

# The *JAK2* 46/1 haplotype predisposes to *MPL*-mutated myeloproliferative neoplasms

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**The 46/1 *JAK2* haplotype predisposes to V617F-positive myeloproliferative neoplasms, but the underlying mechanism is obscure. We analyzed essential thrombocythemia patients entered into the PT-1 studies and, as expected, found that 46/1 was overrepresented in V617F-positive cases ( $n = 404$ ) versus controls ( $n = 1492$ ,  $P = 3.9 \times 10^{-11}$ ). The 46/1 haplotype was also overrepresented in cases without V617F ( $n = 347$ ,  $P = .009$ ), with an excess seen for both *MPL* exon 10 mutated and**

**V617F, *MPL* exon 10 nonmutated cases. Analysis of further *MPL*-positive, V617F-negative cases confirmed an excess of 46/1 ( $n = 176$ ,  $P = .002$ ), but no association between *MPL* mutations and *MPL* haplotype was seen. An excess of 46/1 was also seen in *JAK2* exon 12 mutated cases ( $n = 69$ ,  $P = .002$ ), and these mutations preferentially arose on the 46/1 chromosome ( $P = .029$ ). No association between 46/1 and clinical or laboratory features was seen in the PT-1 cohort**

**either with or without V617F. The excess of 46/1 in *JAK2* exon 12 cases is compatible with both the “hypermutable” and “fertile ground” hypotheses, but the excess in *MPL*-mutated cases argues against the former. No difference in sequence, splicing, or expression of *JAK2* was found on 46/1 compared with other haplotypes, suggesting that any functional difference of *JAK2* on 46/1, if it exists, must be relatively subtle. (*Blood*. 2010;115(22):4517-4523)**

## Introduction

Myeloproliferative neoplasms (MPNs) are frequently characterized by deregulated tyrosine kinase signaling, particularly pathways involving *JAK2*. By far the most common mutation is V617F *JAK2*, which is seen in more than 95% of cases of polycythemia vera (PV) and 50% to 60% of essential thrombocythemia (ET) and primary myelofibrosis (PMF).<sup>1</sup> In addition, diverse mutations involving *JAK2* exon 12 have been identified in V617F-negative PV<sup>2</sup> and abnormalities affecting W515 or, less commonly, S505 of the thrombopoietin receptor *MPL* (which signals through *JAK2*) are found in up to 10% of cases with ET or PMF.<sup>3-6</sup>

Recently, we and others made the unexpected finding that V617F *JAK2* is not acquired randomly but instead arises preferentially on a specific constitutional *JAK2* haplotype, at least in white populations.<sup>7-9</sup> This haplotype, which we refer to as 46/1<sup>7</sup> but others have called GGCC,<sup>8</sup> is found in approximately 50% of normal persons and thus is a common, very low penetrance predisposition allele of the type that is typically detected by genome-wide association studies. We estimated that 46/1 accounts

for 50% of the population attributable risk of developing an MPN described in epidemiologic studies, but it does not account for familial MPNs, which are expected to be caused by rare, highly penetrant variants in genes that remain to be identified.<sup>10</sup> Furthermore, 46/1 was seen at comparable frequencies in different MPN subtypes and thus does not explain the phenotypic diversity associated with V617F *JAK2*.<sup>7</sup>

It is not clear why an acquired mutation as prevalent as V617F is associated with a particular inherited background, but 2 hypotheses have been suggested.<sup>7,8,11</sup> First, 46/1 may be inherently more genetically unstable, acquiring V617F at a faster rate than other haplotypes (hypermutable hypothesis). Second, V617F may arise on all haplotypes at equal rates, but 46/1 may carry an additional factor that either gives a selective advantage to the V617F-positive clone or interacts in some way to increase the likelihood of abnormal blood counts (fertile ground hypothesis). We aimed to explore these hypotheses by detailed genetic analysis of clinically defined patient cohorts.

