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***MPL* Mutations in Myeloproliferative Disorders: Analysis of the PT-1 Cohort**

Running title: *MPL* mutations in ET and IMF

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Abstract

Activating mutations of *MPL* exon 10 have been described in a minority of patients with idiopathic myelofibrosis (IMF) or essential thrombocythemia (ET) but their prevalence and clinical significance are unclear. Here we demonstrate that *MPL* mutations outside exon 10 are uncommon in platelet cDNA and identify four different exon 10 mutations in granulocyte DNA from a retrospective cohort of 200 patients with ET or IMF. Allele-specific PCR was then used to genotype 776 samples from patients with ET entered into the PT-1 studies. *MPL* mutations were identified in 8.5% of *JAK2 V617F*-negative patients and a single *V617F*-positive patient. Patients carrying the *W515K* allele had a significantly higher allele burden than those with the *W515L* allele, suggesting a functional difference between the two variants. Compared to *V617F*-positive ET patients, those with *MPL* mutations displayed lower hemoglobin and higher platelet levels at diagnosis, higher serum erythropoietin levels, endogenous megakaryocytic but not erythroid colony growth and reduced bone marrow erythroid and overall cellularity. Compared to *V617F*-negative patients, those with *MPL* mutations were older with reduced bone marrow cellularity but could not be identified as a discrete clinico-pathological subgroup. *MPL* mutations lacked prognostic significance with respect to thrombosis, major hemorrhage, myelofibrotic transformation or survival.

Introduction

The myeloproliferative disorders, comprising essential thrombocythemia (ET), polycythemia vera (PV) and idiopathic myelofibrosis (IMF), are hematopoietic stem cell disorders characterised by the overproduction of one or more mature myeloid lineages. Although the clonal nature of these disorders was recognised some three decades ago¹, their molecular basis remained obscure until the identification of the activating *JAK2 V617F* mutation²⁻⁵. The vast majority of patients with PV harbour a mutation in *JAK2*, with *JAK2 V617F* seen in around 97%³ and mutations in *JAK2* exon 12 found in many of the remainder⁶⁻⁸. PV patients with *JAK2* exon 12 mutations differ from those with *JAK2 V617F*, presenting at a younger age with higher hemoglobin levels, lower platelet counts and lower white cell counts at diagnosis⁶. The *JAK2 V617F* mutation is present in around half of patients with ET and is associated with features resembling a ‘forme-fruste’ of PV, including increased erythropoiesis, increased granulopoiesis and increased rates of venous thrombosis compared to the *JAK2 V617F*-negative group⁹⁻¹². The *JAK2 V617F* mutation is also present in around half of patients with IMF, and is associated with higher neutrophil and platelet counts at diagnosis, reduced likelihood of transfusion dependence and probably a poorer survival compared to the *JAK2 V617F*-negative group¹³⁻¹⁵.

Mutations in the juxtamembrane region of the thrombopoietin receptor *MPL* have recently been described in IMF and as a rare occurrence in ET^{16,17}. Biochemical and cell line studies indicate an autoinhibitory role for this region, with disruption leading to receptor activation in the absence of thrombopoietin binding¹⁸. Expression of the *MPL W515L* allele resulted in cytokine-independent growth of 32D, UT7 and BaF3 cell lines, together with constitutive phosphorylation of JAK2, STAT3, STAT5, AKT and ERK¹⁶. Transplantation of mice with bone marrow expressing the *MPL W515L* allele resulted in an MPD-like disease characterised by marked thrombocytosis, splenomegaly, splenic infarction and reduced life

expectancy¹⁶. *MPL* mutations in IMF patients have been associated with lower hemoglobin levels at diagnosis and increased risk of transfusion dependence compared to both *JAK2 V617F*-positive and *JAK2 V617F*-negative patients¹⁹. Mutations in *MPL* have been reported in a small minority of patients with ET¹⁷ but their clinical significance remains unclear. Here we describe the prevalence of *MPL* mutations in a retrospective cohort of unselected patients with ET or IMF, and also report the clinical and laboratory features associated with *MPL* mutations in the large prospective Primary Thrombocythaemia 1 (PT-1) cohort.

Materials and Methods

Retrospective cohort: patients and samples

Patients aged 18 years or over, who met the Polycythaemia Vera Study Group (PVSG) criteria for either ET or IMF²⁰ were recruited from MPD clinics in Cambridge, London, Sheffield, Birmingham and Odense. Institutional and Multi-region Ethics Committee approval was obtained and the study was carried out in accordance with the principals of the Declaration of Helsinki.

Granulocytes were prepared by centrifugation of whole blood through a Ficoll density gradient, and T cells were isolated from the mononuclear-cell layer by anti-CD2 magnetic beads (DynaBeads, Invitrogen, UK). Mean purity was greater than 95% for granulocytes and 91% for T cells. Platelets were isolated from whole blood by three rounds of centrifugation at 150g for 20 minutes at room temperature, followed by negative selection of CD45⁺ cells using magnetic beads (DynaBeads, Invitrogen, UK). RNA was prepared from TRI reagent (Sigma, UK) as per the manufacturer's instructions. cDNA was synthesised using M-MLV (Invitrogen, UK) as per the manufacturer's instructions.

PT-1 study: patients and samples

Newly diagnosed and previously treated patients, aged 18 years or over, who met the Polycythaemia Vera Study Group (PVSG) criteria for ET²⁰, were recruited into one of three multicenter PT-1 studies: the Medical Research Council high-risk trial, in which high-risk patients were randomly assigned to either hydroxyurea plus aspirin or to anagrelide plus aspirin²¹; the National Cancer Research Institute intermediate risk study, a randomisation between aspirin alone or hydroxyurea plus aspirin; or the National Cancer Research Institute low-risk study, a prospective observational study of low-risk patients given aspirin alone. Patients entered a higher risk study if they developed appropriate features. The study protocol was approved by institutional ethics committees in all participating centers, and written informed consent was obtained from all patients. This study is registered at <http://isrctn.org> as #72251782 and at <http://eudract.emea.europa.eu/> as #2004-000245-38. Further information regarding the trial can be accessed at <http://www.ctsu.ox.ac.uk/projects/leuk/pt1>.

