influenced by adverse events to CS or other treatments, which may not outweigh the benefit of symptom reduction (Table 1). The development of a disease-specific PRO is important to register and monitor the consequences of eosinophilia and therapeutic interventions. The first important steps have been taken to introduce and validate a questionnaire and to decide on endpoints to be implemented in clinical trials and care (206).

10 Conclusion

The observation of eosinophilia in the differential count or blood smear is common and should always merit a reflection as to why it is present. This may, in almost all cases, be readily explained by the diagnostic workup. However, the remaining cases with persistently increased counts and variable symptoms despite adequate interventions, or without a proper diagnosis, may be discussed in a patient-centered MDT. This activity is justified by the pathophysiologic basis and clinical manifestations involving medical and diagnostic specialties. It remains to be demonstrated that the activity in this forum translates to a clinical benefit in patient QoL, overall management, and prognosis. An MDT at a tertiary center can be instrumental to generate data, providing education, and contributing to research. The function supports not only the hematologic subpopulation of patients but also patients with eosinophilia in general.

Author contributions

GT, CA, HV, and OB designed the project and drafted a major part of the manuscript, Tables 1, 2; Figures 2–5. HL, JD, KA, CM, AK, TH, SB-O, MM, SJ, and OB created Supplementary Table 1. CH and RM-I created Supplementary Table 2. MBM created Figure 1, and MC created Table 2. DEF wrote the section on histiocytosis and IgG4-related disease. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1193730/full#supplementary-material

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Mutations, inflammation and phenotype of myeloproliferative neoplasms

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Knowledge on the myeloproliferative neoplasms (MPNs) – polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF) – has accumulated since the discovery of the JAK/STAT-activating mutations associated with MPNs: *JAK2*V617F, observed in PV, ET and PMF; and the *MPL* and *CALR* mutations, found in ET and PMF. The intriguing lack of disease specificity of these mutations, and of the chronic inflammation associated with MPNs, triggered a quest for finding what precisely determines that MPN patients develop a PV, ET or PMF phenoptype. The mechanisms of action of MPN-driving mutations, and concomitant mutations (*ASXL1, DNMT3A, TET2*, others), have been extensively studied, as well as the role played by these mutations in inflammation, and several pathogenic models have been proposed. In parallel, different types of drugs have been tested in MPNs (JAK inhibitors, interferons, hydroxyurea, anagrelide, azacytidine, combinations of those), some acting on both JAK2 and inflammation. Yet MPNs remain incurable diseases. This review aims to present current, detailed knowledge on the pathogenic mechanisms specifically associated with PV, ET or PMF that may pave the way for the development of novel, curative therapies.

KEYWORDS

myeloproliferative neoplasms (MPN), polycythemia vera, essential thrombocythemia inflammation, therapeutic targets, primary fibrosis (PMF), mutations

Introduction

Normal myelopoiesis depends on the activation of the JAK2/STAT5 pathway by hematopoietic cytokines and their receptors. The JAK2/STAT5 pathway and myelopoiesis are physiologically hyper-stimulated in case of bleeding, hypoxia, or inflammation (1). Other causes of hyperstimulation of the JAK2/STAT5 pathway and myelopoiesis include the chronic Philadelphia-negative myeloproliferative neoplasms (MPNs). MPNs are

characterized by an excessive production of mature cells of the three myeloid lineages. They arise from the acquisition in a multipotent hematopoietic progenitor of a JAK2/STAT5-activating mutation in one of three genes – *JAK2*, *MPL, CALR* – and thus can be seen as clonal versions of myelopoiesis (2–8). Three subtypes of MPNs are distinguished: essential thrombocythemia (ET), where overproduction of megakaryocytes and platelets is predominant; polycythemia vera (PV), which concerns predominantly the erythroid lineage; and primary myelofibrosis (PMF), characterized by severe fibrosis of the bone marrow and splenomegaly (7–9). Among MPN-driving mutations, the V617F mutation of *JAK2* exon 14 (*JAK2*V617F) was discovered first, rapidly followed by the *MPL* exon 10 (W515L, W515K) and *CALR* exon 9 mutations (2–6). *JAK2*V617F is detected in >95% PV cases and in 50-60% of ET and PMF cases, while *CALR* mutations characterize 25-30% ET and PMF cases; *MPL* mutations are found in 5-10% ET and PMF cases. In addition, MPN patients typically present with chronic inflammation (10–13). Logically, numerous inflammatory cytokines are overexpressed by MPN patients; some activate the JAK2/STAT5 pathway (G-CSF, GM-CSF, interleukin 6 (IL-6)) and further increase myelopoiesis, while others activate the JAK1/STAT1-STAT3 pathways (IL-6, interferons (IFN)) and thus enhance cytokine production and facilitate cell survival (13–19). The severity of MPN clinical symptoms – fatigue, fever, night sweats, weight loss, itching – and complications – thrombosis (arterial, venous), splenomegaly, bone marrow fibrosis – typically increase with the level of inflammation, mild in ET, moderate in PV, and severe in PMF (20).

The lack of disease specificity of JAK2/STAT5-activating mutations triggered a quest for finding what precisely determines that a patient develops a PV, ET or PMF phenotype. Over the last decade, the mechanisms of action of MPN-driving mutations, as well as co-occurring mutations (*ASXL1, EZH2, DNMT3A, TET2*), in MPN disease initiation and progression have been extensively studied, *in vitro* and in murine models (21–30). The roles played in inflammation by driving and non-driving mutations, and their chronology, have also been investigated (13–19, 31, 32). In parallel, clinical trials have tested different drugs in MPNs (hydroxyurea, anagrelide, interferons (IFN), azacytidine, JAK inhibitors, some blocking only JAK2, or JAK1 and inflammation, or both), sometimes with unexpected results (33–41). Logically, the JAK inhibitors that significantly inhibited inflammation reduced clinical symptoms and spleen size (34, 36, 42–45). However, JAK inhibitors suppress the MPN clone and mutation load only partially, whereas IFN-α2 therapy leads to durable clinical and hematological remission for >75% MPN patients, as well as molecular remission for ~10% *JAK2*V617F-mutated PV, ET and PMF patients (33, 37, 46–48). Interestingly, IFN-α2 and JAK inhibitors reportedly act in synergy in MPNs (49, 50).

Despite major advances in knowledge and in therapy, MPNs remain incurable. Indeed, to be curative, treatments must counter the initial and other main events responsible for a particular disease. This review summarizes the present knowledge on the pathogenic events associated with the PV, ET or PMF phenotypes, with the aim to identify new therapeutic targets that could lead to curative treatments in the different MPN subtypes.

JAK2/STAT5-activating mutations and MPN phenotype

Certain MPN phenotypes are associated with specific driving mutations or/and mutant allele burden, but none can be explained solely by the patient's *JAK2*V617F load nor by the presence of *CALR* or *MPL* mutation(s). MPN phenotypes clearly do not depend on *JAK2*V617F, since this mutation is found in all MPN subtypes (PV, ET, PMF), as well as in refractory anemia with ring sideroblasts and thrombocytosis (RARS-T) and in splanchnic vein thrombosis (SVT) (Figure 1A). MPN clones can be heterozygous or homozygous for the *JAK2*V617F mutation, after recombination or gain of mutated chromosome 9, and the allele count and *JAK2*V617F load can also increase due to the amplification of the whole chromosome 9 (trisomy 9). Consequently, the size of *JAK2*V617F-mutated clones and the percentage (%) of *JAK2*V617F-mutated alleles varies widely, from 1% to 100%. Of note, 25-50% *JAK2*V617F-mutated alleles are observed in all MPN subtypes. Moreover, if homozygous *JAK2*V617F-mutated clones (*JAK2*V617F load ≥50%) are typical of PV, they are also found in PMF. Heterozygous *JAK2*V617F-mutated clones (*JAK2*V617F loads <50%) are typical of ET and RARS-T, but also observed in PMF, in SVT, and more rarely, in PV (Figure 1A). Yet the *JAK2*V617F mutant load affects clinical presentation: high *JAK2*V617F-mutated allele burdens are associated with increased hematocrit and leukocyte numbers, and more venous thrombotic events (51). In contrast, MPN patients with mutations in *JAK2* exon 12 develop erythrocytosis only.

MPN clones are typically heterozygous for the other driving mutations – *MPL* exon 10 (W515L, W515K) and *CALR* exon 9 mutations – with mutated allele loads close to 50%. Again, *MPL* and *CALR* mutations are found in both ET and PMF, in 5-10% MPN cases for *MPL* mutations, and 25-30% MPN cases for *CALR* mutations (Figure 1A). Compared to *JAK2V617F*-mutated ET or PMF, the presence of *CALR* mutations in ET or PMF is linked to a younger age and high platelet counts (51). The *JAK2/CALR/MPL* mutational status does not affect median survival in ET (19-20 years) (51). However, in PMF, the median survival is longest for patients with *CALR* mutations (15.9 years) compared to patients with *MPL* or *JAK2V617F* mutation (9.9 and 5.9 years, respectively), and worse for patients with no mutation in the *JAK2/CALR/MPL* genes (2.3 years) (52).

Other genetic alterations and MPN phenotype

Genetic predisposition to MPNs

Genetic predisposition to MPN is now established: relatives of MPN patients have about 6-8 fold higher risk of developing a MPN (53–55). Genetic predisposition to MPNs include the 46/1 or GGCC haplotype of *JAK2*, germline *ATG2B* and *GSKIP* duplication or mutations in *RBBP6* or *EPOR* (EPOR-p.P488S), and single nucleotide polymorphisms (SNPs) in the *TERT*, *MECOM*, and

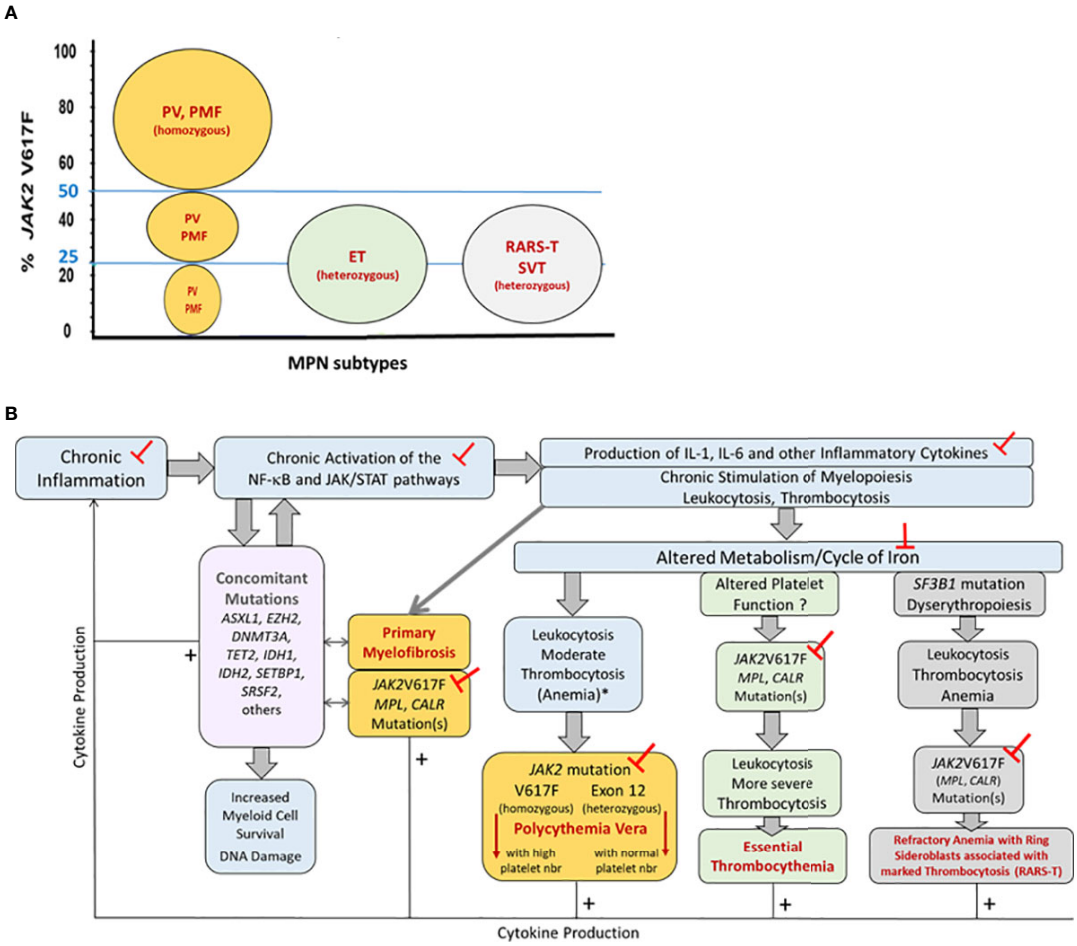

FIGURE 1
Representation of the pathogenesis of MPNs, associated mutations and inflammation, and impact on MPN phenotype. Part **(A)** *JAK2*V617F-mutant allele burdens in the different MPN phenotypes. Patients diagnosed with classic MPNs (PV, ET, PMF) or with refractory anemia with ring sideroblasts and thrombocytosis (RARS-T) or splanchnic vein thrombosis (SVT) can present with 25-50% *JAK2*V617F-mutated alleles in blood cells. The size of *JAK2*V617F-mutated clones varies: MPN clones are typically heterozygous for the *JAK2*V617F mutation in ET and RARS-T, and homozygous in PV and PMF. Note that in PV and in PMF with <50% *JAK2*V617F, clones may be heterozygous or homozygous for *JAK2*V617F. Part **(B)** Pathogenesis of MPNs and impact on MPN phenotype. Chronic inflammation of various causes (smoking, inflammatory diseases, auto-immunity, high-fat diet, metabolism disorders, genetic pre-disposition to inflammation) leads to chronic activation of the NF-κB and JAK/STAT pathways, hereby further increasing the production of inflammatory cytokines and myeloid cells, and facilitating the acquisition of non-driving, concomitant mutations (in the *DNMT3A, TET2, SRSF2, SF3B1* genes) as well as driving JAK/STAT-activating mutations (in the *JAK2, CALR* or *MPL* genes) in myeloid progenitors, resulting in the development of a MPN. The MPN phenotype (ET, PV, PMF, RARS-T) depends in part from the driving *JAK2, CALR* or *MPL* mutation(s) and the presence of concomitant mutation(s), and in part from the level and type of inflammation, iron stocks, and metabolism. The mutation-dependent production of cytokines is indicated by thin arrows and + signs. Concomitant mutations can be found in all MPN phenotypes but are more frequent in PMF, which is indicated by arrows. The ⊤ symbols indicate the different targets potentially useful in MPN prevention and therapy. (*) In case of inflammation and iron deficit.

CHEK2 genes (56–63). Genetic loci associated with a high risk of MPN typically affect the self-renewal of hematopoietic stem cells (*ZNF521, GATA2, MECOM, HMGA1, ATM, FOXO1*) (64). As in sporadic MPNs, mutations in *JAK2*, *CALR* or *MPL* are observed in individuals predisposed to MPNs, *JAK2*V617F being the most frequent driving mutation. The different germline variants or mutations that increase the risk of MPN are not associated with a specific MPN phenotype.

Concomitant mutations

Concomitant mutations found in MPN clones concern mostly the *DNMT3A, TET2, ASXL1, EZH2, SRSF2* and *SF3B1* genes (65). These mutations are not specific for MPNs, and their frequency is low in MPNs compared to other blood malignancies and solid cancers. Mutations in one or more of the *DNMT3A, TET2, ASXL1, EZH2, SRSF2* and *SF3B1* genes concern up to 20% PV, 20% ET, and 40% PMF. They typically occur after acquisition of a MPN-driving mutation, but also occur as early events that facilitate clonal emergence, followed by the acquisition of mutation(s) in *JAK2, MPL* or *CALR*. Concomitant mutations do not directly influence the MPN phenotype but are associated with clonal expansion and disease progression, notably secondary myelofibrosis and leukemic transformation (52, 65–67). *DNMT3A*, *TET2*, *ASXL1* and *EZH2* mutations alter epigenetic regulation; they are more frequent in PMF than in PV and ET. *DNMT3A* and *TET2* mutations appear to

lead to the activation of inflammatory pathways, notably NF-κB signalling (68, 69). In PMF, *ASXL1* mutations are associated with increased white blood cell counts, and reduced survival (70). Mutations in the *SRSF2* gene, which encodes a splicing factor, cause aberrant splicing that enhances differentiation towards the monocyte and megakaryocyte lineages (71, 72). *SRSF2* mutations do not alter MPN phenotype but they are associated with inferior survival in PV, ET and PMF (70–73). Mutations in the *SF3B1* gene, which encodes a splicing factor subunit, alter RNA splicing and are associated with the presence of ringed sideroblasts. *SF3B1* mutations are frequent in patients with refractory anemia with ringed sideroblasts (RARS), with myelodysplastic/myeloproliferative neoplasms with ringed sideroblasts and thrombocytosis (RARS-T), and also in up to 14% PMF patients (73–76). Like *DNMT3A*, *TET2*, *ASXL1* and *EZH2* mutations, *SRSF2* and *SF3B1* mutations are more frequent in PMF than in PV and ET.

Chronic inflammation in MPNs

Chronic inflammation is a long-established hallmark of all MPN subtypes, and PMF is associated with the most severe level of inflammation. Pro-inflammatory cytokines IL-1 and IL-6 stimulate the production of leukocytes and megakaryocytes, which in turn secrete a number of pro-inflammatory molecules (including IL-6), thus reinforcing chronic inflammation and the production of myeloid cells, and increasing the risk of mutation of myeloid progenitors (77, 78).

Mutation-dependent inflammation

The discovery of driving and non-driving mutations in MPNs prompted researchers to investigate whether these mutations could explain the inflammation associated with MPNs. Then JAK inhibitors tested in PMF patients showed efficacy on clinical symptoms, spleen size and inflammation cytokine levels. Of note, most JAK inhibitors block JAK1 as well as JAK2, and JAK1 activation is required for the production of major inflammation cytokines, particularly IL-1 and IL-6 (79). Different pathogenic models were proposed, where MPN-associated inflammation could be either the consequence of the *JAK2*V617F mutation in the MPN clone (i.e. "clonal inflammation"), or an early event predisposing patients to the acquisition of JAK/STAT-activating mutations in myeloid progenitors and the development of MPN (10–13, 31, 32).

In recent years it has been demonstrated that most inflammation-linked cytokines or receptors produced in excess in MPNs were not directly linked to *JAK2*V617F, nor to *CALR* mutations; *in vitro* only IL-1β, IL-1Rα and IP-10 were induced by *JAK2*V617F (17). In turn, increased levels of IL-1β in blood or bone marrow presumably enhance the production of inflammatory cytokines, notably by monocytes and macrophages. This model has been validated in *JAK2*V617F-expressing mice, where the knockout of IL-1β resulted in reduced inflammatory cytokine levels, and decreased megakaryopoeisis and myelofibrosis (80, 81). In contrast, there is no evidence that *CALR* or *MPL* mutations can induce cytokine production: the main cytokines found in excess in *CALR*-mutated ET (IL-4, IL-9, IL-26) are typically produced by non-mutated T-cells (17). Thus mutation-independent inflammation is likely more important in *CALR*/*MPL*-mutated MPNs than in *JAK2*V617F-mutated MPNs, and possibly more frequently an early event in *CALR*/*MPL*-mutated MPNs.

The role played by non-driving mutations in the inflammation associated with MPNs has also be investigated. Several groups reported that mutations in the *DNMT3A, TET2, SRSF2, SF3B1* genes could all result, indirectly, in the activation of the NF-κB signaling pathway (13, 68, 69). NF-κB is a major inducer of inflammatory cytokines (IL-1β, TNFα, TGF-β), and the crosstalk of NF-kB with other signaling pathways and the inflammasome is important (82). In addition, NF-κB regulates essential functions of monocytes and macrophages (M1/M2 polarization, activation, apoptosis). Thus *DNMT3A, TET2, SRSF2* and *SF3B1* mutations may contribute to increase inflammation in the subsets of MPN patients who carry these mutations. Of note, inflammation linked to concomitant mutations may precede the acquisition of mutations in the *JAK2/CALR/MPL* genes.

These findings do not explain MPN phenotype, but they have important consequences for therapy: they imply that in addition to JAK inhibitors, blocking major inflammatory cytokines in MPNs should be considered (80–84). The efficacy of this approach has been proven in JAK2V617F-expressing mice, where inhibition of IL-1β with anti-IL-1β antibody alone or in combination with ruxolitinib had beneficial effects on myelofibrosis and osteosclerosis (81). In fact, important mechanisms of action of IFN-α therapy include the repression of IL-1β and IL-1β-induced cytokines, as well as the NF-κB and c-MET/HGF pathways, which explains that long-term complete remissions can be obtained with IFN-α in both *JAK2*V617F- and *CALR*-mutated MPNs (13, 33, 46–48, 85–88). Consistently, IFN-α2 and JAK inhibitors were reported to act in synergy in MPNs (49, 50, 88). However, *TET2, DNMT3A, ASLX1, EZH2* mutations are associated with inferior responses to IFN-α therapy (88).

Mutation-independent inflammation anterior to MPN

The link between inflammation and cancer is proven, especially for inflammation due to chronic infections (89, 90). Indeed, chronic inflammation may be a consequence of infection, lipid oxidation, metabolism disorders, auto-immunity. In older individuals, clonal inflammation may exist, linked to certain early genetic events (for instance, mutations in *JAK2*, *TET2, DNMT3A, SRSF2, SF3B1*…). Causes of inflammation other than genetic alterations have been investigated in myeloid malignancies. These include smoking, chronic inflammatory diseases, auto-immunity, metabolism disorders (13, 31, 32, 91–95). This field of research is important

since specific causes of mutation-independent inflammation could become useful new targets in MPN therapy for subsets of patients.

Chronic inflammatory conditions or diseases

As a major risk of cell transformation, chronic inflammation likely facilitates the development of subsets of MPNs. During chronic inflammation, high levels of IL-6 stimulate the production of leukocytes and platelets, and increase the levels of hepcidin, a molecule that binds to ferroportin and inhibits iron absorption, thus decreasing the iron level in blood. The iron cycle is significantly disturbed during inflammation, notably *via* the repression of ferroportin expression, and altered synthesis of ferritin (increased) and transferrin (decreased). Thus, chronic inflammation is characterized by mild elevations of leukocyte and platelet counts, and impaired erythropoiesis despite important iron stocks, eventually resulting in anemia (Figure 1B). Acquisition of the *JAK2*V617F mutation in the context of chronic inflammation may counter or correct anemia, and increase leukocytosis and thrombocytosis. In contrast, the effect of *CALR* or *MPL* mutations would be restricted to a strong increase in thrombocytosis. Of note, iron deficiency is typically observed at the time of diagnosis in PV patients, but not in ET patients, and iron depletion is achieved in low-risk PV with phlebotomies (96).

It is now demonstrated that certain chronic inflammatory conditions can precede the development of a MPN: those inflammatory conditions include smoking, obesity, chronic inflammatory diseases such as Crohn disease, inflammatory bowel disease (IBD), polymyalgia rheumatica, giant cell arteritis (31, 32, 91–95, 97). Moreover, the 46/1 haplotype of *JAK2*, possibly a marker of inappropriate myeloid cell response to cytokine stimulation, has been shown to pre-dispose carriers to IBD and myeloid malignancies, notably MPNs (with or without mutation of *JAK2*) and acute myeloid leukemia (98, 99). Interestingly, the *JAK2* 46/1 haplotype contains two other genes, *INSL6* and *INSL4*, in addition to *JAK2*. *INSL6* and *INSL4* encode insulin-like peptides, expressed in brain, gonads, placenta, not in healthy hematopoietic stem cells. In non-hematopoietic cancer cells, *INSL4* expression can result in an autocrine loop, and *INSL4* has been proposed as a cancer prognostic marker (100).

Inflammation may also be due to chronic infection, and infections have been shown to be associated with myeloid malignancies, including MPNs (cellulitis) (101). In addition, chronic infection may lead to myeloid malignancy by facilitating the acquisition of *DNMT3A* mutations, hereby causing clonal myelopoiesis and further inflammation (68, 69, 102).

Auto-immunity

Non-genetic pathogenic mechanisms such as chronic antigen stimulation and antigen-driven selection are implicated in the pathogenesis of blood malignancies. Prior history of any autoimmune disease confers a significant risk of developing a myeloid malignancy, notably a MPN; the autoimmune diseases concerned include immune thrombocytopenic purpura and aplastic anemia (91–93). In MPNs, chronic immune stimulation may facilitate clonal evolution and/or progression toward myelofibrosis.

Recently, autoantibodies reactive against pro-inflammatory glucosylsphingoside (GlcSph), also called lysoglucosylceramide (LGL1), were detected in 20% MPN (especially ET and PMF) patients, and 40% myeloma patients, which implied that an auto-immune process accompanied the development of MPN or myeloma disease in these patients (17, 103, 104). Accumulation of GlcSph is a hallmark of Gaucher disease (GD), where it is a consequence of germline mutations in the glucocerebrosidase (*GBA*) gene; subsets of GD patients develop GlcSph-reactive autoantibodies. Interestingly, GD patients present with chronic inflammation (with high levels of IL-1β, HGF, IL-8, MIP-1β, TNF-α), various clinical manifestations, and an increased risk of blood malignancies (105, 106). Intriguingly, MPN patients have slightly elevated GlcSph levels compared to healthy controls (17). One hypothesis is that anti-GlcSph autoantibodies contribute to reduce the GlcSph level in blood.

Diet and metabolism

Inflammation may also be diet-induced. The influence of dietary factors on the risk of MPN has been investigated: the only finding was that a high intake of caffeine protects against PV (107). In contrast, obesity elevates the risk for clonal hematopoiesis and MPN, especially ET (108, 109). A high-fat diet predisposes to chronic inflammation, leukocytosis and thrombocytosis, whereas adherence to a Mediterranean diet has been shown to reduce symptoms in MPN patients (107–110). Moreover, as stated above, high levels of certain glucolipids in blood are associated with an increased risk of MPN (105, 106).

Discussion

According to present knowledge, MPNs result from the combination of acquired mutations (JAK2/STAT5 activating driving mutations and/or concomitant mutations), chronic inflammation (of various origins, mutation-dependent or independent) and for a minority of individuals, of germline genetic pre-disposition to MPNs. Hence, whenever possible, better addressing the causes of mutation-independent inflammation (smoking, high-fat diet, inflammatory and autoimmune diseases) and iron deficiency) should prevent or reduce the risk of MPN. Moreover, to be curative MPN treatments should target mutations, eliminate disease-initiating stem cells, and suppress the production of inflammatory cytokines and causes of mutation-independent inflammation. Among present treatments, IFN-α2 and JAK inhibitors counter both inflammation and JAK2/STAT5 driving mutations, with partial results for JAK1/2 inhibitors (which do not act on NF-κB-dependent inflammation) and complete remissions for IFN-α2 (which counters inflammation more broadly) (33, 37, 47, 48, 86). Importantly, JAK inhibitors and IFN-α2 can act in synergy (49, 50).

Further studies are needed to demonstrate the interest of using a JAK inhibitor/IFN-α2 combination therapy to eliminate the MPN clone in the early stages of MPN disease.

Presented in Table 1, the different pathogenic events, cytokines and other molecules associated with increased erythropoiesis (increased hematocrit, possible PV phenotype), thrombocytosis (possible ET phenotype), myelofibrosis (primary or secondary), or overproduction of specific inflammatory cytokines, constitute new potential therapeutic targets for those MPN patients who present with such characteristics. For instance, a better knowledge of the iron stocks and iron metabolism of patients, and the correction of iron deficiency, could help prevent the development of ET. Inversely, depletion of iron stocks or reduction of iron availability have been part of the treatment of PV for decades *via* phlebotomies; hepcidin mimetics and ferroportin inhibitors offer new therapeutic options (96).

Importantly, searching for causes of inflammation in patients other than mutations may contribute to improve their response to treatment in case of established MPN, and help reduce the risk of developing a MPN in older individuals. For instance, prevention of smoking should reduce the risk for PV, and prevention of obesity, *via* increased physical activity and an improved diet, would be expected to reduce the risk for ET. A more systematic search for and treatment of undiagnosed chronic inflammatory or/and autoimmune diseases should help reduce inflammation and the associated risk of acquired MPN-driving mutations in healthy myeloid cells, or additional mutations in the MPN clone. In patients with proven autoimmunity against GlcSph, GlcSph could

TABLE 1 Impact of mutations, cytokines and inflammatory conditions on MPN phenotype.

	All MPNs	ET	PMF	Therapeutic Targets
Mutation-Independent Chronic Inflammation				
Cigarette smoking	↑ Leukocytes, ↑ Hematocrit			Smoking prevention
High-fat diet Metabolism disorders	↑ Leukocytes, ↑ Platelets			Mediterranean diet ↑ Physical activity
Iron deficiency Disturbed iron cycle	↓ Hematocrit ↑ Platelets			PV: Iron restriction (Phlebotomy) Hepcidin mimetics Ferroportin inhibitor
Inflammatory diseases	↑ Leukocytes, ↑ Platelets ↓ Hematocrit			Treatment of inflammatory disease
Auto-immunity	Anti-GlcSph auto-antibodies (20% MPNs)			Treatment of auto-immune disease GlcSph reduction?
Inflammatory Cytokines				
IL-1β, IL1-Rα, HGF, IP-10	↑ Neutrophils		↑ Splenomegaly	IL-1β inhibitors (IFN-α, antibodies)
IL2-Rα, SDF1α, IL-7, IL-17	↑ Platelets			
IL-2, IL-4, IL-26		↓ Hematocrit		
GRO-α		Myelofibrosis		
Germline Genetic Predisposition to MPNs				
46/1 haplotype of *JAK2* *ATG2B*, *GSKIP*, *RBBP6*, *EPOR*, *TERT*, *MECOM*, *CHEK2*, *others*	No effect on MPN Phenotype ↑ Inflammation (46/1 haplotype of *JAK2*)			
Mutations				
DNMT3A, ASXL-1, TET2, EZH2	↑ Clonal expansion ↑ IL-1β, TNFα, TGF-β/Inflammation *via* NF-κB ↑ Myelofibrosis, Resistance to IFN therapy			NF-κB inhibitors IL-1β inhibitors (IFN-α, antibodies)
MPL	No effect on cytokine production/Inflammation			
*JAK2*V617F (high mutant burden)	↑ Hematocrit, ↑ Leukocytes ↑ Venous thrombotic events ↑ IL-1β, IL-1Rα/Inflammation *via* JAK1			JAK inhibitors IL-1β inhibitors (IFN-α, antibodies)
CALR	No effect on cytokine production nor inflammation	Young age ↑ Platelets		Vaccination with *CALR* mutant epitopes? (ref. 111-113)
ASXL-1			↑ Leukocytes	
SF3B1	Presence of ring sideroblasts, ↓ Hematocrit			

↑ (increase), ↓ (decrease).

become a new target in MPN therapy, since GlcSph can be reduced with existing treatments (105, 106).

Other new potential therapeutic targets in MPNs include certain cytokines, particularly IL-1β, which can be inhibited efficiently with IFN-α, and also with anti-IL-1β antibodies or NF-κB inhibitors. Finally, because of the immunogenicity of *CALR* exon 9 mutants, patients with *CALR*-mutated ET or PMF may benefit from *CALR* mutant peptide vaccination (111–113).

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

The author declares the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Myeloproliferative neoplasms – blurring the lines between cancer and chronic inflammatory disorder

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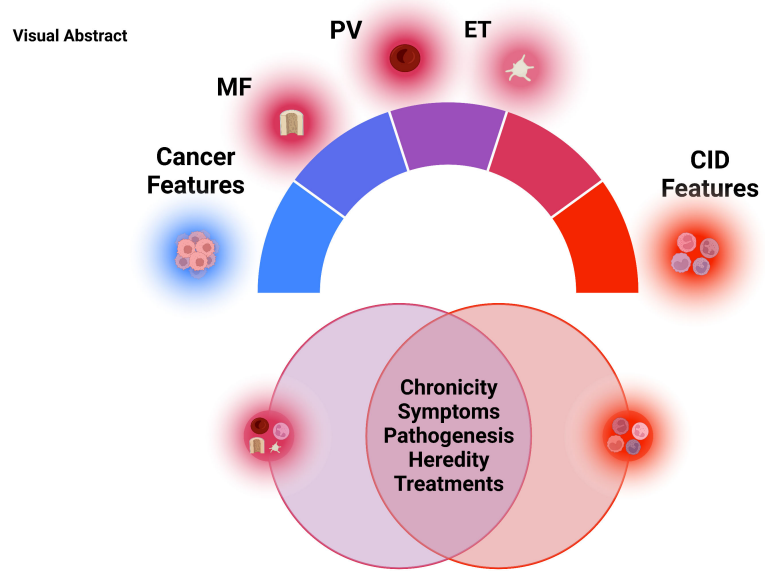
Myeloproliferative Neoplasm (MPN) is a group of chronic blood cancers that arise from a hematopoietic stem cell (HSC) clone with somatic mutations causing constitutive activation of myeloid cytokine receptor signaling. In addition to elevated blood cell counts, MPN typically presents with increased inflammatory signaling and inflammation symptoms. Therefore, while being a clonally derived neoplasm, MPN has much in common with chronic non-cancerous inflammatory conditions, such as rheumatoid arthritis, lupus, and many more. MPN and chronic inflammatory disease (CID) share similar chronicity, symptoms, dependency on the immune system, environmental triggers, and treatments. Overall, we will highlight the similarities between an MPN and CID. We highlight that while MPN is classified as a cancer, its behavior is more aligned to that of a chronic inflammatory disease. We propose that MPN should inhabit a fluid/spectrum between auto-inflammatory disease and cancer.

