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BIOMOLECULAR AND HISTOLOGICAL FEATURES IN PEDIATRIC ESSENTIAL THROMBOCYTHEMIA: ADEQUACY OF WHO DIAGNOSTIC CRITERIA

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ABSTRACT

Myeloproliferative neoplasms (MPN), Essential Thrombocythemia (ET), Polycythemia Vera (PV) and primary myelofibrosis (PMF), are clonal disorders of the hematopoietic stem cell. In the recent years, somatic mutations of *JAK2*, *CALR* and *MPL* genes have been found in these diseases. However, these mutations do not allow the discrimination between the different diseases, being present in more than one form. World Health Organization (WHO) criteria, in fact, request in addition to the molecular study, a complete bone marrow histological evaluation. Pediatric ET is a rare disorder with an incidence about 60 times lower than the adult form albeit with similar blood finds. In children with ET, the incidence of *JAK2*V617F and *CALR* mutations is significantly lower than in adults, while hereditary cases characterized by *MPL* mutations are relatively more common. So far the histological evaluation of the bone marrow has not been exhaustively explored in this setting of patients.

The aim of the present study was to evaluate the adequacy of current WHO criteria in pediatric ET exploring both the incidence of driver mutations and bone marrow features in a large cohort of children with a clinical diagnosis of ET.

Our study was built in two steps: (i) a biomolecular study of a pediatric population with a clinical diagnosis of ET performed by Italian pediatricians, expert in hematological disorders and (ii) a histological evaluation strictly adherent to WHO criteria of BM features of a sub group of these children. Firstly, biomolecular studies of 89 children with a clinical diagnosis of ET, all having a sustained increase in platelet count (>450 x10^9/L) with no demonstrable reactive or secondary cause and no familial history of MPN or thrombocytosis, were evaluated to our central laboratory. In the second phase of our work we collected naïve bone marrow (BM) biopsies of 20 children with a clinical diagnosis

of ET (PedET) and, as controls, BM of 6 children (PedST) with reactive/secondary thrombocytosis, 18 children (Norm) with a normal BM histology and 36 adults (AdsET) with WHO-diagnosed ET. All BM biopsies were reviewed by two MPN's expert pathologists, blinded to the cause of each child's thrombocytosis.

In the biomolecular study we found that 23 patients (25,8%) had a clonal disease. The *JAK2*V617F mutation was identified in 14 children, 1 child had the *MPL*W515L mutation, and 6 had *CALR* mutations. The HUMARA monoclonal X-chromosome inactivation pattern was demonstrate in 6 patients (two with *JAK2*V617F and two with *CALR* mutations). The other 66 patients (74,2%) had persistent thrombocytosis with no clonality. There were no clinical or hematological differences between the clonal and non-clonal patients.

From the histological point of view, while cellularity was increased in all pediatric cases compared to adults ($p<0.001$), megakaryocytes (MK) density was higher in PedET (37.5 MK/mm2) than in PedST (9.2 MK/mm2) ($p<0.001$). Moreover, MK clusters (100%) and BM fibrosis (30%) were observed only in PedET but not in PedST and in Norm. The BM histology was similar in PedET and AdsET. On a whole, BM histology confirmed the diagnosis of ET in 15 children, suggested a PV in 1 child, a PMF in 3 (1 grade 1 and 2 grade 0) and secondary thrombocytosis in one.

Our study shows that children with ET are mostly non-clonal, however, the relative proportion of ET-specific mutations in the clonal children was much the same as in adults. Histological WHO criteria are able to identify ET, PMF and PV and distinguish ST from primary thrombocytosis, also in pediatric population. Therefore, WHO criteria seem suitable in all age groups, making both complete biomolecular evaluation and BM assessment mandatory in children with suspected ET.

RIASSUNTO

Le Neoplasie Mieloproliferative (MPN), sono disordini clonali della cellula staminale emopoietica, caratterizzate dalla proliferazione di una o più linee mieloidi e sono Trombocitemia Essenziale (ET), Policitemia Vera (PV) e Mielofibrosi Idiopatica (PMF). I criteri diagnostici per le MPN dell'adulto si sono evoluti nel tempo di pari passo con l'acquisizione di nuove conoscenze clinico-laboratoristiche e biomolecolari di tali patologie. Negli ultimi anni sono state descritte mutazioni somatiche a carico dei geni *JAK2*, *CALR* e *MPL*, tuttavia queste mutazioni non consentono una distinzione accurata in quanto presenti in più di una MPN. Nei più recenti criteri diagnostici WHO, di conseguenza, accanto allo studio biomolecolare, è necessaria una completa valutazione della biopsia osteo-midollare (BOM).

La ET pediatrica è una malattia rara con un'incidenza stimata di circa 60 volte inferiore alla forma dell'adulto. L'incidenza delle mutazioni di *JAK2* e di *CALR* è significativamente inferiore nei bambini con ET rispetto agli adulti, mentre sono relativamente più numerosi i casi ereditari caratterizzati da mutazioni di *MPL*. Ad oggi in questi pazienti non è stata ancora esplorata esaustivamente la rilevanza della valutazione istologica del midollo.

Lo scopo del nostro studio è stato quello di verificare l'adeguatezza dei criteri WHO nella popolazione pediatrica esplorando sia l'incidenza delle mutazioni principali che le caratteristiche della BOM in un'ampia casistica di bambini con diagnosi clinica di ET.

Il nostro lavoro è stato costruito in due momenti: (i) uno studio biomolecolare in bambini con diagnosi clinica di ET fatta da Pediatri Italiani esperti in disordini ematologici e (ii)

una valutazione istologica della BOM in un sottogruppo di questi bambini, applicando rigorosamente i criteri WHO.

Nella prima parte dello studio abbiamo valutato 89 bambini con diagnosi clinica di ET con un incremento prolungato della conta piastrinica (>450 x10^9/L) in assenza di cause secondarie o reattive e senza familiarità per MPN o trombocitosi. I campioni stati sono stati centralizzati presso il nostro laboratorio per lo studio biomolecolare completo. Nella seconda fase, abbiamo collezionato le BOM di 20 bambini con diagnosi clinica di ET (PedET) e, come controlli, di 6 bambini con trombocitosi reattiva (PedST), 18 bambini (Norm) con istologia midollare nella norma e 36 adulti con diagnosi di ET in accordo con in criteri WHO (AdsET). Tutte le BOM sono state rilette in cieco da due patologi esperti in MPN.

Nello studio biomolecolare in 23 pazienti (25,8%) è stata dimostrata la presenza di un marker di clonalità: 14 bambini erano positivi per la mutazione *JAK2*V617F, 1 bambino aveva la mutazione *MPL*W515L e 6 avevano mutazioni di *CALR*. Inoltre, sei pazienti sono risultate clonali allo studio dell'inattivazione del cromosoma X (due portatrici anche la mutazione *JAK2*V617F e due con mutazioni di *CALR*). Gli altri 66 pazienti (74,2%) presentavano una trombocitosi persistente senza evidenza di clonalità. Non sono state dimostrate differenze clinico-ematologiche tra i pazienti clonali e non clonali.

Dal punto di vista istologico, la cellularità è risultata più alta in tutti i casi pediatrici rispetto agli adulti ($p < 0.001$), mentre la densità megacariocitaria (MK) è risultata più alta nei PedET (37,5 MK/mm2) rispetto a PedST (9.2 MK/mm2) ($p < 0,001$). Inoltre, i cluster di MK (100%) e la fibrosi midollare (30%) sono stati osservati solo in PedET, essendo sostanzialmente assenti sia in PedST che Norm. L'istologia della BOM è risultata pressoché sovrapponibile in PedET e AdsET. Tra i bambini con PedET, l'istologia della

BOM ha confermato in 15 casi la diagnosi di ET, in 1 caso è risultata suggestiva per PV, in 3 casi per PMF (1 grado 1 e 2 grado 0) ed in un caso per trombocitosi secondaria.

Il nostro studio conferma che, sebbene la maggior parte dei bambini non presenti un marker di clonalità, le mutazioni classiche delle MPN sono presenti anche nella ET pediatrica con una proporzione simile a quella delle forme dell'adulto. I criteri istologici WHO sono in grado, anche nella popolazione pediatrica, di identificare ET, PV e PMF e di distinguere forme primitive e secondarie di trombocitosi.

In conclusione, i criteri WHO sembrano adeguati per tutte le fasce d'età. Ciò impone una completa valutazione biomolecolare ed istologica anche nei bambini con sospetta ET.

BACKGROUND

Myeloproliferative Neoplasms (MPN) are clonal disorders of the hematopoietic stem cell characterized by proliferation of one or more myeloid lineages (granulocytic, erythroid, megakaryocytic), and consequent increase of red cell mass, leukocytosis and/or thrombocytosis.

In 1951, William Dameshek first defined "Myeloproliferative Disorders" (MPD) (1), grouping in this category Chronic Myeloid Leukemia (CML), Polycythemia Vera (PV), previously known as Osler-Vasquez disease, Essential Thrombocythemia (ET), Primary Myelofibrosis (PMF) and Di-Guglielmo disease (erythroleukemia); the latter one has been then re-defined as acute erythroid leukemia and consequently excluded from MPNs (2).