Details obtained at trial entry included diagnostic features, such as blood counts, cytogenetics and clinical complications at or preceding diagnosis. Follow-up forms were completed every year by the patient's clinician, documenting medications, blood counts and clinical events for which standard definitions were used²¹. All data were collected prospectively with >99% patients having complete follow-up. The median follow-up in this study was 36.5 months. Bone marrow trephines were independently reviewed by three hematopathologists who were aware of the patient's age and sex but unaware of *JAK2* and *MPL* status, and scored for reticulin grade on a 0–4 scale, cellularity, megakaryocyte clustering and atypical megakaryocyte nuclear morphology²². Where there was disagreement, the mean reticulin score and the mode of other scores were used.

Samples of peripheral blood were requested at trial entry from all patients, and 776 samples were received from the 1022 patients entered. Whole-blood genomic DNA was extracted commercially (Whatman International, Ely, UK) and used for genotyping and mutant allele quantitation.

Mutation detection

JAK2 V617F mutation status was determined by allele-specific PCR as previously described³. *MPL* exon 10 screening was performed by direct sequencing of PCR products from peripheral blood granulocytes. Allele-specific PCR assays were developed for the *MPL W515L*, *MPL W515K* and *MPL S505N* alleles, and the sensitivity of each assay was established by mixing experiments using normal and mutant genomic DNA.

Mutant allele quantitation

Pyrosequencing assays were established for each *MPL*-mutant allele in order to quantitate mutant allele burden. Mixing experiments using cloned PCR products were performed for each assay, and the results used to plot a dilution curve from which the mutant allele burden was read (Supplementary Figure 1). For the *MPL S505N* and *MPL W515L* alleles, the dilution curve was plotted against the allele proportion generated by the pyrosequencing software. As the software is unable to quantitate allele proportion where two or more adjacent bases are changed, *MPL W515Ki* and *MPL W515Kii* alleles were quantitated using the following formulae:

$$MPL\ W515Ki = \frac{\frac{A}{2}}{\frac{A}{2} + T} \qquad MPL\ W515Kii = \frac{\frac{A}{3}}{\frac{A}{3} + T}$$

where A and T are the pyrosequencing peak heights for the mutant and wild-type base respectively (Supplementary Figure 1).

Statistical analysis

Due to the large number of hypothesis tests performed in the paper, we elected to use a threshold of $p < 0.01$ to define statistical significance, thus reducing the risk of false positive results. Pairwise univariate analyses comparing diagnostic variables between the *MPL*-mutant and either *JAK2 V617F*-positive or *JAK2 V617F*-negative groups were performed using the *t* test for continuous variables, Fisher's exact test for 2×2 tables, and Cochran-Armitage test for trend with exact *p* values for ordinal variables. Multivariate analyses for the associations of ordinal variables with mutation status, accounting for the effects of age, were performed using proportional odds logistic regression. Complication rates during follow-up were assessed using Kaplan-Meier life tables and log-rank analyses. Confidence intervals for odds ratios of complications in the year prior to diagnosis were calculated using the asymptotic formula $1/a + 1/b + 1/c + 1/d$ to estimate the variance of the $\log(\text{OR})$. Multivariate survival analyses were performed using Cox proportional hazards models. *S-*plus v7.0 (Insightful Corp, Seattle, WA) was used for all statistical analyses, apart from the exact methods, for which SAS v9.0 (SAS, Cary, NC) was used.

Colony analysis

For erythroid colony analysis, mononuclear cells were plated at 1×10^5 cells/ml in methocult (H4531, Stem Cell Technologies, Canada) in the presence or absence of erythropoietin (2u/ml). For megakaryocyte colony analysis, mononuclear cells were plated at 3×10^5 /ml in Megacult (Stem Cell Technologies) in the presence or absence of cytokines (thrombopoietin 50ng/ml, IL-3 10ng/ml and IL-11 50ng/ml). Cultures were incubated for 14 days at 37°C, high humidity and 5% CO₂. Individual erythroid colonies were placed in water and heated to

95°C for 8 minutes for DNA preparation. Single colonies were genotyped for the *MPL* *W515L* mutation by either pyrosequencing or direct sequencing. Colonies were classified as heterozygous if equal amounts of wild-type and mutant alleles were seen, and as wild-type or homozygous if only the wild-type or mutant allele was present respectively. Whole megakaryocyte cultures were dehydrated and stained for GpIIb/IIIa expression following the manufacturer's instructions (Stem Cell Technologies, Canada).

Results

***MPL* mutations outside exon 10 are uncommon in ET patients negative for the *JAK2* *V617F* mutation**

To investigate the possibility that *JAK2* *V617F*-negative patients might harbour *MPL* mutations other than those previously described^{16,17}, we sequenced the entire *MPL* coding region in 18 patients with ET and 2 patients with IMF. As previous studies have indicated that *JAK2* *V617F*-negative ET patients have a more isolated megakaryocyte proliferation⁹, platelet-derived cDNA was used for mutation screening. One patient with ET carried a mutation in *MPL* exon 10 (*MPL* *W515L*); all other base changes seen were previously reported single nucleotide polymorphisms (data not shown). These results indicate that mutations outside *MPL* exon 10 are not common in *V617F*-negative ET patients, although our data do not exclude the existence of such mutations in a small minority of patients. In order to identify different types of mutations within exon 10, we proceeded to sequence *MPL* exon 10 in granulocyte DNA from 200 patients. Of these, 88 had ET (47 *V617F*-positive and 41 *V617F*-negative) and 112 had IMF (57 *V617F*-positive and 55 *V617F*-negative). Mutations were identified in 11 patients. Two carried *MPL* *S505N* mutations (1 ET and 1 IMF), seven carried *MPL* *W515L* mutations (2 ET and 5 IMF) and two carried *MPL* *W515K* mutations (both IMF; one *W515Ki* and one *W515Kii*). In this cohort, the prevalence of *MPL* mutations was 3.4% in ET and 7.1% in IMF. None of the *MPL*-mutant

patients in this cohort had a coexisting *JAK2 V617F* mutation. Two different mutations resulting in the same *MPL W515K* amino acid substitution were observed (*MPL W515Ki* and *MPL W515Kii* respectively, Figure 1A). The *MPL S505N* mutation has been described as an inherited mutation in a Japanese pedigree with familial thrombocythemia²³. However, in our ET patient, the majority of T cells (Figure 1A) and buccal cells (data not shown) did not carry the mutation, strongly suggesting that it was acquired in this individual.