KEYWORDS

inflammation, myeloproliferative neoplasm, autoimmunity, cytokines, clonal hematopoiesis



GRAPHICAL ABSTRACT

Introduction

Classical/Philadelphia chromosome negative myeloproliferative neoplasms (MPN) are a group of hematologic malignancies including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). Each subset (PV, ET, PMF) has its unique clinical features with a unifying theme of somatic acquisition of a mutation in either Janus Activated Kinase 2 (*JAK2*^{V617F}) (1–5), Calreticulin (*CALR*) (6, 7) or Thrombopoietin Receptor (*TPOR*, *MPL*) in a Hematopoietic Stem Cell (HSC). *JAK2*^{V617F} mutations can be seen in all three subtypes of MPN, whereas *CALR* or *MPL* mutations are restricted to ET and PMF. This change leads to an overproduction of any combination of white cells, red cells, and platelets, with major clinical consequences including increased risk of thrombosis, and constitutional symptoms. Development of MF, primary (PMF) or secondary to PV/ET, results in cytopenias and risk of transformation to acute leukemia. Chronic inflammation is a hallmark feature of MPN, most notably PMF, which plays an integral role in multiple aspects of its pathobiology, including symptomatology, thrombosis, disease progression, and heightened cardiovascular disease risk (8–11). Although MPN is currently classified as a malignancy, many of the aspects of the disease are more like a chronic inflammatory condition rather than a cancer.

In the sections below, we will highlight specific clinical and pathobiological overlaps between inflammatory disease and each subset of MPN, summarized in Figure 1.

Chronicity

Inflammatory disease is the result of immune-mediated damage of self-tissues. It is characterized by the presence of chronic inflammation mediated by loss of self-tolerance mechanisms (commonly from genetic mutations) that would normally regulate self-reactive lymphocytes, or inappropriate activation of innate immune cells commonly triggered by certain environmental exposures (e.g., specific foods, chemicals). Inflammatory disease severity increases with chronic exposure to inflammatory stimuli, causing tissue damage and fibrosis.

MPNs are also chronic in nature. The MPN driver clone emerges decades prior to diagnosis (12, 13), and in some cases arises *in utero* (14). Normal life expectancy is possible in essential thrombocytosis (ET) and polycythemia vera (PV) (15, 16), but in general patients with these diseases have a shorter life expectancy than their age matched counterparts (17). Much of the morbidity and mortality in MPN stems from cardiovascular pathology which is characterized as an inflammatory disease (18). MPN patients are at increased risk for both arterial and venous thromboses. A large population based study found the Hazard Ratios (HRs) of arterial thrombosis among MPN patients compared to controls at 3 months, 1- and 5 years post diagnosis to be 3.0, 2.0, and 1.5, respectively (19). The corresponding HRs for venous thrombosis were 9.7, 4.7, and 3.2, and for myocardial infarction the HRs were 2.5, 1.8, and 1.4 (19).

A large population study found that presence of at least one cardiovascular disease (CVD) risk factor predicted higher risk for thrombosis among MPN patients (20). Accordingly, reduction of CVD risk factors is paramount in MPN, and as such is listed as the initial item on the intervention list for MPN's in the National Comprehensive Cancer Center Network (NCCN) guidelines. However, there are no established strategies for how this risk reduction should be achieved specifically in MPN patients.

Primary Myelofibrosis (PMF), although also a chronic disease, has significantly worse prognosis than ET or PV. Even within PMF

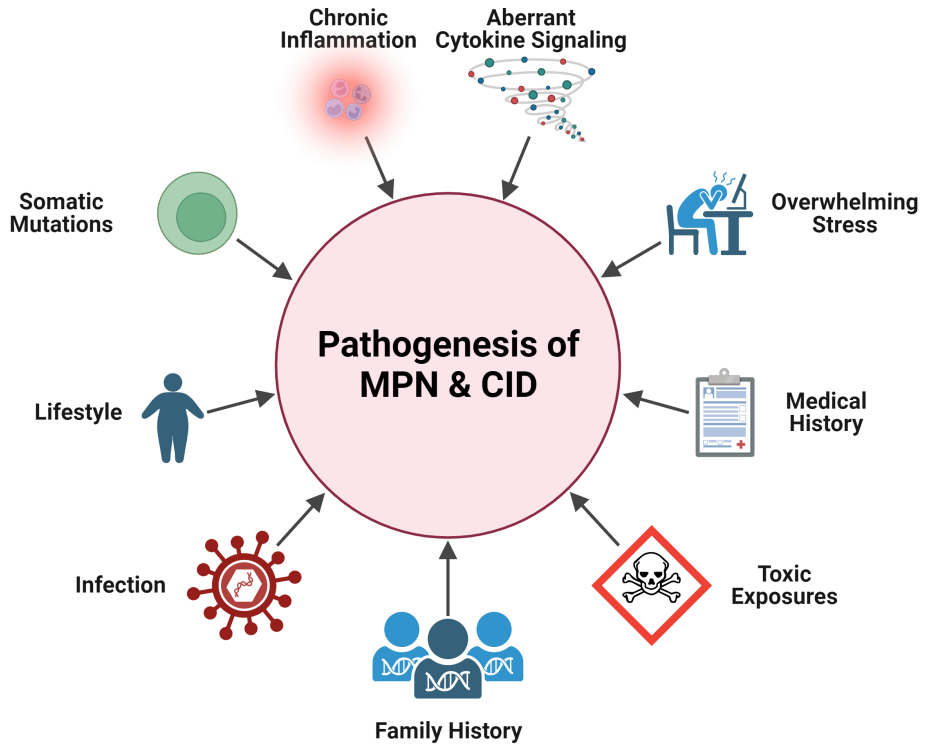


FIGURE 1
Shared pathogenesis features in MPN and CID. Pathogenic elements that are in common between MPN and CID: aberrant cytokine signaling, chronic inflammation, somatic mutations, general stress, lifestyle (e.g., obesity), medical history (e.g., MPN patient having pre-existing CID), family history, and exposures (infectious, toxic). Created with BioRender.com.

there is a wide range of life expectancy and because of this there are multiple scoring tools available to help clinicians prognosticate in PMF. Although the thrombotic risk and CVD risk is present in PMF, goal of care in PMF are more focused on improving symptom burden, splenomegaly and cytopenia as well as surveillance for transformation to acute myeloid leukemia.

Symptoms

Symptom overlap between MPN and CID

There is significant overlap between signs and symptoms in MPN and auto-inflammatory disease (Figure 2). Symptoms are clearly the most impactful clinical feature of PMF with extreme fatigue being prevalent. Weight loss, night sweats, and fever can also be seen in PMF along with abdominal pain from splenomegaly. Symptoms are not restricted to patients with PMF. Headaches, numbness/tingling, and pruritis can also be debilitating in PV and ET. The overall symptom burden is a significant morbidity associated with MPN which negatively affects quality of life (21), leads to impaired work productivity (22, 23), and the need for medical disability leave (24). The central importance of symptom burden in MPN is reflected in the widespread use of the MPN Symptom Assessment Form (MPN-SAF), an internationally validated objective scoring tool to quantify symptom burden in MPN patients (25, 26). Reduction in MPN-SAF score is a key endpoint in MPN clinical trials and reduction in symptom burden was the outcome that led to the FDA approval of the JAK inhibitors ruxolitinib (27), fedratinib (28), and pacritinib (29) for myelofibrosis.

The prevalence and severity of symptoms differ by MPN subtype and even within each MPN subtype symptoms can be variable. For example, a prospective evaluation of 1470 MPN patients, including 622 ET, 519 PV, and 329 PMF patients identified five symptom clusters in PV and ET, respectively, and four clusters in PMF (30). Clinical variables including age, language, gender, the presence of laboratory abnormalities, spleen size, history of hemorrhage, and MPN-SAF score. Despite symptom management being regarded as a high therapeutic priority, symptoms are still inadequately addressed particularly for patients with ET and PV (31).

Symptoms are also integrated into disease scoring tools in autoimmune disease, including the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (32) in SLE, the Simplified Disease Activity Index (SDAI) and Clinical Disease Activity Index (CDAI) (33, 34) in Rheumatoid Arthritis, and the Sjögren's Tool for Assessing Response (STAR) (35) in Sjögren's syndrome.

Symptom management strategies

The effectiveness of currently available therapies may be in part due to their ability to reduce the inflammatory state of MPN patients. Ruxolitinib, a JAK1/2 inhibitor Food and Drug Administration (FDA) approved for MF patients and for PV

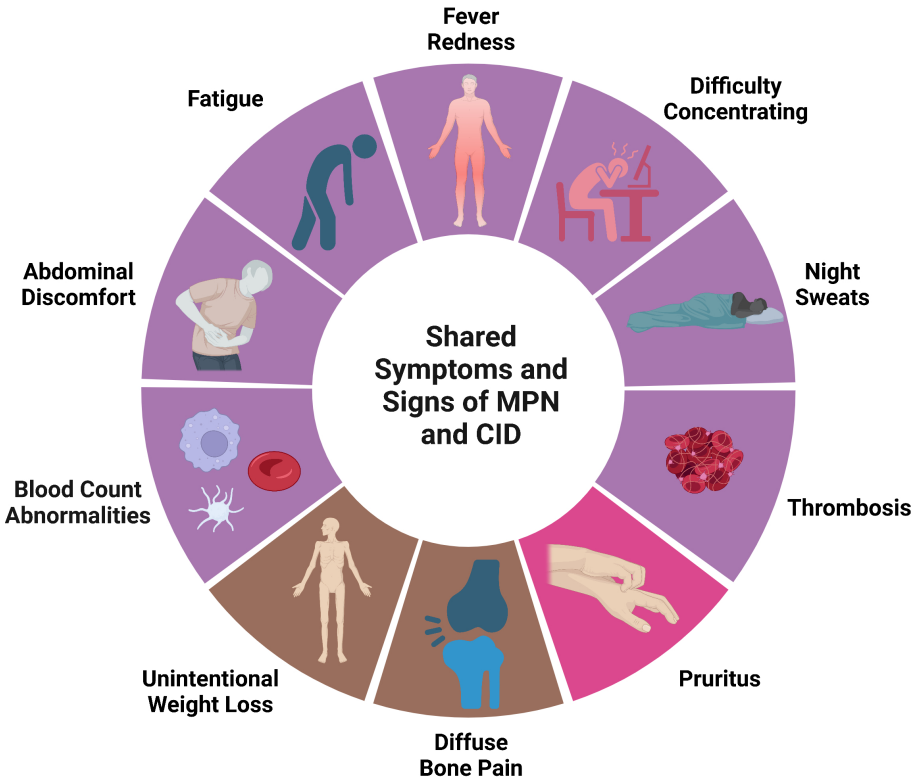


FIGURE 2
Shared signs and symptoms in MPN and CID. Symptoms and signs that can be seen in both MPN and CID. Purple represents signs/symptoms seen in all MPN subtypes, bright pin represents signs/symptoms seen in PV primarily, brown represents signs/symptoms seen primarily in PMF. Created with BioRender.com.

patients intolerant or resistant to hydroxyurea, has anti-inflammatory properties and its administration has been correlated with reductions in plasma levels of C reactive Protein (CRP), interleukin-1 receptor-alpha (IL-1Rα), Macrophage Inflammatory Protein-1-beta (MIP-1β), Tumor Necrosis Factor-alpha (TNF-α) and interleukin-6 (IL-6) (8). However, plasma cytokines remain abnormal following ruxolitinib (36), indicating that JAK inhibitors are not sufficient to normalize cytokines (36). Moreover, JAK inhibitors are not without significant side effects including thrombocytopenia, anemia, increased risk of skin cancer, and immunosuppression. There remains an unmet need in MPN care for low-risk treatment options that improve or control disease related inflammation.

Symptom burden and obesity

Obesity causes chronic low-grade inflammation (37), body mass index (BMI) is a modifiable variable. A U-shaped association between BMI and Total Symptom Burden was observed in a combined analysis of two large cross-sectional surveys, the Danish Population-based Study, MPN health survey (n = 2044), and the international Fatigue Study (n = 1070), with significantly higher mean symptom scores for underweight and obese patients relative to normal weight (38). Interestingly, in an Israeli population study obesity (BMI ≥ 95th percentile) in adolescence significantly predicted increased risk of PMF with HR of 1.81 (95% confidence interval 1.13-2.92, P = 0.014) (39).

In PV patients, the PV-NET Real World Study was conducted to evaluate the impact of Charlson Comorbidity Index (CCI) and BMI on treatment success and survival in 530 PV patients. The study concluded that CCI/BMI can influence the choice between therapies (interferon vs JAK inhibitor), and that quantification of body composition and control of comorbidities can improve PV outcome (40, 41). Therefore, a focus on maintaining a healthy BMI should be emphasized in all subsets of MPN.

Inflammation, the driving symptom of MPN and CID

Inflammation appears as pain (joint or diffuse bone), redness, fatigue, urticaria, fever, and stiffness. These symptoms and any disease-specific signs (e.g., malar rash as seen in lupus) would prompt a blood workup, genetic screening, and imaging for indicators of inflammatory disease. The etiology of inflammation in MPN is likely multifactorial. The $JAK2^{V617F}$ mutant cells not only produce excessive inflammatory cytokines themselves, but also induce other bystander cells to produce inflammatory cytokines (42).

Chronic inflammation is responsible for much of the symptom burden in MPN (43), and also contributes to disease development

and progression by promoting expansion of the neoplastic clone (44, 45). Importantly for symptom management, specific symptoms have been correlated with elevated levels of specific inflammatory biomarkers (43). In treatment-naive PMF patients, increased levels of Interleukin-8 (IL-8), IL-2R, IL-12, IL-15, and Interferon gamma-induced protein 10 (IP-10) were independently predictive of inferior survival (10).

Each subset of MPN may have its own distinct inflammatory signature. For example, ET has a specific inflammatory cytokine signature consisting of Eotaxin, GRO-α, and EGF (46). GRO-α and EGF in ET patients were associated with disease transformation in initial sample collection (GRO-α) or longitudinal sampling (EGF). Therefore, addition of cytokine profiling could potentially add prognostic value for predicting transformation from ET to PMF.

Clonality in MPN and CID

The identification of somatic mutations leading to expansion of a clone transitioned the nomenclature of ET, PV, and PMF from a *disorder* to a *neoplasm*. However, presence of a clone with an MPN driver mutation can be seen in humans without a clinical MPN. *JAK2*^{V617F} is the 5th most common mutation seen in clonal hematopoiesis of indeterminate potential (CHIP) (47). Screening of almost 20,000 Danish citizens revealed presence of somatic *JAK2*^{V617F} and *CALR* mutations in 3.2 and 0.16 percent of the population (48). Screening of >250,000 people who have submitted samples to 23andme personal genome service who denied a history of MPN revealed *JAK2*^{V617F} in approximately 0.2% of these samples (49).

The clonal architecture of MPN can be complex, with additional mutations acquired in the MPN clone either before or after the MPN driver mutation, or in separate clones from the MPN driver clone (50, 51). Mutations in high risk genes including *ASXL1, EZH2, SRSF2* and *IDH* identifies PMF patients who are at risk for premature death or leukemic transformation (52). Patients with an *ASXL1* mutation on its own had no increased prognostic to a worsening outcoming, unless when compounded with one of the high-risk genes (53).

A perceived distinguishing factor between hematologic malignancy and inflammatory disease is somatic mutations, present in hematologic malignancy but absent in autoimmune/inflammatory disorders. However, this line is also blurring. Somatic mutations in hematopoietic cells are being identified in inflammatory conditions. A prime example of somatic mutations of myeloid lineage cells causing a severe inflammatory disorder is VEXAS (vacuoles, E1 enzyme, X-linked, autoinflammatory, somatic) syndrome (54). VEXAS is caused by somatic mutations in UBA1 that result in systemic inflammation and progressive bone marrow failure that presents late in life.

Patients with other non-malignant autoimmune disorders harbor somatic mutations in T cells. Individuals with newly diagnosed, untreated Rheumatoid Arthritis (RA) are found to harbor somatic mutations in clonally expanded CD8+ T cells (55). Somatic mutations in the CD8+ T cell compartment have also been identified in patients with myasthenia gravis (56).

Overlapping populations of MPN and autoimmune disease

There is a significant overlap in the autoimmune disease and MPN patient populations. A prior history of any autoimmune disease was found to be associated with a significantly increased risk of MPN with an Odd's Ratio (OR) of 1.2 (57). Another study found a significantly increased risk of MPNs in subjects with a prior history of any autoimmune disease (OR=1.86) (58). MPN is likely underappreciated in patients with autoimmune disease, as elevated platelets could be attributed to reactive thrombocytosis in this patient population. Analysis of patients with thrombocytosis (platelets > 450) in an inflammatory bowel clinic revealed that 23% of them harbored JAK2^{V617F} mutations (59).

It is important to distinguish the myeloproliferative neoplasm PMF from autoimmune MF (AIMF). While both conditions cause fibrosis, cytopenia, and elevated inflammatory cytokines, AIMF will present with serological evidence of autoantibodies and lack of MPN driver mutations (60). However, PMF patients may contain autoantibodies, especially from a prior autoimmune disease, requiring a physician to not rely on this test alone (61).

Familial predisposition in CID and MPN

For various auto-immune conditions, a high degree of heritability is seen among family members of the affected patient, especially in first-degree relatives (62, 63). This can be due to several conditions centered around similar genetic makeup and environmental exposures. There is also a familial predisposition to acquire MPN. A study involving 35,037 hematologic malignancies patients and 93,199 of their first degree relatives found an increased risk of developing AML (RR 1.53), MDS (RR 6.87), and the MPN subtypes PV (RR 7.66) and ET (RR 6.3) if a family member has the disease (64). A search for the genetic basis of the predisposition to acquire MPN has revealed single nucleotide polymorphisms (SNPs) associated with MPN, for example the germline haplotype (GGCC, referred to as "46/1") encompassing the 3' region of the JAK2 gene is associated with a three- to four-fold risk of MPN (65). Interestingly, this same JAK2 46/1 haplotype also increases susceptibility to Crohn's disease, an autoimmune inflammatory bowel disease (66). However, the genetic predisposition to acquire MPN has yet to be fully elucidated.

Infectious triggers for CID and MPN activation

A common activator of the disease state in MPN and autoimmune disease may be exposure to a severe inflammatory stimulus such as infection. An emerging theme among mutations associated with myeloid malignancies and clonal hematopoiesis of indeterminate potential is resistance to chronic inflammation. For example, many chronic inflammatory stimuli, including Interferon-

gamma (IFN-γ (67) and TNF-alpha (TNFα) (45, 68) augment the selective advantage of clones with myeloid malignancy associated mutations in mouse models. However, the impact of an acute inflammatory stressors on the emergence of neoplastic clones is an area less studied. An acute inflammatory stimulus may allow for the expansion of a neoplastic clone to reach a critical threshold after which chronic low grade inflammation, either from environmental sources or driven by the neoplastic clone itself maintains the selective pressure in favor of continued expansion of the clone. Further research, both epidemiologic and mechanistic is required to elucidate the impact of acute infectious stressors on the development of MPN.

Epidemiologic data support the notion that infection may promote development of MPN. A large Swedish population-based study demonstrated that a history of infection was associated with an increased risk of AML and MDS (69). Using the Surveillance, Epidemiology and End Result (SEER)-Medicare database Titmarsh et al. found that MPN was significantly associated with a history of cellulitis (70). Infection is a well-established trigger for autoimmune disease. There are different mechanisms by which an infection may trigger autoimmunity, including molecular mimicry, epitope spreading, and bystander activation. Exposures to specific pathogens have been associated with specific autoimmune disease. Increased amounts of antibodies formed against various bacteria have been linked to driving lupus (71). Epstein-Barr virus infection have been implicated in the pathogenesis of multiple sclerosis (72).

Treatment overlaps in MPN and CID

The most notable therapy overlap is the use of JAK inhibitors as a mainstay of treatment in both MPN and autoimmune disease. While in MPN, JAK inhibitors suppress constitutively active JAK/STAT signaling, their ability to reduce inflammation in general may be their broader mechanism of action. The JAK inhibitor ruxolitinib reduces inflammation, however not down to the level of normal (36). This suggests that additional anti-inflammatory drugs, such as those that target the NF-κB pathway could work synergistically with JAK inhibitors to quell inflammation in MPN. Prior to the JAK inhibitor era, anti-TNF agents had been investigated in MPN (73), however not nearly to the depth as anti-TNF agents in autoimmune disease. This highlights the common goal in MPN and CID therapy is to reduce inflammation. Further treatment that addresses anti-inflammation specifically (in combination with JAK inhibitors) is being studied, specifically the targeting of TNF-α receptors 1/2 (TNFR1/2) to help control constitutive symptoms (74).

Interferon-alpha (IFNα) is used in MPN in multiple formulations and has the potential of inducing a molecular response (75–77). IFNα can exacerbate autoimmune conditions (78), demonstrating that some drugs used for MPN may be contraindicated in autoimmune disease. However, other interferons are used for autoimmune disease. Evidence supports an anti-inflammatory and beneficial role of IFNβ locally in the joints of patients with rheumatoid arthritis and in murine arthritis models, and many patients with multiple sclerosis show a clinical response to recombinant IFNβ (79).

Lifestyle modification in MPN

Interventions such as physical activity and yoga have been implicated in reduction of symptom burden and improvement in quality of life in MPN (80–82). A role between increased inflammation and development of depression has been characterized in recent years, encouraging the development of depression reduction techniques (83, 84). Pilot studies with the mindfulness meditation app *Calm* suggest that this may be effective in reducing depression and anxiety symptoms in MPN patients (85–87). A randomized online yoga intervention demonstrated small effects on sleep, pain, and anxiety as well as a moderate effect on depression (80). Ultimately, a multimodal lifestyle approach (diet, exercise, mindfulness) is likely the optimal intervention in MPN, but first we must rigorously interrogate the impact of each modality on its own before combining them into a holistic lifestyle approach to improving health in MPN.

Dietary management may represent a low-risk way to optimize cardiovascular health and reduce inflammation in MPN. The Mediterranean diet, characterized by increased consumption of extra virgin olive oil (EVOO), nuts, legumes, vegetables, fruits, fish, and whole grain products, has proven beneficial in modifying subclinical inflammation in diseases where chronic subclinical inflammation plays a key role (88). Specifically, the randomized interventional PREDIMED (Prevención con Dieta Mediterránea) study demonstrated that a Mediterranean diet supplemented with EVOO reduced the incidence of major cardiovascular events (89). Numerous longitudinal cohort studies show that a Mediterranean style eating pattern was associated with lower risk for cardiovascular disease (CVD), explained in part by reduction in CVD risk factors, most notably inflammation (90). We performed two pilot interventional studies to establish the feasibility of a Mediterranean diet in MPN (Mendez Luque et al, manuscript in preparation). We found that MPN patients can alter their diet toward a Mediterranean diet eating pattern (91). We did not observe significant changes in plasma inflammatory cytokines or gut microbiome over the 10-week intervention (92), although analysis was limited due to the small sample size. Upcoming studies with a larger cohort of MPN patients are required to rigorously test the impact of a Mediterranean diet on inflammatory biomarkers and symptom burden.

Conclusions

Approaching MPN as a disease that lies at the intersection of a neoplasm and chronic inflammatory disorder may better serve patients and the physicians who treat them. Addressing inflammation in MPN will likely reduce symptom burden, ameliorate some of the inflammation-driven morbidity associated

with the disease, and potentially blunt disease progression. It is important, however, to note that other treatment modalities beyond reduction of inflammation are necessary to eradicate the neoplastic clone. Investigating MPN through the scientific lens of an inflammatory disease in addition to a cancer may reveal important aspects of MPN pathogenesis that may be missed by viewing MPN through cancer blinders.

Author contributions

ES and AF together wrote the article and edited it. ES created figures. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Epigenetics in myeloproliferative neoplasms

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The myeloproliferative neoplasms (MPNs) are a group of acquired clonal disorders where mutations drive proliferative disease resulting in increased blood counts and in some cases end-stage myelofibrosis. Epigenetic changes are the reversible modifications to DNA- and RNA-associated proteins that impact gene activity without changing the DNA sequence. This review summarizes mechanisms of epigenetic changes and the nucleosome. The drivers and epigenetic regulators in MPNs are outlined. In MPNs, distinct patterns of epigenetic dysregulation have been seen in chronic and in advanced phases. Methylation age and histone modification are altered in MPNs and by further treatment. The alterations found in methylation age in MPNs and with treatment are discussed, and the changes in histone modification with Janus kinase (JAK) inhibition are evaluated. Currently available therapeutic areas where the epigenome can be altered are outlined. Thus, we review the current knowledge and understanding of epigenetics in MPN and consider further management options. Understanding the epigenome and its alteration in MPNs and epigenetic changes associated with the progression of disease will lead to advances in therapeutic options.

KEYWORDS

epigenetics, myeloproliferative neoplasms, DNA methylation, JAK2, NFE2, histone modification

Epigenetics

Genomic instability is fundamental to the development of malignancy. Acquired mutations may drive the process, but dysregulation of the normal epigenetic mechanism is frequently observed across nearly all forms of hematological malignancy and solid tumors (1). Epigenetic changes include reversible modifications to chromatin structure, histone modifications, and DNA methylation, which dictates the way genes can be expressed or silenced. Maintaining a particular gene transcription profile is critical to the normal function of the cell. Thus, in contrast to changes in the genetic code, with epigenetic dysregulation, there are no DNA sequence changes. Alterations in chromatin structure or modification of histones, DNA methylation, or changes to RNA can result in downstream gene expression changes that may influence the initiation, maintenance, or progression of a malignant cell (2).

In cells, DNA is packed into chromatin in the nucleosome. The DNA is coiled round a histone protein core of eight histone proteins (two each of H2A, H2B, H3, and H4).

Histone H1 further stabilizes the structure. This is not a static structure, as changes in the condensed nature of the chromatin signal for changes in gene transcription (3) (Figure 1). Posttranslational modifications of histone proteins are one of the most studied and best understood mechanisms of epigenetic control. Histone modification predominantly occurs on histone N terminal residues with specific changes driving transcriptional activation or repression. These modifications primarily include acetylation, methylation, and phosphorylation. Other histone posttranslational modifications including butyrylation and sumoylation (4) are increasingly recognized, but their relevance in MPN is unclear and they will not be discussed in this review.

Proteins involved in the modification of histones are broadly classed as writers, erasers, and readers. Writers are responsible for the deposition of a particular chemical modification, while erasers remove these modifications. Readers recognize modifications and recruit additional proteins to enhance or repress transcription. Histone acetylation is reversibly regulated by histone acetyltransferases (writers) and histone deacetylases (HDACs) (erasers). Particularly on histones H3 and H4, acetylation occurs at lysine (K), while HDACs remove acetyl residues from histone tails (5).

Histone acetyltransferases and HDACs are recruited to target genes with specific factors and regulate gene expression. HDACs can be divided into three classes. Class I HDACs (1, 2, 3, and 8) are located in the nucleus, Class II HDACs (4–9) are located in the nucleus and cytoplasm, and Class III HDACs are distinct NAD-dependent enzymes (6). Class I and II HDACs are inhibited by suberoylanilide hydroxamic acid (SAHA) and other HDAC inhibitors inducing growth arrest, differentiation, apoptosis, and inhibition of tumor growth in cancers including hematological malignancy (7).

Methylation of histones takes place on lysine, arginine, and histidine residues. However, in addition, lysine may be mono-(me), di-(me2), or tri-(me3) methylated. S-adenosyl methionine (SAM) donates the methyl group by the action of histone methyltransferase (HMT) (10). Enhancer of Zeste 2 (EZH2) is the enzymatic component of the Polycomb repressive complex 2 (PRC2) and is a prime example of an HMT, which is frequently dysregulated in cancer and may act as a therapeutic target (8). Histone demethylase enzymes including lysine-specific demethylase 1 (LSD1) act as erasers in this context to remove methylation marks (9). Histone methylation marks frequently studied are histone H3 lysine 4 (H3K4) associated with transcription activation in its trimethylated state and histone H3 lysine 9 (H3K9) associated with transcriptional repression. The balance between acetylation and methylation histone H3 lysine 27 (H3K27) is important for determining gene expression. Trimethylation of H3K27 (H3K27me3) is associated with transcriptional repression, while acetylation (H3K27ac) is a mark of active gene transcription (11, 12).

DNA methylation is a well-described epigenetic mechanism where the highly conserved methylation of chromatin components is involved in the regulation of gene expression, DNA repair and replication. Cytosines are methylated by the addition of a methyl group to the pyrimidine ring by the action of DNA methyltransferase (DNMT) enzymes. This occurs mainly at CpG islands (regions where a cytosine nucleotide is followed by a guanine). CpG islands are found across the genome and are present in the promoter regions of the majority of genes in

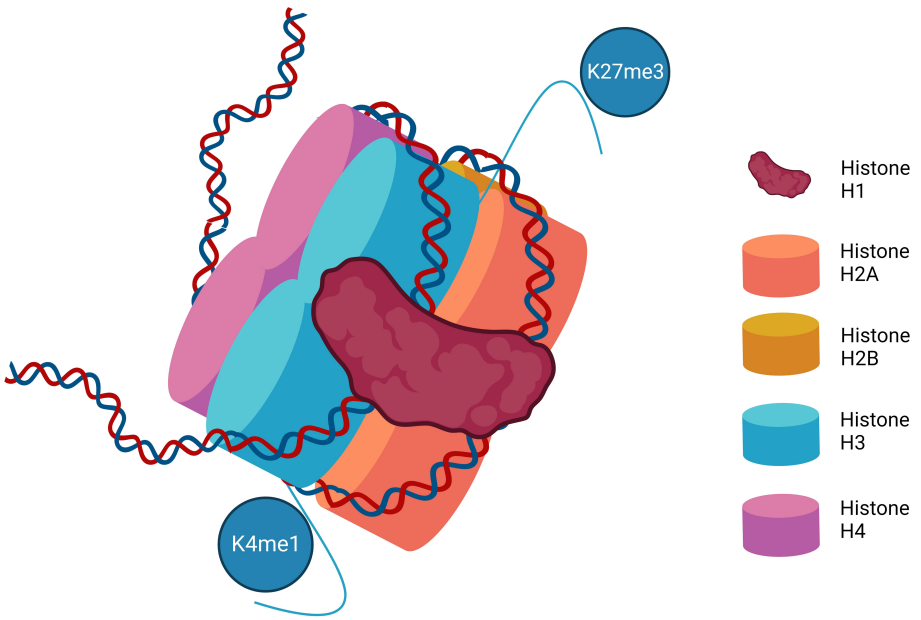

FIGURE 1
The structure of the nucleosome. Represented are the eight histone proteins that form the core of the histone along with histone H1. Various methylation marks are illustrated on the N terminal amino acid chains depicting examples of the more common and best characterized posttranslational histone modifications that impact DNA transcription. K4me1–Lysine 4 mono-methylation is considered mark of active and primed enhancers. K27me3–Lysine 27 trimethylation is a mark of gene repression.

humans. The 5-methylcytosine (5-mc) formed represses gene transcription. SAM donates a methyl group (CH3) and is reduced to S-adenosyl homocysteine (SAH). The ten-eleven translocation (TET) proteins catalyze the conversion of 5-mc to 5-hydroxymethylcytosine (5-hmc), which is an initial step in demethylating DNA. The isocitrate dehydrogenase (IDH) enzymes catalyze the conversion of isocitrate to a-ketoglutarate, a reaction that is required for TET enzyme function. Ultimately, unmethylated DNA with an open chromatin structure is involved in active transcription, whereas with methylated DNA and a closed chromatin structure, transcription is impeded (13) (Figure 2).

RNA changes in regulation can lead to cell cycle arrest and apoptosis. Mutations in the genes controlling RNA regulation, *SF3B1 (Spicing factor 3b subunit2)*, *SFSF2 (Serine and arginine rich splicing factor 2)*, and *IKZF1* (*IKAROS family finger 1*) have been described and may affect these processes in myeloid cancers including MPN (14, 15).

