

# Definition of subtypes of essential thrombocythaemia and relation to polycythaemia vera based on *JAK2* V617F mutation status: a prospective study

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**Background** An acquired V617F mutation in *JAK2* occurs in most patients with polycythaemia vera, but is seen in only half those with essential thrombocythaemia and idiopathic myelofibrosis. We aimed to assess whether patients with the mutation are biologically distinct from those without, and why the same mutation is associated with different disease phenotypes.

**Methods** Two sensitive PCR-based methods were used to assess the *JAK2* mutation status of 806 patients with essential thrombocythaemia, including 776 from the Medical Research Council's Primary Thrombocythaemia trial (MRC PT-1) and two other prospective studies. Laboratory and clinical features, response to treatment, and clinical events were compared for V617F-positive and V617F-negative patients with essential thrombocythaemia.

**Findings** Mutation-positive patients had multiple features resembling polycythaemia vera, with significantly increased haemoglobin (mean increase 9.6 g/L, 95% CI 7.6–11.6 g/L;  $p < 0.0001$ ), neutrophil counts ( $1.1 \times 10^9/L$ ,  $0.7\text{--}1.5 \times 10^9/L$ ;  $p < 0.0001$ ), bone marrow erythropoiesis and granulopoiesis, more venous thromboses, and a higher rate of polycythaemic transformation than those without the mutation. Mutation-positive patients had lower serum erythropoietin (mean decrease 13.8 U/L; 95% CI, 10.8–16.9 U/L;  $p < 0.0001$ ) and ferritin ( $n=182$ ; median 58 vs 91  $\mu\text{g/L}$ ;  $p=0.01$ ) concentrations than did mutation-negative patients. Mutation-negative patients did, nonetheless, show many clinical and laboratory features that were characteristic of a myeloproliferative disorder. V617F-positive individuals were more sensitive to therapy with hydroxyurea, but not anagrelide, than those without the *JAK2* mutation.

**Interpretation** Our results suggest that *JAK2* V617F-positive essential thrombocythaemia and polycythaemia vera form a biological continuum, with the degree of erythrocytosis determined by physiological or genetic modifiers.

## Introduction

The human myeloproliferative disorders consist of three main diseases, essential thrombocythaemia, polycythaemia vera, and idiopathic myelofibrosis.<sup>1</sup> The main clinical features of essential thrombocythaemia are arterial and venous thrombosis, although haemorrhage can also occur.<sup>2–4</sup> In a few patients the disease can proceed to myelofibrosis, acute myeloid leukaemia, myelodysplasia, or polycythaemia vera. The idea that essential thrombocythaemia might be heterogeneous is lent support by studies of X chromosome inactivation patterns,<sup>5,6</sup> *PRV1* mRNA expression,<sup>7,8</sup> myeloproliferative leukaemia expression,<sup>9,10</sup> histological features,<sup>11</sup> and the presence of erythropoietin-independent erythroid colonies.<sup>8</sup> However, where assessed, the concordance between these features is variable,<sup>8</sup> and definition of biologically or pathogenetically distinct subgroups of essential thrombocythaemia has proved difficult.

Recently, a single somatic mutation of the Janus kinase 2 (*JAK2*) gene was reported in most patients with polycythaemia vera, but in only some patients with essential thrombocythaemia or idiopathic myelofibrosis.<sup>12–15</sup> The V617F mutation lies in the auto-

inhibitory JH2 domain, and therefore increases *JAK2* kinase activity, confers cytokine-independent growth on cell lines, and is associated with erythropoietin-independent growth of primary cells. Transplantation of bone marrow cells with mutant *JAK2* results in erythrocytosis *in vivo*.<sup>13</sup> By use of mutation-specific PCR, the *JAK2* mutation was detected in peripheral blood granulocytes from around half of essential thrombocythaemia patients,<sup>12</sup> but whether mutation-bearing patients are biologically distinct from those lacking the mutation is not known, or why the same mutation is associated with different diseases. To address these questions, we have analysed samples from patients enrolled in three large prospective studies of high-risk, intermediate-risk, and low-risk essential thrombocythaemia.