Development of assays for the detection and quantitation of *MPL* exon 10 mutations

In order to investigate the clinical significance of *MPL* exon 10 mutations in patients with ET, we wished to assess the prevalence of these mutations in samples from patients entered into the PT-1 studies, for whom comprehensive diagnostic and prospectively acquired follow-up data is available. However, these samples were from unfractionated whole blood. Since *JAK2* or *MPL* mutations are absent from or found at a low level in lymphocytes, and since a variable proportion of the granulocytes were also likely to be normal, it was important to develop sensitive assays for each mutant *MPL* allele. We therefore developed three separate allele-specific PCR assays to detect the *MPL S505N*, *MPL W515L* and *MPL W515K* alleles, with the *W515K* assay being able to detect both the *W515Ki* and *W515Kii* mutations (Figure 1B). To assess the sensitivity of the allele-specific PCR assays, we quantitated mutant allele burden in patient samples by pyrosequencing (see Materials and Methods and Supp Figure 1). Allele-specific PCR was then performed on dilutions corresponding to 1-9% mutant allele burden. As shown in figure 1C, the assays for *MPL S505N* and *MPL W515L* could detect a mutant allele burden of ~1%, whereas the assay for *MPL W515Ki* and *MPL W515Kii* could detect a mutant allele burden of ~3-5%.

Clinical and laboratory features of *MPL* mutations in ET

Patients in the PT-1 cohort (n=776) were genotyped for the *MPL S505N*, *MPL W515L* and *MPL W515K* mutations using allele-specific PCR. *MPL* mutations were detected in 32 patients, accounting for 4.1% of all ET patients (95% CI 2.9%-5.8%, Table 1) and 8.5% of *JAK2 V617F*-negative ET patients. *MPL W515L* was the most common mutation, seen in 24 patients; 5 patients had *MPL W515K* (all *MPL W515Ki* allele) and 3 patients had *MPL S505N* mutations. In all patients with *MPL W515K* or *MPL S505N* alleles, the presence of the mutation was confirmed by both direct sequencing and pyrosequencing. Of the 24 *MPL W515L* mutations, 19 were detected by both direct sequencing and pyrosequencing, 3 were detected by pyrosequencing but not direct sequencing, and 2 were detected by allele-specific PCR alone. In these 2 patients, the presence of the mutation was confirmed by repeating the allele-specific PCR using an independent blood sample. Of the 32 *MPL*-mutant patients, one also carried the *JAK2 V617F* mutation, and is included in the *MPL*-mutant group in the statistical analysis. No patient was positive for more than one *MPL*-mutant allele.

Laboratory and clinical features of the *MPL*-mutant group were compared to both the *JAK2 V617F*-positive and *JAK2 V617F*-negative groups, both of which lack *MPL* mutations. As shown in Table 1, *MPL*-mutant patients were significantly older at diagnosis than *JAK2 V617F*-negative patients (mean vs. mean; p=0.0001). Compared to *JAK2 V617F*-positive patients, *MPL*-mutant patients had lower hemoglobin and higher platelet levels at diagnosis (mean vs. mean; p=0.0001 and mean vs. mean; p=0.006 respectively). There were no differences, however, in diagnostic blood counts between the *MPL*-mutant and *JAK2 V617F*-negative groups. There were also no significant differences in the presence of splenomegaly or bone marrow cytogenetic abnormalities between the groups.

Bone-marrow trephine biopsies at diagnosis were available from 311 patients, including 13 patients with *MPL* mutations, comprising 2 *S505N*, 2 *W515K* and 9 *W515L* patients. These

were assessed independently by three hematopathologists who were aware of the patient's age and sex but unaware of *JAK2* or *MPL* mutation status (Table 2). Given the known association between age and bone marrow cellularity, we included patient age as a variable in the statistical analyses for cellularity. There were no differences in the mean reticulin grade, megakaryocyte cellularity or the presence of megakaryocyte clusters and atypia between the *MPL*-mutant, *JAK2 V617F*-positive and *JAK2 V617F*-negative groups. However, trephine biopsies from the *MPL*-mutant group were less cellular than both the *JAK2 V617F*-positive and *JAK2 V617F*-negative groups ($p=0.0001$ (0.0003 with age) and $p=0.005$ (0.003 with age) respectively). Compared to the *JAK2 V617F*-positive group, both erythroid and granulocytic cellularity were reduced in the *MPL*-mutant group ($p=0.0008$ (0.0007 with age) and $p=0.009$ (0.02 with age) respectively). Compared to the *JAK2 V617F*-negative group, the *MPL* mutant group showed reduced erythroid cellularity ($p=0.005$ (0.004 with age)). Thus *MPL*-mutant patients exhibited a more isolated megakaryocytic proliferation at diagnosis, with a reduction in overall cellularity compared to both the *JAK2 V617F*-positive and *JAK2 V617F*-negative groups. There was, however, considerable overlap between the histological appearances observed in the three groups of patients, and our data indicate that *MPL* mutations as a whole do not define a distinct histological subtype of ET. It remains formally possible that specific *MPL* mutations are associated with particular histological features, but the number of patients with each individual *MPL* mutation was too small to address this issue.