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## Methods

### Patients and samples

The Primary Thrombocythemia 1 (PT-1) study included newly diagnosed and previously treated patients, 18 years of age or older, who met the Polycythemia Vera Study Group criteria for ET. Patients were recruited into 1 of 3 multicenter 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 aspirin; the National Cancer Research Institute intermediate-risk study, a randomization 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.<sup>12</sup> Details obtained at trial entry included diagnostic features, such as blood counts, cytogenetics, and clinical complications at or preceding diagnosis. Samples of peripheral blood were requested at trial entry from all patients, and 776 samples were received from which DNA was extracted. These samples have been analyzed previously for V617F *JAK2* and *MPL* mutations.<sup>5,13</sup>

Other patients who met the Polycythemia Vera Study Group or World Health Organization criteria for ET or MF were recruited from the United Kingdom, Germany, Italy, Greece, and the United States. Patients were tested for *JAK2* V617F, *MPL* exon 10, and, if appropriate, *JAK2* exon 12 mutations using allele-specific (AS) polymerase chain reaction (PCR), pyrosequencing, or high resolution melt analysis according to local procedures using DNA extracted from purified granulocytes or peripheral blood leukocytes. As controls, we used data generated from 3 population-based studies: the Wellcome Trust Case Control Consortium (WTCCC) United Kingdom blood donor cohort (n = 1500),<sup>14</sup> the German Kooperative Gesundheitsforschung in der Augsburg cohort (KORA; n = 1814),<sup>15</sup> and the Italian Invecchiare in Chianti cohort (InCHIANTI; n = 1200).<sup>16</sup> We also analyzed healthy control persons from Greece (n = 108). The study was approved by the internal review boards and/or ethics committees of all participating institutions, and informed consent was provided according to the Declaration of Helsinki.

### Genotyping

Quantitative genotyping assays for the 46/1 tag single nucleotide polymorphism (SNP) rs12340895, *JAK2* V617F, and *MPL* W515K/L by pyrosequencing have been described previously.<sup>5,17</sup> Primers for other SNPs are listed in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). *JAK2* V617F and *MPL* W515K/L mutations were scored as homozygous if the proportion of the mutant allele was greater than 50%, the maximum expected if a heterozygous mutant clone had expanded to include all cells in the sample. We used previously defined criteria to score homozygosity and heterozygosity for flanking SNPs in the context of skewed allele ratios brought about by acquired uniparental disomy at 1p or 9p.<sup>7</sup> AS PCR between V617F and rs12343867 was performed as described to determine whether the V617F *JAK2* mutation was in *cis* or *trans* to 46/1.

### AS PCR for *JAK2* exon 12 mutations

AS PCR was performed using a forward primer located in *JAK2* intron 11 (*JAK2\_intron11\_F*) in combination with 1 of 2 reverse primers specific for a 46/1 tagging 5-bp insertion/deletion polymorphism rs56241661 (primers plus\_TCTTA\_R and del\_TCTTA\_R). The 398-bp products harboring the whole of *JAK2* exon 12 were directly sequenced to determine whether the exon 12 mutation was present on a 46/1 or non-46/1 allele. Amplification conditions were optimized on DNA from a case carrying the H538\_K539delinsL mutation and normal healthy controls: the PCR contained 25 ng of DNA, 0.5 μM *JAK2\_intron11\_F*, and either plus\_TCTTA\_R or del\_TCTTA\_R, 0.5 mM dNTPs, 25 mM magnesium chloride, 10× AmpliTaq buffer (Applied Biosystems), 1 U of AmpliTaq Gold (Applied Biosystems), and distilled water to a final volume of 25 μL. PCR was performed on a Tetrad thermocycler (Bio-Rad), and the cycling conditions were 95°C for 15 seconds, and 34 cycles of 95°C for 30 seconds, 56°C for

45 seconds, 72°C for 45 seconds, followed by 72°C for 10 minutes. In cases where the exon 12 mutation was at low level and not clearly visible after AS PCR and sequencing, amplification of the wild-type alleles was blocked using a locked nucleic acid-oligonucleotide probe during PCR, thus facilitating the preferential amplification of mutant alleles. This PCR composed 25 ng DNA, 0.5 μM *JAK2\_intron11\_F*, and either plus\_TCTTA\_R or del\_TCTTA\_R, 1 μM *JAK2\_ex12\_locked* nucleic acid oligo (Sigma-Aldrich), 0.5 mM dNTPs, 75 mM magnesium chloride, 10× Stoffel buffer (Applied Biosystems), 1 U of AmpliTaq DNA polymerase Stoffel fragment (Applied Biosystems), and distilled water to a final volume of 25 μL. The cycling conditions remained the same.