Myeloproliferative neoplasms (MPNs)

The typical Philadelphia-negative myeloproliferative neoplasms (MPN)s are polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF). This group of disorders is characterized by acquired clones that drive excess mature myeloid cell production and, in the case of MF, the secondary phenomenon of excess fibrosis. In each of these conditions, driver mutations have been detected that suggest that the acquired clone is accounting for the excess cell production (16).

Cytokines such as erythropoietin (EPO) regulate normal hematopoiesis by stimulating receptors on the cell surface and activating the Janus kinase (JAK) protein. This protein then autophosphorylates and binds to signal transducer and activator of transcription (STAT) protein. STAT proteins then phosphorylate, dimerize, and translocate to the nucleus where they bind at promoter sequences in the genome. This promotes gene transcription that ultimately affects cell functions including cell growth, differentiation, and apoptosis (16). There is then a mechanism to turn off this process *via* the negative regulators including the phosphatase Src homology1 domain (SHP1). In PV, the majority of cases have been found to have a single mutation in the *JAK*2 gene *JAK*2V617F (17), and a small minority have alternative mutations in exon 12 of *JAK*2 (18). These gene mutations lead to constitutively activated proteins that result in upregulation of the JAK-STAT pathway. Activation of alternative intracellular signaling cascades including MAPK and PI3K has also been observed as a result of the JAK2 mutation.

In 50% of those with ET, the *JAK*2V617F mutations can be determined. In cases of ET that do not have this driver mutation, approximately 15% have been found to have mutations in the

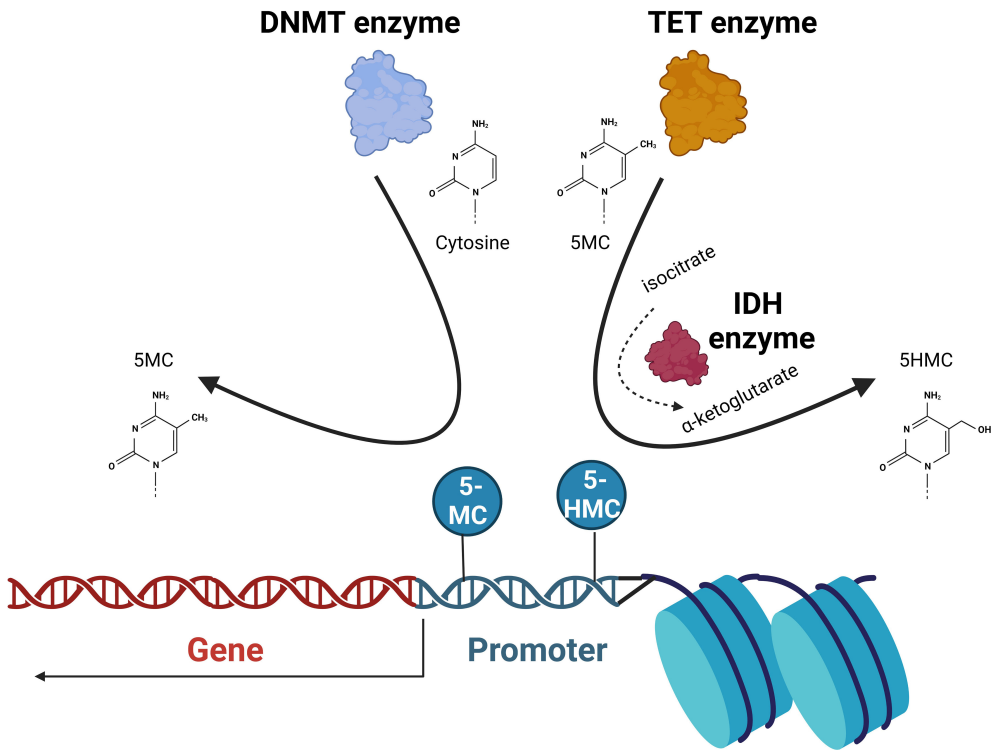

FIGURE 2
DNA methylation. The DNA methyltransferase (DNMT) enzymes are responsible for the conversion of cytosine to 5-methylcytosine (5-MC) that at promoter regions acts to repress transcription. Ten-eleven translocation (TET) enzymes convert 5-MC to 5-hydroxymethylcytosine (5-HMC) that acts to de-repress transcription. Isocitrate dehydrogenase (IDH) proteins convert isocitrate to α-ketoglutarate that the TET2 is dependent on. The balance of unmethylated, methylated, and 5-hydroxy methylated cytosines present in promoter regions will ultimately determine the transcriptional activation of the gene.

myeloproliferative leukemia virus oncogene (*MPL*) (19). This gene encodes the thrombopoietin (TPO) receptor protein. The activated receptor stimulates signaling *via* the JAK-STAT pathway controlling the production of blood cells. Again, mutated *MPL* produces a constitutively activated protein and increased cell production. *JAK*2 and *MPL* mutations have been found also in MF.

In some of the ET and MF patients negative for *JAK*2 and *MPL* changes, mutations in the gene calreticulin (*CALR*) have been identified. In approximately 70%–84% of MPN patients with nonmutated *JAK*2, somatic *CALR* mutations have been described (20). The CALR protein is present in the endoplasmic reticulum where it ensures proper glycoprotein folding, contributes to calcium homeostasis, and plays an important role in the unfolded protein response (UPR). Mutant CALR proteins interact with the TPO receptor (MPL) leading to dimerization and activation of JAK2 and activation of the downstream pathway (21, 22). There remain a few ET and MF cases in which no driver mutations have yet been identified so-called "triple-negative" cases.

JAK2 signaling is involved in numerous biological processes in many cell types. This includes cell cycle progression, mitotic recombination, apoptosis, genetic instability, and alteration of heterochromatin (22–24). It has been shown that a significant fraction of JAK2 is present within the nucleus and that it directly phosphorylates tyrosine 41 on histone H3 (H3Y41). In the nucleus, JAK2 mediates the phosphorylation of H3 and displaces heterochromatin protein 1 alpha (HP1α) from a novel binding site surrounding H3Y41. While the displacement of HP1α is tightly regulated in normal cells, with constitutively activated mutated JAK2, unregulated displacement of chromatin-bound HP1α may override its potential tumor-suppressive functions (25).

In addition, the type II arginine methyltransferase, protein arginine methyltransferase 5 (PRMT5) that mediates dimethylation of arginine residues within histones H2A, H3, and H4, contributes to the JAK2 mutant-induced MPN phenotype. JAK2 mutants bind PRMT5 more strongly than wild-type JAK2, phosphorylating PRMT5 and impairing its ability to methylate its histone substrates. This represents a gain of function, allowing the regulation of chromatin modifications (26).

Mutations in epigenetic regulators

In MPNs, besides the above-described driver mutations, there are a number of genes involved in epigenetic regulation and messenger RNA (mRNA) splicing that are commonly mutated in MPNs. These mutations occur across different myeloid malignancies being not specific to MPNs. Of those, the three most commonly identified pathogenic mutations in MPN patients involve the epigenetic regulators *TET2*, *ASXL1*, and *DNMT3A*, all present at frequencies above 5% in an unselected MPN population (1). *EZH2* mutations are less common in MPN, occurring in approximately 2% of patients overall, but appear particularly important in determining disease progression (27).

TET2 is a member of the α-ketoglutarate-dependent enzyme family that catalyzes the conversion of 5-methylcytosine of DNA to 5-hydromethylcytosine and then induces DNA methylation. *TET*2 mutations have been reported throughout the gene and such loss-of-function mutations result in DNA hypermethylation. *TET*2 mutations have been reported in 12% of a series of MPN patients (28). The prognostic impact of *TET2* mutations is less clear. The sequence of mutation acquisition appears to be important. Hematopoietic stem cells that have a *TET2* mutation occurring first show enhanced self-renewal but lack the proliferative drive to produce excess mature cells. A *JAK2* V617F second hit is then required to produce the MPN phenotype. The opposite is seen in *JAK2* V617F first, *TET2* second cells. Individuals with *TET2* first tend toward an ET phenotype, whereas those *JAK2* V617F first cells tend toward a PV phenotype (29). DNA methyltransferase 3A (*DNMT3A*) encodes for the protein that is responsible for the *de novo* methylation of CpG dinucleotides. Loss-of-function mutations are found in various MPN types (30). It is thought that the epigenetic deregulation resulting from these mutations leads to upregulation of the hemopoietic stem cell (HSC) fingerprint genes (31).

Genes involved in histone methylation are also mutated in MPNs. PcG EZH2 is the catalytic component of the PRC2 and is involved in the trimethylation of histone H3 lysine 27 resulting in transcriptional repression. In MPNs, *EZH*2 mutations tend to be loss-of-function mutations (32) that result in derepression of numbers of oncogenes and are associated with increased HSC self-renewal (33). This is in contrast to the gain-of-function mutations more commonly observed in lymphoid malignancy. *EZH2* mutations are more commonly observed in primary MF. In *JAK2* V617F murine models of MPN, *EZH2* mutations induce a myelofibrosis-like phenotype. This is associated with the loss of H3K27me3 and an epigenetic switch to H3K27ac (34). In this mouse model, the addition of an *EZH2* mutation was observed to drive a bias toward megakaryopoiesis. This highlights the potential for epigenetic changes to control the differentiation capacity of the mutant stem and progenitor cells and ultimately change the resulting disease phenotype.

Additional sex combs like 1 (ASXL1) is an enhancer of the polycomb complexes and regulates both PRC1 and PRC2 (35). *ASXL*1 mutations are associated with the reduction of H3K27 methylation and *HOXA* upregulation, which are both linked to impaired recruitment of the PRC2 complex in particular of EZH2 (36) and occur in MF (37). In a mutant ASXL1-induced model of myelodysplasia (MDS), depression of *HOXA9* was also observed to be related to a reduction in H3K27 methylation (38). Isocitrate dehydrogenase 1 and 2 (*IDH*1 and *IDH*2) catalyze the conversion of isocitrate to α-ketoglutarate. Mutations in a small percentage of MPN cases have been described (39). These mutations result in the production of 2-hydroxyglutarate that inhibits Jumonji-C domain histone methylases leading to histone hypermethylation. While the presumed effects of IDH1/2 mutations also include the hypermethylation of DNA promoter sequences and gene repression, the exact consequences for individual promoters and gene transcription are less clear (40).

Ikaros, a Kruppel-like zinc finger transcription factor, interacts with the histone deacetylase repressor complexes and resulting in a repressive effect on genes involved in myelopoiesis. The *IKZF1* influences maturation and differentiation. Deletions in *IKZF*1 have

been found in 21% of MPN patients in blast phase but only in 0.2% of chronic phase patients (41), making it highly possible that *IKZF*1 has a role in leukemic transformation in MPN.

Therefore, a number of mutations found in MPNs affect the regulation of DNA and histone methylation in the HSC compartment. The importance of these mutations is also reflected in the prognostic relevance of each. *ASXL1, EZH2, IDH1/2*, and *TET2* have all been implicated in the progression to fibrotic or leukemic transformation and reduced survival (27).

Nuclear factor erythroid 2 (NFE2)

Nuclear factor erythroid 2 (NFE2) is a transcription factor that is overexpressed in the majority of MPN patients (42, 43). NFE2 is functioning in chromatin remodeling and gene transcription. *In vivo* mouse models with elevated NFE2 levels have an MPN phenotype (44). In MPN patients, insertions and deletions of NFE2 have been described in approximately 2% of patients. *NFE2* mutations were identified as a predictive variable in determining the risk of fibrotic transformation in a large cohort of individuals with MPNs with chronic stage disease (1). These mutations result in truncated NFE2 proteins that enhance wild-type NRE2 function. NFE2 mutant cells have a proliferative advantage (45). The target genes of NFE2 include the histone demethylase, Jumonji domain containing C (*JMJD1C*), resulting in elevation of JMJD1C levels in PV and MF. Levels of the histone marks H3K9m1 and H3K9me2 are decreased. JMJD1C and NFE2 participate in an autoregulatory loop. NFE2 is independently regulated through the JAK2 epigenetic pathway by phosphorylation of H3Y41 (46). The exploration of these pathways may lead to the discovery of durable targets.

Epigenetic studies of MPN

In general, DNA methylation has been studied on a gene-by-gene basis as well as using comprehensive methylation profiling aiming at a genome-wide characterization of epigenetic markers in MPNs. In a cohort study of PV, ET, and MF patients, global DNA methylation profiling was investigated, including some patients who had transformed to acute myeloid leukemia (AML) (71 in chronic phase and 13 transformed). MPN samples showed an aberrant methylation pattern compared to control samples, but patterns were similar in all three MPN types. The gene network involved in the NF-κB pathway showed enrichment of differentially methylated regions. The transformed AML cases had an increased number of differentially methylated regions compared to the chronic cases. Therefore, altered DNA methylation may have a role in the pathogenesis of leukemic transformation in MPNs (47). A previous study of 35 MPN patients found homogeneous methylation patterns in MPN subtypes and controls (48). Another study of 29 MPN chronic phase patients showed that PV and ET were characterized by aberrant promoter hypermethylation, whereas MF was epigenetically distinct with aberrant hypo and hypermethylation. Cases with *ASXL1* and *TET2* mutations had distinct epigenetic signatures revealing methylomic signatures for these mutations (49).

Methylation age

DNA methylation (DNAm) is a well-defined epigenetic mechanism of transcription modification. It is affected by aging, lifestyle, diet, and disease. It is possible to calculate the methylation age of a range of tissues (3). Vorinostat (MK-0683) (suberoylanilide hydroxamic acid) is a pan-histone deactylase inhibitor (HDACi) that has been shown to induce tumor cells to undergo growth arrest, differentiation, and apoptosis (50–52). This agent has been trialed in patients with PV and ET with some responses including decreased leukocyte and platelet counts and modest reductions in *JAK*2V617F allele burdens. Treatment discontinuation rates were high due to toxicity (53).

Methylation age (MA) may be a more accurate reflection of disease than chronological age (CA). Using the aging signature of Weidner et al. (54) to generate individual MA, it was explored whether DNAm is altered in MPN and whether the HDACi vorinostat altered the MA in PV and ET patients who were treated in the trial.

Having verified the aging signature, an older MA was observed in patients with a higher *JAK*2V617F allelic burden and in those with longer duration of disease. PV had an older MA than predicted, whereas it was younger than predicted in ET, perhaps related to the mutant allele burden. Treatment with vorinostat resulted in a younger MA in PV patients and an older MA in ET patients resulting overall in a trend to normal CA. Comparing MA and response, nonresponse was associated with a younger than predicted MA in ET patients and an older than predicted MA in PV patients. There would appear to be a link between MA and *JAK*2 mutant allele burden implying that the allelic burden is not only influencing the clinical phenotype, and disease evolution, but also the overall methylation landscape of the MPN cells (55).

Histone modification with JAK inhibition

Therapeutic options for MPNs are limited, and the main drug licensed to treat MF is the JAK1/2 inhibitor ruxolitinib. This drug is effective in reducing symptom burden, cell counts, and spleen volume in PV and MF (56). Disease-modifying effects are modest with some reductions in *JAK*2 allele burden (57), but transformation to myelofibrosis and acute leukemia is not altered by ruxolitinib (58). Epigenetic dysregulation may have a role in MPN, and the effect of JAK inhibitor therapy on the epigenetic landscape is of interest in understanding the benefits of treatment. Histone modification was therefore explored in MPN cell lines and in patient samples from the randomized controlled trial MAJIC, which evaluated the efficacy of ruxolitinib versus best available therapy in a second-line setting in PV and ET (59).

After establishing a dose of ruxolitinib in MPN cell lines that showed inhibition of phosphorylation of STAT3 and STAT5 and therefore being sufficient to exert molecular responses in cell lines but not inducing cell death, histone modification was investigated using a 100-nM concentration of ruxolitinib. An increase in methylation marks was seen in the MPN cell lines SET-2, UKE-1, and HEL, showing that ruxolitinib treatment of cell lines could induce modification of histones. The effect of ruxolitinib treatment was quantified with a screen of 21 histones. In the combined cell line analysis, H3K27me3 and H3K36me2 were significantly increased. For validation of histone methylation, three histone marks of primed and active transcription (H3K4me2, H3K4me3, and H3K27ac) were studied in detail with immunoprecipitation and sequencing. A clear differential was seen for H3K4me3 and H3K27ac as two marks of active transcription. Overall, results in cell lines showed that ruxolitinib had an immediate effect on the histone landscape with a significant increase in methylation and acetylation. However, the transcriptome of the cell showed a reduction in the expression of genes involved in cell signaling pathways. The epigenetic repriming of the cell was not immediately reflected in the transcriptome (60).

MAJIC: A randoMised study of best Available therapy versus JAK Inhibition in patients with high risk Polycythaemia Vera of Essential Thrombocythaemia who are resistant or intolerant to HydroxyCarbamide (ISRCTN61925716) evaluated the efficacy of ruxolitinib versus best available therapy in a second-line setting in PV and ET. With the MAJIC patient samples, the aim was to examine histone modification in MPN patients on ruxolitinib or BAT and to investigate for any association with clinical outcome. Paired samples from 51 patients in the trial were investigated. For some, all methylation histone marks increased with treatment, for others, all decreased, while in others, there were increases and decreases.

There was no change in any histone mark associated with a treatment arm in either disease group. However, when modifications within the same lysine were examined, methylation marks were decreased in follow-up samples compared to trial entry. The decrease in H3K36 marks was significant in ruxolitinib patients but not BAT patients, whereas the decrease in H3K4 marks was significant in the BAT patients but not the ruxolitinib treated. However, when looking at histone marks in isolation, at baseline and follow-up, high levels at trial entry of H3K4m2 and H3K4me3 were associated with lack of response to ruxolitinib. This study reflects the heterogeneous patient population, and the evolving histone landscape during prolonged therapy suggests a dynamic process of transcriptional control reflective of the role of therapy to modify transcription and suppress proliferation (60).

Further study

Further investigation of the epigenome in MPNs is certainly warranted to understand the diseases and the factors that are involved in progression to acute leukemia and in the case of PV and ET to MF. A recent study explored the role of high-mobility group A1 (HMGA1), a chromatin regulator, in MPN disease progression in human samples and mouse models. HMGA1 is upregulated in MPN with highest levels after transformation. In *JAK2*V617F mouse models, loss of a single Hmga1 allele prevents progression to MF and HMGA1 depletion enhances responses to ruxolitinib preventing MF and increasing survival in the mice (61). This is of further interest in elucidating the pathogenesis of MPNs.

Epigenetic therapies

As there are differences in the epigenome in MPNs and further alterations associated with progression, therapeutic modulation of epigenetically deregulated pathways may present an opportunity for targeted therapy in MPN patients at various stages of their disease pathway.

There are a number of agents in use or in development that have or may have potential in the future in treating MPNs. Hypomethylating agents such as azacytidine, a cytosine nucleotide analog, is incorporated into RNA inhibiting metabolism and protein synthesis (see hypomethylating agents, Figure 3). However, it has demethylation activity (62). It is widely used in the management of myelodysplastic syndromes and AML. In the context of treatment of progression of MPN to AML, it has some utility currently.

Decitabine, another hypomethylating agent, a deoxycytidine analog, which is incorporated into DNA resulting in binding of methyltransferase and inactivation. However, at lower doses, it has a hypomethylating activity and reactivates silenced genes. It is therefore a useful agent with this function in the treatment of myeloid malignancies (63). In *JAK2*V617F-positive cell lines treated with decitabine, H3Y41ph levels were lowered and H3K9me2 levels increased at the NFE2 locus, normalizing NFE2 expression (46). These hypomethylating agents may therefore have further potential in treatment of MPNs.

Specific inhibitors of the epigenetic regulators *IDH*1/2 and *EZH2* are becoming available. Ivosidenib and enasidenib, IDH1 and 2 inhibitors, have been approved and licensed for AML (64). Tazemetostat is the first EZH2 inhibitor approved by the FDA and targets both wild-type and mutant EZH2 inducing cell cycle arrest. The use of EZH*2* inhibitors to date has been trialed in lymphoid malignancy where mutations are gain of function (65). It will be important to establish if inhibition of EZH2 may potentially drive selection of antecedent myeloid clones to establish MPN, MDS, or AML (see Figure 3, IDH inhibitors and EZH inhibition). The use of these targeted agents in MPN as inhibitors of the epigenetic regulators that are frequently mutated needs careful assessment.

There are a number of HDACis in clinical use (HDAC inhibition, Figure 3). Vorinostat has been trialed in PV and ET with some response, but the doses used in this trial were probably too high (53). Panobinostat, a pan HDACi, is used in the treatment of multiple myeloma, and romidepsin, a class 1 HDACi, is used as monotherapy in cutaneous T-cell lymphoma and in peripheral T-cell lymphoma. Toxicity of HDACi has been a consistent issue in clinical studies. Givinostat may be better tolerated than other HDACis and appears to be efficacious in MPN. PV patients treated with givinostat in phase I/II studies demonstrated clinical

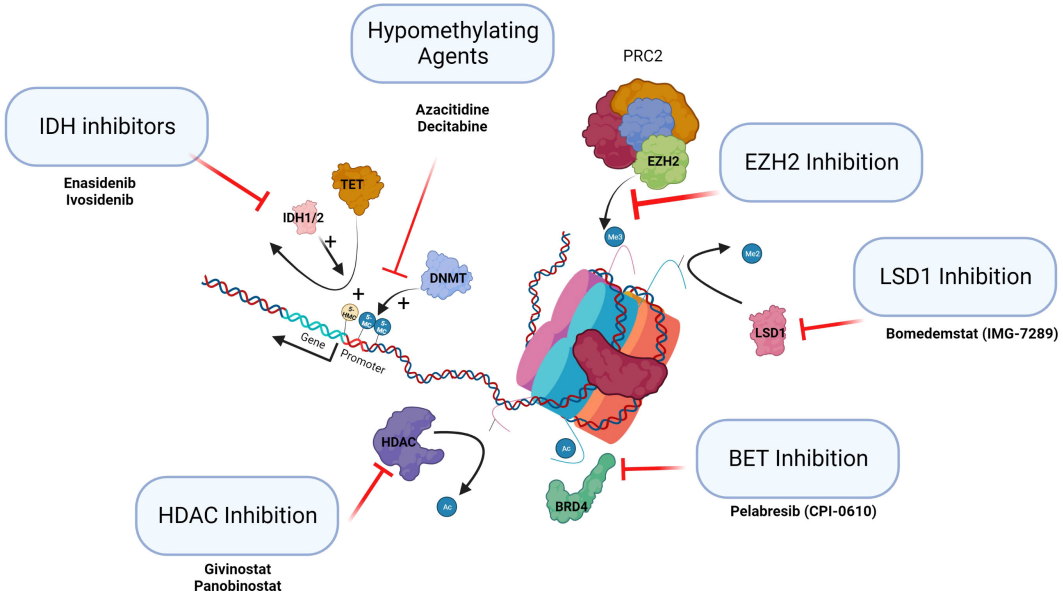

FIGURE 3
Epigenetic therapies in clinical trials in MPN. This figure summarizes the classes of epigenetic drugs being considered for or recently in trial for MPN patients.

benefit in approximately two-thirds of patients initially. Longer-term follow-up of patients with PV who had an initial response to the drug showed a consistent overall response rate of greater than 80% with a 4-year mean follow-up (66). These agents may have a potential future role in the treatment of MPNs by altering the epigenome.

Bromodomain and extra terminal (BET) protein family consists of multiple epigenetic reader proteins that regulate gene transcription through binding of acetylated histones resulting in the control of cellular processes, such as transcription and chromatin remodeling (BET inhibition, Figure 3) (67). In mouse models of MF, BET inhibitors demonstrated responses resulting from the attenuation of NF-κB signaling. These agents alone or in combination with ruxolitinib are now completing clinical trials in MF.

The demethylation of histone H3 lysine 4 and lysine 9 mono and di methylation (H3K4me1/2 and H3K9me1/2) is specifically catalyzed by lysine-specific demethylase 1 (LSD1). Normal LSD1 function is critical for normal differentiation during hematopoiesis (LSD1 inhibition, Figure 3). In mouse models of MPN, inhibition of LSD1 showed prolonged survival and was selective in targeting the disease clone with improvements in hematological parameters (68). These agents are now in clinical trials.

Conclusion

Reversible epigenetic changes and epigenetic regulation are part of the pathogenesis of MPNs both in the disease development and in progression. Epigenetic changes in MPN have been investigated with a variety of results. Effects of aging and treatment on the epigenome are revealing. However, there is room for much greater investigation and understanding of epigenetic changes and events involved in progression. Investigation of the epigenome shows that there are a variety of therapeutic pathways available and that can be explored further leading to targeted therapy.

Author contributions

Both author conceived the concept, wrote the article and checked final version GG designed the figures. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Cigarette smoke stimulates clonal expansion of $Jak2^{V617F}$ and $Tet2^{-/-}$ cells

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Introduction: Somatic mutations in myeloid growth factor pathway genes, such as JAK2, and genes involved in epigenetic regulation, such as TET2, in hematopoietic stem cells (HSCs) leads to clonal hematopoiesis of indeterminate potential (CHIP) which presents a risk factor for hematologic malignancy and cardiovascular disease. Smoking behavior has been repeatedly associated with the occurrence of CHIP but whether smoking is an environmental inflammatory stressor in promoting clonal expansion has not been investigated.

Methods: We performed in vivo smoke exposures in both wildtype (WT) mice and transplanted mice carrying $Jak2^{V617F}$ mutant and Tet2 knockout (Tet-/-) cells to determine the impact of cigarette smoke (CS) in the HSC compartment as well as favoring mutant cell expansion.

Results: WT mice exposed to smoke displayed increased oxidative stress in long-term HSCs and suppression of the hematopoietic stem and progenitor compartment but smoke exposure did not translate to impaired hematopoietic reconstitution in primary bone marrow transplants. Gene expression analysis of hematopoietic cells in the bone marrow identified an imbalance between Th17 and Treg immune cells suggesting a local inflammatory environment. We also observed enhanced survival of Jak2V617F cells exposed to CS in vivo and cigarette smoke extract (CSE) in vitro. WT bone marrow hematopoietic cells from WT/Jak2V617F chimeric mice exposed to CS demonstrated an increase in neutrophil abundance and distinct overexpression of bone marrow stromal antigen 2 (Bst2) and retinoic acid early transcript 1 (Raet1) targets. Bst2 and Raet1 are indicative of increased interferon signaling and cellular stress including oxidative stress and DNA damage, respectively. In chimeric mice containing both WT and Tet2-/- cells, we observed an increased percentage of circulating mutant cells in peripheral blood post-cigarette smoke exposure when compared to pre-exposure levels while this difference was absent in air-exposed controls.

Conclusion: Altogether, these findings demonstrate that CS results in an inflamed bone marrow environment that provides a selection pressure for existing CHIP mutations such as Jak2V617F and Tet2 loss-of-function.

KEYWORDS

inflammation, myeloproliferative neoplasm, reactive oxygen species, cigarette smoke, clonal hematopoiesis

Introduction

Clonal hematopoiesis (CH) describes the dominance of a specific hematopoietic stem cell clone in the peripheral blood. Clonal hematopoiesis of indeterminate potential (CHIP), is an age-related condition where hematopoietic stem cells (HSCs) that have acquired somatic mutations give rise to mature mutant cells in the peripheral blood with a variant allele frequency of at least 2% (1–3). CHIP is very common in the elderly with at least 10% of the population over 65 years being affected (1). CHIP not only presents an increased risk for developing hematologic malignancies but is also associated with a range of co-morbidities that involve inflammation such as cardiovascular disease (4, 5) and gout (6). Somatic mutations in recurrent genes confer a selective growth advantage or increased fitness in mutant HSC clones. DNMT3A, TET2, ASXL1 and JAK2 are among the commonly mutated genes associated with CHIP (1–3).

Although CHIP is very common after the fifth decade of life the emergence of CH is variable and may take several years before a pathogenic mutant can overtake its normal counterparts in the stem cell pool (7). Clonal dominance can be accelerated by cell extrinsic factors including inflammation (8), genotoxicity (9), and expediated HSC proliferation (10). Thus, specific environmental stressors may provide ideal conditions for outgrowth of specific mutant clones that are superiorly adapted for the stressor. Age is the strongest determinant for CHIP indicating that inflammatory stress or inflammaging associated with the natural process of aging contributes to CH (11). Environmental influences such as smoking (12) and cytotoxic chemotherapy (13) have also been repeatedly associated with clonal hematopoiesis.

Myeloproliferative neoplasms (MPNs) are characterized by clonal outgrowth of mutant hematopoietic stem cells contributing to the over-production of circulating mature cells of the myeloid lineage (14). MPNs can be described as a human inflammation model of cancer development where chronic inflammation is a hallmark feature of myeloproliferative neoplasms (MPNs) and is associated with disease initiation and progression (15). The most common somatic mutation in MPN patients occurs in Janus Kinase 2 (JAK2) known as the $JAK2^{V617F}$ mutation (16–19). We have shown that an *in vitro* inflammatory environment facilitates the selective expansion of the $JAK2^{V617F}$ neoplastic clone in human subjects (20). More recently, IL-1b exposure stimulated clonal expansion of $Jak2^{V617F}$ mutant cells and accelerated progression to bone marrow fibrosis (21). Additional extrinsic mechanisms mediating $JAK2^{V617F}$ clonal expansion are lacking. Epidemiological studies such as the UK Million Women Study and the Iowa Women's Health Study have reported a correlation between smoking and the development MPNs (22, 23). Sørensen and Hasselbalch, also described a positive association between smoking behavior and the risk for MPN (24). It is possible, that the systemic inflammation associated with smoking can support clonal expansion of the $JAK2^{V617F}$ malignant clone.

Biological studies have demonstrated that *Tet2*-deficient hematopoietic stem and progenitor cells (HSPCs) expand under inflammatory stimulation. Lipopolysaccharide (LPS) induced acute inflammation (8) and increased IL-6 production by intestinal microbiota (25) promote expansion of $Tet2^{-/-}$ HSPCs in mice while prolonged TNFα exposure *in vitro* increased the clonogenic capacity of Tet-2 deficient murine and TET2-mutant human bone marrow cells (26). More recently, IL-1α exposure was found to expand $Tet2^{+/-}$ hematopoietic cells relative to wildtype cells while IL-1 receptor (IL-1R1) knockout mice failed to increase $Tet2^{+/-}$ cells in response to IL-1α (27). Similarly, loss of IL-1R1 restored the hematologic abnormalities, myeloid-lymphoid balance and systemic inflammation observed in Tet2-deficient mice (28). These studies underscore the importance of inflammatory cytokine signaling in driving TET2 dependent CH. However, the contribution of smoking in promoting clonal expansion of TET2 mutant cells has not been explored.

Cigarette smoking exerts a severe oxidative and inflammatory insult upon the respiratory system leading to the upregulation of several pro-inflammatory cytokines. Smoking causes systemic inflammation and is an independent risk factor for CHIP (11, 12). The inflammatory milieu caused by smoking on the clonal outgrowth of specific CHIP mutations has not yet been fully elucidated.

In this study we tested the impact of a two-month nose-only exposure on the causal relationship between smoking and CH. We assessed hematopoietic stem cell numbers and gene expression profiling in wildtype mice. Then, in competitive repopulation assays we tested how smoke exposure affects the clonal expansion of two common mutations seen in hematologic malignancies and CHIP, JAK^{V617F} and TET2 loss-of-function. We hypothesized that CHIP mutant hematopoietic stem and progenitor cells are more resistant to inflammatory stimuli rendered by smoking than their normal counterparts which leads to their selective expansion in the context of smoke exposure.

Materials and methods

Mice

Wildtype C57BL/6J (CD45.2) (Stock No. 000664) mice at 8 weeks of age were purchased from the Jackson Labs for cigarette smoke exposure. The conditional knockout $Jak2v617f^{fl/+}$ mice were a kind gift from Dr. Ann Mullaly and were crossed to the Vav-iCre mice from Jackson labs (Stock No. 008610) to generate $Jak2^{V617F}$ mutant mice for bone marrow transplants. $Tet2^{-/-}$ mice (Stock No. 023359) and C57BL/6.SJL (CD45.1) (Stock No. 002014) mice were purchased from the Jackson labs. Mice were housed in specific pathogen-free facilities at the University of California, Irvine and maintained on a 12-hour light/dark cycle. All animal procedures were performed under the approval of the Institutional Animal Care and Use Committee at the University of California, Irvine.