Serum erythropoietin levels at trial entry in *MPL* mutation patients were significantly higher than *JAK2 V617F*-positive but not *JAK2 V617F*-negative patients (mean vs. mean; $p<0.0001$ and $p=0.6$ respectively). There were no significant differences in iron status between *MPL*-mutant patients and both comparator groups, as assessed by serum ferritin and erythrocyte mean cellular volume (Table 2).

Cytokine-independent colony formation is one of the hallmark features of the myeloproliferative disorders, with the presence of both endogenous megakaryocyte and erythroid colonies reported in patients carrying the *JAK2 V617F* mutation^{3,24}. To investigate the association of *MPL* mutations and cytokine-independent colony formation, colony assays were performed using peripheral blood from two hydroxyurea-treated ET patients, and an IMF patient receiving transfusion support only, all of whom carried the *MPL W515L* mutation, and all of whom had only heterozygous and wild-type erythroid colonies. These investigations demonstrated the presence of thrombopoietin-independent megakaryocyte colony growth in all three patients studied. In contrast to the *JAK2 V617F* mutation, however, we did not observe endogenous erythroid colonies in any of 5 patients with the *MPL W515L* mutation (4 ET, 1 IMF). These data confirm previous studies in IMF and extend these findings to patients with ET^{25,26}.

Clinical outcome of patients with *MPL* mutations

As part of the PT-1 studies, comprehensive clinical and outcome data were collected prospectively. Clinical events were independently adjudicated according to pre-defined criteria by a panel of experts blinded to treatment allocation²¹. The median follow-up was 36.5 months, although the relatively small numbers of patients in the *MPL*-mutant cohort meant that only a few end-point events were recorded in this group. There were no significant differences between the three comparator groups in rates of arterial thrombosis either before or after trial entry (Table 3). When compared to *JAK2 V617F*-negative patients, *MPL*-mutant patients had a higher rate of venous thrombosis after trial entry (odds ratio 34.3, 95% CI: 1.6-725, $p=0.02$). In view of the large number of hypothesis tests performed, we elected to use a threshold for statistical significance of $p<0.01$. Moreover, the association appeared weaker in multivariate analysis including patient age, sex and past

history of venous thrombosis (Hazard ratio (HR), 4.7; 95% CI, 0.7-29.5; $p=0.09$), and there was no increased rate of venous thrombosis in the year before diagnosis. *MPL*-mutant patients had an increased rate of death compared to the *JAK2 V617F*-negative group on univariate analysis, but this was due to the significantly older age of the *MPL*-mutant patients (HR, 1.2; 95% CI, 0.5-3.5 after correction for patient age; $p=0.7$).

Biological aspects of *MPL* mutations

To investigate possible differences between the *MPL W515L* and *MPL W515K* alleles, mutant allele burden was quantitated by pyrosequencing using whole blood obtained at PT-1 trial entry. Patients with *MPL W515K* had a significantly higher mutant allele burden than those with *MPL W515L*; the number of patients with a mutant allele burden $>50\%$ was 0/24 for *W515L* and 4/5 for *W515K* ($p=0.0002$, Figure 2A). There were no significant differences in age at diagnosis ($p=0.6$), disease duration ($p=0.5$), use of cytoreductive therapy ($p=0.2$) or proportion of peripheral blood neutrophils at time of sampling (*W515L*, 0.67 ± 0.11 ; *W515K*, 0.75 ± 0.05 ; $p=0.2$) to account for this difference. In order to examine the phenotypic correlates of the higher *MPL W515K* mutant allele burden, blood counts at diagnosis were compared between *MPL W515L* and *MPL W515K* patients in the PT-1 cohort. Despite the higher mutant allele burden in the *MPL W515K* group, there were no significant differences in diagnostic hemoglobin level (*W515L*, $13.2 \pm 1.1\text{g/dl}$; *W515K*, $13.5 \pm 1.6\text{g/dl}$; $p=0.7$), neutrophil count (*W515L*, $6.5 \pm 2.0 \times 10^9/\text{L}$; *W515K*, $8.7 \pm 2.7 \times 10^9/\text{L}$; $p=0.07$) or platelet count (*W515L*, $1010 \pm 242 \times 10^9/\text{L}$; *W515K*, $1124 \pm 397 \times 10^9/\text{L}$; $p=0.4$). Moreover, there were no significant differences in blood counts at trial entry (data not shown).

Although none out of 24 patients in the PT-1 cohort carrying the *MPL W515L* mutation had a mutant allele burden of greater than 50% at trial entry, this does not exclude the presence of a subclone homozygous for the mutation²⁷. We therefore genotyped 354 single erythroid

colonies cultured from 4 Hydroxyurea-treated ET patients with *MPL W515L* mutations (Figure 2B). In one of the four patients, a single homozygous colony was found out of 94 colonies analysed; the other three patients had only heterozygous colonies, with or without wild-type colonies. Taken together with the mutant allele quantitation, our data suggest that ET patients carrying the *MPL W515K* allele commonly harbour clones in which the ratio of wild-type to mutant alleles is reduced (for example as a result of mitotic recombination or deletion of the wild-type allele), but that such clones are less common in patients with the *MPL W515L* allele. Of the 5 IMF patients with *W515L* mutations, 3 showed predominance of the mutant allele by direct sequencing (data not shown), indicating that subclones with reduced or lost wild-type allele can occur with this mutation, and may be associated with the development of myelofibrosis.

Three patients in the PT-1 cohort harboured an *MPL S505N* mutation, with mutant allele burdens of 62%, 47% and 17%. Further samples were available from one patient (mutant allele burden 17%) which demonstrated the absence of the mutation in buccal cells (data not shown), and the presence of both wild-type and heterozygous erythroid colonies (Figure 2B), indicative of an acquired mutation.