### Genetic analysis

The LDMap program<sup>18</sup> was used to construct linkage disequilibrium (LD) maps measured in LD units for the entire genome using phase 2 HapMap data from the Centre d'Etude du Polymorphisme Humaine population<sup>19</sup> and genomic locations relative to NCBI build 36.1 of the human genome sequence. For regions of interest, the PHASE program<sup>20</sup> was used to estimate haplotypes from genotypic data derived from the WTCCC blood donor cohort.

### Statistical analysis

The proportion of 46/1 alleles within each patient subgroup was compared with corresponding controls using Fisher exact test (2-tailed). To take into account variation in SNP frequency across control populations, meta-analysis was performed using local control populations for each patient group using StatsDirect, Version 2.7.6. Datasets were compared using the features built into this software to confirm that there was no evidence of noncombinability (Breslow-Day and Cochran Q tests). Deviations from expected allelic ratios of 50% were assessed by the exact binomial distribution, and mutation levels were compared between groups by the Mann-Whitney test. Pairwise univariate analyses comparing diagnostic variables and genotype were performed using the *t* test for continuous variables, Fisher exact test for 2 × 2 tables, and Cochran Armitage test for trend with exact *P* values for ordinal variables. Clinical outcome was assessed by  $\chi^2$  test for trend for events preceding diagnosis and log-rank test for events after trial entry. The relationship between 46/1 haplotype, blood counts, and response to treatment was assessed using the linear mixed-effects models.

## Results

### Characterization of the *JAK2* 46/1 haplotype in the PT-1 cohort

We genotyped patients from the PT-1 cohort for rs12340895, a tag SNP that acts as a surrogate for 46/1 (G allele = 46/1; C allele = not 46/1).<sup>7</sup> Results were obtained from 751 cases and compared initially with mutational status (Table 1). As expected, 46/1 was more frequent in patients compared with controls ( $P = 1.7 \times 10^{-9}$ ), and this was accounted for principally by an excess in cases that were V617F-positive ( $P = 3.9 \times 10^{-11}$ ). The 46/1 haplotype was also overrepresented in cases that tested negative for V617F ( $P = .025$ ), and a relatively high 46/1 frequency was also seen in cases that were positive for *MPL* exon 10 variants (W515L, n = 24; W515K, n = 5; S505N, n = 3), although this did not reach statistical significance ( $P = .06$ ;  $P = .096$  if the 3 cases that were also V617F-positive are excluded).

To confirm our previous finding that V617F probably arises specifically on the 46/1 allele, we performed AS PCR for the *JAK2* mutation and sequenced the products to determine the genotype of a nearby 46/1 tag SNP, rs12343867. Of the 212 V617F-positive cases that were heterozygous for 46/1, results were obtained for 207. Of these, 160 (77%) V617F alleles arose on the 46/1 haplotype ( $P < .001$ , exact binomial test), whereas only 45 (22%) residual

**Table 1. Summary of genotyping results for the 46/1 tag SNP rs12340895 in the PT-1 cohort**

Category	No. of cases	No. of 46/1 alleles	No. of non-46/1 alleles	Frequency of 46/1	P (vs WTCCC controls)	OR (95% CI)
All PT-1 cases	751	511	991	0.341	1.7e-9	1.52 (1.34-1.74)
JAK2 V617F positive	404	302	506	0.374	3.9e-11	1.76 (1.49-2.07)
MPL exon 10 mutation positive*	32	23	41	0.359	.060	1.60 (0.99-2.75)
JAK2 V617F, MPL exon 10 negative	318	189	447	0.300	.025	1.24 (1.03-1.50)
WTCCC controls	1492	757	2227	0.252	—	—

WTCCC indicates Wellcome Trust Case Control Consortium; OR, odds ratio; CI, confidence interval; and —, not applicable.  
 \*Three *MPL* mutant cases also tested positive for V617F.

wild-type alleles were 46/1. In addition, there were 2 V617F-positive cases where the V617F mutation was present on both 46/1 and non-46/1 haplotypes, suggesting that V617F arose at least twice in these cases.