## Methods

### Study population

Newly diagnosed and previously treated patients, aged 18 years or over, who met the Polycythemia Vera Study Group (PVSG) criteria<sup>16</sup> for essential thrombocythaemia, were recruited into one of three multicentre studies: the

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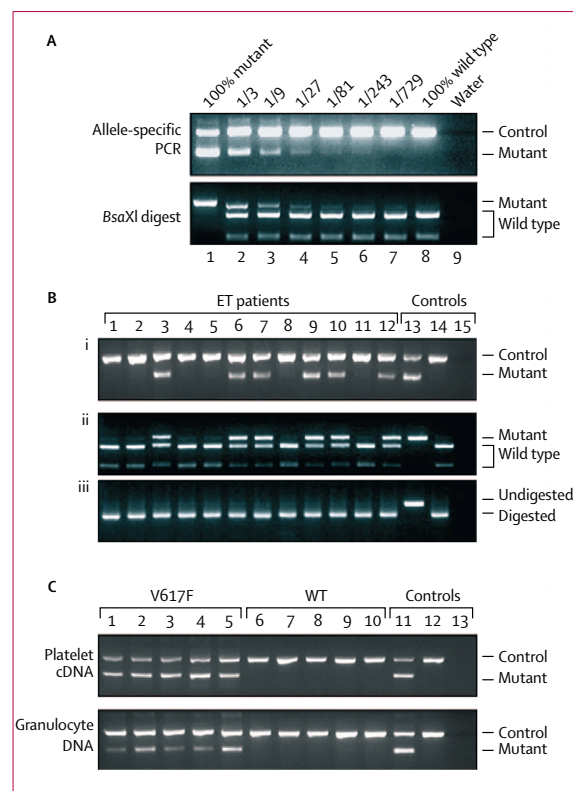
Medical Research Council PT-1 trial,<sup>17</sup> in which high-risk patients were randomly assigned to either hydroxyurea plus aspirin or to anagrelide plus aspirin; the National Cancer Research Institute intermediate risk study, a randomisation between aspirin alone or hydroxyurea plus aspirin; or the National Cancer Research Institute low-risk study, a prospective observational study of patients at low risk 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 centres, and written informed consent was obtained from all patients.

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 used.<sup>17</sup> Bone-marrow trephines were independently reviewed by three haematological pathologists unaware of *JAK2* status, and scored for reticulin grade on a 0–4 scale,<sup>18</sup> cellularity, megakaryocyte clustering, and atypical megakaryocyte nuclear morphology.<sup>19</sup> Where there was disagreement, the mean reticulin score and the mode of other scores were used. For studies of expression of the Polycythemia Rubra Vera 1 (*PRV1*) gene and of erythropoietin-independent erythroid colonies, we recruited patients with essential thrombocythaemia (as defined above) and polycythaemia vera (modified PVSG criteria<sup>20</sup>) attending the myeloproliferative disorders clinic at Addenbrooke's Hospital, Cambridge, UK.

### Procedures

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). Allele-specific PCR and *Bsa*XI digestion were done as described previously.<sup>12</sup> For platelet purification, blood was spun for 20 min up to three times, and the upper 50% of platelet-rich plasma used to extract RNA. Contaminating white cells were less than 1 per 10<sup>6</sup> platelets. The primers and conditions used to detect the V617F mutation in platelet cDNA are available from the authors on request.