Cigarette smoke exposure

Exposures were performed at the Air Pollution Health Effects Laboratory at the University of California, Irvine. Aerosols were

generated using a 2-s puff with a 60-s interval between puffs (35 mL puff volume) based on the ISO standard cigarette puff protocol (ISO 3308:2012) using a custom-built smoking system. This system uses a peristaltic pump to draw in aerosolized smoke from a lit combustion cigarette (1R6F Certified Reference Cigarette, Center of Tobacco Reference Products (CTRP), University of Kentucky) and directs it into a nose-only exposure manifold (In-Tox Products, Moriarty, NM, USA). Control mice were exposed concurrently with the combustion cigarette exposure to air purified over potassium permanganate-impregnated alumina beads, activated carbon, and high-efficiency particulate air (HEPA) filters. The nose-cone inhalation exposure system is advantageous over whole-body exposure systems because it minimizes contamination of fur and potential non-relevant exposure due routine grooming that would occur in a whole-body exposure regime. During exposures, animals were held in individual exposure tubes that were connected to the exposure manifold and positioned with just the snout exposed to the exposure atmosphere. Exhaust ports surrounding each nose cone were under slight negative pressure which directs the flow of fresh aerosol to the animal's breathing zone and exhausts exhaled air thereby minimizing the potential for re-breathing secondary vapor and preventing CO_2 buildup. Exposures occurred 2 hours per day, 4 days/week, for 8 weeks. Between exposures, the mice were housed 4 per cage in an atmosphere-controlled room on a 12-hr light/dark cycle in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited animal housing facility at the University of California, Irvine vivarium.

Cigarette smoke extract

Aqueous cigarette smoke extract (CSE) was prepared from mainstream cigarette smoke using an impinger and following a standard protocol (29). Briefly, mainstream smoke from one 2R4F research cigarette was drawn into serum-free media by continuous negative pressure and filter-sterilized to obtain 100% CSE. Volume/volume percentage (v/v %) dilutions were used for *in vitro* exposures.

Bone marrow transplantation

Adult mice were euthanized before bone marrow harvesting. Euthanasia was performed using an overdose of isoflurane with the isoflurane concentration adjusted at 5% or greater and isoflurane exposure was continued until 1 minute after breathing stopped. Euthanasia was confirmed by cervical dislocation method. Recipient mice were lethally irradiated (single dose of 8Gy) and received 2×10^6 whole bone marrow cells mixed in a 1:1 ratio from either air or smoke exposed mice and Pepcb/Boy CD45.1 mice *via* retro-orbital injection. Peripheral blood chimerism was assessed at regular intervals. For chimeric mice, lethally irradiated CD45.1/CD45.2 recipient mice were transplanted with $2x10^6$ unfractionated bone marrow mixed in a 1:1 ratio from $Jak2^{V617F}$ and CD45.1 mice. Whole bone marrow from $Tet2^{-/-}$ and CD45.1 wildtype mice were mixed in a ratio of 1:10 and a total of $3x10^6$ cells were injected into recipient mice. Chimeric mice were rested post-transplant before cigarette smoke exposure.

Flow cytometry of mouse peripheral blood and bone marrow

Peripheral blood was collected from the saphenous vein and used to obtain complete blood counts using the automated cell counter machine (ABCVet Hemalyzer, scil). To determine peripheral blood chimerism, red blood cells were lysed using ammonium-chloride-potassium buffer stained with the anti-mouse antibodies against CD45.1 (clone A20), CD45.2 (clone 104), Ly6G (clone 1A8) and CD11b (M1/70). Bone marrow hematopoietic Pacific blue-conjugated anti-mouse antibodies against TER-119 (clone Ter119), CD3 (clone 17A2), Gr1 (clone RB6-8C5), CD11b (clone M1/70), B220 (RA3-6B2) were used to detect mature cells (Lineage cocktail). HSPC populations were detected by staining with CD34 (RAM34, BD Biosciences), CD16/32 (clone 93), c-Kit (clone 2B8), Sca-1 (clone D7), CD48 (clone HM48-1) and CD150 (clone TC15-12F12.2). For bromodeoxyuridine (BrdU) incorporation, mice were injected with BrdU at 1mg/kg and euthanized 16 hours later. Bone marrow cells were stained with cell surface markers, fixed, permeabilized and probed for BrdU following manufacturer's instructions in the BrdU flow kit (BD Biosciences, San Jose, CA). For gamma-H2AX, stained, fixed and permeabilized cells were incubated with anti-H2A.X phospho (Ser139, clone 2F3) for 20 minutes at room temperature. All antibodies were purchased from BioLegend unless otherwise stated. Flow cytometry was performed on the Novocyte (ACEA Biosciences) at the UCI Immunology Core facility. Data were analyzed using FlowJo software (Tree Star Inc.).

Colony formation assay

Colony formation assays were performed according to protocols provided by StemCell Technology and as described previously (20). Fresh peripheral blood mononuclear cells or whole bone marrow cells were incubated with increasing concentrations of cigarette smoke extract overnight in the presence of N-Acetylcysteine (100μM), as previously described (30), where indicated. Cells were washed thoroughly and plated in Methocult medium (H4230 andM3224, StemCell Technologies) containing human or mouse IL-3 at 10ng/ml, human or mouse SCF at 50ng/ml and human Epo at 20ng/ml. Colonies were counted 7-12 days later.

Nanostring data analysis

Bone marrow cells from wildtype mice exposed to air or cigarette smoke were harvested and RNA was isolated using the RNeasy micro kit (Qiagen). WT/$Jak2^{V617F}$ chimeric mice exposed to air or CS were euthanized within 24 hours of the last exposure and WT bone marrow cells were sorted on the BD FACSAria Fusion (BD Biosciences) by staining for anti-mouse CD45.1 and CD45.2 antibodies for RNA isolation. The nCounter Mouse PanCancer Immune Profiling Panel

was used to analyze mRNA expression of immune function and inflammatory transcripts. Nanostring data was analyzed by ROSALIND® (https://rosalind.bio/) (ROSALIND Inc, San Diego, CA). Data normalization was performed using the protocol from the nCounter advanced analysis software (NanoString Technologies). Abundance of various cell populations was calculated on ROSALIND using the Nanostring Cell Type Profiling Module. Differential gene expression was calculated following the nCounter® Advanced Analysis protocol to identify targets with significantly increased or decreased expression. P-value adjustment was performed using the Benjamini-Hochberg method of estimating false discovery rates (FDR). Nanostring data are publicly available on ArrayExpress, accession number E-MTAB-13133.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Data are presented as mean with error bars representing the SEM. Comparison between groups was performed using unpaired *t* test. For matched samples, paired t test was used to determine the *P* value. Two-way ANOVA was used to compare more than 2 groups and *P* value adjusted using Bonferroni's correction.

Results

Cigarette smoke exposure induces secondary erythrocytosis in wildtype mice

Wildtype C57BL/6J mice were exposed to nose-only inhalation of conventional combustible cigarette smoke (CS) for 2 hours per day, 4 days a week for 2 months. Cigarette smoke exposure in rodents can be carried out using either whole-body or nose-only exposure systems. The nose-only exposure allows for efficient and targeted exposure while limiting non-respiratory exposure routes such as ingestion and dermal absorption observed with whole-body exposure. Smoke exposure increased erythrocyte parameters such as red blood cell count (Figure 1A) and hematocrit (Figure 1B) in comparison to air exposed mice. However, platelet counts (Figure 1C), white blood cell counts and (Figure 1D) white blood cell differentials (Figure 1E) were not affected by smoke exposure. Body weight was increased by smoke exposure; however, heart, liver, and spleen weights were comparable between the two exposure groups (Supplemental Figure 1). These observations demonstrate that our smoke exposure model system replicates erythrocytosis observed in human smoking behavior.

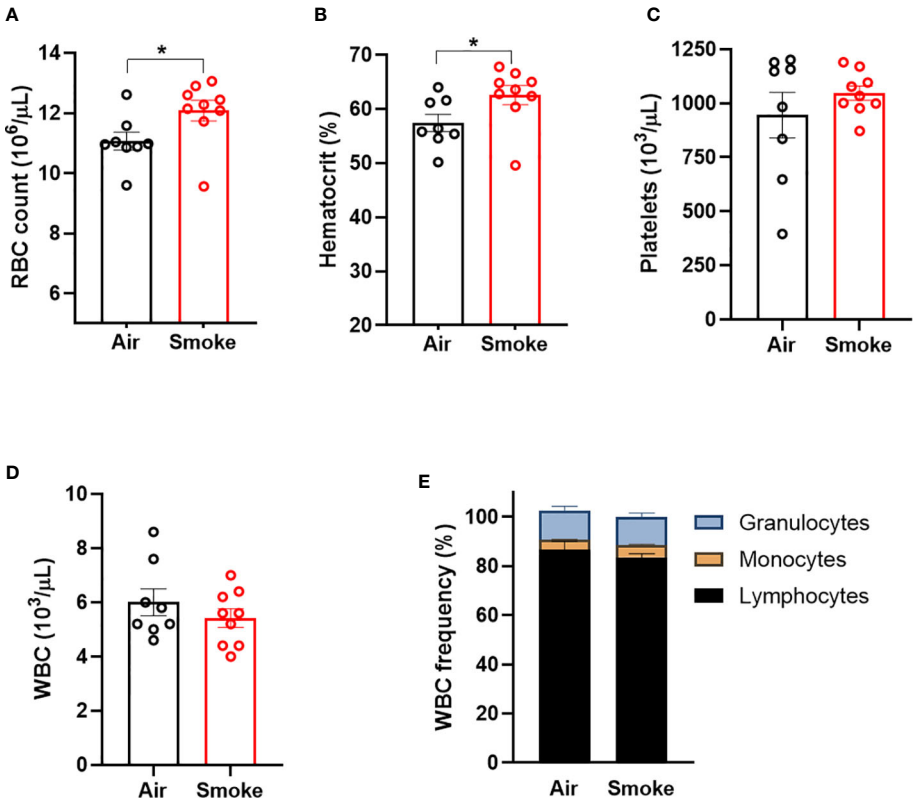


FIGURE 1
Combustible cigarette smoke exposure induces secondary erythrocytosis. Two months of smoke exposure in wildtype mice increased **(A)** red blood cell (RBC) count, and **(B)** hematocrit. **(C)** Platelet counts, **(D)** white blood counts (WBC), nor **(E)** white blood cell differentials were not affected by smoke exposure. Data are shown as mean ± SEM, n=8-9 mice/group. *p<0.05, unpaired student's t-test.

Cigarette smoke exposure induces inflammatory gene expression changes in BM cells

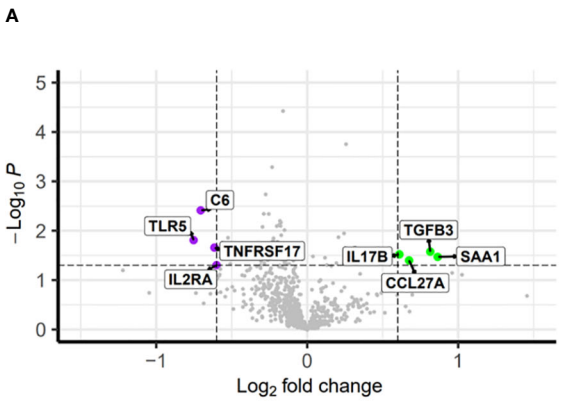
To study the specific gene expression changes in hematopoietic cells exposed to CS, mice exposed to CS or air for 2 months were euthanized and bone marrow was harvested from the long bones for comparative expression analysis using the Nanostring PanCancer Immune Profiling Panel. Genes that were significantly upregulated include transforming growth factor beta 3 (Tgfb3), serum amyloid A1 (Saa1), C-C motif ligand 27 (Ccl27a) and interleukin 17 receptor A (IL17Ra). Interleukin-2 receptor alpha (IL2Ra), Toll-like receptor 5 (Tlr5), complement 6 (C6) and TNF receptor superfamily member 17 (Tnfrsf17) were found to be downregulated (Figures 2A, B). Biological processes associated with the dysregulated genes included T-cell homeostasis (Figure 2C). Saa1 and IL-17A are neutrophil mobilizing mediators, with IL-17A being a major inflammatory cytokine that drives pathological inflammation (31). Increase in IL-17Ra indicates upregulation of inflammatory responses and IL-17Ra has been shown to be involved in CS-induced chronic obstructive pulmonary disease (COPD) (32). IL2Ra or CD25 is expressed on T regulatory cells and its downregulation indicates an imbalance between inflammatory Th17 cells and immunosuppressive T regulatory cells and inability to suppress inflammation associated with CS (33). Interestingly, Saa1 can initiate polarization and maturation of IL-17A expressing cells and the combined upregulation of IL-17Ra and Saa1 serve as markers of persistent inflammation (34). Tgfb3 exerts a suppressive effect on B cells and also induces Th17 cells that display pathologic inflammation (35).

Increased oxidative stress in hematopoietic stem cells exposed to cigarette smoke

Next, we used flow cytometry to quantify the effect of CS on wildtype hematopoietic stem and progenitor cells (Figure 3A). Smoke exposure was associated with a significant increase in reactive oxygen species (ROS) within the lineage$^-$, c-Kit$^+$, Sca1$^+$ (LKS) and LKS-CD150$^+$, CD48$^-$ (LKS-SLAM) or long-term HSCs (LT-HSCs) (Figure 3B) populations. Cigarette smoke lowered the absolute number of LKS cells in the bone marrow (Figure 3C) but did not significantly change the frequency of LKS cells (Figure 3C). We did not detect a statistical difference in the frequency nor absolute numbers of LKS-SLAM, or myeloid progenitor compartments (common myeloid progenitor CMP, granulocyte monocyte progenitor GMP, megakaryocyte erythroid progenitor MEP) when comparing air versus smoke exposed mice (Figures 3B, C).

Smoke exposure does not compromise competitive repopulation ability

To functionally assess the impact of smoke exposure on hematopoietic stem cells we transplanted air-or smoke-exposed



C

Biological Process

Term	p-Elim
positive regulation of cytoskeleton organization	0.01949
positive regulation of activation of membrane attack complex	0.02158
detection of oxygen	0.02158
toll-like receptor 5 signaling pathway	0.02158
regulation of T cell homeostatic proliferation	0.02158

FIGURE 2
Differentially expressed genes in CS exposed bone marrow hematopoietic cells. **(A)** Volcano plot showing difference in expression between air and smoke exposure. Annotated genes are significantly differentially expressed, p<0.05. **(B)** Heatmap depicting the differentially expressed genes. **(C)** Gene ontology biological processes affected by smoke exposure.

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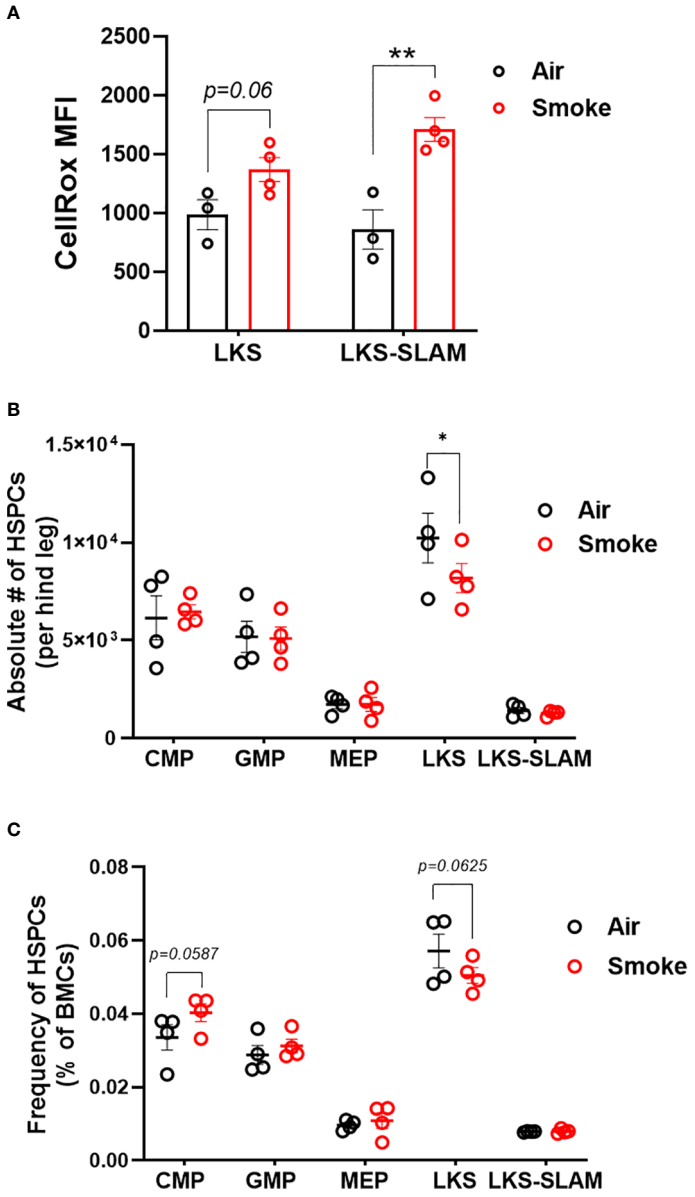

FIGURE 3
HSCs display oxidative stress upon smoke exposure. **(A)** Oxidative stress in LKS and LKS-SLAM cells as determined by CellRox staining. Absolute numbers **(B)** and frequencies **(C)** of hematopoietic stem and progenitor cell population after 2 months of exposure to nose-only smoke or air. Data are shown as mean ± SEM, n=3-4 mice per group. *p<0.05, **p<0.001, unpaired student's t-test.

whole bone marrow cells along with equal numbers of unexposed cells into lethally irradiated mice and followed peripheral blood chimerism (Figure 4A). Competitive ability of bone marrow from mice exposed to smoke was equivalent to that of air exposed mice in primary recipients (Figure 4B). Six months after transplant, recipient mice were euthanized and their bone marrow was analyzed. The contribution of air and smoke exposed CD45.2 donor cells to total donor derived bone marrow leukocytes was equivalent (Figure 4C). To determine if bone marrow from smoke exposed mice display expansion or shrinkage of stem and progenitor cells, we quantified the frequency of lineage negative, c-kit$^+$, Sca-1$^-$ (contains myeloid progenitors), LKS, and LKS-SLAM of total CD45.2 cells (Figure 4D). We found no significant differences in the frequencies of these populations when comparing air versus smoke exposed donors.

In vitro cigarette smoke extract reduces myeloid colony formation partially through oxidative stress

To evaluate the impact of an acute *in vitro* smoke exposure on hematopoiesis bone marrow from wildtype mice and peripheral blood from normal human controls were cultured overnight with 0, 2, 5, or 10% cigarette smoke extract (CSE), washed prior to plating on methylcellulose, and colonies were counted 7-12 days later. We observed a decrease of both mouse (Figure 5A) and human (Figure 5B) myeloid colony formation with overnight exposure to CSE. To determine if this effect was mediated by oxidative stress we included the anti-oxidant N-Acetylcysteine (N-AC) in the overnight CSE culture. N-AC rescued the suppressive impact of

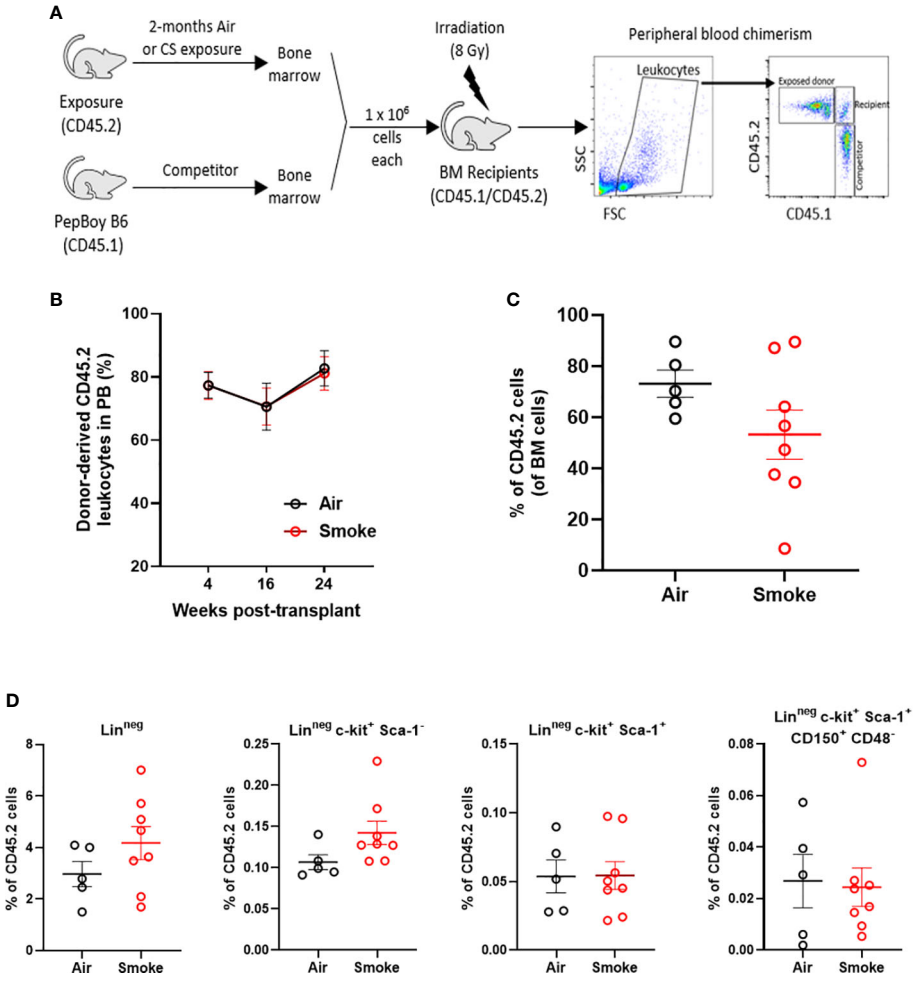

FIGURE 4
Smoke exposure does not compromise competitive repopulation ability. **(A)** Experimental design of competitive repopulation assay used. **(B)** contribution of CD45.2 donor cells to peripheral blood leukocytes of transplanted mice over time and **(C)** contribution of CD45.2 donor cells to bone marrow leukocytes at time of euthanasia, **(D)** frequencies of progenitor and stem cell populations gated on CD45.2 bone marrow cells. Data are shown as mean ± SEM, n=5-7 mice per group.

CSE on colony formation, suggesting that CSE's suppressive effect is mediated *via* oxidative stress (Figures 5A, B).

Smoke exposure enhances competitive ability Jak2^{V617F} mutant cells

Next, we tested whether cigarette smoke impacts the competitive ability of Jak2^{V617F} mutant cells in competitive repopulation assays. C57BL/6J mice were lethally irradiated and transplanted with equal numbers of whole bone marrow from Jak2^{V617F} and WT donors (1:1) (Figure 6A). This knock-in model of Jak2^{V617F} does not display a competitive advantage in a lethally irradiated bone marrow transplant setting and we expect to observe decreasing percentage of Jak2^{V617F} cells in the peripheral blood over time. Mice were rested post-transplant and then exposed to 2 months of combustible cigarette smoke *via* nose-only inhalation. The percentage of mutant cells was low (2-20%) to mimic clonal hematopoiesis without an overt myeloproliferative neoplasm phenotype. Smoke exposure in transplanted mice slightly increased red blood cells, p=0.055, and showed a trend towards increased hematocrit, and leukocytes (p<0.1) (Supplemental Figures 2A-D). There was no significant increase in spleen to body weight ratio (Supplemental Figure 2E) nor liver weight (Supplemental Figure 2F). As expected, mice exposed to air demonstrated a decline in Jak2^{V617F} mutant cells in the peripheral blood over time, however in contrast smoke exposed mice maintained circulating Jak2^{V617F} levels before and after exposure (Figure 6B).

Smoke exposure increases HSC DNA damage and proliferation

We compared the impact of CS on wildtype and Jak2^{V617F} mutant cells in transplanted and exposed mice. We observed a significant reduction in the frequency of total LKS cells (containing Jak2^{V617F} and wildtype cells combined) in transplanted mice exposed to smoke (Figure 7A). To identify the relationship between smoke exposure and HSC cycling, air- and smoke-exposed mice were

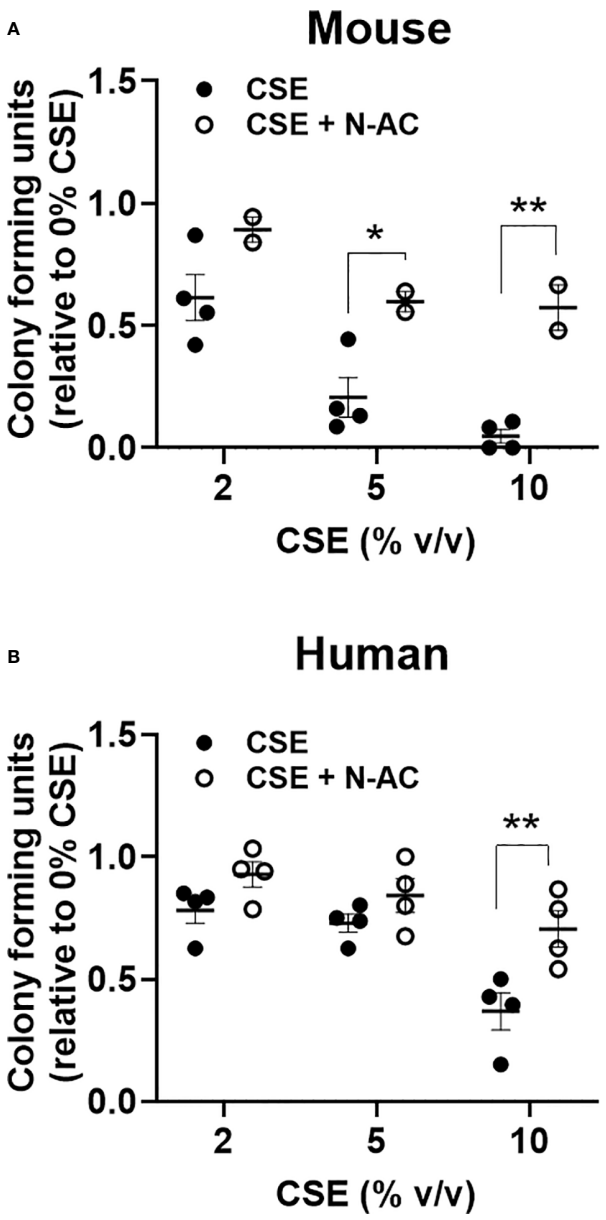

FIGURE 5
Acute *in vitro* exposure reduces myeloid colony formation and is rescued by the anti-oxidant N-Acetylcysteine. **(A)** Wildtype mouse bone marrow cells and **(B)** normal human peripheral blood mononuclear cells were incubated overnight with increasing concentrations of cigarette smoke extract +/- 100nM N-AC, washed twice, then plated in methylcellulose supplemented with SCF, IL-3, and EPO. Colonies were enumerated 7-10 days later. *p<0.05, **p<0.01 unpaired student's t-test.

injected with bromodeoxyuridine (BrdU,1mg/kg) sixteen hours prior to euthanasia to capture proliferation. While it was not technically feasible to assess the phenotype of mutant and wildtype cells separately with this experimental protocol, we were able to observe specific smoke induced changes in all bone marrow cells, irrespective of genotype. We observed a trend towards increased cell division in LKS-SLAM cells exposed to smoke as seen by the increased percentage of BrdU positive cells (Figure 7B), supporting the notion that CS induces low grade chronic inflammation. We also documented increased gamma-H2AX fluorescence in LKS-SLAM cells exposed to smoke indicating DNA damage (Figure 7C).

Smoke exposure induces inflammatory gene expression in wildtype bystander cells in mice containing Jak2^{V617F} cells

To explore how smoke exposure impacts gene expression of wildtype cells when co-existing with a small population of Jak2^{V617F} cells, we compared inflammatory gene expression in wildtype whole bone marrow cells sorted from air and smoke exposed Jak2^{V617F}/WT chimeric mice. The differentially expressed genes in transplanted mice showed overlap with biological processes related to T cell functions as observed in the untransplanted mice. IL-21 is involved in the generation of Th17 cells (36) while programmed cell death 1 (Pdcd1) plays a role in Treg differentiation (37). Cxcl10 is an inflammatory cytokine implicated in COPD in smokers (38). Interestingly, we also identified overexpression of retinoic acid early transcript 1 (Raet1) and bone marrow stromal antigen 2 (Bst2) specific to the transplanted mice (Figure 7D). Raet1 is a ligand for the cytotoxic lymphocyte activating receptor (NGKD) and plays a major role in NK cell function, and is expressed only under cellular stress conditions such as oxidative stress, DNA damage and hyperproliferation (39). Bone marrow stromal antigen 2 (Bst2) is involved in the growth and differentiation of B cells but can also be induced on other cells by the IFN pathway. Interferon gamma (IFNg) stimulation of HSCs induces surface expression of Bst2 leading to displacement of HSCs from their quiescent niche followed by cell cycle activation (40).

Jak2^{V617F} renders cells resistant to the suppressive effects of CSE in colony formation assays

To determine whether JAK2^{V617F} protects hematopoietic progenitors from the suppressive ex vivo effects of acute smoke exposure, we performed methylcellulose colony forming assays on peripheral blood mononuclear cells from MPN patients and normal controls incubated in cigarette smoke extract (CSE). There was significant difference in the clonogenic capacity of CSE exposed cells obtained from MPN patients compared to normal subjects (Figure 8A) suggesting that JAK2^{V617F} renders cells resistant to acute ex vivo smoke exposure. Parallel experiments using Jak2^{V617F} and wildtype mouse bone marrow cells yielded similar results (Figure 8B). Together, with *in vivo* exposure to smoke preserving the competitive ability of Jak2^{V617F} and *in vitro* resistance suggest that smoke exposure promotes Jak2^{V617F} selection.

Cigarette smoke promotes selective outgrowth of Tet2$^{-/-}$ cells

Next, to investigate whether smoke selects for hematopoietic cells with other common mutations seen in CHIP and hematologic malignancies, we exposed mice transplanted with a mixture of Tet2$^{-/-}$ and WT cells to air and CS for two months. Since Tet2$^{-/-}$

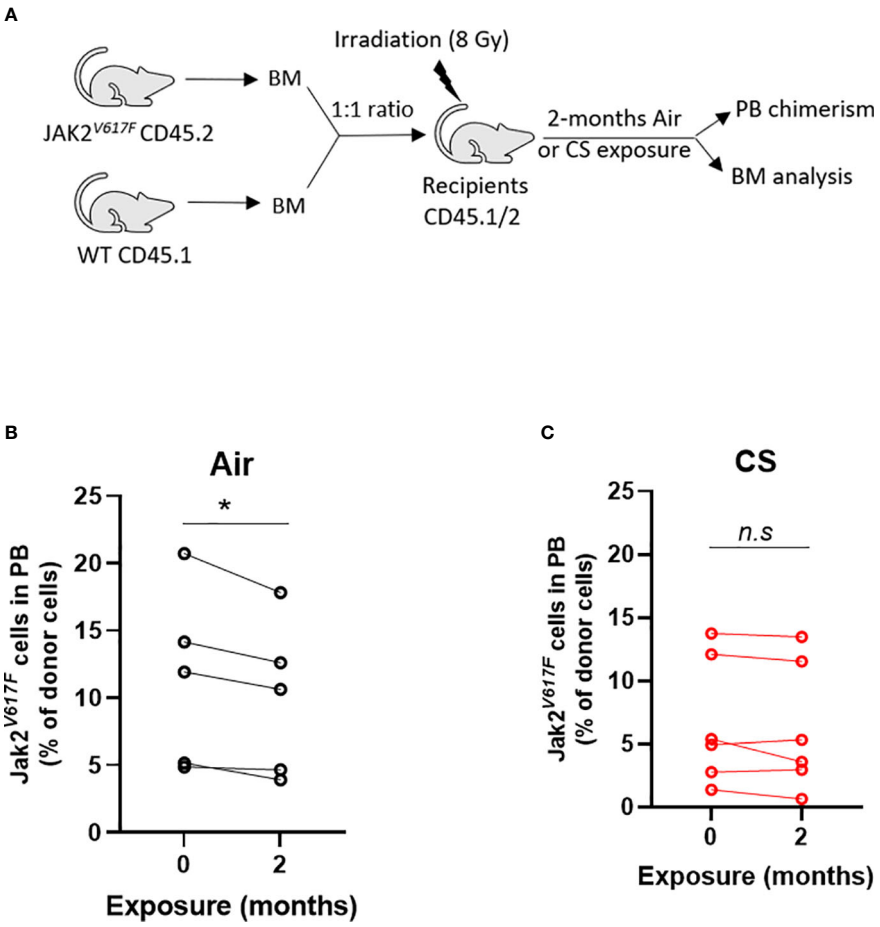

FIGURE 6
Smoke exposure maintains survival of JAK2^{V617F} mutant cells. **(A)** Experimental design. Change in circulating Jak2^{V617F} cells of **(B)** air and **(C)** cigarette smoke exposures. Data are shown as percentage chimerism before and after exposure. n=4-6 mice per group. *p<0.05, paired t-test.

cells display a significant selective advantage in competitive repopulation assays we utilized a low input ratio of Tet2$^{-/-}$ cells (1:10 ratio) to enable assessment of changes in mutant cell populations following extrinsic stressors (Figure 9A). Mice were rested for several months post-transplant and then exposed to two months of CS or air *via* nose-only inhalation. Mice in both air and smoke groups displayed a reduction in lymphocytes with time due the myeloid-lymphoid imbalance that is characteristic of Tet2-deficiency (Figure 9B). Spleen and liver weight were not affected by smoke exposure in Tet2$^{-/-}$ transplanted mice (Figures 9C, D). The contribution of Tet2$^{-/-}$ donor cells to total leukocytes (Figure 9E) as well as myeloid cells (Figure 9F) increased during the two-month exposure in the CS group but remained stable in the air group.