**No association between 46/1 and clinical features or outcome**

If *JAK2* on 46/1 is functionally different from *JAK2* on other haplotypes, we speculated that 46/1 might be associated with specific disease features. We therefore compared clinical, laboratory, and demographic data with 46/1 genotype (both homozygous and heterozygous) for the PT-1 group as a whole, for V617F-positive and V617F-negative cases considered separately, and also for V617F-positive cases where the mutation was known to be on the 46/1 allele. No significant associations were seen with features at diagnosis, including age, blood counts, hemoglobin, erythropoietin, and spleen size (supplemental Table 2). Furthermore, there was no association with survival, arterial thrombosis, venous thrombosis, major hemorrhage or transformation to myelofibrosis, myelodysplastic syndrome, or acute leukemia (supplemental Table 3). There was an apparent association with transformation from ET to PV; however, the number of cases is extremely small (n = 6); furthermore, we saw no association between haplotype status and changes in hemoglobin, platelet, or white cell counts over time.

**Association between 46/1 and MPL W515 mutations**

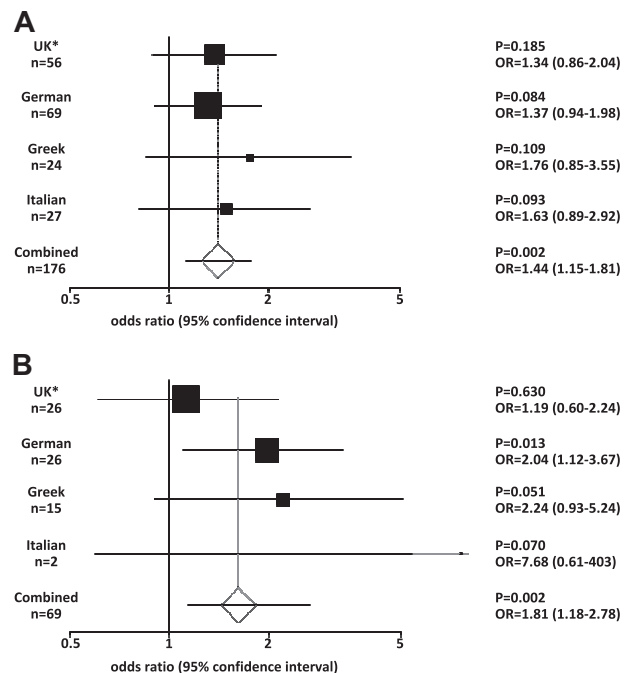
To explore the possible association of 46/1 with *MPL* mutations, we further investigated an independent cohort of 176 cases with W515L (n = 110), W515K (n = 58), other W515 variants (n = 4), or S505N (n = 4). All 176 cases were specifically selected as being negative for V617F using an assay with a sensitivity of 1% to 2%. As shown in Figure 1 and supplemental Table 4, the frequency of 46/1 as assessed by rs12340895 genotype was higher in *MPL*-mutated cases compared with controls from the same country for each of the 3 populations. Although these differences did not reach significance for each population considered in isolation, meta-analysis to control for population variation in SNP frequencies demonstrated that 46/1 was indeed more frequent in *MPL*-mutated cases (P = .002, odds ratio [OR] = 1.44). Even considering the worst case scenario, that is, all *MPL*-mutated cases compared with the control population with the highest frequency of 46/1 (Italians), the difference is still significant (P = .012). No difference in the frequency of 46/1 was seen on comparison of cases with W515L or W515K. To further confirm that 46/1 predisposes to *MPL*-mutated MPNs, we analyzed a second 46/1 tag SNP (rs10118390; located 20 kb downstream of *JAK2*). As shown in supplemental Table 5, this SNP is also significantly overrepresented in *MPL*-mutated, V617F-negative cases (P < .001; OR = 1.51; n = 176).