Quantification of *PRV1* expression was done with real-time PCR in duplicate as previously described<sup>8</sup> with granulocyte cDNA from five healthy individuals, 19 patients with polycythaemia vera, and 24 patients with essential thrombocythaemia. Haemopoietic progenitor assays were done.<sup>12</sup> The presence of erythropoietin-independent erythroid colonies was assessed only when at least 40 erythroid colonies grew in the presence of erythropoietin. Serum samples taken at trial entry were analysed by automated chemiluminescence immunoassays for concentrations of erythropoietin



**Figure 1: JAK2 genotype analysis**

ET=essential thrombocythaemia. WT=wild type. (A) Sensitivity of allele-specific PCR and *Bsa*XI methods. Serial dilutions of 100% V617F-homozygous granulocyte DNA into normal control DNA assessed by allele-specific PCR (upper) and *Bsa*XI digestion (lower). Lane 1 100% V617F-positive DNA; lanes 2–7 serial three-fold dilutions of mutant DNA; lane 8 100% V617F-negative DNA; lane 9 water. In allele-specific PCR, control primers amplify from both wild-type and mutant DNA, giving a 364 bp product. Mutation-specific primer and common reverse primer amplify a 203 bp product only from mutant DNA. (B) Allele-specific PCR (i) and *JAK2* *Bsa*XI digestion (ii) were used to genotype DNA from unfractionated peripheral blood leucocytes from patients with essential thrombocythaemia (ET). Lane 13 granulocyte DNA from V617F-homozygous control; lane 14 granulocyte DNA from V617F-negative control; lane 15 water. (iii) Digestion control with *SCL* genomic fragment containing a *Bsa*XI site. Lane 13 no enzyme control; lane 14 with enzyme control; lane 15 water. (C) Upper: platelet cDNA; Lower: granulocyte genomic DNA. Lanes 1–5 V617F-positive patients; lanes 6–10 V617F-negative patients; lane 11 V617F-positive control; lane 12 V617F-negative control; lane 13 water.

(Nichols Advantage, San Clemente, CA, USA) and ferritin (Bayer Centaur, Newbury, UK).

### JAK2 genotype analysis

X chromosome inactivation patterns indicate that, in patients with essential thrombocythaemia, lymphocytes and a variable proportion of peripheral blood granulocytes do not derive from the malignant clone.<sup>5,6</sup> Two sensitive methods were therefore developed to detect the V617F mutation in a minority of unfractionated peripheral blood cells. Mixing experiments showed that both methods were significantly more sensitive than sequencing. Allele-specific PCR detected a homozygous mutation at a one in 81 dilution in normal DNA

(equivalent to one heterozygous mutant cell in 40), and the *Bsa*XI digestion method detected the mutation at a one in 27 dilution (figure 1, A).

Both genotyping methods were used to analyse DNA extracted from unfractionated blood cells obtained from 776 patients with essential thrombocythaemia entered into the three prospective studies (figure 1, B). Genotypes obtained independently by these methods were concordant for 750 samples (concordance 97%; kappa score 0.93). In 23 of the 26 discrepant results, allele-specific PCR was reproducibly positive for the V617F mutation, which is consistent with the greater sensitivity of this method. In the 26 discrepant cases, the allele-specific PCR result was therefore used to assign *JAK2* status.

### Statistical analysis

We used Cohen's kappa score to estimate agreement between the two genotyping methods. Univariate analyses comparing diagnostic variables between *JAK2* V617F-positive and V617F-negative patients were done with the *t* test for continuous variables, Pearson's  $\chi^2$  test with Yates' continuity correction for 2×2 tables, and  $\chi^2$  test for trend for ordinal variables. Ferritin concentrations were compared with the Wilcoxon rank-sum test because data were heavily skewed. The erythropoietin-haemoglobin dose-response curves were modelled by fitting a regression model to log (erythropoietin) entering haemoglobin concentration (within a month of serum sample), haemoglobin-squared, age, sex, and treatment as explanatory variables. We assessed complication rates during follow-up using Kaplan-Meier life tables and log-rank analyses.