In 1960, Nowell and Hungerford discovered the Philadelphia Chromosome (Ph1) in Chronic Myeloid Leukemia (CML)(3). This chromosome is the result of a mutual translocation of chromosome 9 to 22 leading to the constitution of BCR-ABL1 fusion gene, that encodes for a protein with tyrosin-kinase activity (4,5). Therefore, MPN were distinguished in Ph-1 positive and negative forms, the latter including PV, ET and PMF, then known as "Classic" Ph-1 negative myeloproliferative diseases (CMPDs) (6).

MPN diagnostic criteria have been repeatedly updated along time. Louis Wasserman in 1967 created the Polycythemia Vera Study Group (PVSG) that proposed diagnostic criteria for PV (7) and in 1986 those for ET, all based on the exclusion of mechanisms able to sustain clinical-laboratory findings. For instance, in ET, diagnosis implied absence of evidence for reactive thrombocytosis and exclusion of the other myeloid malignancies (8).

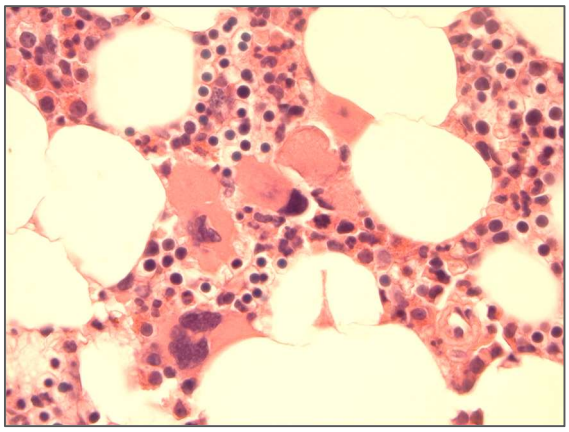
In 2001, the World Health Organization (WHO) released a new classification of myeloproliferative neoplasms (9), introducing histopathological features in bone marrow biopsy (BM) able to discriminate among different forms of MPN. For the first time in this classification early/prefibrotic primary myelofibrosis (pre-PMF) was considered. Patients labeled with pre-PMF are clinically undistinguishable from those with ET, but they have a different prognosis histologically based (10-11) (Figures 1,2). However, some Authors (12) claim that current histologic criteria are not sufficient to permit routine separation of ET into biologically distinct subsets.

Figure 1. Histologic characteristics of megakaryocytes (MK) of ET and pre-PMF. ET is characterized by loose clusters of MK (a), staghorn MK (b) or normal MK (c). Pre-PMF is characterized by tight cluster of MK (d) and bulbous MK (e). (pictures from our archives)

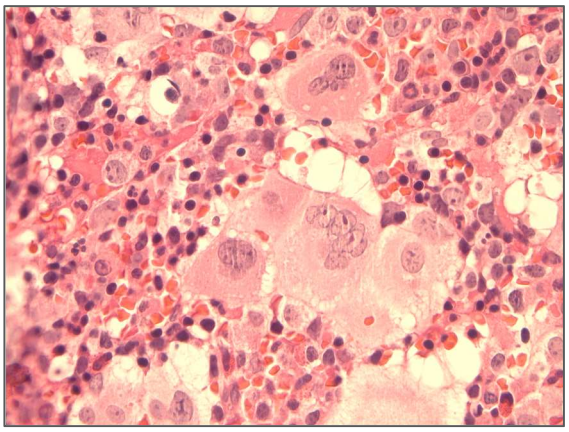
ET

pre-PMF

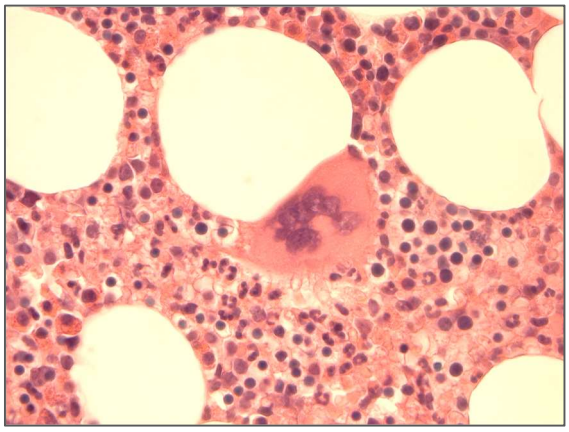
a.



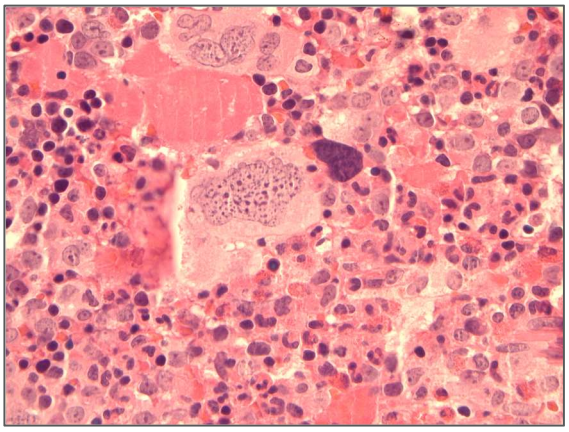
d.



b.



e.



c.

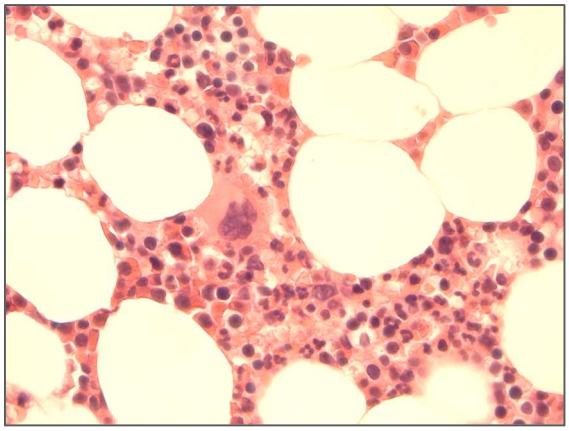
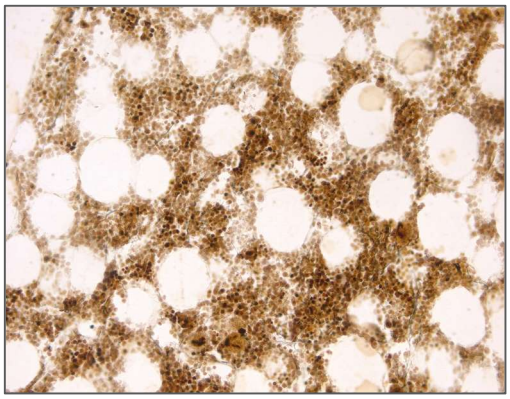
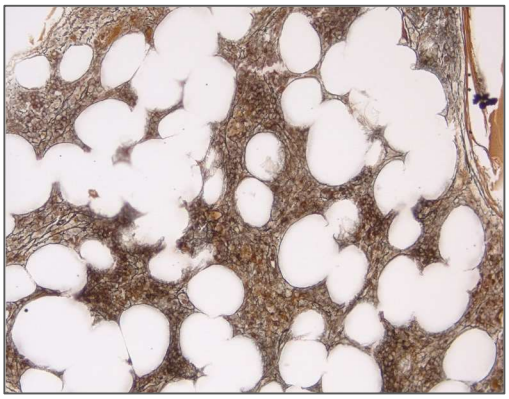


Figure 2. BM grading of fibrosis. (pictures from our archives)