**No association between MPL haplotype and MPL mutations**

To investigate the possibility that *MPL* mutations might be associated with a specific *MPL* haplotype, we first determined the

genetic structure of this locus using the 8 SNPs genotyped by the WTCCC in the blood donor cohort. Analysis using LDMapper and PHASE showed that the entire *MPL* gene resides in a single LD block, with 4 haplotypes accounting for 88% of alleles (Table 2). These 4 haplotypes could be captured by 3 SNPs (rs1199038, rs11210838, and rs839757), which we analyzed by pyrosequencing in 138 *MPL*-mutated cases. Because *MPL* mutations may be affected by acquired uniparental disomy at chromosome 1p,<sup>21</sup> we quantified mutation levels in all cases by pyrosequencing and identified 29 cases with evidence of a homozygous clone (mutation level > 50%). We used previously defined criteria to take into account the possibility of SNP allele skewing in these persons but found no difference in the allele frequencies between cases and controls (Table 3).

As we have shown previously for V617F *JAK2*, the skewing of heterozygous flanking SNP ratios in cases with a homozygous mutant clone make it possible to read directly the haplotype on which the *MPL* mutation arose, as well as the residual wild-type haplotype.<sup>7</sup> Of the 29 cases with a homozygous clone, we were able to assign the mutant allele to 1 of the 4 common haplotypes in 23 cases and the wild-type allele in 21 cases. As shown in Table 2, the mutant allele was not seen preferentially on any haplotype, and



**Figure 1. Meta-analysis of genotype data.** (A) *MPL*-mutated cases. (B) *JAK2* exon 12 mutated cases. The plots show the odds ratio (OR) and 95% confidence interval (CI) within each patient subgroup compared their corresponding population controls. The combined data show the OR and CI derived by meta-analysis of all the data after confirmation of the comparability and homogeneity of the respective datasets. \*The United Kingdom *MPL* and *JAK2* exon 12 cohorts include 1 and 2 cases, respectively, of European origin from the United States.

**Table 2. *MPL* haplotypes in the United Kingdom population (n = 1500) and *MPL* mutated cases with a homozygous clone (n = 23)**

Haplotype no.	rs1467809	rs1098182	rs12076751	rs1199038*	rs11210838*	rs4141740	rs4141741	rs839757*	WTCCC frequency	Expected (n = 23)	Observed	
											<i>MPL</i> W515L/K allele (n = 23)	Wild-type allele (n = 21)
1	G	C	T	T	A	A	T	A	0.132	3	3	4
3	G	C	T	T	C	A	T	A	0.204	5	9	4
29	A	C	C	C	C	T	C	A	0.269	6	4	7
30	A	C	C	C	C	T	C	G	0.271	6	6	4

WTCCC indicates Wellcome Trust Case Control Consortium.

\*SNPs that were genotyped in *MPL* mutated cases.

there was no significant difference in haplotype distribution on comparison of the mutant and wild-type alleles. Furthermore, the number of mutant and wild-type haplotypes did not differ significantly from the distribution expected from analysis of the WTCCC control cohort. These data therefore are consistent with the hypothesis that *MPL* mutations arise randomly on different *MPL* haplotypes.

### Higher *MPL* mutation loads in MF

Having determined the *MPL* mutation load in W515K/L cases, we compared the results to clinical phenotype. Of the 138 cases (ET, n = 99; MF, n = 36; other MPN, n = 3), the median W515K/L mutation levels in ET (21%) were significantly lower than those seen in MF (46%;  $P < .001$ ; Figure 2A). The 29 homozygous cases had a diagnosis of primary or secondary MF (n = 15), ET (n = 12), or accelerated/transformed MPN (n = 2); 17 cases had W515L and 12 had W515K. Overall, mutation levels in W515L cases (n = 106; median = 25%) were lower than those seen in cases with W515K (Figure 2B; n = 32; median = 37%;  $P = .029$ ), confirming our previous observations in a much smaller number of cases.<sup>5,22,23</sup>