We calculated 95% CIs for odds ratios for events before diagnosis using  $(1/a+1/b+1/c+1/d)$  to estimate variance of log (odds ratio), and for events after trial entry using Peto's odds ratios  $(\exp[(O-E)/V \pm 1.96/\sqrt{V}])$ .<sup>21</sup> To model response to therapy, mixed-effects models were fitted to medication dose, blood count and response variables at 3-month intervals, treating time as a categorical variable, with an autoregressive moving average model ARMA(1,1)<sup>22</sup> of within-patient correlation. Blood counts within a month of each 3-month time-point were included, and analyses were done on the basis of both intention-to-treat and per-protocol bases (apart from medication-dose models, which were per-protocol only). Age, sex, baseline blood counts, and whether the patient was newly or previously diagnosed were entered as covariates in each model. The main hypothesis of interest was whether V617F-positive and V617F-negative patients differed in their response to therapy (drug-mutation interaction). V617F-time interactions were included only in the haemoglobin model, and no third or higher order interactions were included. To test for an interaction between randomised treatment and mutation status for

time-to-event data, we used Cox proportional hazards models with an interaction term. All analyses were done with S-Plus v 6.0.

### Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

### Results

The V617F mutation was present in over half of patients (table 1), giving an overall frequency of 53%. We considered the possibility that essential thrombocythaemia patients without the V617F mutation in peripheral blood leucocytes might nonetheless carry the mutation in the megakaryocyte lineage. To investigate this possibility, we used allele-specific PCR to detect the mutation in purified platelet cDNA from ten patients. The results were completely concordant with those obtained with granulocyte DNA from the same patients (figure 1, C), which indicated that patients negative for the V617F mutation in their granulocytes do not have the mutation in cells of the megakaryocyte lineage.

The proportion of V617F-positive patients was not significantly different in patients at high, intermediate, or low risk of vascular events, or in high-risk patients allocated to receive hydroxyurea or anagrelide (table 1),

	JAK2 V617F	JAK2 wild-type	p
Number	414	362	
Percentage (95% CI)	53.4% (49.8–56.9)	46.6% (43.1–50.2)	
<b>Risk category</b>			
Low*	26	31	
Intermediate†	68	50	
High‡	342	298	
Hydroxyurea + aspirin	169	151	0.8
Anagrelide + aspirin	173	147	
<b>Demographics</b>			
Female (number, %)	258 (62%)	206 (57%)	0.1
Male (number, %)	156 (38%)	156 (43%)	
Age (years, median, range)	60 (39–77)	52 (32–75)	<0.0001
Duration of disease§ (months, median, range)	38 (0–1131)	51 (0–2541)	0.06
<b>Laboratory and clinical features at diagnosis</b>			
Haemoglobin (g/L) – (mean, SD)	145 (14)	135 (14)	<0.0001
(median, range)	145 (128–163)	135 (117–153)	
White cells ( $\times 10^9/L$ ) – (mean, SD)	10.6 (3.4)	9.3 (2.6)	<0.0001
(median, range)	10.0 (7.0–14.5)	8.8 (6.3–13.0)	
Neutrophils ( $\times 10^9/L$ ) – (mean, SD)	7.4 (3.0)	6.2 (2.2)	<0.0001
(median, range)	6.8 (4.2–11.0)	5.8 (3.8–9.2)	
Platelet count ( $\times 10^9/L$ ) – (mean, SD)	902 (276)	1030 (343)	<0.0001
(median, range)	846 (632–1222)	962 (668–1535)	
Splenomegaly	11/329 (3%)	11/286 (4%)	0.9
Abnormal cytogenetics	14/314 (4%)	10/285 (4%)	0.7

Data are number of patients (%), unless otherwise indicated. \*Includes four patients (two vs two) who subsequently enrolled in the intermediate risk group, and ten (seven vs three) in the high risk group. †Includes four (two vs two) patients who were previously enrolled in the low risk group and 25 (13 vs 12) who subsequently enrolled in the high risk group. ‡Includes ten (seven vs three) and 25 (13 vs 12) who were previously enrolled in the low and intermediate risk groups, respectively. §Time elapsed between diagnosis and trial entry. Median range=10th–90th percentile.