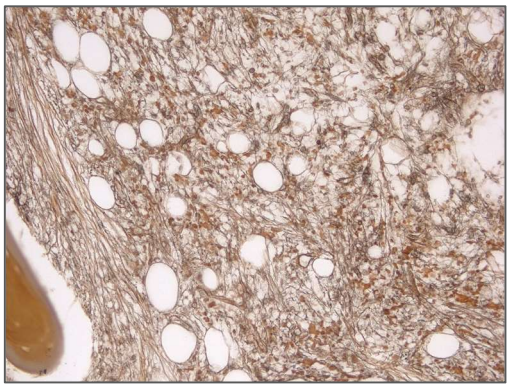
Grade 0



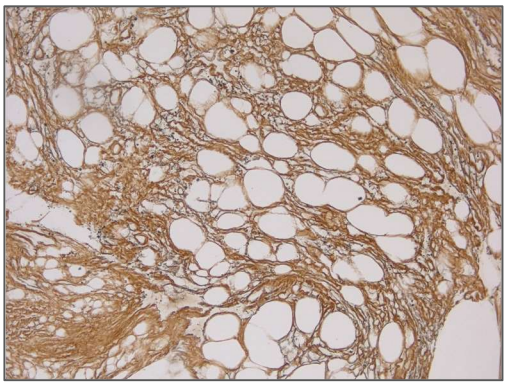
Grade 1



Grade 2

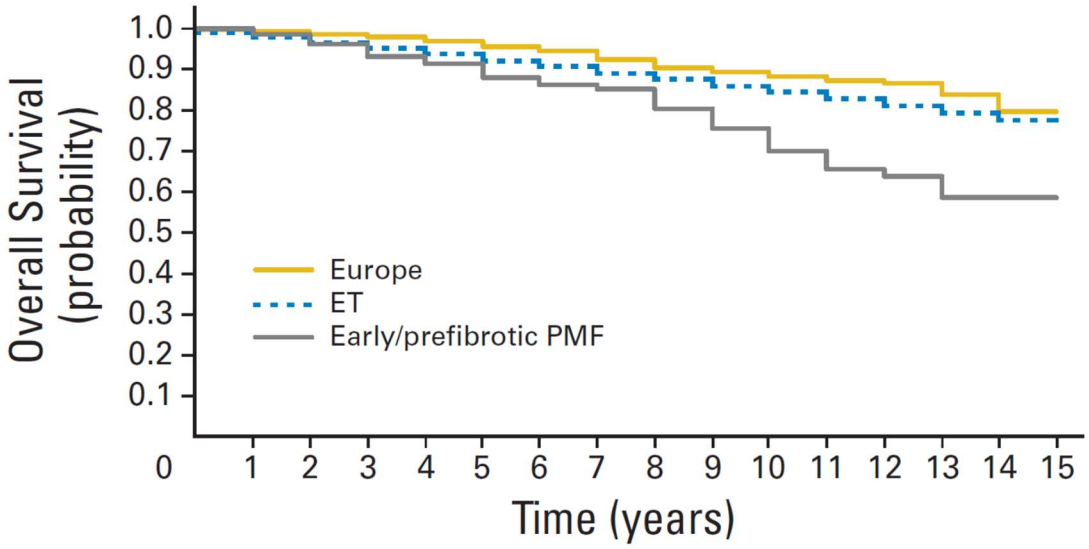


Grade 3



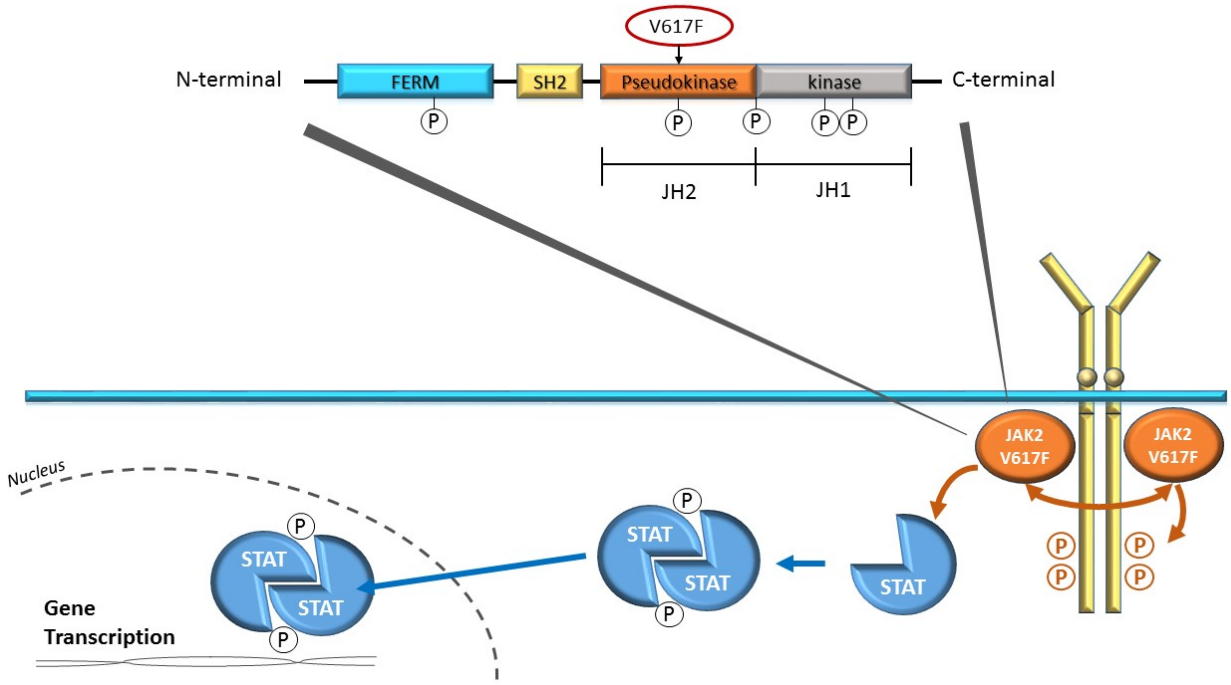
The clinical relevance of a correct evaluation of BM histology and morphology was later determined: in fact, it has been shown that pre-PMF patients display lower overall survival and higher rate of progression into overt myelofibrosis compared to “true” ET (Figure 3) (13).

Figure 3. Survival estimates for patients with ET and pre-PMF were compared with those obtained by applying the 2008 Eurostat age- and sex standardized incidence rates for all causes of death to the general population, using the indirect standardization procedure (13).



In April 2005 five different groups (14-18) demonstrated the presence of a somatic mutation in exon 14 of *JAK2* gene located in 9p24 chromosome, causing the replacement of a Guanine with a Thymine and thus the encoding of Phenylalanine instead of a Valine in position 617 of JH2 domain of JAK2 protein (*JAK2*V617F). JAK2 protein is a tyrosine-kinase implied in the hematopoiesis and the *JAK2*V617F mutation causes constitutive activation of JAK2 and transduction of the signal, particularly for STAT5/Bcl-xL pathway (Figure 4).

Figure 4. Representation of *JAK2*V617F mutation and effects.

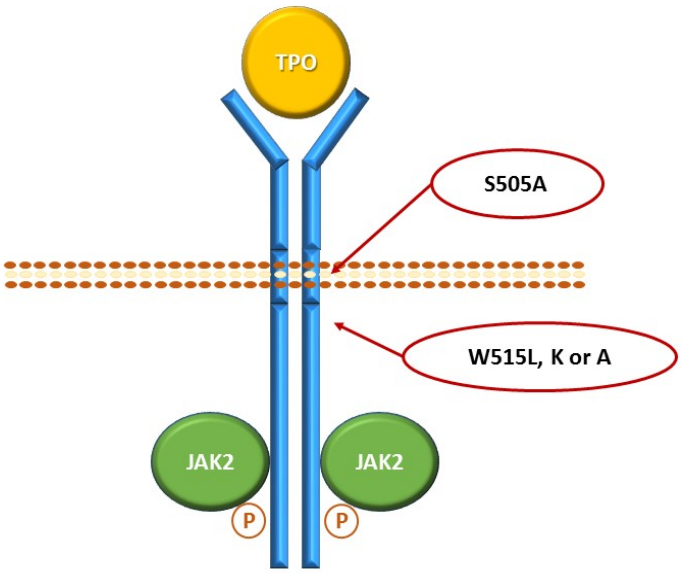


*JAK2*V617F mutation is detectable in 90% of PV and in 50-60% of ET and MF patients. Screening for this mutation became rapidly mandatory in MPN evaluation, making both the PVSG and the 2001-WHO criteria out of date (19).

In 2006, a somatic point mutation in exon 10 of *MPL* (Myeloproliferative Leukemia Virus) gene in chromosome 1p34, encoding thrombopoietin (TPO) receptor, was described. This mutation is responsible for the substitution of Trp→ Leu or Lys or Ala (*MPL*W515L, K or A) in the transmembrane domain of TPO receptor (TpoR), resulting in a gain-of-function and constitutive activation of JAK/STAT signaling pathway. These genetic abnormalities are found in 5-10% of PMF and 1-3% of ET cases (20,21).

Another mutation in *MPL* gene, resulting in substitution of Ser→Asn in codon 505 (*MPL*S505N), has been initially identified in familial thrombocytosis, and then demonstrated in sporadic cases of ET (22) (Figure 5).

Figure 5. Representation of c-MPL and its mutations.



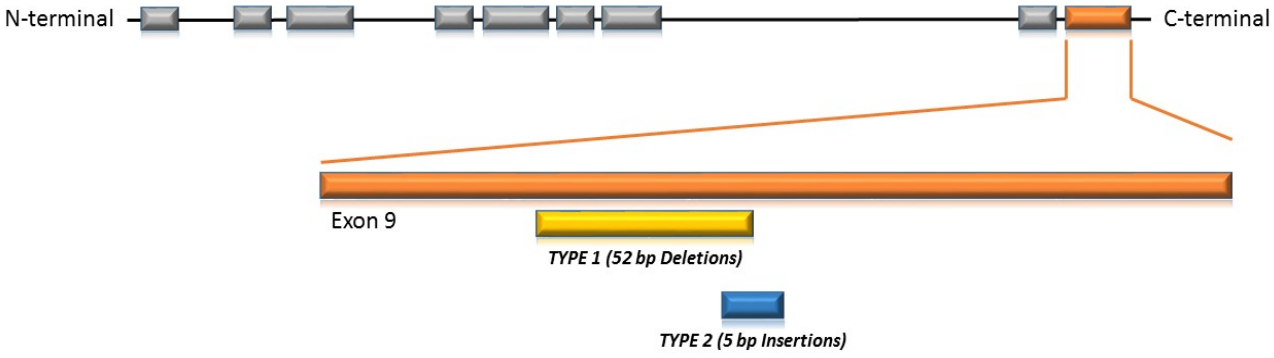
In 2008, WHO published the new classification of MPN: CMPDs are renamed as myeloproliferative neoplasms, underlying the clonal origin of these diseases (23), as yet demonstrated by Adamson and Fialkow (24,25), and the presence/absence of biomolecular markers was included as a major criterion for diagnosis of MPN (26).