### The 46/1 predisposes to *JAK2* exon 12 mutations

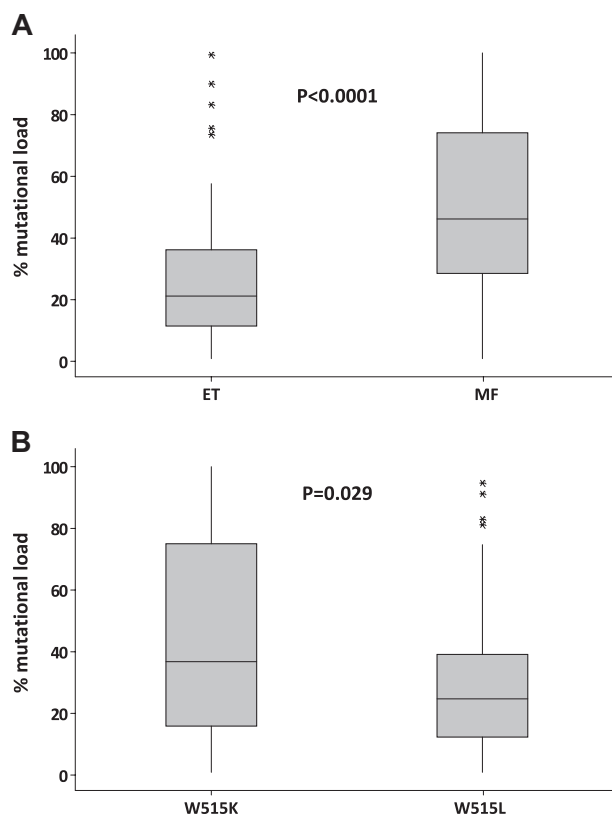
To determine whether the 46/1 haplotype predisposes to acquisition of other MPN-associated mutations, we investigated its association with mutations in exon 12. A total of 69 cases with PV that tested positive for an exon 12 mutation but negative for V617F were genotyped for rs12340895. Cases were from Italy (n = 2), Germany (n = 26), Greece (n = 15), and the United Kingdom/United States (n = 26); and, as performed for *MPL*, each group was compared with local control populations (Figure 1B; supplemental Table 6). Of the 138 alleles, 54 (39%) were 46/1, and meta-analysis indicated that this was significantly higher than controls ( $P = .002$ , OR = 1.81).

Of the 69 cases, 6 were homozygous for 46/1 and 42 were heterozygous. For the heterozygous cases, we investigated whether *JAK2* exon 12 mutations arose in *cis* or *trans* to the 46/1 haplotype using AS PCR. To do this, we exploited a 5-bp insertion/deletion polymorphism (rs56241661) downstream of exon 12 that we found

to be tightly linked to 46/1 (supplemental Figure 1). Of the 31 informative cases, 22 (71%) exon 12 mutations arose on the 46/1 haplotype, whereas only 9 (29%) residual wild-type alleles were 46/1 ( $P = .029$ ).

### No differences between *JAK2* on 46/1 compared with other haplotypes

We have previously demonstrated that the mRNA expression level of *JAK2* on 46/1 is no different from the expression of *JAK2* on other haplotypes, at least in peripheral blood leukocytes. To determine whether *JAK2* on 46/1 might be functionally different, we sequenced all exons, including those encoding the 5' and 3' untranslated regions, in 8 MPN cases with V617F on the 46/1 allele, 2 MPN with exon 12 mutations, and 8 healthy controls that were negative for 46/1. No sequence variants were detected, except for the 2 previously described silent polymorphisms



**Figure 2. Higher *MPL* mutation loads in MF and cases with W515K.** Box-and-whisker plots comparing the percentage of *MPL* W515K/L alleles in essential thrombocythemia (ET) versus myelofibrosis (MF; A) and in cases with W515K versus those with W515L (B).  $P$  values were obtained by Mann-Whitney analysis. \*Outliers.

**Table 3. Minor allele frequencies for 3 *MPL* SNPs in all *MPL* cases compared with controls**

SNP	<i>MPL</i> mutated cases (N = 138)	WTCCC controls (N = 1500)	$P$
rs1199038	0.391	0.388	1.0
rs11210838	0.141	0.147	1.0
rs839757	0.344	0.341	.9

WTCCC indicates Wellcome Trust Case Control Consortium.

**Table 4. Frequencies of other SNPs in PV and ET versus controls**

	SNP minor allele frequency			
	rs318699 EpoR	rs2279784 3q21	rs12500918 4q31	rs1524395 7p11
PV	0.310 (n = 163)	0.088 (n = 152)	0.446 (n = 147)	0.303 (n = 152)
ET	0.342 (n = 753)	0.085 (n = 763)	0.436 (n = 763)	0.315 (n = 762)
Controls	0.374 (n = 1495)	0.076* (n = 224)	0.466 (n = 1491)	0.309 (n = 1499)
<b>P</b>				
PV versus ET	.122	.824	.797	.685
PV versus controls	.025	.587	.501	.986
ET versus controls	.245	.560	.054	.659

PV indicates polycythemia vera; ET, essential thrombocythemia; and WTCCC, Wellcome Trust Case Control Consortium.