Discussion

In this paper we report the existence of four mutant *MPL* alleles in patients with a *JAK2 V617F*-negative MPD. No mutations were detected outside exon 10 despite the analysis of platelet cDNA, which excludes the possibility that such mutations might be restricted to the megakaryocyte lineage. Our results are consistent with and extend two previous studies using genomic DNA from 14 adults with an MPD²⁸ and 9 children with ET²⁹, both of which failed to identify *MPL* mutations outside exon 10. Our results demonstrate that mutations outside *MPL* exon 10 are not a common cause of *V617F*-negative ET but do not exclude the

existence of such mutations in a small minority of patients. Changes elsewhere in *MPL* have recently been reported, although it is not yet clear if they are acquired mutations or inherited polymorphisms³⁰. Previous studies have reported the *W515L* and *W515K* allele in IMF^{17,19} but only the *W515L* allele in ET¹⁷. Here we demonstrate the presence of the *W515K* allele in patients with ET and also report two different mutations capable of generating a *W515K* substitution. We also report 2 patients with ET in whom an *MPL S505N* allele, previously reported as an inherited mutation^{23,31}, was detected in granulocytes but was absent from buccal cells. Moreover in one of the patients the mutation was only present in a minority of erythroid colonies, and the other patient had a normal platelet count over several years prior to presentation with ET, making somatic mosaicism unlikely. Taken together, these findings strongly suggest that the *MPL S505N* allele can occur as both an inherited and acquired mutation. Mutations in the *KIT* gene have been reported in patients with both sporadic and familial mast cell proliferations. However the alleles involved are different, with inherited mutations not seen in acquired disease and *vice versa*³²⁻⁴¹. We believe this is the first example of a single allele being associated with both acquired and inherited forms of an MPD.

In IMF our results demonstrate the presence of *MPL* mutations in 7% of all patients and 15% of *JAK2 V617F*-negative patients. These data are similar to previous reports describing a prevalence of 5.5% or 8.5% in all patients^{17,19} and 9% in *JAK2 V617F*-negative patients¹⁶. In ET the prevalence of *MPL* mutations was similar in our retrospective and prospective cohorts. In our retrospective group of patients we found the prevalence of *MPL* mutations to be 3.4% overall and 6.4% in *JAK2 V617F*-negative patients. In the prospective PT-1 cohort, the prevalence was 4.1% overall and 8.5% in *JAK2 V617F*-negative patients. These results are somewhat higher than the 1.3% overall prevalence previously reported¹⁷, but are in keeping with data presented in abstract form⁴².

Mutant allele burden was measured in the PT-1 cohort using whole blood obtained at trial entry. ET patients with the *W515K* allele had significantly higher allele burdens than those with the *W515L* allele. This result is reminiscent of the observation that most PV patients with a *JAK2 V617F* mutation have homozygous erythroid colonies²⁷, whereas such clones are rarer in patients with a *JAK2* exon 12 mutation⁶. *JAK2* exon 12 mutations appear to signal more strongly than *V617F* mutations⁶ but it is not clear whether the difference between the *W515L* and *W515K* mutations reflects quantitative or qualitative alterations in signalling. Interestingly a peripheral blood allele burden of >50% was found in 0/24 ET patients with the *W515L* allele, but in 3/5 IMF patients. An allele burden of >50% implies the existence of one or more additional events giving rise to homozygous or hemizygous mutant clones. Our results would therefore be consistent with the concept that patients labelled as having IMF may be presenting in an accelerated phase of a previously undiagnosed MPD⁴³. Patients with a longer disease duration, or in whom additional mutations may have increased genomic instability would be expected to have a greater probability of undergoing a second event.

The development of allele-specific PCR assays for each *MPL* mutation allowed analysis of samples from the PT1 cohort together with an assessment of the clinical and laboratory features associated with *MPL* mutations. This large cohort includes ET patients in all risk categories, with centralized review of endpoints, comprehensive follow-up and the participation of a large number of secondary and tertiary centers. As such, these results are likely to be of general relevance to ET patients with *MPL* mutations. Compared to *JAK2 V617F*-positive patients, those carrying an *MPL* mutation exhibited lower hemoglobin levels and higher platelet counts at diagnosis, higher serum erythropoietin levels and reduced bone marrow erythroid and overall cellularity. Moreover it was possible to grow thrombopoietin-

independent megakaryocyte colonies but not erythropoietin-independent erythroid colonies from ET patients with an *MPL* mutation, an observation consistent with previous data from patients with IMF^{25,26}. Taken together, these features suggest that patients with *MPL* mutations have a lower drive towards erythroid differentiation, accompanied by a more isolated thrombocytosis. There were no consistent differences in other histological features including megakaryocyte morphology and reticulin grade. Compared to *JAK2 V617F*-negative patients, those carrying an *MPL* mutation were older at diagnosis with reduced bone marrow cellularity that remained significant when patient age was taken into account. However, it was not possible to identify other clinical, histological or additional laboratory features which allowed the *MPL*-mutant subgroup to be distinguished from the remaining *JAK2 V617F*-negative patients. Furthermore, *MPL*-mutant patients did not exhibit altered rates of thrombosis, major hemorrhage, transformation or death when compared to *V617F*-positive or *V617F*-negative patients.

In this paper, we describe 44 patients with *MPL* mutations, more than doubling the number thus-far reported in peer-reviewed journals. Within the PT-1 cohort, however, the number of clinical events in each category was small and statistical analyses were associated with large confidence intervals. Our data should therefore be interpreted acknowledging the possibility of false negative results. Our analysis also included a large number of hypothesis tests, and to minimise the risk of false positive results, we applied a threshold of $p < 0.01$ for statistical significance.

Known mutations in *MPL* and *JAK2* account for 57% of the PT-1 patients presented here. Comparisons between this group and the remaining 43% who lack an identified mutation did not reveal any significant differences in the prevalence of splenomegaly, abnormal cytogenetics, myelofibrotic transformation or acute myeloid leukaemia. There were also no

differences between the mutation-negative and mutation-positive groups in histological features other than cellularity. Taken together these findings suggest that mutation-negative patients do have a myeloproliferative disorder, the molecular basis for which remains obscure.