**Table 1: Laboratory and clinical features at diagnosis**

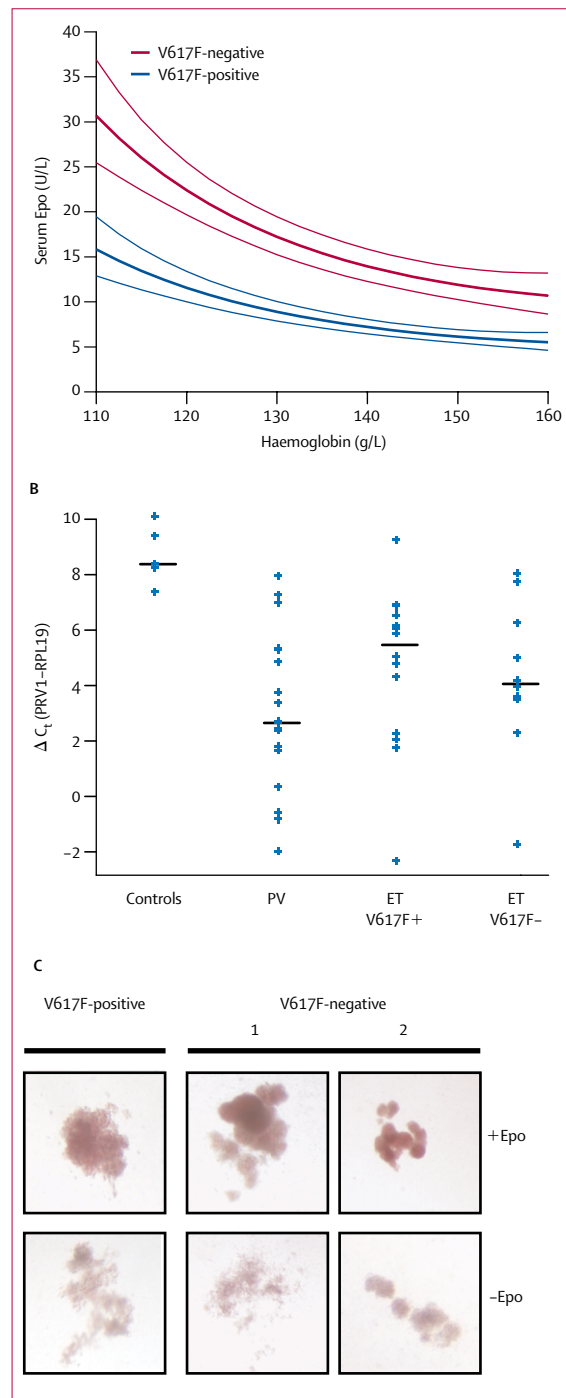
	JAK2 V617F (n=209)	JAK2 wild-type (n=184)	p
<b>Bone-marrow trephine histology</b>			
Reticulin grade (mean, SD)	1.8 (0.8)	1.8 (0.9)	0.6
<b>Megakaryocyte clusters and nuclear morphology</b>			
Clusters (absent/loose/tight)	30/113/66	26/88/70	0.3
Pyknotic (absent/present)	69/140	72/112	0.2
Staghorn (absent/present)	39/170	32/152	0.8
Cloud-like (absent/present)	55/154	59/125	0.3
<b>Cellularity*</b>			
Overall (dec/normal/inc)	1/47/161	5/64/115	0.0008
Erythroid (dec/normal/inc)	4/107/98	8/118/58	0.001
Granulocytic (dec/normal/inc)	4/92/113	7/103/74	0.005
Megakaryocytic (normal/+/++/+++)	1/35/113/60	1/40/88/55	0.6
<b>Erythropoietin concentrations and iron stores</b>			
Erythropoietin† (U/L) – mean (SD)	9.8 (10.8)	23.6 (28.0)	<0.0001
median (10th–90th centile)	6.9 (3.2–17.3)	15.0 (7.9–42)	
Mean cell volume‡ (fL) – mean (SD)	87.5 (6.5)	89.5 (6.0)	<0.0001
median (10th–90th centile)	88.2 (79.2–94.2)	89.4 (84.0–95.4)	
Mean cell volume <80 fL‡ – number (%)	43/414 (10.4%)	4/362 (1.1%)	<0.0001
Ferritin§ (µg/L) – mean (SD)	90 (91)	110 (81)	0.01
median (10th–90th centile)	58 (22–192)	91 (28–212)	