In 2013, novel mutations in exon 9 of calreticulin (*CALR*) gene (27,28) have been described in 15% of ET and PMF. *CALR* gene encodes for a cytoplasmic protein of endoplasmic reticulum. The *CALR* mutations cause a frameshift to alternative reading frames: the resulting mutant *CALR* proteins share a novel amino acid sequence at the C-terminal that contains a number of positively charged amino acids, whereas the non-mutant *CALR* C-terminal is largely negatively charged (27). There are two types of *CALR* mutations: type 1 is a 52-bp deletion that results in loss of most of all negative charged amino acids; type 2 is a 5-bp insertion that maintains about 50% of normal amino acid sequence in C-terminal (28). Type 1 and 2 together represent about 80% of identified *CALR* mutations, the other 20% are rarer mutations.

It has been recently demonstrated that the two most common MPN-associated *CALR* mutants induce constitutive, ligand-independent activation of TpoR: the interaction between *CALR* mutants and TpoR directly leads to close dimerization of JAK2 kinase domain and its activation. The extracellular domain of TpoR is crucial for activation by *CALR* mutants but their way of interaction is not yet exactly known. (29). It has been shown how TpoR is able to signal from several dimeric interfaces. It has been supposed that *CALR* mutant might stabilize one of the many active interfaces of TpoR (30,31), resulting in a constitutive activation of the receptor. Both *CALR* mutants (del52 and ins5) might stabilize two related, but not identical, active dimeric interfaces of TpoR, leading

to differences in the phenotype, as suggested (32, 3). Furthermore, binding of *CALR* mutant to TpoR and its activation do not require TPO-binding.

Figure 6. Representation of *CALR* gene and locus of mutations.



The newly identified molecular features have yielded new perspectives regarding diagnostic and prognostic markers that provide novel insights for the understanding of MPN. Furthermore, the improved characterization and standardization of morphological and histological features have increased the reliability and reproducibility of diagnoses, aiding in the differentiation of these diseases. Therefore, in May 2016, a new revision of WHO criteria based on an integrated approach that includes hematologic, morphologic, cytogenetic and molecular genetic findings has been released and for the first time pre-PMF has been described as an apart entity (Table I-IV) (34).

Table I. WHO-2016 criteria for PV (34).

MAJOR CRITERIA	1	Hemoglobin > 16,5 g/dl (men), > 16,0 g/dl (women), *or* Hematocrit > 49% (men), > 48% (women), *or* increased red cell mass more than 25% above mean normal predicted value
	2	BM biopsy showing hypercellularity for age with trilineage growth (panmyelosis) including prominent erythroid, granulocytic, and megakaryocytic proliferation with pleomorphic, mature megakaryocytes (differences in size)
	3	Presence of *JAK2*V617F or *JAK2* exon 12 mutation
MINOR CRITERION	1	Subnormal serum erythropoietin level
Diagnosis of PV requires meeting either all 3 major criteria, or the first 2 major criteria and the minor criterion		

Table II. WHO-2016 criteria for ET (34).

MAJOR CRITERIA	1	Platelet count ≥ 450 x10^9/L
	2	BM biopsy showing proliferation mainly of the megakaryocyte lineage with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei. No significant increase or left shift in neutrophil granulopoiesis or erythropoiesis and very rarely minor (grade 1) increase in reticulin fibers
	3	Not meeting WHO criteria for *BCR-ABL1*$^+$ CML, PV, PMF, myelodysplastic syndromes, or other myeloid neoplasms
	4	Presence of *JAK2*, *CALR*, or *MPL* mutation
MINOR CRITERION	1	Presence of a clonal marker or absence of evidence for reactive thrombocytosis
Diagnosis of ET requires meeting all 4 major criteria or the first 3 major criteria and the minor criterion		

Table III. WHO 2016 criteria for pre-PMF (34).

MAJOR CRITERIA	1	Megakaryocytic proliferation and atypia, without reticulin fibrosis > grade 1, accompanied by increased age-adjusted BM cellularity, granulocytic proliferation, and often decreased erythropoiesis
	2	Not meeting the WHO criteria for *BCR-ABL1*$^+$ CML, PV, ET, myelodysplastic syndromes, or other myeloid neoplasms
	3	Presence of *JAK2*, *CALR*, or *MPL* mutation or in the absence of these mutations, presence of another clonal marker,† or absence of minor reactive BM reticulin fibrosis
MINOR CRITERIA	Presence of at least 1 of the following, confirmed in 2 consecutive determinations:	
	1	Anemia not attributed to a comorbid condition
	2	Leukocytosis ≥ 11 x10^9/L
	3	Palpable splenomegaly
	4	LDH increased to above upper normal limit of institutional reference range
Diagnosis of pre-PMF requires meeting all 3 major criteria, and at least 1 minor criterion		

Table IV. WHO 2016 criteria for overt PMF (34).

MAJOR CRITERIA	1	Presence of megakaryocytic proliferation and atypia, accompanied by either reticulin and/or collagen fibrosis grades 2 or 3
	2	Not meeting WHO criteria for ET, PV, *BCR-ABL1*$^+$ CML, myelodysplastic syndromes, or other myeloid neoplasms
	3	Presence of *JAK2*, *CALR*, or *MPL* mutation or in the absence of these mutations, presence of another clonal marker, or absence of reactive myelofibrosis
MINOR CRITERIA	Presence of at least 1 of the following, confirmed in 2 consecutive determinations	
	1	Anemia not attributed to a comorbid condition
	2	Leukocytosis ≥ 11 x10^9/L
	3	Palpable splenomegaly
	4	LDH increased to above upper normal limit of institutional reference range
	5	Leukoerythroblastosis
Diagnosis of overt PMF requires meeting all 3 major criteria, and at least 1 minor criterion		

MPN are typical diseases of middle-advanced age, with a median age at diagnosis of 60-65 years; they can affect both sexes, with a mild prevalence of males with PV and of females with ET (35). In 10-20% of cases, diagnosis occurs before age of 40 (36).

These diseases are very rare in children and adolescents.

While a reactive/secondary thrombocytosis is a common finding in pediatric age, especially in younger children, either because of the immaturity of their innate and/or adaptive immunity, or because they have more frequently infections (37), ET, despite being the most represented MPN in children, is uncommon. Under the age of 14, ET has an estimated incidence of 1/10 000 000 children/year (38), about 100x less than that of adults (39). It is also important to remember that familial ET is responsible in many pediatric cases, as seen in a large Italian series (40). PV has only been reported anecdotally in pediatrics and is possibly observed in teenagers or young adults (40).

Due to the rarity of ET in pediatric age, few is known about the disease in this setting of patients. However, in the recent years some papers were published regarding ET in children. Unlike in adults, the majority of children with a clinical diagnosis of ET do not have clonal markers and seem to have different biological characteristics from adult cases (41). Varying frequencies of the *JAK2*V617F mutation have been reported in childhood ET, ranging from 0% to 30%, representing in any case an incidence lower than in adults (40-48). Interestingly, in a study of Veselovska et al. (47) within 5 children with ET and growth of erythrocytes (EEC) and granulocytes (CFU-GM) spontaneous colonies, while *JAK2*V617F mutation was undetectable in peripheral blood, it was present in two cases in EEC and in one in CFU-GM. This observation suggests that the inability to detect the *JAK2*V617F mutation in purified cell populations may be due to the rarity of *JAK2*V617F sub-clones, with an allele burden below the level of PCR detection limits. Therefore, the

incidence of the *JAK2*V617F mutation might be underestimated in children (47). The observed differences in clinical and hematological features between adults with or without *JAK2* mutation and the several correlations of MPN phenotype with the *JAK2*V617F allele burden have not been confirmed in children (40,49).

The *MPL* mutations are anecdotal in children with ET: on my best knowledge, there are only two published cases of pediatric non-familial ET patients carrying a *MPL* mutation (50,51), while in hereditary thrombocytosis *MPL* mutations (especially in position 505) are not rare (52,53).

In a recent paper, Giona et al. describes *CALR* mutations in 44% of *JAK2* and *MPL* wild-type patients and 23% of all series in a cohort of ET patients younger than 20 years (54). Furthermore, *CARL*-mutated children were more frequently asymptomatic at diagnosis in comparison with those carrying *JAK2*V617F mutation (54). In contrast, Langabeer et al. failed to detect *CALR* mutations in a small series of ET patients younger than 15 years (55). On the whole, pediatric patients with a clinical diagnosis of ET, i.e. a sustained and prolonged increase in platelet count without any identifiable cause of thrombocytosis, seem to carry fewer driver mutations than adults.

Therefore, the WHO criteria appear not adequate in children with clinical ET (44), but so far there is a lack of information regarding bone marrow (BM) histology in these patients (40,56). Pediatricians are reluctant to perform BM biopsy in the absence of a defined malignancy in children because it requires profound sedation.

AIM

The aim of our study was to validate current WHO criteria in pediatric ET (i) exploring the incidence of driver mutations in a wide cohort of pediatric patients with ET (ii) and evaluating the BM histological features in children with a clinical diagnosis of ET.

MATERIALS & METHODS

Our study was built in two stages: (i) a biomolecular study aimed to evaluate the prevalence of driver mutations in the pediatric population with a clinical diagnosis of ET and (ii) a histological study for the evaluation of BM features in pediatric ET in comparison both to adults affected by ET, strictly diagnosed in agreement with WHO criteria, and to pediatric ad-hoc collected controls.

The Ethical Committee of Padua Hospital approved the study, an informed consent was obtained from each patient and controls. Children's parents gave also their informed consent for their babies. The procedures adopted for the study were according to the Helsinki Declaration.