Combining our retrospective and prospective cohorts, we identified *MPL* mutations in 36 patients with ET, only 1 of whom carried a *JAK2 V617F* mutation. This is in marked contrast to the only previous report in which 2 out of 4 ET patients with an *MPL* mutation carried a *JAK2 V617F* mutation¹⁷, and suggests that the frequency of such double positive patients is much less than previously thought. We also identified *MPL* mutations in 8 IMF patients, none of whom harboured a *JAK2 V617F* mutation. This observation is not significantly different from previous studies in which 4 of 16¹⁷ or 4 of 18¹⁹ patients with an *MPL* mutation carried a *JAK2 V617F* mutation. If the prevalence of patients with both *MPL* and *JAK2* mutations does prove to be higher in IMF compared to ET, this would be consistent with the concept that IMF represents patients presenting in an accelerated phase of a pre-existing MPD⁴³.

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We have no conflict of interest to declare.

Author contribution

PAB designed and applied *MPL* mutation detection and pyrosequencing assays, processed and genotyped hematopoietic colonies, performed statistical analysis and co wrote the manuscript; PJC carried out *JAK2 V617F* mutation and statistical analyses; LMS carried out *JAK2 V617F* mutation analysis and processed and genotyped hematopoietic colonies; AJB carried out *JAK2 V617F* mutation analysis and managed patient samples; WNE, DB and BSW reviewed the trephine biopsies; JTR and HCH provided patient samples and clinical information; RB assisted with design and interpretation of pyrosequencing assays; KW and GB provided statistical support for the PT-1 trials; CNH is principal co investigator the PT-1 trials and provided patient samples and clinical information; ARG is principal co investigator the PT-1 trials, designed experiments and co wrote the manuscript. All authors have reviewed the manuscript.

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Table 1. Laboratory and clinical features at diagnosis of 776 ET patients enrolled in the MRC PT1 studies.

	<i>MPL</i> mutant	<i>MPL</i> negative		p value (vs <i>V617F</i> pos)	p value (vs <i>V617F</i> neg)
		<i>V617F</i> positive	<i>V617F</i> negative		
Number	32 ¹	411	333		
Percentage – (95% CI)	4.1% (2.9-5.8)	53.0% (49.4-56.5)	42.9% (39.4-46.5)		
Risk category					
Low risk ²	0	26	31	0.2	0.09
Intermediate risk ³	4	68	46	0.8	1.0
High risk ⁴	28	339	273	0.6	0.6
Hydroxyurea + Aspirin	13	168	139	0.9	0.8
Anagrelide + Aspirin	15	171	134		
Demographics					
Female – no. (%)	17 (53%)	255 (62%)	192 (58%)	0.3	0.7
Male – no. (%)	15 (47%)	156 (38%)	141 (42%)		
Age (yr.)					
– median (10 th -90 th centile)	67 (48-77)	60 (39-77)	52 (32-74)	0.09	<0.0001
Disease duration ⁵ (mth.)					
– median (10 th -90 th centile)	18 (0-956)	38 (0-1106)	57 (0-2699)	0.2	0.7
Laboratory and clinical features at diagnosis					
Hemoglobin (g/L)					
– mean ± SD	133 ± 12	145 ± 14	135 ± 14	<0.0001	0.3
– median	136	145	136		
– (10 th -90 th centile)	(119 - 147)	(128 - 163)	(117 - 153)		
White cells (x10 ⁹ /L)					
– mean ± SD	9.9 ± 2.4	10.6 ± 3.4	9.3 ± 2.7	0.2	0.2
– median	9.7	10.0	8.8		
– (10 th -90 th centile)	(7.0 - 13.3)	(7.0 - 14.6)	(6.2 - 12.8)		
Neutrophils (x10 ⁹ /L)					
– mean ± SD	6.7 ± 2.1	7.4 ± 3.0	6.2 ± 2.2	0.3	0.2
– median	6.9	6.8	5.8		
– (10 th -90 th centile)	(4.4 - 9.6)	(4.2 - 11.0)	(3.8 - 9.1)		
Platelet count (x10 ⁹ /L)					
– mean ± SD	1040 ± 272	900 ± 274	1032 ± 350	0.006	0.9
– median	962	840	963		
– (10 th -90 th centile)	(752 - 1505)	(632 - 1221)	(666 - 1550)		
Splenomegaly – no. (%)	0/27 (0%)	11/326 (3%)	11/262 (4%)	0.7	0.6
Abnormal cytogenetics – no. (%)	2/24 ⁶ (8%)	14/312 (4%)	10/262 (4%)	0.3	0.2

¹ One patient had both *JAK2 V617F* and *MPL W515L* mutations, and is included in the *MPL*-mutant column

² Includes 4 patients who subsequently enrolled in the intermediate risk arm, and 10 in the high risk arm.

³ Includes 4 patients who were previously enrolled in the low risk arm and 25 who subsequently enrolled in the high risk arm.

⁴ Includes 10 and 25 who were previously enrolled in the low and intermediate risk arms, respectively.

⁵ Refers to time elapsed between diagnosis and trial entry.

⁶ Comprises patient with *MPL S505N* and karyotype 45,XY,add(6)(q?15),-7 [6], 46,XY [25] and patient with *MPL W515L* and karyotype 47,XY,+14 [4], 46,XY [16], both at diagnosis.

Table 2. Bone marrow trephine histology, erythropoietin levels and iron stores in patients enrolled in the MRC PT1 studies.