Data are number of patients (%), unless otherwise indicated. \*Dec=decreased; inc=increased; +, ++, +++, increasing cellularity †Based on 707 serum samples. Normal range for serum erythropoietin, 5–25 U/L. ‡Based on all 776 patients from table 1. Normal range for mean cell volume, 80–100 fL. §Based on serum samples taken within 3 months of diagnosis (n=182). Normal range for ferritin, 20–300 mg/L.

**Table 2: Bone-marrow trephine histology, erythropoietin, and iron stores.**

and there was no difference in disease duration before trial entry. There was no difference in the female to male ratio or in the frequencies of reported splenomegaly or abnormal cytogenetics, but V617F-positive patients were significantly older at presentation than those without the mutation (table 1).

There were striking differences in blood counts and bone marrow histological changes between V617F-positive and V617F-negative patients (table 1). At diagnosis, patients with the mutation had significantly higher haemoglobin concentrations (mean increase 9.6 g/L, 95% CI 7.6–11.6 g/L;  $p < 0.0001$ ), neutrophil counts ( $1.1 \times 10^9/L$ ,  $0.7$ – $1.5 \times 10^9/L$ ;  $p < 0.0001$ ), and total white cell counts ( $1.3 \times 10^9/L$ ,  $0.9$ – $1.7 \times 10^9/L$ ;  $p < 0.0001$ ) than had patients without the mutation. By contrast, V617F-positive patients had lower platelet counts than V617F-negative patients (mean decrease  $127 \times 10^9/L$ ,  $83$ – $172 \times 10^9/L$ ;  $p < 0.0001$ ). Bone-marrow trephines from 393 patients were assessed independently by three haematologists who did not know JAK2 status (table 2). There were no differences in mean reticulin grade; the presence, size, or type of megakaryocyte clusters; or the presence of pyknotic, staghorn, or cloud-like megakaryocytic nuclei between patients with and without the V617F mutation. However, overall cellularity was significantly increased in patients with the mutation, indicating increased granulocytic and erythroid cellularity. Thus, patients without the mutation tended to present with a more isolated thrombocytosis than did those with the mutation, but those with the mutation had increased



**Figure 2: Assessment of serum erythropoietin concentrations, PRV1 expression, and erythropoietin-independent colonies**  
 (A) Mean (95% CI) erythropoietin (Epo) according to haemoglobin in V617F-positive and V617F-negative patients with essential thrombocythaemia. (B) PRV1 expression in granulocyte cDNA from healthy controls, and patients with polycythaemia vera (PV), V617F-positive essential thrombocythaemia (ET) or V617F-negative ET.  $\Delta C_t$  (PRV1-RPL19)=difference in number of cycles to reach detection threshold between PRV1 transcripts and transcripts of the control gene, RPL19. (C) Erythroid colonies grown in the presence (upper) or absence (lower) of erythropoietin in a patient with V617F-positive (upper) and two patients with V617F-negative essential thrombocythaemia.

haemoglobin, neutrophil counts, and bone-marrow erythropoiesis and granulopoiesis, which are all features of polycythaemia vera.

The essential characteristic used to distinguish polycythaemia vera from essential thrombocythaemia is a raised red cell mass, usually accompanied by a high haemoglobin. In view of the phenotypic similarities between V617F-positive essential thrombocythaemia and polycythaemia vera we measured erythropoietin homeostasis and iron stores, which are two factors that might constrain erythropoiesis.