Biomolecular Study

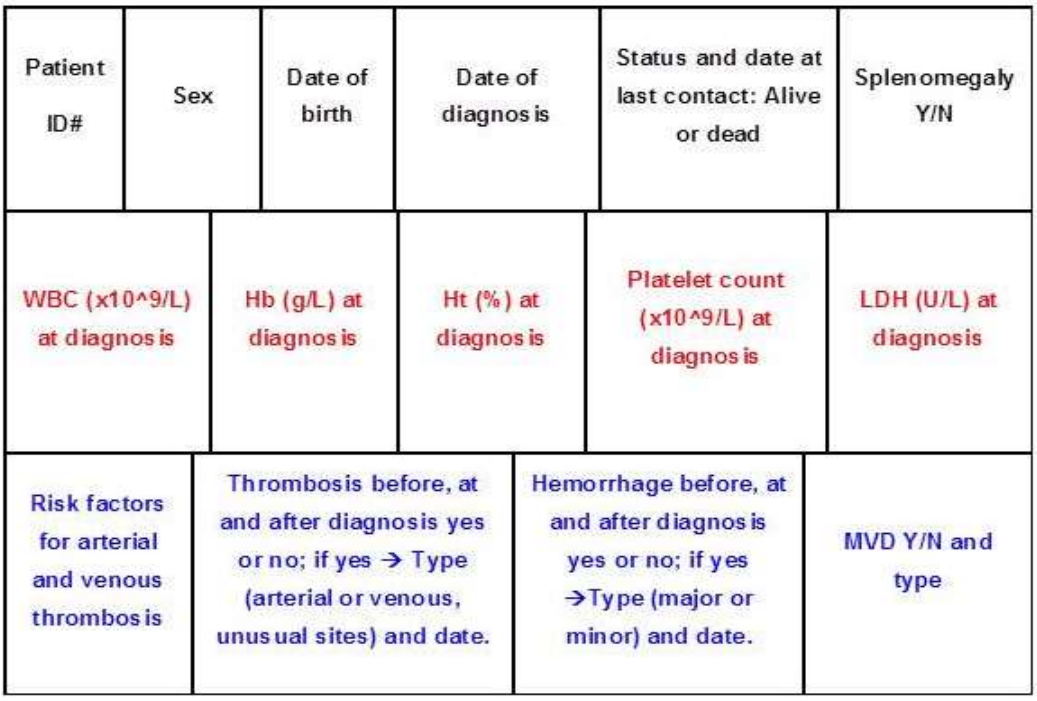
We collected 89 children (58 females and 31 males) with a clinical diagnosis of ET established at one of the 14 tertiary hematology centers linked to the Italian Pediatric Hemato-Oncology Association (AIEOP), which were consecutively referred to our central laboratory in Padua for bio-molecular studies, who were younger than 18 years old at the time of their diagnosis of ET (median age 8,7 years, range 6 months to 16,5 years), with a median follow-up of 6,3 years (range 1–19 years). All the children had a sustained increase in platelet count (>450 x10^9/L) lasting at least 12 months, with no demonstrable reactive or secondary cause (i.e. iron deficiency, acute or chronic inflammation, autoimmune diseases, Kawasaki syndrome, asplenia, neoplastic diseases or any medication), and no familial history of MPN or thrombocytosis. None of the children met the WHO criteria for other myeloid neoplasms.

The research of mutations was conducted on genomic DNA extracted from granulocytes of peripheral blood sample, separated through Ficoll-Paque Plus solution. (42)

The *JAK2*V617F mutation was genotyped using allele-specific polymerase chain reaction (PCR) and the mutant allele burden was measured by quantitative real-time PCR (42,49). The *MPL* and *CALR* mutations were sought by direct sequencing (27,57). The X-chromosome inactivation pattern (X-CIP) in granulocytes was studied in females using the human androgen receptor gene (AR, also termed HUMARA) polymorphism method, as reported in literature (42).

An ad-hoc spreadsheet was built to collect several parameters at diagnosis and during follow-up, including main clinical characteristics, blood counts and vascular events (Figure 7).

Figure 7. Collected data.



Histological Study

For the histological study, we collected all the treatment-naïve BM specimens (prepared in hematoxylin-eosin staining and silver impregnation after Gomori or Gordon-Sweet) of children with a clinical diagnosis of ET (PedET; 7 females and 13 males) available in AIEOP centers. Almost all of them are also included in the biomolecular study. The diagnosis of ET (PedET) was performed by a Pediatrician expert in hematology and based on the WHO criteria. The BM biopsies were initially analyzed by local pathologists, therefore centralized and then reviewed by two expert pathologists according to WHO histologic criteria, first separately and then jointly.

Assessed parameters were:

1. Cellularity (defined as ratio between hematopoietic marrow and adipose tissue)
2. Myeloid-to-erythroid ratio
3. Myeloid and erythroid maturation
4. Megakaryocyte (MK) number and morphology
5. Presence of loose or tight MK clusters
6. MK density (MKD = number of MK per square millimeter of BM)
7. Grade of fibrosis

The pathologists were blinded to each child's clinical and molecular diagnosis.

As controls we evaluated BM specimens of:

- 6 children (PedST; 4 females and 2 males) with reactive/secondary thrombocytosis: 5 had lymphomas and BM biopsy was performed as part of the staging process; one had a prolonged thrombocytosis >650 x10^9/L that resolved

spontaneously after 1 year. All tested cases were negative for *JAK2*, *MPL* or *CALR* mutations.

- 18 children (Norm; 5 females and 13 males) with a normal BM histology, performed for the staging of solid tumors; none of them had splenomegaly and all had normal platelets number.
- 36 adults (AdsET, 26 females and 10 males) with ET diagnosed strictly according to the WHO criteria.

Main clinical characteristic of patients and controls enrolled in the histological study are resumed in Table V.

Table V. Characteristics of patients and controls.

	PedET n=20	PedST n=6	AdsET n=36	Norm n=18
Median age at diagnosis/ BM Biopsy (percentile 5-95th)	10 (5.1 – 16.1)	7.8 (2.6 – 10.7)	51.8 (28.7 – 79.8)	10.5 (2 – 16)
Males, n (%)	13 (65)	2 (33.3)	10 (27.8)	13 (72.2)
Hb, g/L Median (percentile 5-95th)	132 (113 – 141)	113 (73 – 125)	135 (109 – 155)	129 (106 – 160)
Ht, % Median (percentile 5-95th)	40 (37.4 – 43.3)	37 (23.3 – 38.8)	41.4 (34.4 – 47.8)	39.1 (30 – 47.5)
WBC, x 10^9/L Median (percentile 5-95th)	8.2 (5.5 – 11.8)	9.1 (7 – 10.6)	7.9 (4.7 – 11.1)	7.6 (4 – 15.5)
Plts, x 10^9/L Median (percentile 5-95th)	1254 (528 – 1824)	502 (478 – 657)	681 (487 – 1117)	296 (195 – 375)

Statistical analysis

All statistical analyses were performed on the parameters recorded at the time of a patient's diagnosis. Nominal variables were compared with the X^2 or Fisher's exact tests, as appropriate. Continuous variables were compared with the T-test or the Mann-Whitney U test when indicate. A two-tailed P value of less than 0.05 was considered significant. All statistical analyses were performed using the SPSS (Statsoft Inc., Tulsa, OK, USA) software v.23.

RESULTS

Biomolecular Study

In this study we evaluate 89 subjects.

Out of the 89 evaluated *JAK2*V617F mutation was found in 14 children (15,7%), with an allele burden of 26,26 ± 9,78%, the *MPL*W515L mutation in 1 and *CALR* mutations in 6 (8%; 4 had *CALR* type 1 and 2 had *CALR* type 2) cases. None of the patients had more than one mutation. In contrast, 68 children (76,4%) were triple negative (3NEG). Clonality assays were available for 21 girls: 6 (28,5%) were found monoclonal, and 4 of them carried *JAK2*V617F or *CALR* mutations, while 2 were 3NEG (Figure 8). The main clinical findings divided by clonal and non-clonal patients are summarized in Table VI.

Figure 8. Molecular findings. Among *CALR* mutated patients 4 were type 1 and 2 were type 2.

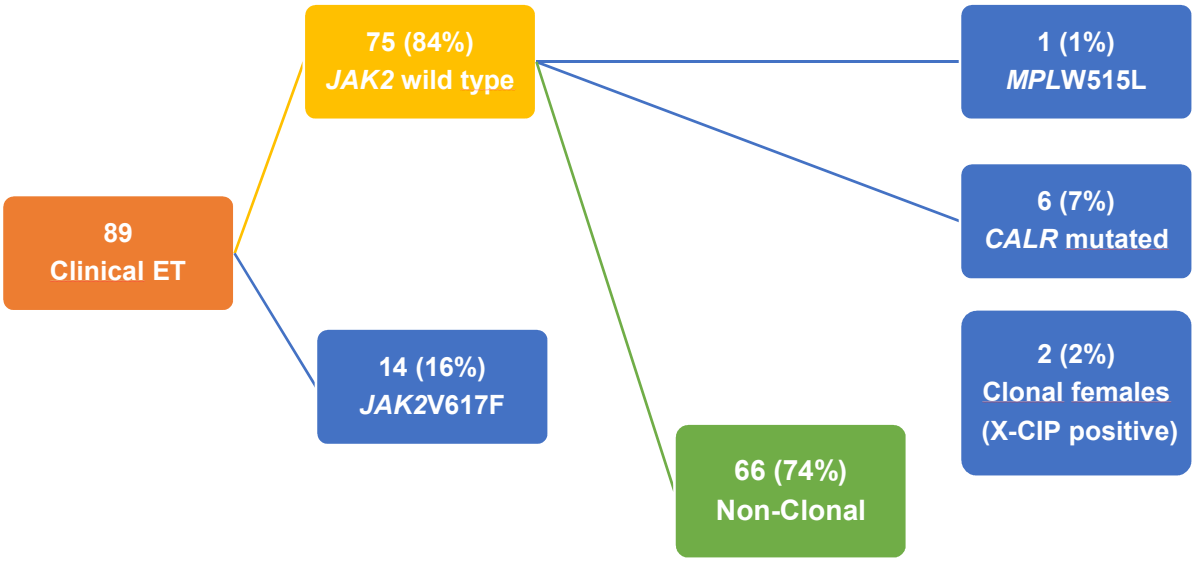


Table VI. Main clinical and laboratory characteristics at diagnosis of the 89 patients divided by clonal and non-clonal.