Discussion

In this study, we performed physiological cigarette smoke inhalation exposures in mice to demonstrate that CS can promote the selective expansion of specific CHIP mutant clones. Our results provide evidence that environmental exposure to cigarette smoke can promote the expansion of pre-existing Jak2^{V617F} and Tet2-deficient clones. The mechanisms by which CS selects for clonal selection is likely *via* induction of low-grade chronic inflammation and oxidative stress. Tet2$^{-/-}$ cells have been shown to expand under several different environmental stressors including inflammatory stimulation (8), space flight (41), atherogenic diet (5), hyperglycemia (42), and sleep fragmentation (10). Many of these external stressors do not occur independently but can co-exist due to lifestyle behaviors. It would be important to assess the synergistic or additive effect of co-stressors along with CS on the augmentation of CH. The short course of the exposure (2 months) allowed us to assess the immediate impact of active smoke exposure, however prolonged effects of a remote smoking exposure was not assessed. Specifically, we did not address whether smoke exposure may increase the likelihood of progression from Tet2$^{-/-}$ CHIP to a bona fide hematologic malignancy. Likely the impact of a remote exposure has a long-lasting effect on clonal selection supported by the increased incidence of CHIP in people with previous history of smoking.

We observed a diminished pool of phenotypically defined HSPCs (LKS cells) in both our untransplanted as well as transplanted mouse models. It is likely that this reduction in the LKS population is mediated *via* direct effects of nicotine through the α7 nicotinic acetylcholine receptor (α7nAChR). Acetylcholine has been recently described to control steady-state hematopoiesis and

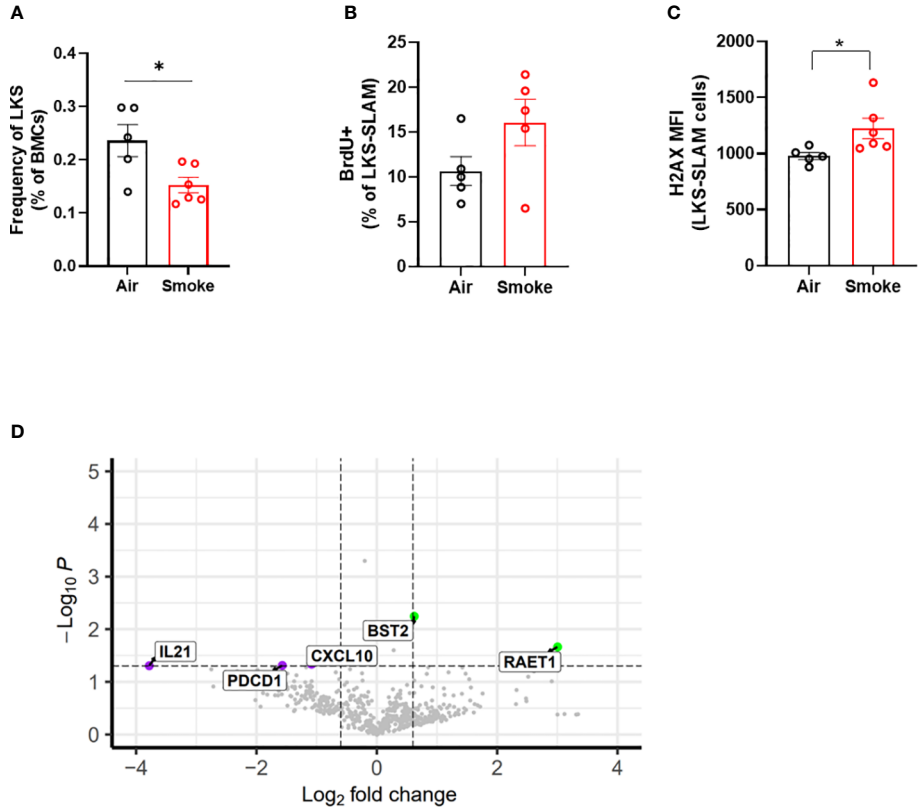

FIGURE 7
Cigarette smoke increases DNA damage in HSCs. **(A)** LKS frequency, **(B)** BrdU incorporation and **(C)** ϒH2AX MFI in LKS-SLAM cells from WT : $Jak2^{V617F}$ transplanted mice. **(D)** Volcano plot of relative gene expression in WT cells sorted from air and smoke exposed transplanted mice. Data are shown as mean ± SEM. *p<0.05, unpaired student's t-test.

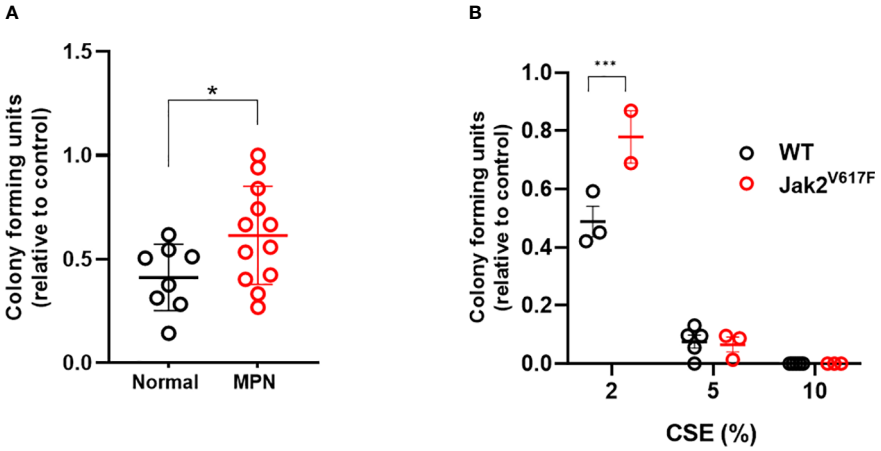

FIGURE 8
MPN cells are resistant to the suppressive effects of CSE. **(A)** Human peripheral blood mononuclear cells were incubated overnight with 0 or 2% CSE, then washed prior to plating in methylcellulose supplemented with SCF, IL-3, and EPO, and enumerated 7-10 days later. **(B)** Mouse bone marrow cells were incubated overnight in 0, 2, 5 or 10% CSE, washed and then plated in methylcellulose. Data are shown as mean ± SEM. *p<0.05, unpaired student's t-test. ***p<0.001, 2-way ANOVA with Bonferroni's correction.

preserve stem cell quiescence (43, 44) while, CHRFAM7A, a dominant negative inhibitor of α7nAChR, leads to an increased reservoir of HSCs in the bone marrow (45). Other smoke exposure studies in mice have also described reduced HSPCs as early as 3 days post exposure (46) and as long as 9-month exposures (47). We have also observed LKS suppression in mice exposed to electronic (E-) cigarette vape containing nicotine (48). HSCs from smoke exposed animals showed increased reactive oxygen species (ROS) and evidence of DNA damage, and cycling compared to air exposed mice. Inflammatory and oxidative stressors are known to drive

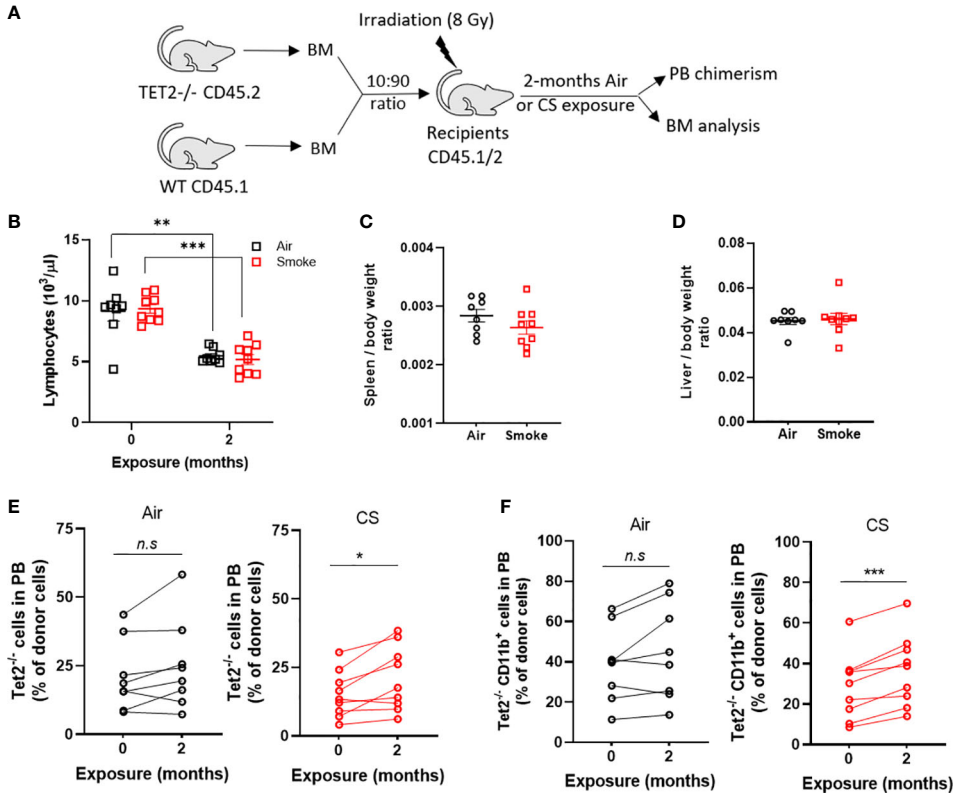

FIGURE 9
Cigarette smoke promotes the selective expansion of Tet2$^{-/-}$ cells. **(A)** Experimental design. **(B)** Deceased lymphocytes in air and smoke exposed mice over time. **(C)** Spleen and **(D)** liver body weight ratio in exposed mice. Change in peripheral blood chimerism of Tet2$^{-/-}$ **(E)** leukocytes and **(F)** myeloid specific cells. **(B–D)** Data are shown as mean ± SEM. n=8-9 mice per group. **p<0.01, ***p<0.001, unpaired student's t-test. (E, F) Data are shown as percentage chimerism before and after exposure, *p<0.05, ***p<0.001, paired t-test.

cycling and myeloid bias of HSCs implying that the different constituents of cigarette smoke can induce opposing effects on HSCs. Our findings of a decreased LKS compartment, increased ROS, DNA damage, and cycling of the HSC compartment in smoke exposed animals did not translate into an observable defect in competitive repopulation ability in primary recipients. It is possible that a short *in vivo* exposure to smoke leads to subtle functional defects in HSC that would only be revealed with expanded proliferative stress such as secondary or tertiary transplants.

We identified a smoke induced pro-inflammatory gene expression signature in bone marrow hematopoietic cells from wildtype mice. We found evidence for increased inflammation involving both the myeloid and lymphoid lineages. Serum amyloid A (Saa1) was increased which is an acute-phase protein that plays a role in the initiation and maintenance of inflammation. Saa1 leads to neutrophilia and production of inflammatory cytokines TNF-α, IL-6 and IL-17 (34). TNF-a and IL-1b also induce the production of Ccl27a, a cytokine involved in T-cell inflammation (49), which we also observed to be upregulated by smoke exposure. Tgfb3, another upregulated gene, plays a pro-inflammatory role by inducing pathogenic Th17 cells (35). We found downregulation of genes that are involved in dampening of an immune response, including IL-2ra. IL-2ra or CD25 is highly expressed on regulatory T-cells (Tregs) and its downregulation indicates immune dysfunction. Tnfrsf17 belongs to the Tnf superfamily receptors and is required for B cell development and immune responses. Tnfrsf17 was previously identified as a blood-based smoke exposure gene signature in humans (50), corroborating our observations in mice.

We also quantified changes in gene expression in wildtype cells co-existing in an environment containing Jak2^{V617F} mutant cells. Our intent was to investigate how the presence of Jak2^{V617F} may modulate or augment the impact of smoke exposure on inducing a pro-inflammatory gene signature in bystander wildtype cells. While the B cell abundance score was suppressed in both untransplanted and transplanted mice following smoke exposure, the presence of Jak2^{V617F} cells increased the cell abundance of neutrophils, indicating an inflammatory phenotype. However, we cannot rule out the possibility that these mice were lethally irradiated and thus display distinct changes compared to naïve wildtype mice. We also observed different gene expression signatures in the wildtype mice and Jak2^{V617F}/WT transplanted mice exposed to cigarette smoke. One reason for this discrepancy could be the age of the mice at the time of cell collection. Wildtype mice were 4 months old at the end of the exposure while the Jak2^{V617F}/WT transplanted mice were between 12 to 15 months old when exposures were completed and bone marrow was harvested. It is possible that the aging bone marrow environment in the older mice influences inflammatory gene expression differently upon smoke exposure. Another reason for the observed differences could be the impact of whole-body irradiation and the consequent inflammation and stress hematopoiesis associated with bone marrow transplant.

Inflammatory processes and regulatory immune mechanisms that are impacted by radiation could alter the gene expression signature induced by cigarette smoke in transplanted mice. Finally, we also speculate that the presence of $Jak2^{V617F}$ mutant cells in the bone marrow environment could result in altered inflammatory responses of the bystander wildtype cells. Studies have shown that the presence of $JAK2^{V617F}$ and MPL^{W515L} mutant cells leads to aberrant cytokine production in bystander non-malignant cells (51).

$Jak2^{V617F}$ mutant progenitors utilize DUSP1 activity to resist inflammation induced DNA-damage and tightly regulate ROS to promote their survival in an inflammatory environment (52). We previously demonstrated that *JAK2*^{V617F} cells are resistant to TNF-α (20). We speculate that the survival of $Jak2^{V617F}$ cells from transplanted mice and from MPN patients in a cigarette smoke environment display increased survival due to their ability to evade apoptotic cues.

Clonal expansion of $Tet2^{-/-}$ hematopoietic cells has been investigated under different inflammatory stressors (8, 10, 28) but the impact of cigarette smoke on $Tet2^{-/-}$ selection has not been studied. We identified a significant increase in $Tet2^{-/-}$ cells upon CS exposure demonstrating that CS exerts a selection pressure for the outgrowth of $Tet2^{-/-}$ cells. However, when determining systemic inflammation using plasma samples from CS and air-exposed mice, we did not observe differences in TNF-α or IL-6 (data not shown). It is possible that changes in immune populations and inflammatory cytokines in the local bone marrow environment exert a selection pressure. Future studies are aimed at investigating the inflammatory phenotype of individual bone marrow immune cell types. An important caveat to note is that the *Tet2-/-* mouse model is lacking TET2, whereas CHIP associated TET2 mutations in humans are usually heterozygous point mutations.

Mutant Asxl1 has been specifically associated with smoking suggesting that CS-induced inflammation promotes the outgrowth of Asxl1 mutant clones (12). Since, the type of stressor is proposed to determine the type of clone that will expand, we hypothesize that Asxl1 mutants will be strongly responsive towards CS exposure and we are currently investigating this using bone marrow transplanted mice containing mouse Asxl1 knockout cells.

In summary, we demonstrate that short-term exposure to cigarette smoke is sufficient to stimulate the expansion of Tet2-loss-of-function hematopoietic cells and maintain survival of $Jak2^{V617F}$ clones. The underlying mechanisms driving clonal expansion need to be investigated with possible mechanisms including HSC resistance to apoptosis, increased HSC differentiation and inflammatory phenotype of mature mutant cells.

Data availability statement

The data presented in the study are deposited in the ArrayExpress repository, accession number EMTAB-13133.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board of the University of California, Irvine. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Institutional Animal Care and Use Committee at the University of California, Irvine.

Author contributions

GR developed experimental plan, performed experiments, analyzed data, created figures, wrote the manuscript; JC and NM performed experiments, analyzed data, created figures and edited the manuscript; TT, AH, JM, BB, DH performed experiments; EM analyzed data; MK and AF developed experimental plan, oversaw research, analyzed data, and edited the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1210528/full#supplementary-material

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Characterization of engraftment dynamics in myelofibrosis after allogeneic hematopoietic cell transplantation including novel conditioning schemes

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Introduction: Myelofibrosis (MF) is a rare hematopoietic stem cell disorder progressing to bone marrow (BM) failure or blast phase. Allogeneic hematopoietic cell transplantation (HCT) represents a potentially curative therapy for a limited subset of patients with advanced MF, who are eligible, but engraftment in MF vs. AML is delayed which promotes complications. As determinants of engraftment in MF are incompletely characterized, we studied engraftment dynamics at our center.

Methods: A longitudinal cohort of 71 allogeneic HCT performed 2000–2019 with >50% after 2015 was evaluated.

Results: Median time to neutrophil engraftment ≥0.5x109/l was +20 days post-transplant and associated with BM fibrosis, splenomegaly and infused CD34+ cell number. Engraftment dynamics were similar in primary vs. secondary MF and were independent of MF driver mutations in JAK2, CALR and MPL. Neutrophil engraftment occurred later upon haploidentical HCT with thiotepa-busulfan-fludarabine conditioning, post-transplant cyclophosphamide and G-CSF (TBF-PTCy/G-CSF) administered to 9.9% and 15.6% of patients in 2000-2019 and after 2015, respectively. Engraftment of platelets was similarly delayed, while reconstitution of reticulocytes was not affected.

Conclusions: Since MF is a rare hematologic malignancy, this data from a large number of HCT for MF is essential to substantiate that later neutrophil and platelet engraftment in MF relates both to host and treatment-related factors. Observations from this longitudinal cohort support that novel conditioning schemes administered also to rare entities such as MF, require detailed evaluation in larger, multi-center cohorts to assess also indicators of long-term graft function and overall outcome in patients with this infrequent hematopoietic neoplasm undergoing allogeneic transplantation.

KEYWORDS

myelofibrosis, myeloproliferative neoplasms, allogeneic hematopoietic cell transplantation, engraftment, reconstitution

Introduction

Allogeneic hematopoietic cell transplantation (HCT) is a potentially curative treatment for hematopoietic stem cell disorders including rare entities such as intermediate to high-risk myelofibrosis (MF). However, it is associated with significant morbidity and mortality, particularly early non-relapse mortality. Specifically, median time to hematopoietic engraftment appears to be later in MF (1–4) compared to acute myeloid leukemia (5–10), presumably due to pooling of infused stem cells by splenomegaly and/or an altered marrow microenvironment (11, 12). Prolonged cytopenia increases the patients' risk for complications, particularly infections and bleeding events, as well as transfusion dependency, which has been associated with increased risk of relapse and higher mortality (13).

Tolerability of allogeneic HCT has substantially improved over recent years. Specifically, the advent of reduced-intensity conditioning (RIC) regimens has decreased peri-transplant complications and has made allogeneic HCT amenable for patients with higher age and/or comorbidities. This has increased the availability of allogeneic HCT for these populations, including the patients with intermediate to high risk MF, who are in important need of curative treatment options (14, 15). In addition, novel options of graft-vs-host-disease (GvHD) prophylaxis, such as e.g. with post-transplant cyclophosphamide (PTCy), have led to improved tolerability of haploidentical HCT, thus expanding the pool of potential HCT donors. While the number of haploidentical HCT in MF is still limited, emerging retrospective studies have assessed haploidentical HCT as overall safe (16–19). However, an influence on engraftment has not been thoroughly characterized in the setting of MF. Here, we sought to evaluate determinants of engraftment dynamics in MF upon allogeneic HCT in a longitudinal cohort of patients at our center, which also included first patients with preparatory regimens emerging more recently for MF.

Methods

Patient selection

We conducted a single-center retrospective study of engraftment dynamics in patients transplanted for primary or secondary MF. All patients undergoing allogeneic HCT at University Hospital Basel between 09/2000 and 09/2019 were screened. One single patient transplanted for MF with cord-blood as a stem cell source was excluded. Of 60 patients, 11 underwent a second HCT within the study period. Data was extracted from electronic patient files. The study was approved by the ethical committee Nordwest- und Zentralschweiz (EKNZ, no.2016-01930) and informed consent was obtained from all patients.

Definitions

We evaluated determinants of time to engraftment for neutrophil, platelet and red cell lineages. Neutrophil engraftment was defined as first of 3 consecutive days with $\geq 0.5 \times 10^9$/l neutrophils in peripheral blood, platelet engraftment as first of 3 consecutive days with $\geq 20 \times 10^9$/l platelets in the absence of transfusions within the 7 preceding days, and red cell engraftment as first day with $\geq 30 \times 10^9$/l reticulocytes. Conditioning was classified as reduced intensity (RIC) or myeloablative (MAC) conditioning according to Bacigalupo (20). Since thiotepa-busulfan-fludarabin (TBF) conditioning (thiotepa 10mg/kg, busulfan 6.4 mg/kg and fludarabine 150 mg/m^2) was not included in this classification and consistently associated with haploidentical HCT at our center, it was evaluated separately. TBF was followed by haploidentical HCT, subsequent PTCy 50 mg/kg on days +3/+4 for GvHD prophylaxis and granulocyte colony-stimulating factor (G-CSF) support at 0.5 Mio units/kg from day+5 (TBF-PTCy/G-CSF) in this cohort. G-CSF support at 0.5 Mio units/kg/day was also applied to 5 patients with RIC and 1 patient with MAC regimens at variable time-points in the post-transplant course at the discretion of the treating physicians to promote reconstitution. Donor cell chimerism was assessed at 1, 3 and 6 months after HCT in peripheral blood and/or bone marrow. Poor graft function was evaluated as previously described (12, 21–24) with cytopenia in at least two hematopoietic lineages incl. neutrophils ≤ 1.5 G/l, platelets ≤ 30 G/l and/or Hb ≤ 85 g/l lasting for > 2 consecutive weeks following documented engraftment beyond d+14 in the presence of full donor chimerism and the absence of severe acute or chronic GvHD, relapse, drug-related or CMV reactivation-related myelosuppression. Patients' disease risk category was determined according to respective prognostic scoring systems, primarily DIPSS plus (25), DIPSS (26), IPSS (27), and MYSEC (28) scores, as well as Cervantes (29) and MIPSS70 plus (30) scores in one patient each. Infections in the post-transplant period as well as thrombocyte concentrate (TC) and erythrocyte concentrate (EC) transfusion requirements were assessed in the first six months after allogeneic HCT.

Statistical analysis

Descriptive statistics were used for patient and transplantation characteristics. Time to engraftment, time to full donor cell chimerism and overall survival were estimated by Kaplan-Meier method and log-rank test used to assess statistical significance between groups. Estimates are given as median with 95% confidence interval (CI). Patients were censored at death, secondary graft failure or second transplantation. Univariate Cox regression was used to evaluate spleen size as a continuous variable. Key disease characteristics such as splenomegaly and BM fibrosis and factors significant in univariate analysis were implemented in multivariate forward-conditional Cox regression. Results are given as hazard ratio (HR) with 95% CI. Cumulative incidence function was used for the estimation of non-relapse mortality (NRM) as well as for acute and chronic GvHD with relapse calculated as the competing event. P-values of ≤0.05 were considered statistically significant. Data were analyzed and figures generated with SPSS 28.0.0 (IBM, Armonk, NY, USA), GraphPad Prism 9.2.0 (GraphPad Inc., San Diego, CA, USA) and NCSS 2020 package (NCSS, LLC, Kaysville, Utah, USA).

Results

Patient and treatment characteristics

We retrospectively evaluated 71 allogeneic HCTs conducted in 60 MF patients in 2000–2019 with >50% performed after 2015 (Table 1, Supplementary Table 1). Median age at transplantation was 60 (31–72) years, increasing over time from 55.5 (31–61) to 58 (40–70), and 61 (34–72) years in the periods 2000–2009, 2010–2014, and 2015–2019, respectively, with a majority of patients being males. Median follow-up of survivors was 61 (24–198) months. Most patients (62.0%) suffered from primary MF (PMF), while 22.5% and 12.7% were transplanted for secondary MF evolving from polycythemia vera (PPV-MF) or essential thrombocythemia (PET-MF) and one patient each for unclassifiable myeloproliferative neoplasm (MPN-U) and MDS/MPN overlap syndrome (Figure 1A). *JAK2* V617F, calreticulin (*CALR*), and thrombopoietin receptor (*MPL*) driver mutations were detected in 66.2%, 19.7%, and 2.8% of patients, respectively, while 4.2% were triple negative for either mutation (Figure 1B). A majority of patients had intermediate-2 or high-risk disease (46.7% and 30%, respectively) as assessed by prognostic risk scoring, similarly in patients with PMF and secondary MF including PPV-/PET-MF (Supplementary Figure 1). BM fibrosis was advanced in most patients with 56.3% showing grade 3 fibrosis. Splenomegaly was prevalent with a median spleen size of 19 (11–32) cm at transplantation as assessed by sonography. Transplantations after previous splenectomy were uncommon with only 3 transplantations in 2 patients occurring in the setting of absent spleen. Ruxolitinib therapy before HCT was administered in a majority of 38/71 cases (53.5%) of the overall cohort with 30 patients receiving ruxolitinib up to transplantation and 8 patients with ruxolitinib discontinuation >1 month before HCT. Median time on ruxolitinib therapy before HCT was 10 months (range 1-78 months) and median spleen size at HCT did not significantly differ between ruxolitinib-treated and untreated patients (p=0.67). In our cohort, 7 patients received ruxolitinib after allogeneic HCT, mostly (5/7) after engraftment as a treatment of acute GvHD, while two patients with extensive splenomegaly were on ruxolitinib through the peri-transplant period. Myeloablative (MAC) or reduced-intensity (RIC) conditioning followed by cyclosporine and methotrexate or mycophenolate-mofetil with or without anti-thymocyte globuline (ATG) for GvHD prophylaxis was applied in 32.4% and 56.3% of allogeneic HCT, respectively. The novel preparatory regimen of haploidentical HCT with PTCy as GvHD prophylaxis, which beyond AML is also increasingly used in specific, rare entities such as in MF patients at our center, accounted for 15.6% of patients from 2015, but just 9.9% from 2000 (Figure 1C). In these patients, haploidentical HCT followed conditioning with thiotepa-busulfan-fludarabine, while subsequent PTCy on day +3/+4 was given for GvHD prophylaxis and granulocyte colony-stimulating factor (G-CSF) from day+5 for regeneration support (TBF-PTCy/G-CSF). Median number of infused CD34+ cells was 7.2 (1.94–18.8) x10^6/kg body weight. Peripheral blood stem cells (PBSC) represented the prevalent CD34+ cell source (87.3%), while a minority of non-haploidentical (6.3%) and a majority of haploidentical HCTs (57%) used BM as source. Second allogeneic HCT was performed in 11 patients to manage graft failure, relapse or persistent disease.

Engraftment in first vs. second transplantations

Given that hematopoietic engraftment, which is known to be delayed in MF vs. AML (4, 10), impacts on the susceptibility for infectious and bleeding complications, we evaluated factors influencing engraftment in the neutrophil, megakaryocytic and erythroid lineages. Neutrophil engraftment occurred at a median of 20 (11–36) days post-transplant, while one patient experienced graft failure and one deceased before engraftment. Time to neutrophil engraftment was similar over the different time periods 2000-2009, 2010-2014 and 2015-2019 (p=0.374, Figure 2A) and was analogous in first and second HCTs (p=0.72, Figures 3A, B). Median time to platelet engraftment was 26 (0–121) days, including 6 patients maintaining platelets ≥20x10^9/l throughout the peri-transplantation period. Platelet engraftment was faster in 2000–2009 vs. later periods (p<0.001), but remained stable 2010–2014 and 2015–2019 (p=0.931, Figure 2B), and was analogous in first and second transplantations (p=0.535, Figures 3C, D). Reticulocyte engraftment occurred at a median of 21 (12–236) days with no relevant difference among the three time periods (p=0.687, Figure 2C) or between first and second transplantations (p=0.64, Figures 3E, F). Donor cell chimerism 1, 3 and 6 months after HCT was similar in 2010-2014 and 2015-2019, while lower 2000-2009 at 1-3 months, and was analogous in first and second HCTs (Supplementary Figures 2, 3). Given that hematopoietic reconstitution times in all three lineages were analogous between first and second transplantations, as well as the limited number of second transplantations, first and second transplants were pooled for further analyses.

Engraftment relates to MF- and transplant-related factors

To explore potential determinants of engraftment, we evaluated MF disease characteristics including primary vs secondary MF, driver mutations in JAK2, CALR and MPL, prognostic risk group, splenomegaly and BM fibrosis as well as ruxolitinib therapy before HCT. Transplant-related factors with potential impact on engraftment dynamics were also assessed including the type of conditioning regimen, GvHD prophylaxis, type of donor, CD34+ cell number and source, administration of G-CSF support, and donor-recipient relation for blood group and CMV status. PMF vs secondary MF (median 20 vs 19 days; p=0.65), driver mutation status (p=0.57) as well as prognostic risk group (p=0.21) did not influence kinetics of neutrophil regeneration. In line with the notion that alterations of the microenvironment could interfere with engraftment (11), we observed that BM fibrosis significantly influenced neutrophil engraftment (median 15 vs 20 days, grade 1 vs 3, p=0.017; median 15 vs 19 days, grade 1 vs 2, p=0.054;

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TABLE 1 Baseline characteristics of patients' allogeneic hematopoietic cell transplantations for myelofibrosis.