	<i>MPL</i> mutant n=13	<i>MPL</i> negative		p value (vs <i>V617F</i> pos)	p value (vs <i>V617F</i> neg)
		<i>V617F</i> positive n=168	<i>V617F</i> negative n=130		
Bone marrow trephine histology¹					
Reticulin grade – mean ± SD	1.8 ± 1.0	1.8 ± 0.8	1.9 ± 0.9	0.9	0.6
Megakaryocyte clusters and nuclear morphology					
Clusters (absent/loose/tight)	1 / 6 / 6	22 / 99 / 47	12 / 67 / 51	0.3	0.8
Pyknotic (absent/pres/predom)	6 / 6 / 1	55 / 112 / 1	48 / 79 / 3	0.8	0.9
Staghorn (absent/pres/predom)	2 / 10 / 1	31 / 132 / 5	23 / 102 / 5	0.7	0.7
Cloud-like (absent/pres/predom)	5 / 8 / 0	46 / 118 / 4	44 / 85 / 1	0.4	0.8
Dysplastic (absent/pres/predom)	4 / 8 / 1	57 / 110 / 1	48 / 80 / 2	0.6	0.6
Cellularity²					
Overall (dec/normal/inc)	3 / 5 / 5	1 / 36 / 131	2 / 43 / 85	<0.0001	0.005
Erythroid (dec/ normal/inc)	3 / 9 / 1	5 / 90 / 73	6 / 77 / 47	0.0008	0.005
Granulocytic (dec/normal/inc)	3 / 6 / 4	3 / 74 / 91	2 / 76 / 52	0.009	0.07
Megakaryocytic (+/+/+/+)	3 / 9 / 1	32 / 88 / 47	25 / 58 / 47	0.3	0.2
Erythropoietin levels and iron stores					
Erythropoietin ³ (U/L)					
– mean ± SD	20.7 ± 16.2	9.8 ± 10.8	23.8 ± 28.9	<0.0001	0.6
– median (10 th -90 th centile)	17.4 (6.3–33.8)	6.9 (3.2–17.3)	14.9 (7.9–42.7)		
Mean cell volume ⁴ (fL)					
– mean ± SD	89.6 ± 5.5	87.6 ± 6.4	89.4 ± 6.1	0.08	0.8
– median (10 th -90 th centile)	89.5 (83.1–96.9)	88.3 (79.8–94.2)	89.4 (83.5–95.2)		
Mean cell volume < 80fL ³					
– no. (%)	2/32 (6.3%)	41/391 (10.5%)	4/312 (1.3%)	0.4	0.2
Ferritin ⁵ (µg/L) – mean ± SD	94 ± 79	90 ± 91	90.7 ± 90	0.8	0.9
– median (10 th -90 th centile)	54 (19 – 213)	58 (23 – 192)	91 (29 – 201)		

¹ All bone marrow trephine biopsies were obtained at diagnosis

² Dec, decreased; inc, increased; +, ++, +++, increasing cellularity

³ Based on 707 serum samples from trial entry. Normal range for serum erythropoietin, 5-25U/L.

⁴ Normal range for mean cell volume, 80-100fL.

⁵ Based on serum samples taken within 3 months of diagnosis (n=182). Normal range for ferritin, 20-300µg/L.

Table 3. Thrombotic, hemorrhagic and transformation events after trial entry and in the year before diagnosis.

	<i>MPL</i> mutant n=32	<i>MPL</i> negative		OR vs <i>V617F</i> pos (95% CI)	OR vs <i>V617F</i> neg (95% CI)	p value ¹ (vs <i>V617F</i> pos)	p value ¹ (vs <i>V617F</i> neg)
		<i>V617F</i> pos n=411	<i>V617F</i> neg n=333				
Arterial thrombosis							
In year before diagnosis²	4	38	20	1.4 (0.5-4.2)	2.2 (0.7-7.0)	0.5	0.2
Myocardial infarction	0	6	5				
Stroke	1	10	4				
Transient ischaemic attack	3	23	13				
After trial entry²	2	25	19	1.0 (0.2-4.3)	1.1 (0.2-5.2)	0.9	0.9
Myocardial infarction	1	7	6				
Unstable angina	0	5	1				
Stroke	1	7	7				
Transient ischaemic attack	1	5	6				
Other ³	0	2	1				
Venous thromboembolism							
In year before diagnosis²	1	10	2	1.3 (0.2-10.4)	5.3 (0.5-60.6)	0.6	0.2
Deep vein thrombosis	0	3	1				
Pulmonary embolism	0	3	1				
Splanchnic vein thrombosis	0	2	0				
Retinal vein thrombosis	1	2	1				
Cerebral sinus thrombosis	0	1	0				
After trial entry²	2	11	3	2.6 (0.4-19.3)	34.3 (1.6-725)	0.3	0.02 (0.09)⁴
Deep vein thrombosis	2	5	3				
Pulmonary embolism	0	5	0				
Splanchnic vein thrombosis	0	2	0				
Major haemorrhage							
After trial entry²	2	18	12	1.3 (0.3-6.8)	1.9 (0.3-12.1)	0.7	0.5
Gastrointestinal	2	6	6				
Intracranial	0	3	4				
Epistaxis	0	5	0				
Other ⁵	0	4	2				
Death	5	34	18	1.8 (0.6-5.5)	4.5 (1.1-18.4)	0.3	0.04 (0.7)⁶
Haematological transformation							
Myelofibrosis⁷	2	7	11	6.2 (0.6-66.6)	2.5 (0.3-17.7)	0.1	0.4
Acute leukaemia/ MDS	0	5	2	0.3 (0.02-7.2)	0.3 (0.01-47.0)	0.5	0.7
Polycythaemia vera	0	6	0	0.3 (0.02-6.5)	NA	0.5	NA

¹ Assessed by Pearson's chi-squared test with Yates' continuity correction for events preceding diagnosis and log-rank test for events after trial entry

² Indicates total patients rather than total events; some patients had more than one event

³ Lower limb arterial embolus (2); upper limb arterial thrombosis

⁴ Multivariate analysis including patient age and previous history of venous thrombosis

⁵ Pericardial (2), urinary (2), post-operative and obstetric

⁶ Multivariate analysis including patient age and previous history of arterial thrombosis

⁷ *MPL*-mutant group comprises one patient with *MPL S505N* and one patient with *MPL W515K*