	Clonal	Non Clonal	P
Patients, n (%)	23 (25,8)	66 (74,2)	
Males/females	8/15	23/43	NS
Median age, years (range)	10,25 (0,5-17,5)	8,3 (0,5-16,5)	NS
Age cut-off			NS
<11 years, n	14	46	-
<15 years, n	20	59	-
15–18 years, n	3	7	-
Median follow-up, years (range)	7,55 (1-11,5)	6,14 (1-20)	NS
Driver mutations			-
*JAK2*V617F, n	14	0	-
*JAK2*V617F Allele burden, % (mean ± SD)	26,26 ± 9,78	-	-
*MPL*W515L, n (56 tested)	1	0	-
CALR mutations, n (74 testes)	6	0	-
X-CIP positive (23 females tested)	6 (2 *JAK2*V617F; 2 *CALR*)	0	-

Table VI (continue). Main clinical and laboratory characteristics of the 89 patients divided by clonal and non-clonal.

	Clonal	Non Clonal	P
Major thrombotic events	3 (13) 2 Budd Chiari Syndrome 1 Cerebral vein Thrombosis	0	0.03
Minor bleeding episodes	3 (13)	5 (7,5)	NS
Patients with microvascular disturbances	8 (34,7)	19 (28,8)	NS
Headache	5	18	-
Paraesthesia	2	7	-
Erythromelalgia	2	1	-
Palpatory splenomegaly, n (%)	9 (39,1)	17 (25,7)	NS
Median platelet count, x10^9/L (range)	1192 (512-3200)	1239 (503-4440)	NS
Median WBC count, x10^9/L (range)	8,94 (5,5-11,8)	9,02 (5,25-19)	NS
Mean Hb level, g/L (range)	138 (105-160)	125 (80-148)	NS

Three different age groups were analyzed: younger children (less than 11 years old), young adolescents (up to 15 years old) and older adolescents (15–18 years old). No differences of the distribution of clonal versus non-clonal cases emerged when we divided the children in three groups of age (Table VI).

Main clinical and laboratory findings of patients with a clonal marker are summarized in Table VII.

Table VII. characteristics of the patients grouped by molecular marker.

	*JAK2*V716F	*MPL*	*CALR*	Monoclonal
Patients, n	14	1	6	2
Males/females	6/8	1/10	1/5	0/2
Median age, Years (range)	10,5 (2,5-17,5)	5,05	12,7 (7,9-13,9)	4,1 (0,5-7,5)
Median follow-up, Years (range)	8 (5-9)	3	6,2 (5,4-11,45)	6,55-9
X-CIP positive/studied	2/8	-	2/4	2/2
Major thrombotic events, n	3	0	0	0
Minor bleeding episodes, n	1	0	2	0
Patients with microvascular disturbances, n	3	1	4	0
Headache, n	3	0	2	0
Paresthesia, n	0	0	2	0
Erythromelalgia, n	0	1	1	0
Splenomegaly Y/N	6/4	0/1	2/4	1/1
Median platelet count, x10^9/L (range)	1042 (512-1710)	1444	1424 (748-3200)	1223-1553
Median WBC count, x10^9/L (range)	9,1 (5,5-11,8)	9,6	10,1 (6,6-16,5)	6,9-7,5
Mean Hb level, g/L (range)	137 (111-157)	113	122 (105-160)	125-144

No statistically significant difference was seen comparing sex distribution, white blood cells and platelets count between *JAK2* positive and *CALR* positive patients (p =NS). Hemoglobin levels show a tendency, not statistically significant, to be higher in *JAK2* compared to *CALR* positive patients (p=0.054).

Three children presented with major thromboses (two Budd–Chiari syndromes and one cerebral vein thrombosis) and they all carried the *JAK2*V617F mutation. Minor hemorrhages occurred in one child with the *JAK2*V617F mutation and in two with a *CALR* mutation. Three *JAK2*-mutated, four *CALR*-mutated patients and the one with the *MPL* mutation had microvascular symptoms, mainly headache (Table VII).

Data on the administered treatments were available for 19 clonal and 51 non-clonal cases (Table VIII).

Table VIII. Treatment administration in clonal and non-clonal cases.

	Clonal (N=23)	Non-Clonal (N=66)
Patients with available data	19	51
No treatment	3 (15,8%)	12 (23,5%)
Aspirin	11 (57,9%)	32 (62,7%)
LMWH/Warfarin	3 (15,8%)	0
Cytoreductive drugs	13 (68,4%)	20 (39,2%)
ANA	6	8
IFN	0	2
HC	4	4
Multiple cytoreductive drugs*	3	6

LMWH, low molecular weight heparin; IFN, interferon alpha; HC, hydroxycarbamide; ANA, anagrelide.
*Six patients were given ANA + HC, one ANA + IFN and two were given HC + ANA + IFN.

Antiplatelet and anticoagulant drugs were commonly used in combination with cytoreductive treatments. In the clonal group, 3 children (15,8%) received no treatment, 11 were given low-dose aspirin (acetylsalicylic acid, ASA), 3 anticoagulant therapy, and 13 (68,8%) received cytoreductive drugs [hydroxycarbamide (HC) in 36,4%, anagrelide (ANA) in 47,3% and interferon-alpha (IFN) in 5,2%]. Three of these 13 patients (15,8%) received multiple cytoreductive drugs (2 ANA plus HC; 1 ANA plus IFN and HC). Twelve (23,5%) patients in the non-clonal group were never treated while 32 (62,7%) received ASA, 20 (39,2%) cytoreductive drugs (8 ANA, 4 HC, 2 IFN), and 6 (11,7%) had multiple cytoreductive drugs. (Table VIII).

Six children had persistently high platelet counts (over 1000 x10^9/L) for many years: four of them received low dose ASA, two were given IFN (for 1 and 2 years), and one girl was treated for less than a year with ANA. Surprisingly, the platelet counts in these patients became normal over the course of a long-term follow-up (median 15 years, range 5– 20) without cytoreductive drugs.

Histological Study

The main clinical and laboratory data of the children with ET or ST and of adults with ET are summarized in Table IX.

Table IX. Clinical and laboratory features of children with ET (PedET), adults with ET (AdsET), children with secondary/reactive thrombocytosis (PedST) and comparison between PedET and AdsET.

	PedET	AdsET	P PedET vs AdsET	PedST
Nr. of patients	20	36	-	6
Median age at diagnosis, Y (5^{th}-95^{th} percentile)	10 (5.1 – 16.1)	51.8 (28.7 – 79.8)	-	7.8 (2.6 – 10.7)
Median follow-up, Y (5^{th}-95^{th} percentile)	4.6 (0.5 – 10.9)	10 (0.5 – 19.3)	-	-
Splenomegaly, n (%)	11 (55)	7 (19.4)	0.017	1 (16.7)
MVD, n (%)	8 (40)	8 (22.2)	NS	1 (16.7)
Abdominal pain, n (%)	4 (20)	0 (0)	0.005	0 (0)
Thrombosis, n (%)	3 (15)	8 (22.2)	NS	0 (0)
Hemorrhage, n (%)	2 (10)	7 (19.4)	NS	0 (0)
Median Hb, g/L (5^{th}-95^{th} percentile)	132 (113 – 141)	135 (109 – 155)	NS	113 (73 – 125)
Median Ht, % (5^{th}-95^{th} percentile)	40 (37.4 – 43.3)	41.4 (34.4 – 47.8)	NS	37 (23.3 – 38.8)
Median WBC, x 10^9/L (5^{th}-95^{th} percentile)	8.2 (5.5 – 11.8)	7.9 (4.7 – 11.1)	NS	9.1 (7 – 10.6)
Median Plts, x 10^9/L (5^{th}-95^{th} percentile)	1254 (528 – 1824)	681 (487 – 1117)	0.001	502 (478 – 657)
*JAK2*V627F, n (%)	5 (25)	14 (38.9)	NS	0 (0)
CALR, n (%)	2 (10)	9 (25)	NS	0 (0)
*MPL*W515L, n (%)	1 (5)	2 (5.5)	NS	0 (0)
3NEG, n (%)	12 (60)	11 (30.5)	NS	6 (100)

Splenomegaly was found more often in children than in adults (PedET vs AdsET p=0.017), and abdominal pain was only reported in pediatric age (PedET vs AdsET p=0.005). No significant differences were found between the PedET and AdsET cases in terms of MVD, major thrombosis or hemorrhage. Median platelet count differed significantly, being higher in children than in adults (PedET vs AdsET p=0.001; Table IX).