Patient and transplantation characteristics	Number of transplantations n (%) unless otherwise specified
Time period	
2000-2009	6 (8.5)
2010-2014	20 (28.2)
2015-2019	45 (63.4)
Sex	
male	43 (60.6)
female	28 (39.4)
Age at transplantation	
<55 years	20 (28.2)
55 - 64 years	32 (45.1)
>64 years	19 (26.8)
median age (range)	60.0 (31 - 72) years
Diagnosis	
PMF	44 (62.0)
Secondary MF	25 (35.2)
PET-MF	*9 (12.7)*
PPV-MF	*16 (22.5)*
MPN/MDS overlap syndrome	1 (1.4)
MPN-U	1 (1.4)
Driver mutation	
JAK2 V617F	47 (66.2)
CALR	14 (19.7)
MPL	2 (2.8)
triple negative	3 (4.2)
n.a.	5 (7.0)
Fibrosis grade	
grade 1	2 (2.8)
grade 2	15 (21.1)
grade 3	40 (56.3)
n.a.	14 (19.7)
Spleen	
splenomegaly (≥13 cm)	65 (91.5)
no splenomegaly	3 (4.2)
prior splenectomy	3 (4.2)
median size (range)	19 (11 - 32) cm
Ruxolitinib before HCT	
no	33 (46.5)
yes	38 (53.5)

(Continued)

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TABLE 1 Continued

Patient and transplantation characteristics	Number of transplantations n (%) unless otherwise specified
median time (range)	10 (1 - 78) months
Donor relation	
matched related	26 (36.6)
haploidentical	7 (9.9)
matched unrelated	35 (49.3)
mismatched unrelated	2 (2.8)
n.a.	1 (1.4)
Stem cell source	
PBSC	62 (87.3)
BM	8 (11.3)
n.a.	1 (1.4)
Stem cell dose	
<6 x10^6/kg	30 (42.3)
6 - 8 x10^6/kg	15 (21.1)
>8 x10^6/kg	24 (33.8)
n.a.	2 (2.8)
CD34+ cells (range)	7.2 (1.94 - 18.8) x10^6/kg
Conditioning regimen	
RIC (FluBu, FluTBI, FluMel)	40 (56.3)
MAC (CyBu, CyTBI)	23 (32.4)
TBF	7 (9.9)
n.a.	1 (1.4)
GvHD prophylaxis	
CyA MTX +/- ATG	59 (83.1)
CyA MMF +/- PTCy	10 (14.1)
CyA	1 (1.4)
n.a.	1 (1.4)
ATG	
no	25 (35.2)
yes	45 (63.4)
n.a.	1 (1.4)
PTCy	
no	63 (88.7)
yes	7 (9.9)
n.a.	1 (1.4)
G-CSF	
no	57 (80.3)
yes	13 (18.3)

(Continued)

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TABLE 1 Continued

Patient and transplantation characteristics	Number of transplantations n (%) unless otherwise specified
n.a.	1 (1.4)
CMV risk	
D-/R-	27 (38.0)
D+/R+	20 (28.2)
D-/R+	14 (19.7)
D+/R-	9 (12.7)
n.a.	1 (1.4)
Blood-group barrier	
no	48 (67.6)
minor	4 (5.6)
major	13 (18.3)
bidirectional	5 (7.0)
n.a.	1 (1.4)

Characteristics of patients undergoing a total of 71 allogeneic hematopoietic cell transplantations in 2000-2019 are indicated in absolute numbers with relative proportions in brackets. Continuous variables are indicated as median and range in brackets. n.a., not available; PMF, primary myelofibrosis; MF, myelofibrosis; PPV-/PET-MF, post-polycythemia vera/post-essential thrombo-cythemia myelofibrosis; MPN-U, unclassifiable myeloproliferative neoplasm; PBSC, peripheral blood stem cells; BM, bone marrow; RIC, reduced-intensity conditioning; MAC, myeloablative conditioning; TBF, thiotepa-busulfan-fludarabin conditioning; GvHD, graft versus host disease; CyA, cyclosporine A; MTX, methotrexate; MMF, mycophenolate mofetil; ATG, anti-thymocyte globulin; PTCy, post-transplantation cyclophosphamide; G-CSF, granulocyte colony-stimulating factor; CMV, cytomegalovirus; D/R, donor/recipient

Supplementary Table 2). In addition, splenomegaly impacted on engraftment of the neutrophil lineage as reflected by reconstitution times significantly relating to spleen size by univariate Cox-regression analysis (p=0.032, Supplementary Table 2). The shortened engraftment times observed in the setting of splenectomy should be interpreted with caution given that only a small minority of 3 transplantations in 2 MF patients of the entire cohort were performed after a splenectomy based on the rarity of this intervention. Ruxolitinib therapy before HCT did not significantly impact on neutrophil engraftment in our cohort (p=0.27). CD34+ cell dose significantly influenced neutrophil engraftment with a median of 18 days if >8x10^6 cells/kg were infused versus 20 days with low cell numbers <6x10^6 cells/kg (p=0.037) consistent with effects observed in similar cohorts (31, 32). We explored a potential impact of conditioning schemes used as preparatory regimens for allogeneic HCT in MF. We did not observe a difference in time to neutrophil engraftment for RIC vs MAC protocols (p=0.819). However, a potential effect of TBF

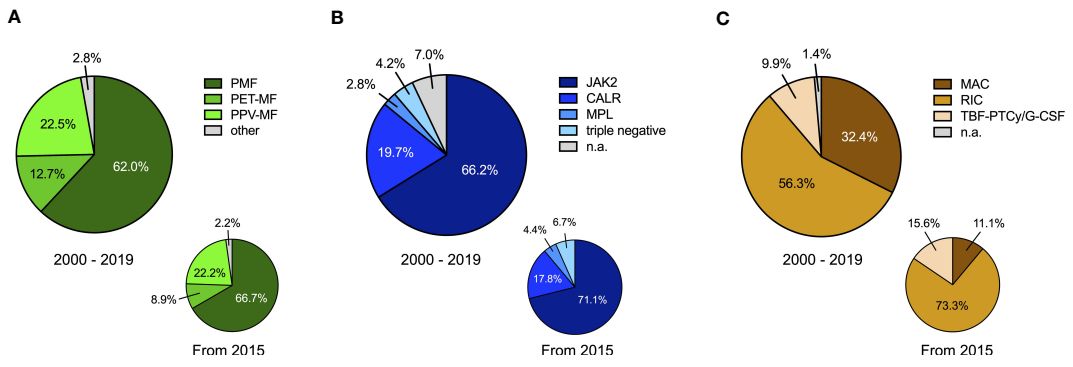

FIGURE 1
Characteristics of myelofibrosis patients undergoing allogeneic hematopoietic cell transplantation (HCT). **(A)** Subtype of myelofibrosis is indicated as primary myelofibrosis (PMF), post-essential thrombocythemia myelofibrosis (PET-MF), post-polycythemia vera myelofibrosis (PPV-MF) or other (including one patient with unclassifiable myeloproliferative neoplasm and one patient with MDS/MPN overlap syndrome) for the overall time-period 2000-2019 (main chart) and from 2015 onwards (small chart). **(B)** The frequencies of genetic driver mutations in JAK2, calreticulin (CALR) or the thrombopoietin receptor (MPL) as well as of triple negative cases non-mutated for JAK2, CALR and MPL are indicated for the overall time-period 2000-2019 (main chart) and from 2015 onwards (small chart). n.a. not available. **(C)** Proportions of the administered conditioning therapies for HCT in MF patients is indicated for the overall time-period 2000-2019 (main chart) and from 2015 onwards (small chart). MAC myeloablative conditioning, RIC reduced-intensity conditioning, TBF-PTCY thiotepa-busulfan-fludarabin conditioning followed by post-transplantation cyclophosphamide.

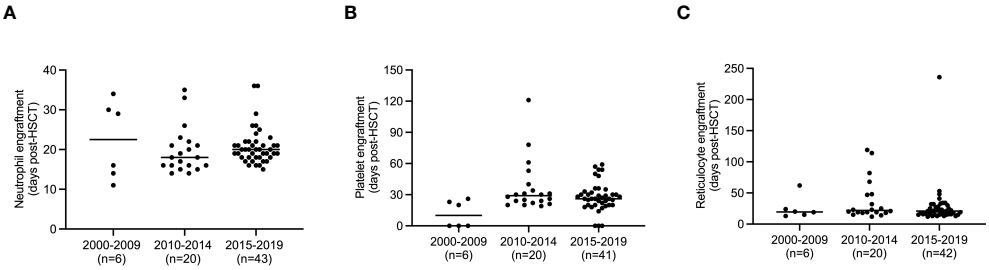

FIGURE 2
Time to engraftment in hematopoietic lineages in different time periods. **(A)** Time to engraftment of neutrophils after hematopoietic cell transplantation (HCT) for myelofibrosis (MF) defined as the first of 3 consecutive days with ≥0.5x10^9/l neutrophils in peripheral blood is indicated for early (2000-2009), intermediate (2010-2014) and recent (2015-2019) time periods. **(B)** Time to engraftment of platelets after HCT for MF defined as the first of 3 consecutive days with ≥20x10^9/l platelets in the absence of transfusions within the 7 preceding days is indicated for early (2000-2009), intermediate (2010-2014) and recent (2015-2019) time periods. **(C)** Time to red cell engraftment after HCT for MF defined as the first day with ≥30x10^9/l reticulocytes is indicated for early (2000-2009), intermediate (2010-2014) and recent (2015-2019) time periods. The number of performed HCT in each time period is shown in brackets, patients who failed to engraft the respective lineage are not displayed.

conditioning, which was used exclusively for haploidentical HCT in our cohort, was detectable with TBF associated with later neutrophil engraftment (median 21 vs 19 days, p=0.017; Figure 4A). This finding should be cautiously noted given the low absolute number of TBF-conditioned haploidentical HCT in this longitudinal cohort, and should promote evaluations in larger, multicentric cohorts of MF undergoing HCT. While ATG as GvHD prophylaxis related to shortened engraftment (p=0.028), PTCy which was exclusively used for TBF-conditioned haploidentical HCT in our cohort, associated with longer neutrophil reconstitution times. This was similarly reflected in HCT from haploidentical as compared to HLA matched donors (21 vs 20 days, p=0.019), while matched related and unrelated donors behaved analogously (p=0.176). BM as CD34 + cell source, which was used in the majority of haploidentical TBF-PTCy HCTs, and post-transplant G-CSF consistently used in TBF-PTCy HCTs in our cohort, analogously associated with later engraftment of neutrophils (median 26 vs 19 days, p=0.007 for BM vs. PBSC; median 21 vs 19 days, p=0.004 for post-transplant G-CSF; Figures 4B, C). Given that TBF conditioned haploidentical transplants overlap with the use of BM as a stem cell source and administration of G-CSF support, these findings may reflect an overall different behavior of engraftment dynamics with this procedure.

Similar to the findings for neutrophil engraftment, reconstitution of platelets did not reveal differential effects in primary vs secondary MF, with JAK2, CALR and MPL driver mutations or different prognostic risk group, but splenomegaly significantly prolonged platelet engraftment (p=0.002, HR: 0.930, Supplementary Table 2). Consistent with an impact of splenomegaly, the few HCT after previous splenectomy associated with earlier platelet engraftment (p<0.001). Similar to the influence of BM fibrosis on neutrophil regeneration, fibrosis grade also showed a trend for later platelet engraftment (p=0.088). Ruxolitinib therapy before HCT did not show a significant effect (p=0.07). As for transplant-related factors, RIC and MAC regimens led to comparable engraftment dynamics (p=0.579), while later platelet engraftment was observed upon TBF conditioning with PTCy as GvHD prophylaxis (p=0.015; Figure 5A) and administration of post-transplant G-CSF (p=0.001), while BM vs PBSC showed a similar trend (p=0.148; Figures 5B, C). Higher CD34 + cell doses significantly correlated with faster engraftment (p=0.021, Supplementary Table 2) as described (11), while blood-group barrier and CMV serology status did not influence platelet reconstitution. Overall, red cell engraftment as reflected by reticulocytes ≥30x10^9/l was less dependent on the evaluated determinants as compared to neutrophil and platelet lineages including prognostic risk groups and pre-transplant ruxolitinib treatment (p=0.97). However, red cell engraftment still related to splenomegaly, which prolonged engraftment time (p=0.008, HR: 0.942; Supplementary Table 2). In line with the notion that not only regeneration but also consumption by e.g. hemolysis might affect reconstitution of the red cell mass, we observed later reticulocyte engraftment upon major (p=0.032), minor (p=0.013) and bidirectional (p=0.047) blood group barriers.

Development of full donor cell chimerism established at a median of 35 days after HCT (range 18-156 days), did not show significant differences relating to CD34+ cell source and number, type of conditioning regimen, use of ATG or PTCY as well as G-CSF, donor/recipient constellation or pre-transplant ruxolitinib therapy (Figure 6). Similarly, disease-specific factors including MF subtypes PMF and PET-/PPV-MF, driver mutation status or splenomegaly did not significantly impact on the dynamics of full chimerism development, whereas higher fibrosis grade associated with increased time to full chimerism (median 58 days, 33 days and 18 days for grade 3, grade 2 and grade 1 fibrosis, respectively, p<0.001, Figure 6).

Multivariate analyses of novel conditioning schemes

To consolidate these findings, we performed multivariate analyses, including age at transplantation, gender, MF disease characteristics, and factors with significant effects in univariate analysis. Upon correction for splenectomy, fibrosis grade, conditioning, use of ATG, G-CSF, donor type, CD34+ cell dose and source, age and gender, an impact of splenectomy on neutrophil engraftment was confirmed, although only 3

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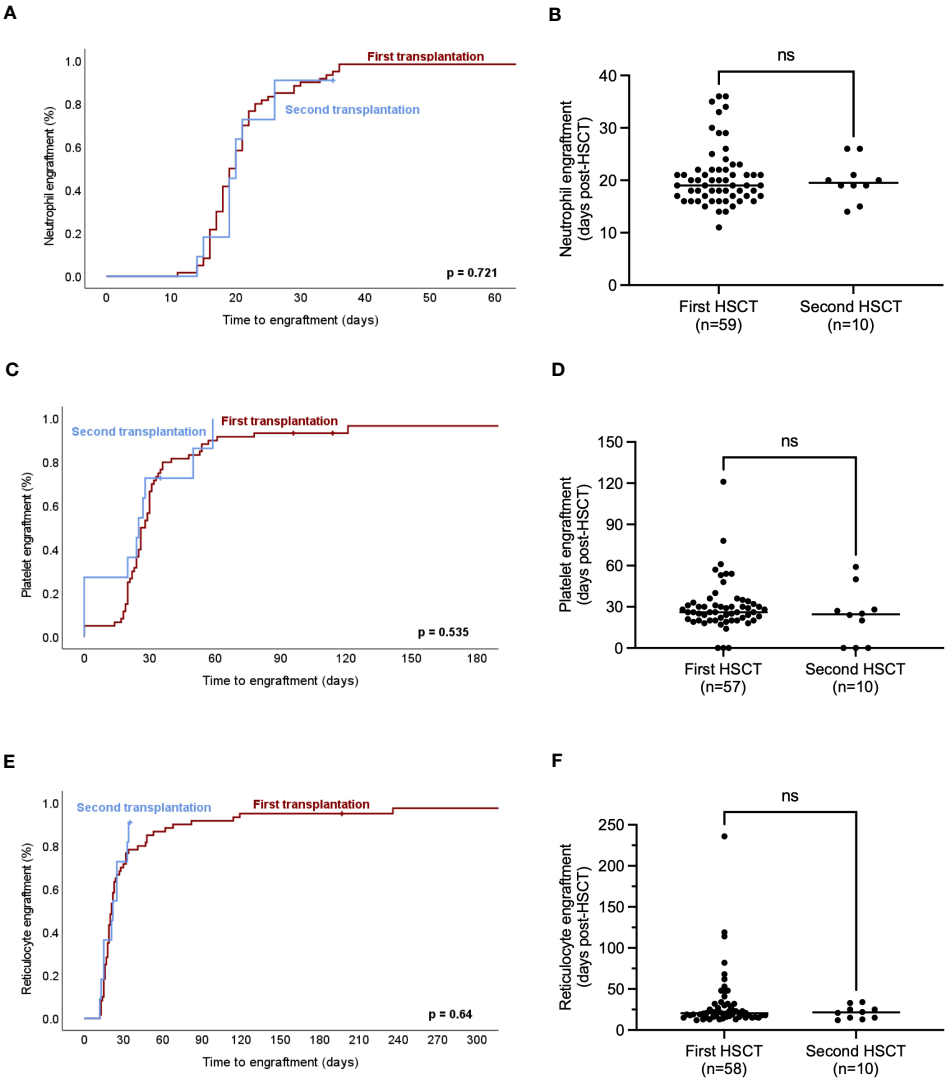

FIGURE 3
Engraftment dynamics in hematopoietic lineages after first and second hematopoietic cell transplantation (HCT). **(A)** Proportion of neutrophil engraftment over time is shown for first as compared to second HCT for MF. **(B)** Time to engraftment of neutrophils in peripheral blood is similar in first and second HCT in MF. **(C)** Proportion of platelet engraftment over time is shown for first as compared to second HCT for MF. **(D)** Time to engraftment of platelets in peripheral blood is similar in first and second HCT in MF. **(E)** Proportion of red cell engraftment over time is shown for first as compared to second HCT for MF. **(F)** Time to red cell engraftment of platelets in peripheral blood is similar in first and second HCT in MF. Median and individual data points are indicated and compared by student's t-test with p-values ≤0.05 considered statistically significant **(B, D, F)**. Kaplan-Meier estimates were assessed by log-rank test **(A, C, E)**. ns, non-significant.

transplantations in 2 patients were performed after previous splenectomy in our cohort (p<0.001, HR: 107.67). Given the rarity of splenectomy in our cohort (4.2%), conflicting data on its effects on outcome (33, 34), and decreasing relevance of this procedure, we omitted splenectomy as a factor from further multivariate analyses. Consequently, post-transplant G-CSF support (p=0.02, HR: 0.415) and BM as CD34+ cell source (p=0.039, HR: 0.421) were confirmed to associate with later neutrophil engraftment (Supplementary Tables 3, 4). However, this finding should be cautiously interpreted considering the substantial overlap of these factors with TBF-conditioned haploidentical HCT in our cohort and the limited number of patients, although substantial for a single center longitudinal cohort of higher risk, transplant-eligible MF.

Similarly, multivariate analysis for determinants of platelet engraftment confirmed the effects of post-transplant G-CSF administration (p<0.001, HR: 0.273), grade 3 vs grade 1 BM fibrosis (p=0.013, HR: 0.145) and splenectomy (p=0.011, HR: 7.044) as seen in univariate analysis. When splenectomy, which was only performed in 2 patients, was omitted from the model, post-transplant G-CSF (p=0.002, HR: 0.301) and grade 3 vs grade 1 BM fibrosis (p=0.026, HR: 0.179) maintained significant effects along with splenomegaly (p=0.021, HR: 0.94, Supplementary Tables 3, 4) in line with similar cohorts (11, 32). For erythroid reconstitution, multivariate analyses confirmed the influence of a major blood group barrier (p=0.019, HR: 0.449) and splenomegaly (p=0.012, HR: 0.942) on reticulocyte engraftment dynamics. Factors associated with haploidentical TBF-conditioned HCT were not

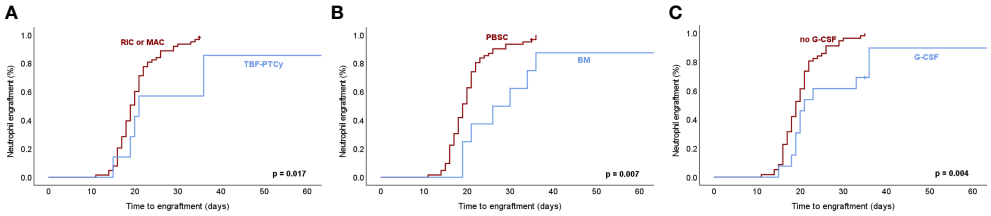

FIGURE 4
Neutrophil engraftment dynamics in association with transplant-related factors in myelofibrosis. **(A)** Neutrophil engraftment after hematopoietic cell transplantation (HCT) for myelofibrosis (MF) defined as the first of 3 consecutive days with ≥0.5x10^9/l neutrophils in peripheral blood associates significantly with thiotepa-busulfan-fludarabine conditioning followed by post-transplant cyclophosphamide (TBF-PTCy) as a preparatory regimen of HCT as compared to reduced-intensity (RIC) or myeloablative (MAC) conditioning therapies. **(B)** Bone marrow (BM) as CD34+ cell source used in a majority of TBF-PTCy haploidentical HCT associates with later neutrophil engraftment. **(C)** Post-transplant G-CSF, which is administered in all TBF-PTCy haploidentical HCT as well as in a minority of HLA-matched HCT associated with later neutrophil engraftment. Kaplan-Meier estimates were assessed by log-rank test with p-values ≤0.05 considered statistically significant. PBSC, peripheral blood stem cells; G-CSF, granulocyte-colony stimulating factor.

evident, confirming the finding from univariate analyses that red cell reconstitution is not as much impacted by novel preparatory regimens (Supplementary Tables 3, 4).

Longer-term outcomes including poor graft function

To explore potential long-term effects of delayed engraftment, we assessed overall survival (OS) in MF after first allogeneic HCT, which was 6.7 years (2460 days) in our cohort in line with previous studies (35–38). Patients with neutrophil engraftment later than the median showed a trend towards reduced OS without statistical significance (median OS 2.7 years, 95% CI 0-5.8 years, p=0.099), while platelet or red cell engraftment after the median did not impact on OS in our cohort. A potential impact of delayed engraftment on OS after second allogeneic HCT was not assessed given the limited number of patients. The cumulative incidence of non-relapse mortality (NRM) in our cohort at 5 years after first HCT was 19.3% and not significantly different in patients with delayed engraftment when assessed as engraftment time greater than the median of the cohort (95% CI 11.3-33.2%, p=0.84, Supplementary Figure 4). Cumulative incidence of relapse in the overall cohort was 26.9% at 5 years (95% CI 17.7-40.9%). Acute graft versus host disease (GvHD), as assessed for grades 2-4, occurred at a cumulative incidence of 33.1% at 180 days after HCT without a significant difference upon delayed engraftment (95% CI 23.7-46.2%, p=0.48, Supplementary Figure 5), while chronic GvHD was documented in 46.7% (95% CI: 35.7-61.3%) at 5 years after HCT and was similar in patients with delayed neutrophil engraftment (p=0.28, Supplementary Figure 6). Infections within 6 months post-transplant were assessed from 2010 onwards, as only a limited number of transplantations (n=6) were performed 2000-2009. While a majority of patients suffered from post-transplant infections in 2010-2014 and 2015-2019 (80.0% and 64.4%, respectively), patients with delayed neutrophil engraftment when assessed as engraftment time greater than the median of the cohort, showed similar rates of post-transplant infections as compared to patients with engraftment time shorter than the median in both time periods (75.0 vs. 83.3% and 56.5% vs. 70.0%, respectively). Within 6 months after HCT, a median of one infection per patient occurred with a range of 0-5 post-transplant infections per patient similarly in patients with delayed engraftment (Supplementary Figure 7).

Notably, delayed engraftment impacted on transfusion requirements after allogeneic HCT. We observed that

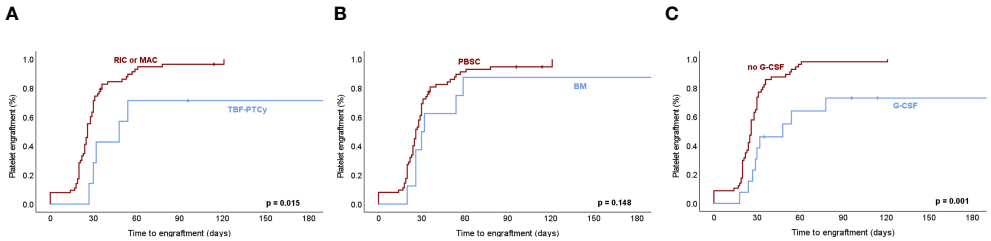

FIGURE 5
Platelet engraftment dynamics in association with transplant-related factors in myelofibrosis. **(A)** Platelet engraftment after hematopoietic cell transplantation (HCT) for myelofibrosis (MF) defined as the first of 3 consecutive days with ≥20x10^9/l platelets in the absence of transfusions within the 7 preceding days associates significantly with thiotepa-busulfan-fludarabine conditioning followed by post-transplant cyclophosphamide (TBF-PTCy) as a preparatory regimen of HCT as compared to reduced-intensity (RIC) or myeloablative (MAC) conditioning therapies. **(B)** Bone marrow (BM) as CD34+ cell source used in a majority of TBF-PTCy haploidentical HCT associates with later platelet engraftment. **(C)** Post-transplant G-CSF, which is administered in all TBF-PTCy haploidentical HCT as well as in a minority of HLA-matched HCT associated with later platelet engraftment. Kaplan-Meier estimates were assessed by log-rank test with p-values ≤0.05 considered statistically significant. PBSC, peripheral blood stem cells; G-CSF, granulocyte-colony stimulating factor.

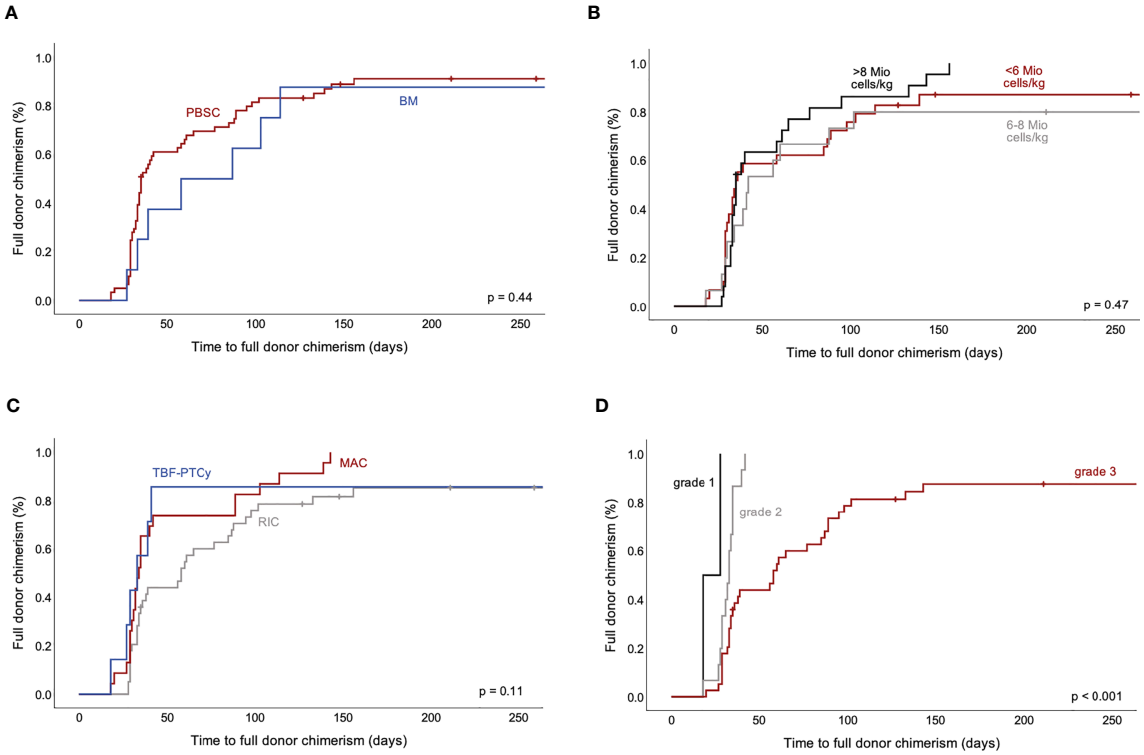

FIGURE 6
Time to full donor cell chimerism after allogeneic HCT for MF. Impact of selected factors on development of full donor cell chimerism are displayed. Time to 100% donor cell chimerism in peripheral blood did not show significant differences for CD34+ cell source **(A)** and cell number **(B)** nor for type of conditioning regimen **(C)**. More extensive bone marrow fibrosis associated with later establishment of full donor cell chimerism **(D)**. HCT, hematopoietic cell transplantation; MF, myelofibrosis.

thrombocyte concentrate (TC) transfusion dependence at day 100 after HCT was > 5-fold more prevalent upon delayed platelet engraftment when assessed as engraftment time longer than the median of the cohort (20% vs. 3% of patients). While delayed neutrophil engraftment also related to somewhat higher TC transfusion dependence at day 100 (15.2% vs. 9.7%), delayed reticulocyte engraftment did not have an impact. For erythrocyte concentrate (EC) transfusion dependence at day 100 after HCT, we observed that it was not affected by delayed neutrophil engraftment (35.5% vs. 36.4%), but increased upon prolonged reticulocyte and platelet engraftment (50.0% vs. 21.2% EC transfusion dependent upon delayed red cell engraftment and 42.9% vs. 24.2% upon delayed platelet engraftment, respectively). Given these findings, we specifically assessed the total number of TC and EC transfusions until engraftment and found that the number of required TC transfusions was significantly higher upon delayed platelet engraftment (median 29 vs. 6 TC, $p < 0.0001$, Supplementary Figures 8A-C). The number of required EC transfusions was significantly increased in settings of delayed reticulocyte (median 18 vs. 8 EC, $p < 0.0001$) and also delayed platelet engraftment (median 16.5 vs. 8 EC, $p = 0.0006$, Supplementary Figures 8D-F). We confirmed these findings over a more extended time period of 6 months after HCT suggesting a relevance of engraftment dynamics for transfusion requirements for such a longer period. We found that the number of TC transfusions within 6 months after HCT was significantly increased in settings of delayed platelet (median 32 vs. 7 TC, $p < 0.0001$) and also neutrophil engraftment (median 23.5 vs. 15 TC, $p = 0.04$, Supplementary Figures 9A-C). Four patients did not engraft platelets and required 86-200 TC transfusions within 6 months after HCT (not shown). For EC, the number of required transfusions within 6 months after HCT was higher upon delayed reticulocyte (median 24 vs 11 EC, $p < 0.0001$) as well as platelet engraftment (median 23 vs. 10.5 EC, $p < 0.0001$), but not affected by neutrophil engraftment dynamics (Supplementary Figures 9D-F). Three patients did not engraft reticulocytes and required 24-58 EC transfusions within 6 months after HCT (not shown).

In addition, poor graft function as previously defined (12, 21–24) was observed in 8 patients within 6 months after HCT. Median onset was at 39 days (range 29-95 days) after HCT and lasted for a median duration of 58 days (range 21-203 days). In all but one patient all three hematopoietic lineages incl. neutrophils, platelets and reticulocytes were affected (Supplementary Table 5). Median age of patients with poor graft function was similar to the entire cohort (64 years, range 46-70 years). A majority of 7/8 patients were transplanted for PMF (88%) and calreticulin mutations were overrepresented as driver mutations in 5/8 (63%) patients. Splenomegaly was observed in all patients with poor graft function at median spleen size of 18 cm (range 15-27 cm) at HCT similar to the overall cohort. Bone marrow fibrosis was pronounced with grade 3 fibrosis in most patients. Infused CD34+ cells were predominantly of peripheral origin and median dose was somewhat lower as compared to the entire cohort at 6.1

Mio/kg body weight. A major donor-recipient blood group barrier was present in 5/8 patients with poor graft function and thus more prevalent than in the overall cohort (63%).

Discussion

Allogeneic HCT represents the sole treatment option with curative potential for patients with higher risk MF, a rare hematologic neoplasm with patients in high need for effective therapeutic approaches (39). The subset of intermediate and high risk MF patients eligible for allogeneic HCT represents a limited population, which might be challenging to study at larger numbers. However, studies on transplantation approaches specifically in MF are essentially required since MF patients display characteristics, which are different from other myeloid malignancies and pose particular challenges to successful allogeneic HCT. Particularly, hematopoietic cell engraftment in MF has been shown to be compromised by adverse BM microenvironment features such as e.g. BM fibrosis, as well as through cell pooling by the prevalent splenomegaly (11, 12, 32), thus mediating an increased risk for post-transplant infections, bleeding and iron overload in MF. Here we report on the factors relating to engraftment dynamics in a longitudinal patient cohort of MF patients after allogeneic HCT between 2000 and 2019 at a major transplant center in Switzerland to provide additional evidence on the aspect of delayed engraftment in the neutrophil, platelet and red cell lineage in this infrequent patient group. While the overall cohort has a substantial size of 60 patients undergoing a total of 71 transplantations, the subsets of patients evaluable for specific factors relating to recent developments such as e.g. novel conditioning schemes may still be limited in number given the rarity of MF patients with high risk features and eligibility for HCT. However, we believe it is instrumental that these findings are reported timely to provide a basis and promote further evaluations in larger, multi-centric studies of MF.

Since engraftment dynamics in MF after allogeneic HCT are incompletely characterized, we evaluated potential determinants of reconstitution in the neutrophil, platelet and erythroid lineages. Median time to neutrophil engraftment in our MF cohort was comparable to other studies of allogeneic HCT in MF such as e.g. Kunte et al. reporting engraftment at a median of 20 days after haploidentical HCT (18). However, since PBSC were used as cell source in the vast majority of transplants (>85%) in that study, comparability to our cohort of transplanted MF patients is limited. Regarding platelet engraftment, variable definitions are used hampering comparisons across studies, and red cell engraftment as reflected by reticulocytes has only been reported by one study using a different cutoff (40). While engraftment failures were rare in our cohort, we assessed determinants of engraftment dynamics, which associated with later reconstitution in neutrophil, platelet, and erythroid lineages. The significance of key factors known to impact on engraftment in MF, such as splenomegaly or BM fibrosis, were evident in our study concordant with similar cohorts, thus highlighting the validity of our cohort as compared to other reports (11, 32). First, splenomegaly associated with later engraftment of neutrophils, platelets, and reticulocytes, highlighting that the prevalent finding of enlarged spleen warrants attention upon transplantation for MF. In addition, we observed significantly faster engraftment of neutrophil, platelet, and red cell lineages after pre-transplant splenectomy, although only 3 HCT in 2 splenectomized patients were performed. Similar findings were also reported by Polverelli et al., which included faster engraftment after splenectomy and delayed engraftment upon gross splenomegaly (33). At the time of HCT, patients with and without ruxolitinib therapy showed similar spleen size and ruxolitinib therapy, which was given in a majority before HCT, did not significantly influence engraftment dynamics. In line with the notion that BM fibrosis contributes to a microenvironment, which hinders engraftment (11), we observed longer reconstitution times for neutrophils and platelets in settings with higher-grade BM fibrosis as well as later establishment of full donor cell chimerism. It should be noted though that since MF is transplanted in advanced phases of the disease, sample size for grade 1 fibrosis was small. Of note, the disease-modifying potential of therapies including the reduction of BM fibrosis, represents an increasing interest of clinical studies for novel therapeutic approaches with targeted inhibitors in MF. Thus, such novel agents or combination therapies might represent suitable options as bridging therapy to allogeneic HCT, which would favor engraftment through pre-transplant reduction of BM fibrosis.