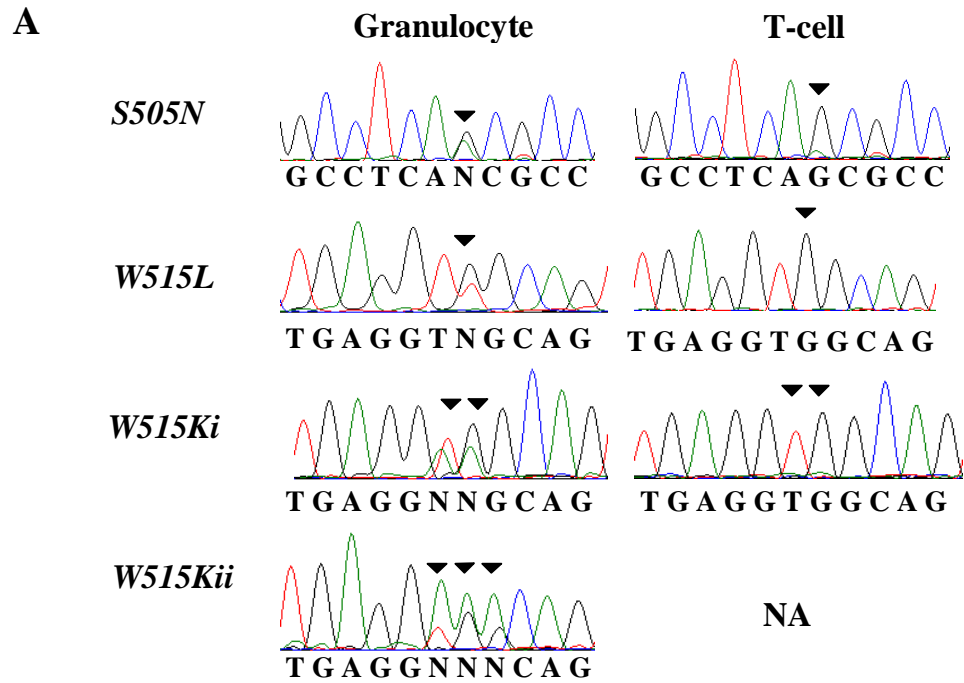
Figure legends

Figure 1. Detection of *MPL* exon 10 mutations. (A) Sequence traces of the four *MPL* exon 10 mutant alleles, showing two different mutations leading to the same *MPL* W515K substitution (*MPL* W515Ki and *MPL* W515Kii), and *MPL* S505N as an acquired mutation. (B) Allele-specific PCR strategy showing the common forward and reverse intronic primers and the allele-specific primer within *MPL* exon 10. (C) Mixing experiments with normal and mutant DNA demonstrating the sensitivity of the allele-specific PCR assays used for mutation screening. The images were captured on a Gel Doc 200 imager (BioRad, California) using Quantity One software (BioRad, California). NA: T cell sample not available from this patient, Pt: patient sample, WT: wild-type, NTC: no template control.

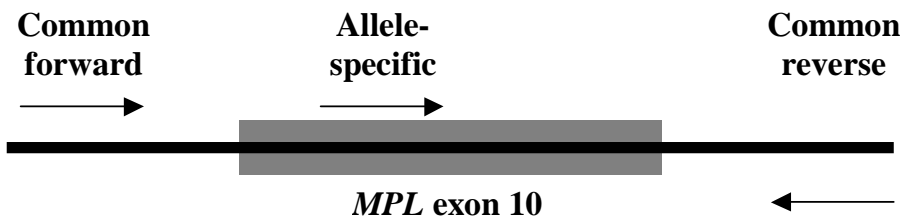
Figure 2. Biological aspects of *MPL* W515L and *MPL* W515K mutations.

(A) Whole blood mutant allele quantitation at trial entry showing significant difference in mean mutant allele burden for *MPL* W515L (mean mutant allele burden $17\% \pm 11\%$), and *MPL* W515K (mean mutant allele burden $66\% \pm 26\%$, $p < 0.0001$). (B) Genotyping of single erythroid colonies from 5 ET patients, 4 with *MPL* W515L and 1 with *MPL* S505N; a single homozygous colony was identified in patient 3.

Figure 1



B



C

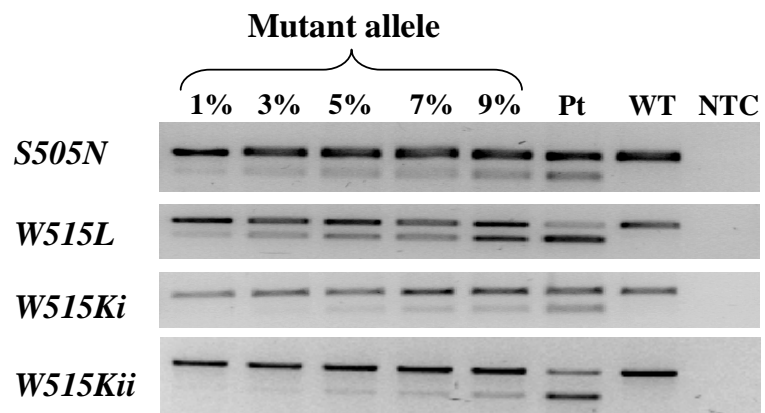
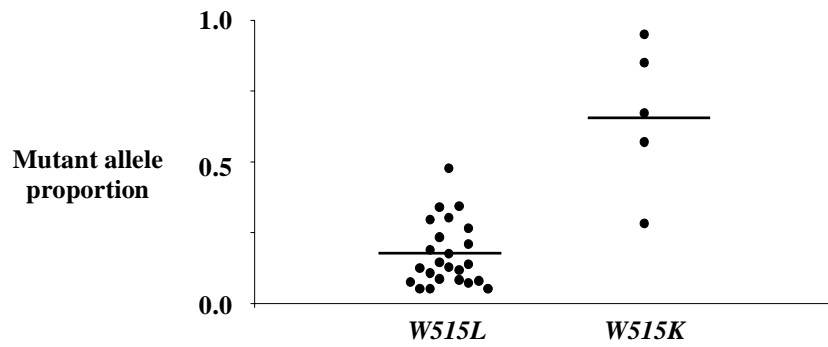


Figure 2

A



B