Among children with ET, *JAK2*V617F mutation was detectable in 25% of cases, 10% carried a *CALR* mutation (one type 1 and one type 2), 1 was *MPL* positive and the remaining 60% was 3NEG (Figure 9). Comparison between histological features of children with ET and controls are summarized in Table X.

Figure 9. Distribution of MPN driver mutations in children with ET.

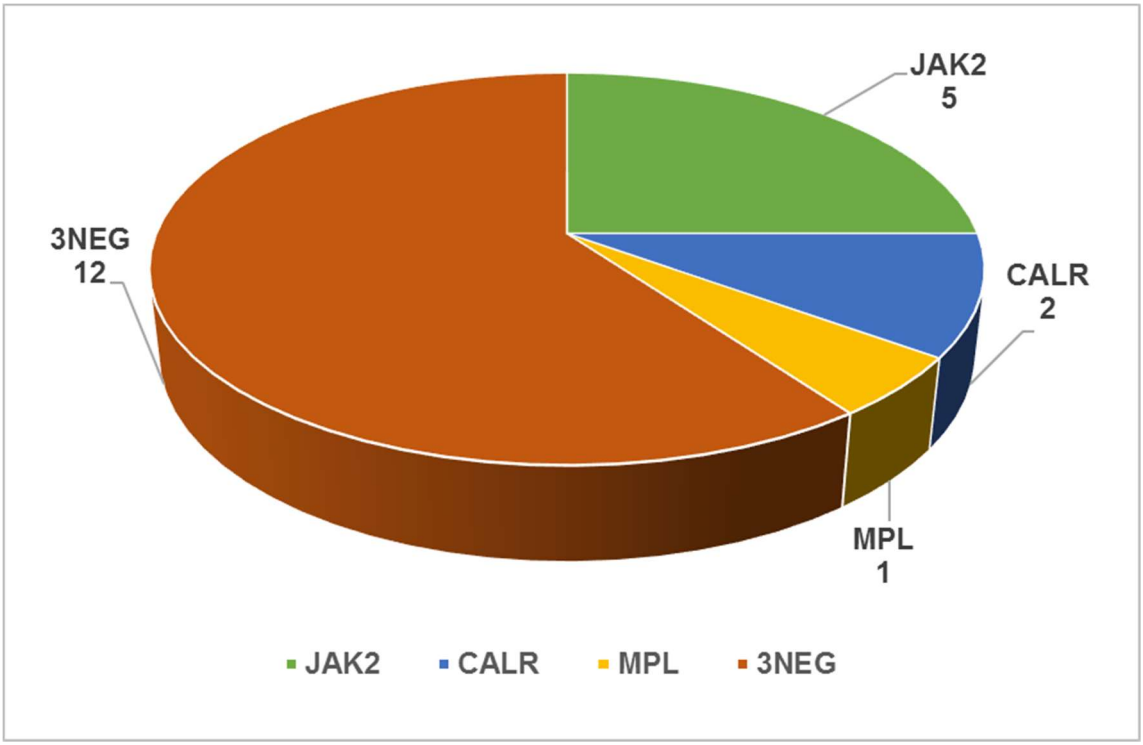


Table X. Comparison between histological findings of study groups.

	PedET (A) n=20	PedST (B) n=6	P A vs B	AdsET (C) n=36	P A vs C	Norm (D) n=18	P A vs D
Cellularity, % Median (percentile 5-95th)	80 (35 – 95)	80 (50-80)	ns	35 (20 – 80)	<0.001	65 (50-90)	ns
MK morphologic alterations, n (%)	17 (85)	0 (0%)	<0.001	36 (100%)	ns	0 (0%)	<0.001
MKD, MK/mm^2 Median (percentile 5-95th)	37.5 (10.5 – 107.1)	9.2 (5.6-13.6)	<0.001	18.3 (9.4 – 55.2)	<0.001	10.4 (2.2-14.0)	<0.001
MKD/cellularity, Median (percentile 5-95th)	46.6 (12.7-133.9)	15.6 (11.2-19.4)	<0.001	45.8 (15.2-105.4)	ns	15.0 (3.1-22.3)	<0.001
Presence of MK clusters, n (%)	20 (100)	0	<0.001	36 (100)	ns	0	<0.001
Presence of dense MK clusters, n (%)	3	0		0		6	
Presence of BM fibrosis, n (%)	6 (30)	0	ns	6 (16.7)	ns	3 (17%)	ns

MK= megakaryocytes; MKD= megakaryocytes density

Cellularity was higher in all pediatric cases than in adults ($p<0.001$). Children with PedET were characterized by a higher MKD (37.5 MK/mm2) than those with PedST (9.2 MK/mm2) ($p<0.001$), and by MK clusters (100%) and BM fibrosis (30%), which were substantially absent both in PedST and in Norm.

All relevant pathological features of the BM biopsies were similar in PedET and AdsET. MKD was higher in PedET than in adult cases, but this difference disappeared when MK were normalized for BM cellularity.

Table XI contains a detailed analysis of the 20 PedET cases.

Three *JAK2*V617F (1M, 3F and 16F) children had a major thrombotic event (2 transient ischemic attacks and 1 Budd-Chiari syndrome). One child had BM features suggestive of PV, and one of PMF-1. All the other children with mutations exhibited a BM picture of true ET.

Among the 3NEG children, two had a histology suggesting pre-PMF, with cloud-like MK in tight clusters but no BM fibrosis. In 10 other children, the histological pattern was consistent with ET, in agreement with the clinical picture. One 3NEG child had BM features of ST.

Table XI. Detailed analysis of the 20 PedET cases.

Patient	Clinical	Molecular	Histology
1M	TIA, headache, minor hemorrhage, occasional increase in HB and HT, splenomegaly	*JAK2*	PV/ET
2F	Headache, erythromelalgia, minor hemorrhage, splenomegaly	*CALR type 1*	ET
3F	Budd Chiari, portal thrombosis, OLT, splenomegaly	*JAK2*	PMF-1
4M	Asymptomatic, splenomegaly	*CALR type 2*	ET
5M	Erythromelalgia	*MPL*	ET
6M	Headache, splenomegaly	*JAK2*	ET
7M	Headache, abdominal pain, splenomegaly	3NEG	ET
8F	Headache, abdominal pain, splenomegaly	3NEG	ET
9M	Asymptomatic, splenomegaly	3NEG	Pre-PMF
10F	Headache, occasional increase in hemoglobin and hematocrit	3NEG	ET
11M	Headache, abdominal pain	3NEG	ET
12F	Asymptomatic, splenomegaly	3NEG	ET
13M	Abdominal pain	3NEG	ET
14F	Asymptomatic	3NEG	ET
15M	Asymptomatic	3NEG	ET
16F	TIA, splenomegaly	*JAK2*	ET
17F	Asymptomatic, splenomegaly	3NEG	ST
18M	Asymptomatic	3NEG	Pre-PMF
19M	Asymptomatic, splenomegaly	*JAK2*	ET
20M	Asymptomatic	3NEG	ET

DISCUSSION

Myeloproliferative Neoplasms (MPN) are clonal disorders of the hematopoietic stem cells. MPN, in the most recent WHO classification, are those not carrying BCR/ABL1 rearrangement (Philadelphia-negative) and are divided in classic (PV, ET, PMF) and rare (Chronic neutrophilic leukemia, Chronic eosinophilic leukemia not otherwise specified, MPN unclassifiable and Mastocytosis) (3,58) The discovery of *JAK2*V617F mutation, detectable in 90% of PV and about two-thirds of ET and PMF patients, led to the knowledge of a new relevant feature in diagnosis of MPN. However, the presence of the mutation alone cannot identify a specific MPN (59). This is true also for the more recent described mutations in the *MPL* and *CALR* genes (20,21,27) that are found both in ET and in PMF patients. Therefore, histological features of the BM are now considered necessary to distinguish PV, ET and PMF, mainly in patients with asymptomatic thrombocytosis without a specific clinical-biochemical profile (13). Today, a correct evaluation of the BM histology and morphology is a cornerstone in the diagnostic tool of patients with ET, because it can discriminate between pre-PMF and "true" ET. In the new WHO classification ET and pre-PMF are reported as two distinct MPN (34) because, in spite of an undistinguishable clinical phenotype, the prognosis of the two forms is different (13).

The main clinical features of patients with MPN are thrombotic and hemorrhagic complications (60-64). Thromboses represent the major cause of morbidity and mortality but it must be taken into account that progression into myelofibrosis or transformation in acute leukemia occur respectively in 1.6 per 1000 pats/y and 1.2 per 1000 pats/y in ET and both in 5 per 1000 pats/y in PV (65,66). These transformations may occur as a natural evolution of the disease or may be due to the use of cytoreductive drugs (67-70).

Classical MPN are diseases typical of the median-advanced age, while they are uncommon in young adults and even more rare in adolescents and children (36,71), with an estimated annual incidence of newly-diagnosed cases around 1 per 10 million of children younger than 14 years (38). In contrast, thrombocytosis is a common finding in children (37), given that inflammatory diseases (i.e. upper respiratory inflammation, allergy, exanthematous diseases) are usual in pediatric age, both in hospital and in the outpatient setting (72). Such cases of reactive/secondary thrombocytosis (ST) relate to increased levels of cytokines, especially IL6 (37), and represent an inflammatory reaction carrying no thrombotic or hemorrhagic risk, destined to return to the normal spontaneously. Rare hereditary thrombocytoses have been described (57,73) as well.