As to transplant-related factors, a correlation between CD34+ cell dose and neutrophil engraftment has been reported and was also evident in our cohort (31, 32). For red cell reconstitution, it has been established that engraftment may be delayed upon major blood group barriers but not in settings of minor, bidirectional or without ABO mismatch (41, 42). This has been attributed to residual circulating antibodies mediating hemolysis of donor-derived red cells, which was also found to prolong erythrocyte but not neutrophil engraftment in this study (43). In regard to preparatory regimens, reduced intensity conditioning (RIC) was most prevalent in the entire cohort and was applied to the vast majority of patients transplanted after 2015. Refinement of conditioning therapy such as by RIC approaches has largely improved tolerability of allogeneic HCT also in MF similarly to other hematologic malignancies and such modified conditioning regimens are widely used (44–47). More recently, the advent of TBF conditioning followed by PTCy as GvHD prophylaxis has improved the tolerability of haploidentical HCT and has enlarged the donor pool. Thus, TBF-conditioned haploidentical HCT with PTCy was also increasingly used in MF patients at our center. However, engraftment upon haploidentical HCT is incompletely characterized for MF so far, which relates at least in part to the challenges of studying new approaches in populations as rare as advanced, transplant-eligible MF. So far, two studies focused on haploidentical/mismatched family donors and found engraftment rates >90% (17, 18). Bregante et al. analyzed matched related, unrelated and alternative donors including mismatched family

donors, and found improved engraftment rates after introduction of TBF conditioning (16). Battipaglia et al. reported lower engraftment rates at prolonged times with haploidentical donors, but without implications for survival outcomes (19), while Angelucci et al. related reduced survival to graft failure in haploidentical transplants for MF (48). TBF conditioning followed by PTCy as GvHD prophylaxis has been specifically used as a preparatory regimen for haploidentical HCT. We observed that HCT after TBF-PTCy as a preparatory regimen showed slower reconstitution dynamics for neutrophils and platelets in our MF cohort and this same finding also related to the donor type with delayed regeneration in HCT from haploidentical donors, which converge with the TBF-PTCy regimen at our center. Similarly, later engraftment associating with BM as a CD34+ cell source or use of G-CSF support may be in part driven by these effects, since haploidentical TBF-PTCy HCT was overrepresented both among BM transplants and transplants with G-CSF support. These observations are supported by Battipaglia et al., who showed delayed neutrophil engraftment with haploidentical stem cell grafts (19), and Ballen et al. who described lower engraftment rates in patients receiving grafts from partially matched or mismatched family donors (49). In contrast, we found no differential effects on engraftment when RIC or MAC preparatory regimens were used. While our study highlights that haploidentical TBF-PTCy HCT is feasible in MF, a differential impact on engraftment dynamics in both the neutrophil and platelet lineages might be at play and should be evaluated in the appropriate larger-scale, multi-center studies to allow for conclusive assessments of such effects specifically in HCT for MF given the risks relating to later reconstitution and prolonged neutropenia and thrombocytopenia.

Inherent limitations of our study primarily relate to the number of MF patients undergoing allogeneic HCT. Although overall it is substantial for a single transplant center given the rarity of higher risk MF eligible for HCT, it remains rather limited when specific aspects such as e.g. influences of more recent conditioning schemes should be assessed. In addition, multivariate analyses of previously reported determinants of post-transplant engraftment such as e.g. splenomegaly and BM fibrosis may be compromised by the limited patient numbers. However, since post-transplant reconstitution in MF is prolonged as compared to other myeloid malignancies, it is imperative to initiate studies of factors, which could further compromise reconstitution and to highlight the need for larger studies which will require collaborative efforts in the field. A second limitation concerns the convergence of the TBF-PTCy treatment with haploidentical donor HCT at our center, which also partly overlaps with the use of BM as stem cell source and G-CSF support. The strong association of TBF-conditioned haploidentical HCT and PTCy in our cohort hinders a dissection of which factor(s) would interfere with engraftment. Overall, the performance of TBF conditioning and PTCy use for HCT in MF patients has remained controversial so far, particularly in regard to engraftment dynamics. While no negative effect of TBF conditioning on engraftment has been shown in two recent studies (19, 50), the use of PTCy has been associated with significantly lower engraftment rates in one (19), but not in another study (17). Therefore, we believe it is important to amend these data from a major Swiss transplant center. Clearly, additional and larger studies are required to consolidate the findings of multivariate analyses in single center cohorts so far and to clarify, whether determinants of engraftment time in MF also affect graft function and overall outcome of MF patients in the longer term.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: Retrospective analysis of electronic patient files. Requests to access these datasets should be directed to sara.meyer@insel.ch.

Ethics statement

The studies involving humans were approved by Ethical committee Nordwest- und Zentralschweiz, Switzerland (EKNZ, no.2016-01930). The studies were conducted in accordance with the local legislation, institutional requirements and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The participants provided their written informed consent to participate in this study.

Author contributions

SJ, JH and SM contributed to the study concept and design. SJ, KG, FA, MM, AB, and JP collected data, provided essential administrative or technical support, or analyzed and interpreted data. SJ and SM drafted the manuscript with contributions of all co-authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

S.J. and F.C.A. hold shares in Novartis. S.C.M. has consulted for and received honoraria from Celgene/BMS, Novartis and GSK and receives research support from Ajax.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1205387/full#supplementary-material

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The clinical relevance of broad mutational screening of myeloproliferative neoplasms at diagnosis

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Introduction: Myeloproliferative neoplasm (MPN) is a heterogenous group of hematological malignancies including polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). *JAK2*V617F is the most frequent driver mutation in all three entities, but in PMF and ET mutations in *CALR* and *MPL* are also frequent. Mutations seen in additional genes are also often the same regardless of subtype of MPN. The aim of this study was to analyze a population based MPN cohort for genetic variants with prognostic value that can guide clinical decisions.

Methods: MPN patients from Western Sweden diagnosed between 2008-2013 (n=248) were screened for mutations in 54 genes associated with myeloid malignancy.

Results: Mutations in the genes *SRSF2* and *U2AF1* correlated significantly with impaired overall survival but did not correlate to increased risk for vascular events, neither before nor after diagnosis. Rather, mutations in these genes showed an association with disease transformation. Several recurrent gene variants with allele frequency close to 50% were confirmed to be germline. However, none of these variants was found to have an earlier onset of MPN.

Discussion: In conclusion, we identified gene mutations to be independent markers of impaired survival in MPN. This indicates the need for more individualized assessment and treatment of MPN patients and a wider gene mutation screening already at diagnosis. This could ensure the identification of patients with high-risk mutations early on. In addition, several genetic variants were also identified as germline in this study but gave no obvious clinical relevance. To avoid conclusions from non-informative genetic variants, a simultaneous analysis of normal cell DNA from patients at diagnosis should be considered.

KEYWORDS

MPN, *JAK2*, *CALR*, *MPL*, germline, mutation, prognosis

Introduction

Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) all belong to the Philadelphia chromosome negative myeloproliferative neoplasm (MPN) category. These three entities share the same characteristics of causing proliferation of bone marrow cells, resulting in an increase of blood cells of myeloid lineage in the bone marrow and in peripheral blood. Advanced stages of PMF, on the other hand, is characterized by increase of reticulin fibers leading to decreased blood cells (1–3). The complications of these three entities are also similar regarding vascular events, i.e., thrombosis and bleeding. Furthermore, all three entities can transform into acute leukemia and have an impact on survival, however with large differences in frequency. Despite the common driver mutations in *JAK2, CALR* and *MPL* (4), the clinical presentation, risk and frequencies of complications and survival differ wildly between individual patients. Prognostic tools are therefore desired in clinical practice for follow-up and treatment decisions already at diagnosis. Modern sequencing techniques have given the opportunity to simultaneously analyze several mutations in blood malignancies. It has become widely used in both research and clinical practice (5). We and other groups have published data on risk mutations using this approach, but several studies analyze data on separate MPN entities or driver mutation groups. Since both driver mutations and several of the additional mutations found are shared between the subtypes of MPN, and since the occurrence of some additional mutations are rather rare, we hypothesize that analysis of mutations in MPN as one group has a potential to extend the prognostic value of genetic markers. Furthermore, there is a growing number of hereditary gene variants that have been linked to predisposition for development of hematological disorders, including MPN (6–11). This also represents a challenge when analyzing large amount of sequencing data, especially if comparison with normal non-malignant cells is not available. We also need to get more information regarding gene variants of unknown significance, to avoid overestimation of their importance but also to identify variants that might influence disease development and prognosis. In this study, we analyzed a well-defined, population based MPN cohort, regardless of subtype, for genetic variants. The aim was to search for additional prognostic markers that can be used to guide clinical decisions, as well as to investigate the potential impact of germline variants detected in the sequence data.

Materials and methods

Patients

All patients diagnosed with PV, ET or PMF according to the 2008 WHO diagnostic criteria (3) in Western Sweden at the Sahlgrenska University Hospital or NU Hospital Group between 2008 and 2013 and reported to the Swedish national blood cancer registry were identified. Basically, all patients in our health care region with suspected MPN are referred to and treated at these two hospitals. Thus, this cohort cover patients in the geographic area without any selection. Of this cohort, 248 patients were included in the study based on informed consent, availability of DNA sample from the time of diagnosis, and review of diagnosis. Data from a subset of this patient cohort have been published previously (12, 13). Details are outlined in Supplementary Table 1. Clinical characteristics, clinical course, vitality status, vascular complications, disease transformation and co-existing cancers were collected from the medical records of all patients. Each of the patient's hospital records were searched after emergency care consultation and hospital admission records that is related to bleeding or thrombotic complications. Follow-up was done from diagnosis until June 2021. The study was performed in accordance with the Declaration of Helsinki after ethical approval.

Screening for myeloid mutations

Genomic DNA from whole blood from the same sample that was analyzed at diagnosis for the presence of *JAK2*, *CALR* or *MPL* mutation was screened for gene variants in 54 genes or mutational hot spots associated with myeloid malignancies. The TruSight Myeloid Sequencing panel (Illumina FC-130-1010) which also was used for diagnostics in the clinical laboratory at the time of the study, was used, and sequencing was performed on a MiSeq instrument (Illumina) according to manufacturer's instructions. Secondary analysis was performed with MiSeq Reporter, v.2.4.60.8, using Burrows-Wheeler Aligner mapper and somatic variant caller (Illumina). Data were filtered and mapped to the human genome reference hg19 using Variant studio v3.0 (Illumina), where global filtering was set to >3% and coverage had a minimum of 500 reads. Variants causing missense, frameshift, an altered stop/initiation codon, in-frame insertion/deletion or variants affecting splice site were regarded as mutations. Variants with quality >Q30 and allele frequencies of at least 5% were considered positive for mutation. Known sequencing artefacts and variants previously found in normal controls were excluded from further analysis according to filter strategies used in the clinical laboratory. BAM files from secondary analysis were used to analyze selected variants by Integrative Genomics Viewer (www.broadinstitute.org). Variants in areas with difficult reads were excluded. Previously analyzed data was reanalyzed according to updated bioinformatic settings to make the results comparable regardless of time for sequencing.

Confirmation of germline variants

Blood sample was taken from patients with variants in *CDKN2A* (rs3731249), *ETV6* (rs145477191), *NOTCH1* (rs61751489) or *MPL* (rs41269541), with a variant allele frequency close to 50%. Blood was enriched for CD3+ cells, using MACS® cell separation kit StraightFrom™ Whole Blood CD3 MicroBeads (Miltenyi Biotech) and Whole Blood Column Kit (Miltenyi Biotech), according to the manufacturer's protocol. Genomic DNA from CD3+ enriched cells were extracted using QIAamp DNA Blood Mini Kit (Qiagen) and 10 ng of DNA were genotyped using TaqMan SNP genotyping assay (Applied

biosciences, Life technologies) according to manufacturer's protocol. The following assays were used: *CDKN2A* (assay ID: C_25611114_10), *ETV6* (assay ID: C_162058060_10), *NOTCH1* (assay ID: C_90123839_10) and *MPL* (assay ID: ANFV4EK). All samples were analyzed in triplicates using the QuantStudio 3 Real-Time PCR system (ThermoFisher Scientific). Genotypes were determined automatically based on dye component fluorescent emission data depicted in the X–Y scatter plot using Taqman genotyper software v.1.6.0. The gnomAD database v2.1.1 (https://gnomad.broadinstitute.org/) was used to compare frequencies in the MPN cohort with a normal Swedish population.

Statistical analysis

Fisher's Exact Test was used to compare differences in frequencies between groups. To estimate overall survival (OS), defined as time from diagnosis to last follow up or death from any cause, the Kaplan Meier Log-rank test was used initially. For multivariable analysis, logistic regression and Cox Regression was used. P-values <0.05 were considered statistically significant. The statistical software used were Analyze-it v.6.15.4 (Microsoft Excel), GraphPad Prism v.9.4.0 and SPSS v29.0.0.0.

Results

A population-based cohort

Between 2008 and 2013, 300 patients were diagnosed with MPN at Sahlgrenska University Hospital and NU Hospital Group in Western Sweden. Of these, 83% (n=248; PV n=84, ET n=123, PMF n=41) were included in the study. All included patients fulfilled the 2008 WHO diagnostic criteria. Age, gender, and blood counts from the time of diagnosis, for the whole MPN group and for the sub entities, are presented in Table 1. The distribution of driver mutations found at diagnosis was consistent with expected findings in the different subgroups of MPN (Figure 1). One patient with PMF was found to harbor both *JAK2* V617F as well as mutation in the *CALR* gene. Patients not included in the study either declined to participate, had another diagnosis when their medical records were reviewed, or diagnostic material was missing. The median age of these patients was slightly lower (66 years vs. 69 years) but there was no other significant difference between these and the included patients.

Mutations and survival

A sequencing panel including 54 genes associated with myeloid malignancies was used to screen for genetic variants that could be used as prognostic markers. Variants regarded as mutations other than the diagnostic driver mutations (*JAK2*, *CALR* or *MPL)* were found in 37 genes in at least one patient and in 27 genes in at least three patients (Figure 2A; Supplementary Table 1). During analysis of the gene data, several recurrent gene variants with allele frequency close to 50% were noted, which implied a hereditary variant. Therefore, analysis of the most common variants *CDKN2A* (NM_001195132.1:c.442G>A), *NOTCH1* (NM_017617.3: c.6853G>A) and *ETV6* (NM_001987.4:c.602T>C) as well as a variant close to a splice site in the *MPL* gene (NM_005373.2: c.1565 + 5C>T) were also analyzed in separated T-cells from new blood samples. This confirmed the variants to be germline. Therefore, these variants were excluded from analyses of prognostic impact. Sixty-three percent of the MPN cases had other mutations in addition to the diagnostic driver mutation (Figure 2B). Presence of at least one additional mutation was found to be associated with inferior survival (Figure 2C). To investigate if it was mutations in general or mutations in particular genes that had impact on survival, all genes with mutations detected in at least three patients were correlated to survival with the Kaplan Meier Log-rank test. For the whole MPN group, only mutations in five genes correlated significantly with inferior overall survival, *ASXL1* (P=0.0005), *SRSF2* (P<0.0001), *U2AF1* (P<0.0001), *CBL* (P=0.01) and *SF3B1* (P<0.0001) (Figure 3). These were further tested with multivariable analysis using Cox regression where also age and type of diagnosis were taken into consideration. When the five genes were grouped together, they still correlated to OS (P=0.002) with a hazard ratio 3.248, and it was not dependent on type of diagnosis (interaction 0.592). Also, age at diagnosis correlated to OS (P<0.001) as expected. However, it should be noted that all cases with *CBL* mutation also harbored mutation in another of the four genes (Supplementary Table 1). Therefore, the genes were also tested separately. When these were adjusted for both

TABLE 1 Age, gender and laboratory findings at diagnosis in 248 patients with MPN.

Diagnosis	Age (years)	Gender	Hemoglobin (g/L)	Hematocrit (%)	WBC (10^9/L)	Platelets (10^9/L)	EPO (IU/L)
MPN n=248	69 (27-94)	131 F 117 M	145 (60-217)	46 (21-67)	9.5 (1.0-42.7)	689 (5-2412)	4.75 (0.5-40.8)
PV n= 84	70 (37-94)	41 F 43 M	169 (120-217)	53 (37-67)	11.6 (5.2-31.4)	615 (200-2412)	2.1 (0.5-13.2)
ET n=123	68 (27-90)	69 F 54 M	139 (99-170)	43 (31-52)	8.9 (2.9-19.8)	844 (467-2061)	6.1 (1.7-40.8)
PMF n=41	70 (41-85)	21 F 20 M	112 (60-156)	37 (21-49)	8.4 (1.0-42.7)	386 (5-1412)	18 (3.3-578)

Median values with ranges within parenthesis.

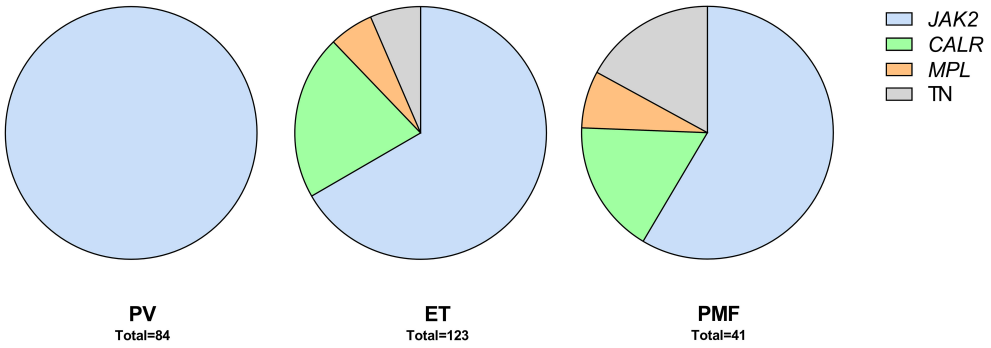

FIGURE 1
Patients by diagnosis and driver mutations. TN, triple negative.

age and type of diagnosis, only mutations in *SRSF2* and *U2AF1* correlated significantly to OS (Table 2).

Mutations and vascular events

Vascular events in MPN are potentially life-threatening. The vascular complications are either thrombosis or bleeding where the co-existence of MPN is a contributing factor. The most common incidences are those that are discovered at the time of diagnosis and the most common thrombotic events were myocardial infarction (n=27), cerebrovascular infarction (n=24), pulmonary embolism (n=19), transient ischemic attack (n=10) and deep vein thrombosis (n=8). The most frequent hemorrhagic complications were gastro-intestinal (n=11) and cerebral bleedings (n=6). Fisher's Exact Test and logistic regression were used to analyze if *SRSF2* or *U2AF1*

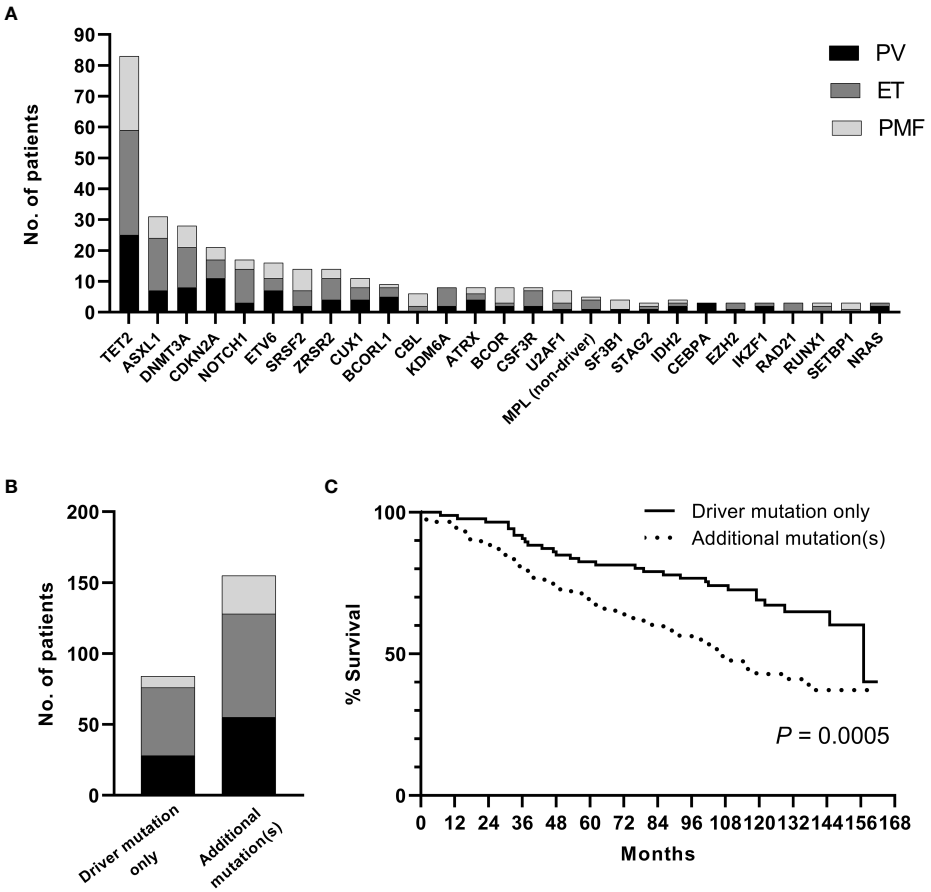

FIGURE 2
(A). Frequency of additional mutations. **(B)**. Distribution of patients with driver mutation only or addition mutation(s) when confirmed germline variants have been excluded. **(C)**. OS in patients with driver mutation only or with addition mutation(s) (confirmed germline variants excluded).

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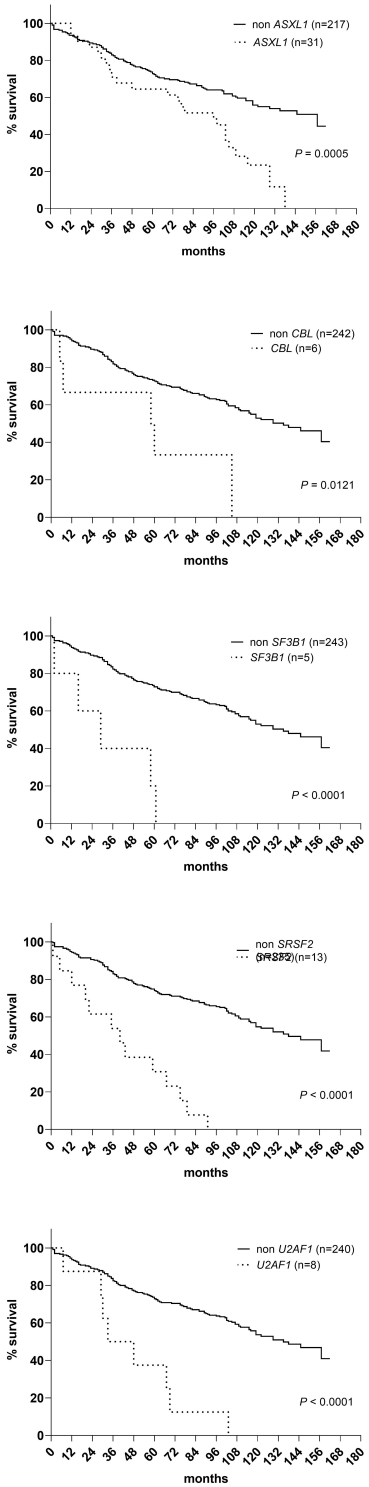

FIGURE 3
OS in patients with mutations in *ASXL1*, *CBL*, *SF3B1*, *SRSF2* and *U2AF1* respectively according to Kaplan Meier Log-rank test.

which correlated with shorter OS also correlated with occurrence of vascular events before or after diagnosis or in total. However, no such correlation was seen, neither when only the mutated genes were tested, nor when they were combined with age and type of diagnosis.

Mutations and disease transformation

All MPNs have a risk of transformation into secondary acute myeloid leukemia (AML). In our cohort, 17 cases had transformed to AML. Both mutated genes with correlation to OS were tested with logistic regression. This showed that mutations in *SRSF2* correlated with AML transformation (P=0.002), but this was not the case for *U2AF1* (P=0.236). The analysis was extended to find genes correlated to fibrotic transformation and co-existence with other myeloid hematological malignancies. There were 18 patients that had secondary myelofibrosis transformation from PV and ET. Other myeloid hematological malignancies that co-existed with MPNs included two chronic myelomonocytic leukemia and one myelodysplastic syndrome. Logistic regression showed that mutations in both *SRSF2* and *U2AF1* correlated with co-existing myeloid hematological malignancies (*SRSF2* P=0.05 and *U2AF1* P=0.014).

Gene germline variants

Identified germ line variants indicated a possible hereditary predisposition of MPN. Comparison of the frequency in our MPN cohort to a normal Swedish population cohort in the gnomAD variant database was performed. Only the variant found in *ETV6* was more frequent in the MPN group (0.0282 vs. 0.00975 in allele frequency). This difference was statistically significant (Fischer's exact test, p=0.0006). However, there was no correlation between any of the variants and occurrence of early onset MPN. We further used logistic regression to test if any of the variants correlated with occurrence of other cancers (both solid tumors and hematological malignancies outside the MPN group). These cancers occurred about the time and after diagnosis of MPN and were noted upon reviewing the patient´s hospital records. In total, 19 patients with non-hematologic cancers were found. The most common types were colon cancer (n=6) and pancreatic cancer (n=4). However, no significant correlation was seen.

Discussion

The serious risks all MPNs impose, although at various frequencies, are vascular complications, transformation to more severe hematologic malignancies and ultimately negative impact on OS. It is thus a priority to identify high risk patients in clinical practice. Age at diagnosis as well as occurrence of vascular complications have been reported as risk factors (14, 15). Access to an abundance of genetic data allows genetic profiling to further broaden prognostic information. Mutational status has progressively taken a big role in clinical practice. Occurrence of mutations have also been used to create scoring systems for MPN (16, 17). The initial focus on gene mutations in MPN was on the driver mutations' importance on disease development. These mutations are found in the genes *JAK2*, *CALR* and *MPL* which all are involved in JAK-STAT signaling (18). Notably, the same *JAK2*

TABLE 2 Mutation impact on OS in univariate or adjusted (including age and type of diagnosis) analysis.

	Univariate		Adjusted	
	Hazard ratio	P	Hazard ratio	P
ASXL1	2.183	<0.001	0.969	0.911
CBL	2.987	0.017	1.504	0.533
SF3B1	5.459	<0.001	2.525	0.223
SRSF2	5.110	<0.001	3.271	<0.001
U2AF1	4.491	<0.001	4.991	<0.001

mutation is found in both PV, ET and PMF, and mutations in *CALR* and *MPL* are seen in both ET and PMF. Thus, the mutation itself does not seem to determine the MPN phenotype, instead, allele burden has been reported as one factor behind the phenotypic differences (19). The order of acquisition of the driver mutation in relation to additional mutations may also have influence (20, 21). If the *JAK2* mutation precedes mutation in *DNMT3A* or *TET2*, the phenotypic picture would likely be PV. If mutations instead occur in reverse order, the MPN phenotype would likely be ET (22). Host factors also contribute to the development of disease (22, 23). Several predisposing gene variants have been identified that may influence not only the risk of developing disease but also the course of the disease (17, 24–26). It is therefore reasonable to investigate the whole MPN cohort as a group independent of diagnosis when analyzing it from a genetic point of view.

Aside from the driver mutations, several additional mutations have also been reported in MPN. These are subclassified to gene families: epigenetic regulators (*ASXL1, EZH2, TET2, IDH1/2, DNMT3A*), spliceosome (*SRSF2, SF3B1, U2AF1, ZRSR2*), transcriptional regulators (*TP53, RUNX1, IKZF1*), general cell signaling genes (*KRAS, PTPN11*) as well as specific negative regulators of JAK/STAT signaling (*CBL*) (27). Occurrence of additional mutations correlated significantly with inferior OS (Figure 2C). Genetic profiling has raised the question if the number of mutations in a particular case is more important for the occurrence of complications or OS rather than in what genes or type of gene the mutations are present. Our results suggest it is not the number of mutations but rather the presence of certain gene mutations that are more informative for prognostic guidance. Mutations in *SRSF2* and *U2AF1* correlated significantly with worse OS in our patient cohort. Although they were more frequent in PMF, which is well known to have an impaired survival compared to patients with PV and ET, mutations in these two genes correlated to worse OS regardless of MPN subtype.

Previous studies, including a subpopulation of our analyzed MPN cohort, have shown that triple negative MPN without *JAK2*, *CALR* or *MPL* mutation have worse prognosis (12, 28). It has also been shown that the presence of several other mutations in addition to a driver mutation correlate with survival (2, 5, 24, 26, 28–32). In this study we initially identified mutations in five non-driver genes (*ASXL1*, *SRSF2*, *U2AF1*, *SF3B1* and *CBL*) to be significantly correlated to impaired OS. Mutations in both *ASXL1* and *SRSF2* have previously been classified as high-risk mutations in both PMF and PV (26, 33). Moreover, mutations in *U2AF1* and *SF3B1* have been identified as genetic risk factors in ET (17). When age at diagnosis as well as type of diagnosis was taken into consideration only mutations in *SRSF2* and *U2AF1* remained associated with shorter OS. Mutations in *CBL* were only found in those patients who harbored mutations in one or more of the four other genes, suggesting that mutated *CBL* might just be a passenger rather than a disease driver. Mutations in *ASXL1* is commonly seen in clonal hematopoiesis, which increases with age (34). This could be an explanation why the presence of *ASXL1* mutation no longer significantly correlated with OS when age was taken into consideration.

Since vascular complications are associated with impaired survival we wanted to investigate if the detected mutations correlated also to vascular events in our MPN cohort. However, neither mutations in *SRSF2* nor *U2AF1* correlated to vascular events before or after diagnosis or in total. Another complication with MPN is transformation to myelofibrosis for PV and ET or to secondary AML for all three MPN. In the present cohort, a significant correlation between mutations in *SRSF2* and transformation to AML was found. Furthermore, mutations in both *SRSF2* and *U2AF1* correlated with transformation from PV and ET to myelofibrosis and development of other hematological malignancies. This is in line with previous findings were mutations in *SRSF2* and *U2AF1* have been reported to serve as prognostic markers for rapid blastic progression in newly diagnosed MPN (35). Moreover, mutations in *SRSF2*, *U2AF1* and *SF3B1* detected at presentation of disease have been associated with rapid fibrotic progression in PMF. This was not demonstrated for mutations in *ASXL1*, *DNMT3A* or *TET2* (36).

Aside from the acquired mutations in our MPN cohort, several specific variants were identified which turned out to be germline. A five- to sevenfold higher risk of MPN among first-degree relatives to MPN patients have previously been reported in Sweden, which suggest a genetic predisposition (37). Also in other myeloid malignancies, the question for germline variants involved in disease have come into focus (38–40). Four variants in our study were more closely investigated, their allele frequency was close to 50%, which could imply a hereditary variant. These genes were *CDKN2A* (NM_001195132.1:c.442G>A), *NOTCH1* (NM_017617.3: c.6853G>A) and *ETV6* (NM_001987.4:c.602T>C) as well as a variant close to a splice site in the *MPL* gene (NM_005373.2: c.1565 + 5C>T). The most frequent occurring *CDKN2A* mutation

leading to a p.A148T substitution has been reported as an inherited coding variant associated with leukemic transformation of hematopoietic progenitor cells (41). Comparison of the frequency to a normal Swedish population cohort, however, only revealed the *ETV6* variant to be more common in the MPN patient cohort. This variant did not correlate to earlier onset of disease, which could be expected for an inherited predisposition. On the other hand, in the Landgren study the mean age at diagnosis did not differ between affected relatives and controls (37). The ethical approval of the current study did not include testing of relatives, but it would of course be of interest to see if any of these variants are associated with an increased incidence of hematological or non-hematological malignancies within these families. Nevertheless, it is important to correctly identify germline gene variants to avoid drawing conclusions from non-informative genetic variants but also to provide genetic counseling when called for. In this study we used CD3+ selection of T-cells from collected blood samples to get constitutive DNA, which turned out to be easiest for both referring doctors and gave acceptable DNA yield for the laboratory but might of course misdiagnose somatic variants that are also present in lymphoid cells. Another alternative is a skin biopsy but this may be considered too much of an intervention for some patients.

In conclusion, our study on a population based MPN cohort strengthens previous reports about prognostic value of genetic data in MPN. Thus, a wider gene profiling at diagnosis is of value. In addition, several genetic variants were also identified as germline in this study but gave no obvious clinical relevance. To avoid conclusions from non-informative genetic variants, simultaneous analysis of normal cell DNA from patients at diagnosis should be considered.

Data availability statement

The original contributions presented in the study are included in the supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Etikprövningsmyndigheten (previously research ethics committee at University of Gothenburg, Sweden - Dnr 425-14 and 2021-05747-01). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

JAs, BA, PJ and LP designed the research study. JAd and JAs performed laboratory work and data analysis. BA, PJ and HP provided patient samples. HP, JAd, JAs, BA, PJ, SN and LP analyzed the combined data and wrote the paper. All authors approved of the final version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1190305/full#supplementary-material

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A germline *JAK2* exon12 mutation and a late somatic *CALR* mutation in a patient with essential thrombocythemia

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Background: It has been discovered that Janus kinase 2 (*JAK2*) exon12 mutations lead to the polycythemia vera (PV) phenotype, while somatic mutations of calreticulin (*CALR*) are associated with essential thrombocythemia (ET) or primary myelofibrosis. In this article, we report a case of ET with coexistence of *JAK2* exon12 and *CALR* mutations. The objective of this study was to elucidate the pathogenicity mechanism of a *JAK2* exon12 mutation (*JAK2*N533S) and the role of the coexistence of mutations on the hematological phenotype.