\*Allele frequency derived by pyrosequencing of 224 healthy United Kingdom persons; other controls are from the WTCCC.

rs10429491 and/or rs2230724 in exons 6 and 19, respectively. To determine whether these exonic SNPs or other unknown intronic variants might affect splicing, we performed overlapping reverse-transcriptase PCR to cover all exon-exon junctions. Sequencing of the products did not reveal any differences between *JAK2* mRNA in MPNs with V617F on 46/1 (n = 8) and controls without 46/1 (n = 8).

#### Role of other inherited polymorphic variants

Previous targeted and genome-wide analysis has identified other loci that may be associated with the phenotypic diversity of MPNs. Pardanani et al<sup>24</sup> found that an SNP in the *EPOR* gene (rs318699) was more common in PV than controls, whereas Kilpivaara et al<sup>9</sup> identified 3 variants (rs1524395 at 7p11, rs2279784 at 3q21, and rs12500918 at 4q31) that were more common in PV compared with ET. We sought to determine whether these associations were reproducible by analysis of these 4 SNPs in ET (n = 763), PV (n = 163), and controls. As shown in Table 4, the frequency of the *EPOR* SNP rs318699 was lower in PV ( $P = .025$ ; OR = 0.75; 95% confidence interval [CI] = 0.59-0.96) but not ET compared with controls. No significant difference between the frequency of rs12500918 (4q31) or rs1524395 (7p11) was seen on comparison of PV and ET or either disorder and controls, although the frequency in ET versus control approached significance for rs12500918. For rs2279784 (3q21), more than 10% of the WTCCC controls were scored as failures, suggesting a genotyping problem for this SNP. We therefore determined the frequency in 224 healthy controls, which was no different from that seen in PV and ET.

## Discussion

The finding that the V617F *JAK2* mutation is acquired preferentially on a specific constitutional *JAK2* haplotype was unexpected, and the mechanism underlying this observation remains unexplained. Two hypotheses have been postulated: (1) “hypermutability” of *JAK2* on 46/1 compared with other haplotypes and (2) a functional difference of *JAK2* on 46/1 that positively interacts with V617F and thus provides “fertile ground” for development of an MPN. Data from our current study give some support to both these hypotheses, but neither is able to explain all the available facts.

Hypermutability of *JAK2* on 46/1 was proposed initially by Olcaydu et al<sup>8</sup> because of their finding that V617F was seen in *cis* to both alleles of a nearby heterozygous SNP in 3 of 109 (3%) MPNs. In this study, we made the same observation in 2 of 207 (1%) ET cases; furthermore, occasional cases with both *JAK2* V617F and *JAK2* exon 12 mutations have been reported.<sup>25</sup> Because the acquisition of V617F is a rare event and the probability of an exon 12 mutation is considerably less, the

chance of acquiring 2 completely independent *JAK2* mutations is miniscule. Although the fertile ground hypothesis cannot explain multiple occurrences of *JAK2* mutations, there are alternative explanations that have some supporting evidence. First, other acquired or inherited genetic variants may predispose to the acquisition of *JAK2* mutations, thus greatly increasing the probability of 2 separate mutations in the same person. An enhanced propensity to acquire *JAK2* mutations is already accepted in the context of familial MPNs where multiple affected persons independently acquire V617F as a consequence of inheriting an as yet uncharacterized, high penetrance genetic variant.<sup>26,27</sup> Although documented MPN families are relatively uncommon, a recent study estimated that as many as 8% of cases have a family history,<sup>28</sup> and thus a substantial minority of affected persons may have an elevated, inherited propensity to acquire multiple *JAK2* mutations. Second, V617F *JAK2* has been reported to increase the rate of homologous recombination as well as the mutation rate.<sup>29</sup> It is conceivable that the observed linkage of *JAK2* to 2 SNP alleles might be explained by an initial acquisition of V617F followed by a recombination event between the mutation and the flanking SNP. Although this seems implausible over a distance of only 410 bp, it is striking that a recent study found that V617F appeared to have been acquired at least twice in 10 of 11 (91%) ET cases.<sup>30</sup> This unexpected finding has not been independently confirmed and appears to be at odds with the fact that mitotic recombination (and thus homozygosity for V617F) is rare in ET; however, the use of an SNP that is 8 kb distal to V617F might have afforded a greater opportunity for recombination between the 2 markers.