When a child shows prolonged and sustained thrombocytosis, this raises the question of whether it is a sign of chronic inflammation or an ET (74).

Interestingly, pediatricians are rather reluctant to perform a complete workup for the diagnosis of ET comprehending BM biopsy, while they have extensive used cytoreductive drugs to obtain a normal platelet count (74-76). Therefore, BM biopsy data in pediatric population with MPN are poor (40,56).

Considering that most children with ET do not reveal clonal markers and that pediatric ET seem to have different biological characteristics from adults (41), even in the absence of histological BM data, the WHO criteria have been considered inadequate in children (45).

In our biomolecular study, in agreement with the data of the literature (37,42,75) *JAK2*V617F mutation was found in less than one in four cases, and the *MPL*W515L mutation was only identified in one patient, recently described also elsewhere (50). We

found six children carrying *CALR* mutations: four were type 1 and two were type 2 (33). While a recent small study (55) detected no *CALR* mutations in six children with ET, other authors (54) found eight *CALR*-mutated children in their monocentric series of 34 children. Given the relatively low sensitivity of sequencing for mutation detection purposes, low-level *MPL* and *CALR* mutants may go undetected (77, 41). While adult patients with *JAK2*V617F mutation are reportedly to be older, with a male prevalence, higher hemoglobin levels and white cell counts, and lower platelet counts than patients with *CALR* mutations (78), we found no such differences among our children.

Major venous thromboses at unusual sites (involving the splanchnic veins) occurred in two children in the present cohort, all carrying *JAK2*V617F mutation (74). In the literature, among adults with *JAK2*V617F mutation, higher rates of venous thrombosis at uncommon sites have been reported in large cohorts (79,80). The risk of major thrombotic events has been described as low for *CALR* mutated adults (27,81-83), while our *CALR*-mutated children suffered more often from mild bleeding and minor microvascular disturbances than their *JAK2*V617F-positive counterparts (though the difference was not statistically significant). No other significant differences were found when we compared patients with different mutations.

On the whole, 23 of the 89 patients reported in the biomolecular study had clonal disease, while the other 66 had persistent thrombocytosis with no markers of clonality. No significant differences in hematological and biological features has been demonstrated when we compared clonal versus non-clonal cases. The proportions of cases with clonal versus non-clonal disease were substantially the same for children and adolescents (84).

Considering the mutated cases, the relative proportions of the three known ET-specific mutations in our sample (*JAK2* vs. *MPL* vs. *CALR*: 60% vs. 4,3% vs. 26%) is like the

proportions found in adults (27,82), so pediatric clonal ET is presumably the same as the disease encountered in adults.

Children with no clonal markers accounted for a large proportion of our sample. We observed a remarkably high difference of prevalence of non-clonal cases in our cohort in comparison to adult reports (74% vs. 10–15%) (78), and this did not vary when children were compared with adolescents (55).

It is worth noting that we observed a "spontaneous remission" in two children with non-reactive sustained thrombocytosis (about 2000 x10^9/L) over a period of more than 15 years: they were both 3NEG cases and X-CIP-negative. Two of such cases had already been reported in the literature (85). In our series, four other girls with a non-clonal pattern have been followed up for more than 10 years now, and have not developed neither new mutations nor a monoclonal pattern. These observations may suggest that in some cases at least, a different non-clonal mechanism may underlie to megakaryocyte proliferation and excessive platelet production posing many questions also about the correct therapeutic approach (86,75).

Considering that, while adult's triple negative cases are still considered MPN because of a complete histologic study that fulfills the WHO criteria (34), our non-clonal children, in the absence of a BM histology, actually should not be definitively assumed to be MPN cases, even if presenting with a clinical picture suggestive of MPN (minor bleeding episodes, microvascular disturbances and splenomegaly).

Therefore, in the second phase of the present work, we explore the histology of BM in children with a clinical diagnosis of ET to evaluate, also in this setting of patients, the well-known histologic characteristics, stated in the WHO criteria to perform a diagnosis

of MPN (10,34). We addressed this issue in 20 children whose BM biopsy performed at diagnosis were available in our collaborating centers. At present, the available data about BM histology in children with MPN are very limited. Giona et al (40) described 5 BM biopsies from children with primary familial thrombocythemia, which revealed histological features no different from those of children with sporadic thrombocythemia. Fu et al (41) reported 63 children with ET whose BM biopsy findings were "consistent with ET". A detailed description of pediatric BM histology that meets Thiele's requirements (10) has only been done by Roy (56) in a single patient, making our 20 children cohort the largest with a complete depiction of BM features.

Among our 20 children with clinical ET, our centralized histologic review identified 19 BM morphologically consistent with a myeloproliferative neoplasm, corresponding to 15 "true" ET, 1 PMF-1, 2 pre-PMF and 1 case more suggestive for PV; 1 child with 3NEG status had a histological picture of ST. In contrast, all 6 children with ST showed only a mild increase in MK number, in keeping with Thiele's observation in adults with a secondary/reactive increase in platelet count (10).

In this subset of patients, we found also a low incidence of driver mutations and about two-thirds of our children with clinical ET does not reveal a clonal marker, while the mutated cases, although few, seem to respect the known proportion of *JAK2*, *CALR* and *MPL* mutations (74). All but one of our 3NEG children had both clinical and histological features of ET. Irrespective of their mutational status, the children with a histologically confirmed diagnosis of ET had a picture similar to that of adult ET, although with a higher cellularity. Megakaryocytes density (MKD) (87) was higher in pediatric ET too, but when the MK number was normalized to cellularity (MKD/cellularity), we found a similar ratio

in children and adults. None pediatric control (PedST and Norm) displays MK abnormalities or MK clusters.

The BM of one *JAK2*V617F boy was suggestive of PV. It has been recently described in adults carrying *JAK2*V617F mutation, a "new" nosological entity called "masked PV" (88), characterized by a mild increase of hemoglobin and hematocrit not meeting the required levels of WHO criteria for PV (39). Masked PV has higher risk of thrombosis (89) compared both to ET and to overt PV, it is more common in young males and can be associated with very high platelet count and mild marrow fibrosis (88). The sum of clinical and histological features seen in our case points to masked PV because the patient had an increase in the number of erythroid cells in the BM, a transient increase in hemoglobin and hematocrit (> 95th percentile for age), and a transient ischemic attack.

One girl with *JAK2*V617F mutations, reported also in the biomolecular study, had Budd Chiari syndrome. She had a histological picture of PMF-1, with an increased number of MK with cloud-like nuclei, often arranged in tight clusters, and grade 1 fibrosis. Her clinical course was complicated, resembling PMF, and she underwent liver transplantation for hepatopulmonary syndrome, portal thrombosis, progressive splenomegaly and low peripheral cells counts. It is worth noting that the first BM biopsy was performed after12 years of follow-up. It is therefore not clear whether the histological findings are attributable to the primary MF or to an evolution of the ET. It is also worth emphasizing the evidence of other two 3NEG patients with a histologic picture of pre-PMF, which supports the impression that PMF can occur in pediatric age, albeit very rarely (90).

In one 3NEG child, our histology review identified features more suggestive of ST than of ET, reinforcing the hypothesis that several children with sustained and prolonged

thrombocytosis may not have a primitive disease (74). One PedST case was initially diagnosed clinically as ET, but his thrombocytosis resolved spontaneously within a year. His BM showed a histological picture of ST. On the whole, these experiences underline, in suspected ET cases, the importance of BM biopsies evaluation in MPN expert centers (91) taking in mind that not all pathologists agree with the reproducibility of Thiele's criteria (Wilkins).

The therapeutic goal for ET patients is to avoid the occurrence of major vascular events while minimizing the side effects induced by medicine. Consensus regarding the management of adult ET is a risk-adapted strategy (92). Low-dose aspirin is recommended in low risk ET patients, while in high-risk patients cytoreductive therapy should be added (93,94).

The decision to start cytoreductive therapy in children with ET requires careful thought, bearing in mind not only the patients' platelet counts but also their clinical, molecular and histological features (41,63,83), as in adults (93,94). There is insufficient evidence to guide the management of childhood ET, but a conservative approach is recommended (40,74,76,95).

In conclusion, our data clearly show that a complete workup for MPN diagnosis, comprehensive both of biomolecular and histological study, is fundamental in children with suspected MPN. Due to the low incidence of driver mutations, the complete biomolecular study is not sufficient to identify all MPN patients and cannot distinguish among different forms. Moreover, the clinical phenotype of children with non-clonal sustained thrombocytosis is identical to that of clonal ET and, even in case with long follow-up, it may not be able to discriminate between children with real 3NEG ET (78) and non-myeloproliferative patients.

Therefore, BM histology is mandatory to the diagnosis of MPN in children as in adults. A correct BM assessment can both discriminate among ET, PMF and PV, and distinguish secondary/reactive from primary thrombocytosis. An important question remains unanswered, however: how long should a 3NEG child with a high platelet count be kept under observation before performing a BM biopsy? National guidelines in Italy (unpublished data) suggest an interval of 6 to 12 months, depending on the platelet count. The present findings need to be confirmed.

It has been repeatedly claimed that pediatric MPN need tailored diagnostic criteria (45,95). However, our data clearly demonstrate that the in-use WHO criteria for MPN are suitable in all age groups, being not only adequate, but also accurate even in pediatric population.

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