Methods: We designed a colony analysis of tumor cells obtained from this patient, and attempted to identify mutant genes using DNA from hair follicles. Mutation impairment prediction and conservative analysis were conducted to predict the mutation impairment and structure of *JAK2*N533S. In addition, we conducted a functional analysis of *JAK2*N533S by constructing Ba/F3 cell models.

Results: Three distinct tumor subclones, namely *JAK2*N533S^{het+}/*CALR*type1^{het+}, *JAK2*N533S^{het+}/*CALR*^{wt}, and *JAK2*N533S^{het+}/*CALR*type1^{hom+}, were identified from the 17 selected erythroid and 21 selected granulocyte colonies. The analysis of hair follicles yielded positive results for *JAK2*N533S. According to the bioinformatics analysis, *JAK2*N533S may exert only a minor effect on protein function. Functional studies showed that *JAK2*N533S did not have a significant effect on the proliferation of Ba/F3 cells in the absence of interleukin-3 (IL-3), similar to wild-type *JAK2*. Notably, there were no increased phosphorylation levels of *JAK2*-downstream signaling proteins, including signal transducer and activator of transcription 3 (STAT3) and STAT5, in Ba/F3 cells harboring the *JAK2*N533S.

Conclusion: Our study revealed that the *JAK2*N533S^{het+}/*CALR*type1^{het+} subclone was linked to a significant expansion advantage in this patient, indicating that it may contribute to the development of the ET phenotype. We further demonstrated that *JAK2*N533S, as a noncanonical *JAK2* exon12 mutation, is a germline mutation that may not exert an effect on cell proliferation and protein function. These results and the present body of available data imply that certain noncanonical *JAK2* mutations are not gain-of-function mutations leading to the development of myeloproliferative neoplasms.

KEYWORDS

essential thrombocythemia, JAK2 exon12 mutation, CALR type1 mutation, distinct phenotype, myeloproliferative neoplasms

1 Introduction

The classic Philadelphia-negative myeloproliferative neoplasms (MPN), which include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis, are a diverse group of clonal disorders characterized by the excessive production of mature cells in the peripheral blood (1–3). Somatic mutations in genes, such as V617F mutation in Janus kinase 2 (*JAK2*), *JAK2*V617F, *JAK2* exon12, calreticulin (*CALR*) exon9, or *MPL* exon10, exhibit a high prevalence (almost 90%) among patients with MPN, and are the underlying etiology of these disorders (4–8). Initially, researchers thought that these mutations were mutually exclusive in patients with MPN. However, a few cases of MPN associated with multiple driver mutations, most commonly the coexistence of *JAK2*V617F and *CALR* mutations, have been reported (9–12).

In this study, we present the case of a patient with ET who carries both *JAK2* exon12 mutation (*JAK2*N533S) and *CALR* mutation (*CALR*type1). Clinical observations revealed that *JAK2* exon12 mutations are found in PV (13). It is puzzling that this patient presents with a *JAK2* exon12 mutation and exhibits an ET phenotype, but lacks the characteristic PV phenotype. Additionally, the biological function of *JAK2*N533S, an atypical *JAK2* mutation, remains largely unexplored. Therefore, further investigation is warranted to elucidate the mechanism by which the coexistence of mutations contributes to the hematological phenotype in this case.

2 Materials and methods

2.1 Patient

The patient was diagnosed with MPN according to the 2016 World Health Organization criteria in the Second Hospital of Shanxi Medical University. Mutational investigation in this patient was performed using a DNA sample from fresh bone marrow or peripheral blood samples. Patient history and clinical data were extracted from the medical records. This patient provided written informed consent, and this study was conducted in accordance with the tenets stipulated in the Declaration of Helsinki.

2.2 Colony-forming assays

A peripheral blood sample of this patient was collected, and peripheral blood mononuclear cells were isolated. The cells were plated at a density of 1×10^5cells/mL, which provided an optimal density for colony selection without the risk of contamination by neighboring colonies. Erythroid colony-forming units were cultured in methylcellulose-based medium (STEMCELL Technologies,VAN,CAN) containing additional 3 U/mL erythropoietin for 15 days. Granulocyte colony-forming units were incubated in methylcellulose-based medium containing an additional 100 ng/mL granulocyte colony-stimulating factor for 15 days. Erythroid and granulocyte colonies were selected, and DNA was extracted, amplified using a 2×STA Master Mix Kit (BBI Life Science,HK,China), and sequenced for *JAK2* exon12 (sense 5'TGG GCCGAAGTCTGA CCCTTT 3' and antisense 5' ACAGAGCGAA CCAATGC 3') and *CALR* exon9 (sense 5' TGGGGCGTAACAA AGGTG AG 3' and antisense 5' TGAAAGTTC TCGAGTCTCA CAGA 3'). After purifying the polymerase chain reaction product, Sanger sequencing (The Beijing Genomics Institute,SZX,China) was used to identify mutation sites.

2.3 Bioinformatics analysis

We performed pathogenicity analyses for the novel missense variant using Mutation Taster score (https://www.mutationtster.org/), E-SNPs&GO (https://esnpsandgo.biocomp.unibo.it/), Polymorphism

Phenotyping version 2 (PolyPhen2; http://genetics.bwh.harvard.edu/pph2/), and Sorting Intolerant From Tolerant (SIFT; https://sift.bii.a-star.edu.sg/). Alignment of homologous sequences of *JAK2* protein from various species was performed using the Unipro UGENE software (49.1version, http://ugene.net/) to assess the conservation of amino acid residues at different sites. Protein three-dimensional (3D) model of wild-type *JAK2* (*JAK2*^{wt}) was used the homodimeric JAK2 pseudokinase-protein tyrosine kinase (PK-PTK) model by JAK2 PK-PTK model (ma-evjj8). The five models of *JAK2*S533 was constructed based on the above ma-evjj8 by AlphaFold (https://alphafold.com/) and ColabFold (https://github.com/sokrypton/ColabFold) (14, 15). For further analysis, we used the *JAK2*S533 model with the highest per-residue confidence score (predicted local distance difference test) compared with the other models. The PyMOL system (4.5.0 vision) was used to visualize the results of the protein model. Detailed data of the model and score are provided in Supplementary Material 1.

2.4 Plasmid construction and lentiviral infection

Complementary DNAs (cDNAs) for human *JAK2*N533S and control genes (*JAK2*^{wt}, *JAK2* K539L, and *JAK2*N542-E543del) were synthesized by GenScript (NKG,CN). All cDNAs were cloned into a pCDH-CMV- MCS-EF1-CopGFP-Puro lentiviral-vector. Lentiviral particles were produced in 293T cells to infect Ba/F3 cells. The efficiency of gene transfer into Ba/F3 cells was assessed by green fluorescent protein (GFP) using laser confocal microscopy (Olympus, Tokyo, Japan) and flow cytometry (Beckman, CA,USA).

2.5 Cell culture and Cell Counting Kit-8 assay

Ba/F3 cells were cultured in RPMI 1640 medium (Gibco, USA) containing 15% fetal calf serum (Thermo Fisher Scientific,NK,USA) and 10 ng/mL interleukin-3 (PeproTech,NJ, USA). Cell viability assay was performed using a CCK8 Assay Kit (DOJINDO, kumamato, Japan). For the CCK8 assay, 3,000 cells were seeded in each well of a 96-well plate. Each cell line was cultured for 5 days in the absence of IL-3.

2.6 Western blotting

Ba/F3 cells were deprived of IL-3 for 2 days. Total protein was extracted, processed using radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, NK,USA), and supplemented with protease and phosphatase inhibitor mixture tablets (Thermo Fisher Scientific,NK,USA). Western blotting was performed by Simple Western (Protein Simple Technology,SV, USA). Antibodies against signal transducer and activator of transcription 3 (STAT3), STAT5, phospho-STAT3 (pSTAT3), and pSTAT5 were obtained from Cell Signaling Technologies (MA,USA).

3 Results

3.1 Patient clinical characteristics

A 75-year-old female patient presented with a gradual increase in platelet counts over 3 years. In December 2016, she was referred to the Second Hospital of Shanxi Medical University for evaluation of this marked thrombocytosis (Figure 1A). Despite the elevated platelet count, the patient was in good physical condition and did not experience any clinical symptoms, such as fever, itchy skin, facial flushing, bone pain, splenomegaly, or others. Furthermore, she did not have any complications, such as bleeding, thrombosis, or cerebrovascular disease.

A bone marrow biopsy was conducted to investigate the underlying reason for the thrombocytosis. The findings indicated normal cellularity with a normal ratio of myeloid to erythroid cells without fibrosis. However, there was a significant increase in megakaryopoiesis. Chromosomal analysis of 20 karyotypes revealed that the patient had a normal karyotype of 46, XX. Mutational analysis using peripheral blood revealed that the patient carried a *JAK2* exon12 mutation (*JAK2*N533S) and a *CALR* mutation (*CALR*type1). The patient was diagnosed with ET based on the platelet count (707 per mm^3), proliferation mainly of the megakaryocytic lineage, and the presence of *CALR*type1. However, there was no evidence of PV apart from the presence of *JAK2*N533S. The patient's blood routine returned to normal and remained stable after treatment with hydroxyurea. Currently, she remains in good health despite carrying *JAK2*N533S and *CALR*type1 (Figures 1A, B).

3.2 Clonal relationship of JAK2N533S and CALRtype1

The clonal relationship between *JAK2*N533S and *CALR*type1 was further investigated in this study. Peripheral blood mononuclear cells were isolated, and colony-forming assays were performed for erythroid cells and granulocytes. All 17 selected erythroid colonies exhibited a heterozygous *JAK2*N533S mutation (*JAK2*N533S^{het+}). Of those, 14 colonies (82%) also carried a heterozygous *CALR*type1 mutation (*CALR*type1^{het+}), one colony (6%) harbored a homozygous *CALR*type1 mutation (*CALR*type1^{hom+}), and two colonies (12%) exhibited a wild-type *CALR* (*CALR*^{wt}) genotype. In addition, all 21 selected granulocyte colonies exhibited a *JAK2*N533S^{het+}. Among those, 20 colonies (95%) had a *CALR*type1^{het+}, and one colony (5%) exhibited a *CALR*^{wt} genotype (Figures 1C, D).

Because *JAK2N*533S^{het+} was present in all colonies, *JAK2*N533S was further tested using DNA extracted from hair follicles (Figure 1E). This analysis yielded positive results for the presence of *JAK2*N533S^{het+}, indicating that this is a germline mutation. In contrast, among these colonies, 20 (95%) exhibited a *CALR*type1^{het+}, while one (5%) had a *CALR*^{wt} genotype. This evidence further indicated that *CALR*type1 was a late somatic event and occurred in a multipotent hematopoietic stem cell compartment capable of generating both myeloid and erythroid progeny.

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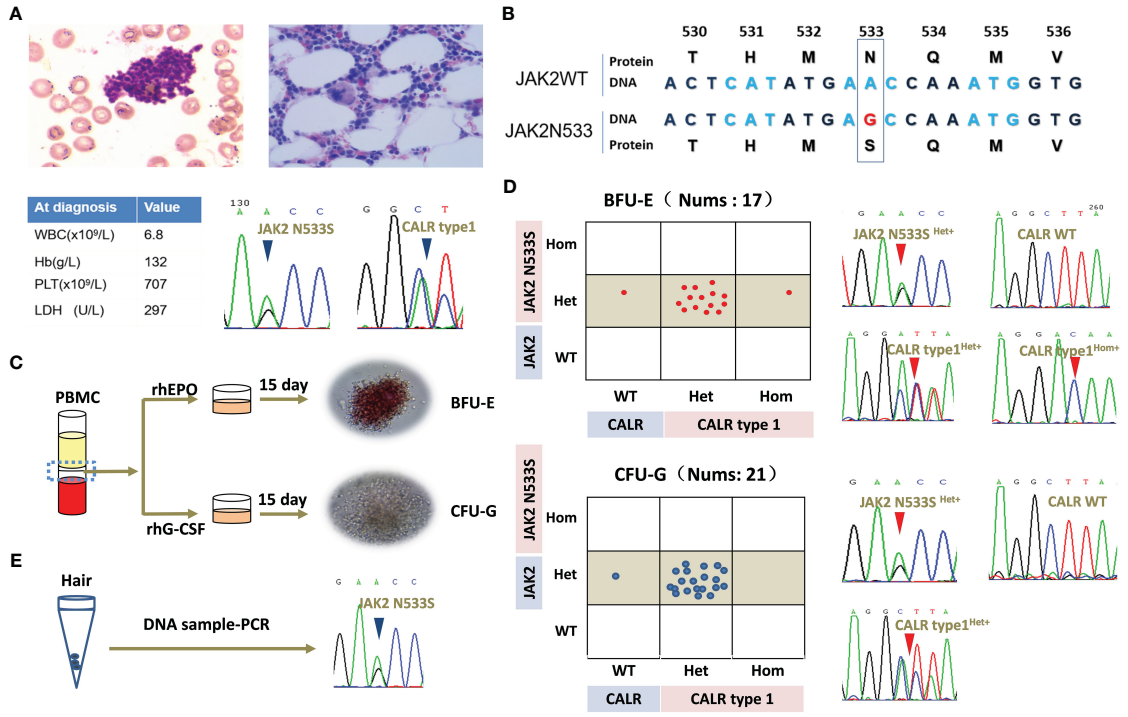

FIGURE 1
Clinical data of the patient and clonal evolution analysis for *JAK2*N533S and *CALR*type1. **(A)** Representative image of bone marrow biopsy and blood counts at diagnosis. Sanger Sequencing Charts of heterozygous *JAK2*N533S and *CALR*type1 using a peripheral blood samples at diagnosis. **(B)** Diagram of *JAK2*N533S. The substitution of base A with base G results in the change of asparagine to serine. **(C)** Schematic representation of erythroid and granulocyte colony-forming units. **(D)** Analysis of single colonies and sequencing chromatograms for *JAK2*N533S and *CALR*type1. **(E)** Sequencing chromatograms for *JAK2*N533S using DNA obtained from hair follicles. CALR, calreticulin; JAK2, Janus kinase 2.

Individual colony analysis revealed three distinct tumor subclones, namely *JAK2* N533S^{het+}/*CALR*type1^{het+}, *JAK2*N533S^{het+}/*CALR*^{wt}, and *JAK2*N533S^{het+}/*CALR*type1^{hom+}. Notably, significant expansion was observed in the *JAK2*N533S^{het+}/*CALR*type1^{het+} clone compared with the *JAK2*N533S^{het+}/*CALR*^{wt} clone. This finding suggested that the acquisition of *CALR* mutation provides a growth advantage over the presence of *JAK2*N533S alone (Figures 1D, E).

3.3 Mutation impairment prediction and conservative analysis

We sought to better understand the pathogenicity of *JAK2*N533S. Hence, a bioinformatics analysis was conducted to determine the function of *JAK2*N533S. In this analysis, various mutation impairment prediction tools were utilized to predict the impact of Asn533Ser substitution on the *JAK2* protein. The Mutation Taster score indicated that Asn533Ser substitution was pathogenic, suggesting a detrimental effect on protein function. However, other tools (e.g., E-SNPs&GO, PolyPhen-2, and SIFT) classified the N533S variant as benign, indicating that it is unlikely to significantly impact the protein function. The contradictory results obtained from the pathogenic prediction analysis of *JAK2*N533S are perplexing (Figure 2A). It is important to consider that different prediction methods may be characterized by inherent biases and limitations, which could contribute to discordant conclusions.

Additionally, a conservation analysis was performed to assess the level of conservation of Asn533 across different species. It was found that Asn533 is conserved in the *JAK2* gene among species, such as humans, *Pan troglodytes*, *Macaca mulatta*, etc. However, of note, in *Danio rerio*, Asn533 can naturally be replaced with Ser (Figure 2B).

Furthermore, a comparison of the three-dimensional model of the homodimeric JAK2 PK-PTK model containing N533 and S533 was conducted (Figures 2C, D). The S533 mutation does not appear to significantly alter the structure of the *JAK2* protein based on the merge of the JAK2 PK-PTK model of N533 and S533 (Figure 2C). The substitution of Asn with Ser could not trigger significant structural changes due to the slightly shorter length of Ser compared with Asn and their similar polar nature. Hence, it may cause subtle and confined alterations in *JAK2* structure.

Previous research has determined that the *JAK2*K539L mutation in *JAK2* exon12 is a pathogenic mutation that leads to *JAK2* constitutive activation. This activation occurs by disrupting the highly charged region of D620 and altering the salt bridge interaction between residues D620 and E621. Moreover, the change of salt bridging among Lys539, Glu592, and Glu596 in the periphery of the hydrophobic interface could potentially lead to the stabilization of a constitutive active dimer (15–18). Could the N533S mutation influence them by establishing or disrupting the surrounding salt bridging and hydrogen bonds to promote *JAK2* activation? There were no changes observed in the surrounding

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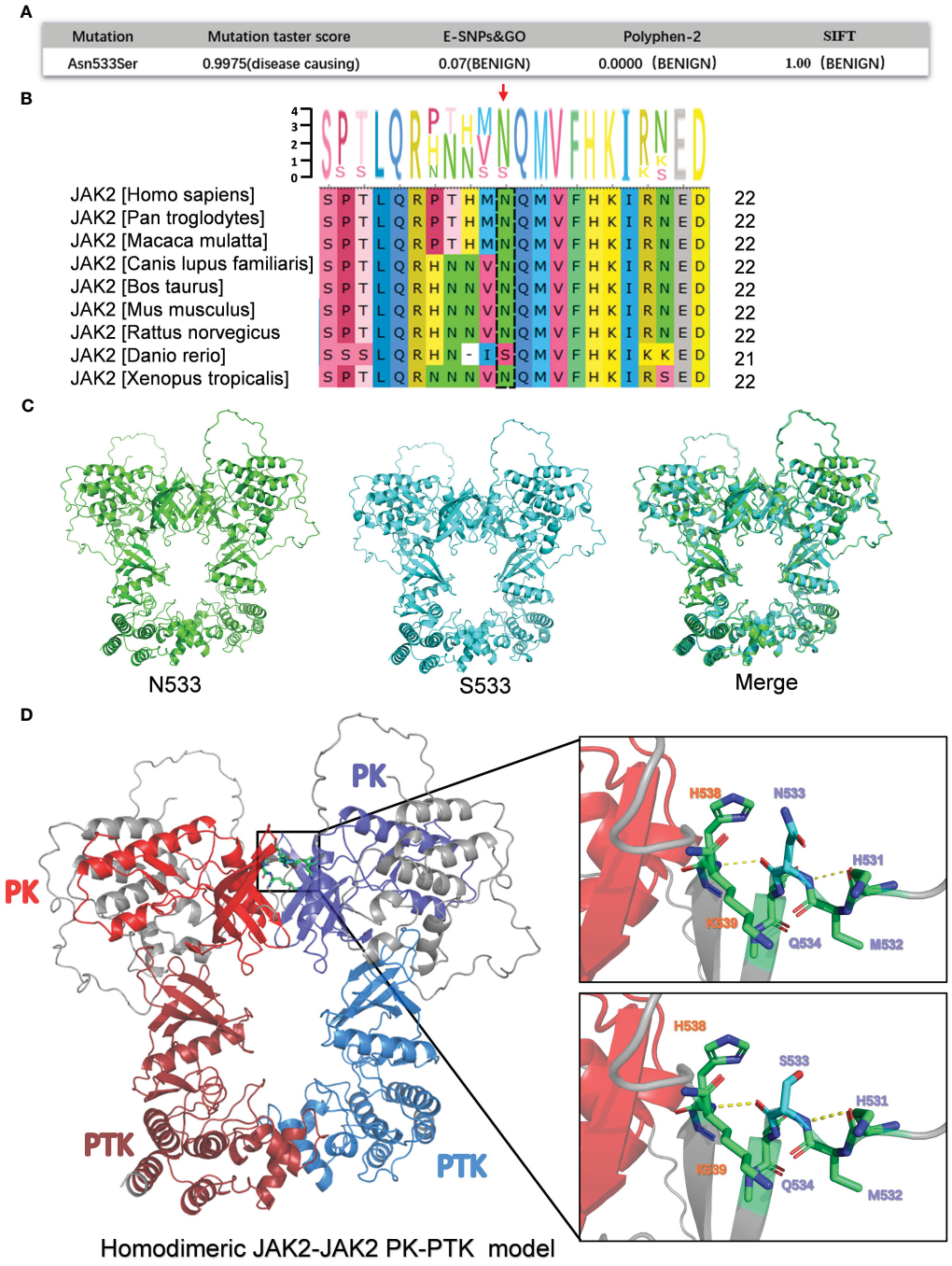

FIGURE 2
Bioinformatics analysis of the pathogenicity of *JAK2*N533S. **(A)** Prediction results for the pathogenicity of N533S based on the Mutation Taster score, E-SNPs&GO, PolyPhen-2, and SIFT. **(B)** Conservative analysis diagrams of the *JAK2* 533 site across different species. **(C)** Homodimeric *JAK2* PK-PTK model of *JAK2*N533 (green) and *JAK2*S533 (blue). **(D)** Left, homodimeric *JAK2* PK-PTK model of *JAK2*S533; the PK domain and PTK domain are marked using different colors. Right, zoom-in image showing the amino acids within 6 Å of N533 or S533. JAK2, Janus kinase 2; PK, pseudokinase; PTK, protein tyrosine kinase.

hydrogen bonds, and the N533S mutation did not establish a salt bridge interaction with residues D620, E621, and K539, as the distance between them exceeded 5 Å.

Collectively, based on the results of the bioinformatics analysis, *JAK2*N533S may exert only a minor effect on protein function. Further investigation and experimental studies are required to fully understand the functional consequences of this substitution.

3.4 Impact of JAK2N533S on Ba/F3 cell proliferation

To investigate the functional effects of *JAK2*N533S, we cloned the cDNAs of *JAK2*N533S and control genes (*JAK2*^{wt}, *JAK2*K539L, and *JAK2*N542-E543del) into a lentiviral expression vector with an GFP and puromycin; of note, *JAK2*K539L and *JAK2*N542-E543del are the

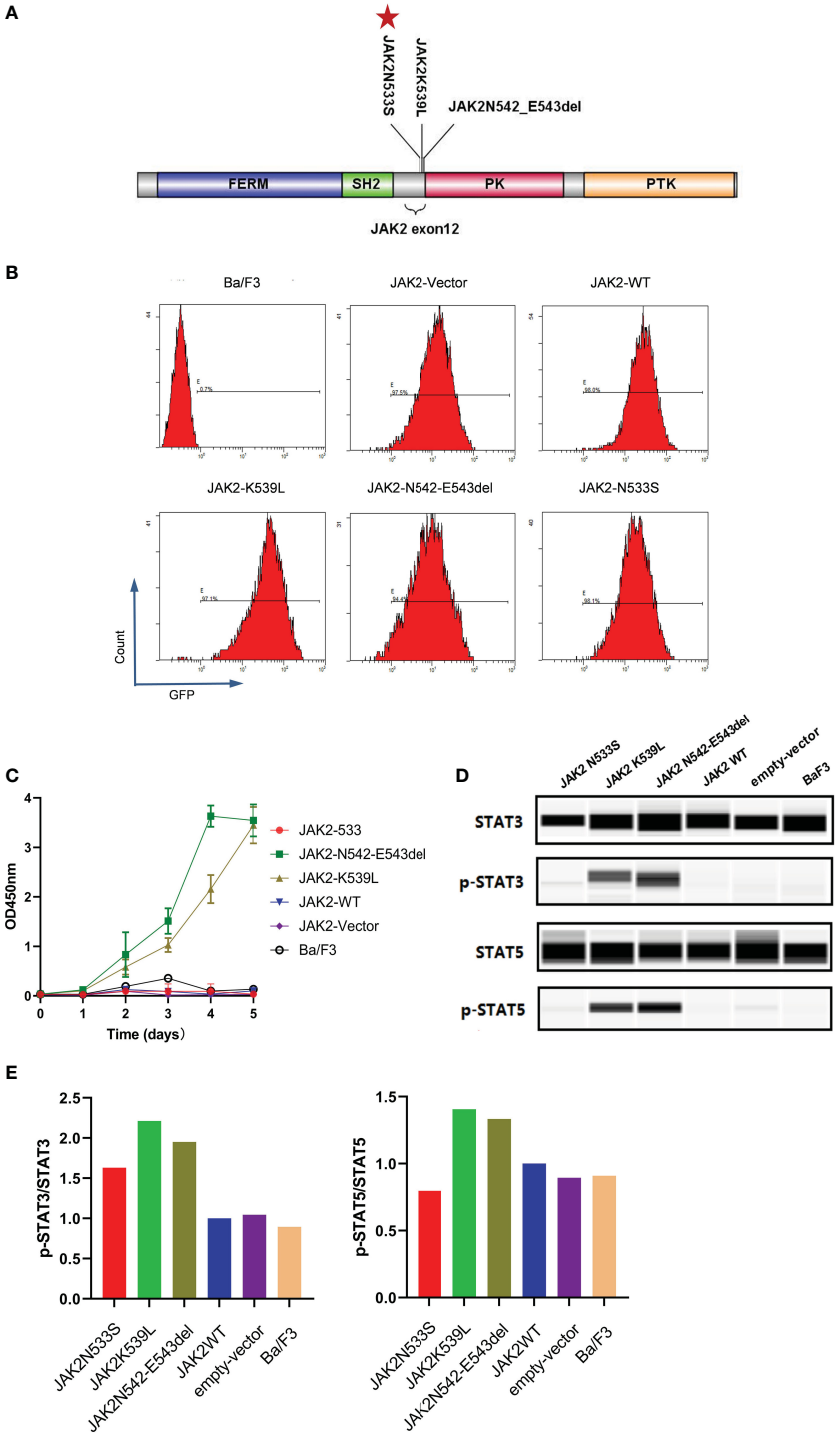

FIGURE 3
Functional analysis of *JAK2*N533S. **(A)** Schematic representation of the *JAK2* domain and *JAK2* exon12 mutations. Red stars in the panel indicate the positions of *JAK2*N533S. **(B)** Detection of GFP in different cell lines through flow cytometry. **(C)** IL-3-independent proliferation of *JAK2* mutation Ba/F3 models, detected by CCK8 assay. Data are presented as the mean ± standard deviation (SD) of three independent experiments; error bars indicate SD. **(D)** Lower phosphorylation levels of STAT3 and STAT3 in cells carrying *JAK2*N533S compared with cells harboring *JAK2*K539L and *JAK2*N542-E543del, detected by Simple Western. **(E)** Quantification of the expression of phospho-STAT3 (p-STAT3) and p-STAT5. Left, levels of p-STAT3 determined by densitometry of protein bands and normalized to those of STAT3. Right, levels of p-STAT5 determined by densitometry of protein bands and normalized to those of STAT5. CCK8, Cell Counting Kit-8; GFP, green fluorescent protein; IL-3, interleukin-3; STAT3/5, signal transducer and activator of transcription 3/5.

most common gain-of-function mutations in *JAK2* exon12 (Figure 3A). We used a lentiviral transduction to transfect the JAK2 gene with different mutation sites into Ba/F3 cells. We sorted the transgene-positive cells utilizing puromycin (2 ng/mL) and detected the positive rate of GFP by flow cytometry (Figure 3B). Next, we measured the IL-3-independent proliferation of cells. Each cell line was cultured for 5 days in the absence of IL-3. The experiment was conducted in triplicate. Cell viability assay was performed using CCK8. The control cells expressing *JAK2*K539L and *JAK2*N542-E543del showed significant accumulation, whereas there was no difference observed between cells carrying *JAK2*N533S and control cells expressing $JAK2^{wt}$, empty-vector, and untransfected Ba/F3 cells (Figure 3C). We further examined the levels of phosphorylated *JAK2*-downstream signal proteins, including STAT5 and STAT3. Cells were deprived of IL-3 cytokine for 2 days and analyzed by western blotting (ProteinSimple Technology) for phosphorylation of STAT5 and STAT3. Control cells carrying *JAK2*K539L and *JAK2*N542-E543del presented increased phosphorylation of STAT5(Tyr694) and STAT3(Tyr705), whereas those harboring *JAK2*N533S, $JAK2^{wt}$, empty-vector, and untransfected Ba/F3 cells did not show obvious activation of STAT5 and STAT3 (Figures 3D, E). The data obtained from the functional analysis indicated that *JAK2*N533S did not alter the function of *JAK2* protein.

4 Discussion

In this article, we have described a case with coexistence of *JAK2*N533S and *CALR*type1. Mutational analysis of a single colony allowed us to distinguish three distinct tumor subclones in this patient. Among these subclones, the *JAK2*N533S^{het+}/*CALR*type1^{het+} clone had a significant expansion advantage over the other two clones. Thus, the *JAK2*N533S^{het+}/*CALR*type1^{het+} clone was significantly associated with the ET phenotype of this patient. In contrast, we observed a small number of *JAK2*N533S^{het+}/*CALR*type1^{hom+} subclones. It is unlikely that two independent mutation of *CALR* occurred at the exact same position (*CALR*type1). Therefore, we inferred that *CALR*type1^{hom+} could arise from the original *JAK2*N533S^{het+}/*CALR*type1^{het+} clone by the loss of the $CALR^{wt}$ allele through deletion or uniparental disomy. The percentage of such homozygous colonies was very low. A possible reason is that loss of $CALR^{wt}$ may not provide a competitive advantage. In contrast, an alternative likely could be explained by the fact that the *CALR*type1^{hom+} subclone emerged shortly before the diagnosis. Therefore, the number of *JAK2*N533S^{het+}/*CALR*type1^{hom+} clones was relatively low.

Furthermore, considering that only part of the selected colonies carried *CALR*type1, we inferred that *CALR*type1 is an acquired somatic mutation. All selected colonies carried *JAK2*N533S, indicating that this may be a germline mutation. We further tested this mutation using DNA samples obtained from hair follicles, indicating its potential emergence as a germline mutation. Regrettably, it was not possible to locate samples obtained from the parents of this patient. Hence, we were unable to strictly determine that *JAK2*N533S is a germline mutation.

According to the currently available data, *CALR*type1 is sufficient to induce the ET phenotype. Thus far, *JAK2* exon12 mutations have only been associated with the PV phenotype; *JAK2*K539L and *JAK2*N542-E543del are the most common gain-of-function mutations in *JAK2* exon12. In contrast, *JAK2*N533S (as a noncanonical *JAK2* exon12 mutation) has been rarely reported. In this investigation, we demonstrated that *JAK2*N533S did not exert an effect on the proliferation of cells through protein function prediction and cellular model function assays. The current data indicated that *JAK2* N533S did not contribute to MPN in this patient. Nevertheless, it is possible that this variant serves as a basis for *CALR* driver mutations. Moreover, the coexistence of *CALR*type1 and *JAK2*N533S could induce a stronger growth advantage to promote proliferation. Future research should focus on the effects of this coexistence.

In recent years, an increasing number of other noncanonical *JAK2* mutations have been identified in patients with MPN through next-generation sequencing (19–22). However, it has been observed that some of these *JAK2* mutations (e.g., G335D, F556W, Y590E, G571S, and Y613F) do not play a role in the pathogenesis of MPN (6, 20, 21, 23, 24). Our findings and other available data suggest that certain noncanonical *JAK2* mutations are not gain-of-function mutations leading to the development of MPN (1, 23–25). Therefore, it is recommended to assess the functional impact of noncanonical *JAK2* mutations in MPN cases at the time of diagnosis. This study on *JAK2*N533S can be used a reference in clinical practice.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by Ethics Committee of the Second Hospital of Shanxi Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from primarily isolated as part of your previous study for which ethical approval was obtained. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements. The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

ZH: Data curation, Formal analysis, Software, Writing – original draft, Writing – review & editing. JL: Formal analysis, Conceptualization, Writing – review & editing. FG: Investigation, Methodology, Conceptualization, Writing – review & editing. WR: Methodology, Writing – review & editing. XL: Methodology,

Software, Writing – review & editing. JF: Methodology, Software, Writing – review & editing. CZ: Formal analysis, Investigation, Software, Writing – review & editing. SB: Methodology, Writing – review & editing. JXi: Methodology, Writing – review & editing. ML: Data curation, Methodology, Writing – review & editing. JMC: Data curation, Formal analysis, Methodology, Writing – review & editing. WY: Data curation, Formal analysis, Methodology, Writing – review & editing. RH: Data curation, Methodology, Validation, Writing – review & editing. DM: Writing – review & editing. JXu: Formal analysis, Methodology, Writing – review & editing. JXC: Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. XC: Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. HW: Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1265022/full#supplementary-material

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