We initially favored the fertile ground hypothesis because of the observation that 46/1 is associated with lower numbers of myeloid colony-forming units in normal persons<sup>7</sup> and also because a 46/1 tag SNP showed robust association with Crohn disease,<sup>31</sup> a nonmalignant disorder that is thought to have an inflammatory cause.<sup>32</sup> Genome-wide association studies in Crohn disease also detected significant associations with genes encoding the IL-23 receptor and STAT3, thereby strongly implicating functional differences in the IL23/*JAK2*/STAT3 pathway in the pathogenesis of this disorder. In this study, we demonstrated that both *JAK2* exon 12 and *MPL* mutations are associated with 46/1. The association with *JAK2* exon 12 mutations confirms the findings reported by Olcaydu et al,<sup>33</sup> and is compatible with both the fertile ground and hypermutability hypotheses. The finding of an excess of 46/1 alleles in *MPL*-mutated cases is difficult to explain by hypermutability of *JAK2*. Set against the fertile ground hypothesis, however, we did not detect any association with 46/1 and hematologic or clinical parameters, either with or without V617F. Furthermore, we found no expression difference in peripheral blood leukocytes or sequence difference of *JAK2* on 46/1 compared with other haplotypes.

It was not known whether the association of *JAK2* mutations with 46/1 reflects something unusual about the *JAK2* locus or

whether acquired driver mutations in cancer usually arise on specific inherited haplotypes. We therefore tested whether there was any association between *MPL* exon 10 mutations and *MPL* haplotype but found that the mutations appeared to arise randomly. Although this does not preclude the possibility that somatic mutations in other genes might occur preferentially on particular haplotypes, it does indicate that this is not a general phenomenon.

The mechanism by which V617F *JAK2* is associated with diverse clinical phenotypes remains unexplained. Increased dosage of V617F is associated with PV as opposed to ET in both patients and mouse models<sup>34-36</sup>; however, this clearly does not completely explain the 2 phenotypes because many patients with PV have levels of V617F consistent with a predominant heterozygous clone (although colony analysis usually reveals the presence of both heterozygous and homozygous clones in these cases).<sup>36</sup> In a small genome-wide association study, the frequency of 3 SNPs at 3q21, 4q31, and 7p11 was reported to differ between PV and ET, suggesting that they might contribute to phenotypic diversity.<sup>9</sup> We were unable to replicate these findings in a larger series of cases, suggesting that these associations were either spurious or much weaker than originally proposed. In an analysis of candidate genes, Pardanani et al reported that the allele frequency of rs318699, an SNP close to the *EPOR* gene at 19p13, differed between PV (but not ET) and controls, suggesting that it may specifically predispose to PV.<sup>24</sup> We also found a small but significant difference between PV, but not ET, and controls ( $P = .025$ ); however, in our study, the minor allele frequency was reduced in PV, whereas Pardanani et al<sup>24</sup> found that it was increased. We therefore consider that the relevance of this SNP is questionable.

In conclusion, our data provide some evidence that argues against the fertile ground hypothesis and some that argues against hypermutability. If a functional difference does exist, it must be very subtle, for example, variation in expression of *JAK2* in a hemopoietic precursor cell. A subtle functional difference would also be consistent with the observation that 46/1 is not present in approximately 25% of *JAK2*-mutated MPNs,<sup>7,8</sup> indicating that this haplotype is not required for disease development.

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## Authorship

Contribution: A.V.J. and N.C.P.C. designed the study; A.V.J., P.J.C., P.A.B., L.M.S., W.T., P.S., A.J.C., and N.C.P.C. performed laboratory and data analysis; and P.A.B., S.S., A.M.V., K.Z., M.J.P., M.F.M., L.M.S., R.T.S., D.O., C.N.H., H.G., A.K., and A.R.G. provided clinical samples and data.

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