Erythropoietin concentrations at trial entry were significantly lower in V617F-positive patients with essential thrombocythaemia than in V617F-negative patients (table 2; mean decrease 13.8 U/L; 95% CI, 10.8–16.9 U/L;  $p < 0.0001$ ). Furthermore, for a particular haemoglobin concentration, V617F-positive patients had a lower serum erythropoietin than did those without the mutation (figure 2, A), even after correction for age, sex, and drug therapy ( $p < 0.0001$ ), suggesting that feedback suppression partly compensates for an increased erythropoietic drive mediated by the *JAK2* mutation.

Overt iron deficiency can mask the high haemoglobin and raised red cell mass associated with polycythaemia vera. In patients with essential thrombocythaemia, the mutation-positive subgroup had lower mean red cell volumes at diagnosis (2.0 fL; 1.1–2.9 fL;  $p < 0.0001$ ), contained more patients with a mean cell volume below the normal range (43 vs four patients;  $p < 0.0001$ ), and had significantly lower ferritin concentrations within 3 months of diagnosis ( $n=182$ ; median 58 vs 91  $\mu\text{g/L}$ ;  $p=0.01$ ) than those without the mutation.

Raised *PRV1* levels and the growth of erythropoietin-independent erythroid colonies have been said to identify a distinct subset of patients with essential thrombocythaemia,<sup>7</sup> although these findings have not been confirmed by other reports.<sup>8,23</sup> We therefore studied the relation of these two variables to *JAK2* mutation status. *PRV1* expression was higher in patients with polycythaemia vera than in controls (median  $\Delta\text{C}_t$ , 2.7 vs 8.4;  $p=0.0001$ ) (figure 2, B). However, there was no significant difference in *PRV1* expression between essential thrombocythaemia patients with and without the V617F mutation ( $p=0.7$ ), although both groups had significantly higher *PRV1* expression than controls ( $p=0.001$ , V617F-positive;  $p=0.003$ , V617F-negative). Similarly, erythropoietin-independent erythroid colonies were present in nine of 16 (56%) V617F-positive essential thrombocythaemia patients and four of 12 (33%) V617F-negative patients ( $p=0.4$ ; figure 2, C).

Table 3 shows clinical outcome according to *JAK2* status in the entire cohort of patients. Rates of arterial thrombosis did not differ between V617F-positive and V617F-negative patients, either in the year before diagnosis, or after trial entry. By contrast, the rate of venous thrombosis was significantly higher in V617F-positive patients in the year before diagnosis. Similarly,

	JAK2 V617F (n=414)	JAK2 wild-type (n=362)	Odds ratio (95% CI)	p*
<b>Arterial thrombosis</b>				
In year before diagnosis	38	24	1.4 (0.8–2.4)	0.2
Myocardial infarction	6	5		
Stroke	10	5		
Transient ischaemic attack	23	16		
After trial entry	25	21	1.1 (0.6–2.0)	0.8
Myocardial infarction	7	8		
Unstable angina	5	1		
Stroke	7	8		
Transient ischaemic attack	5	7		
Other†	2	1		
<b>Venous thromboembolism</b>				
In year before diagnosis	11	2	4.9 (1.1–22.3)	0.04
Deep vein thrombosis	3	1		
Pulmonary embolism	3	1		
Splanchnic vein thrombosis	2	0		
Retinal vein thrombosis	3	1		
Cerebral sinus thrombosis	1	0		
After trial entry	12	4	2.6 (1.0–6.9)	0.06
Deep vein thrombosis	6	4		
Pulmonary embolism	5	0		
Splanchnic vein thrombosis	2	0		
<b>Major haemorrhage</b>				
After trial entry	18	14	1.2 (0.6–2.4)	0.6
Gastrointestinal	6	8		
Intracranial	3	4		
Epistaxis	5	0		
Other‡	4	2		
<b>Death</b>				
	34	23	1.5 (0.9–2.4)	0.2
<b>Haematological transformation</b>				
Myelofibrosis	7	13	0.5 (0.2–1.3)	0.2
Acute leukaemia/myelodysplasia	5	2	2.5 (0.5–10.2)	0.3
Polycythaemia vera	6	0	6.8 (1.4–33.7)	0.01

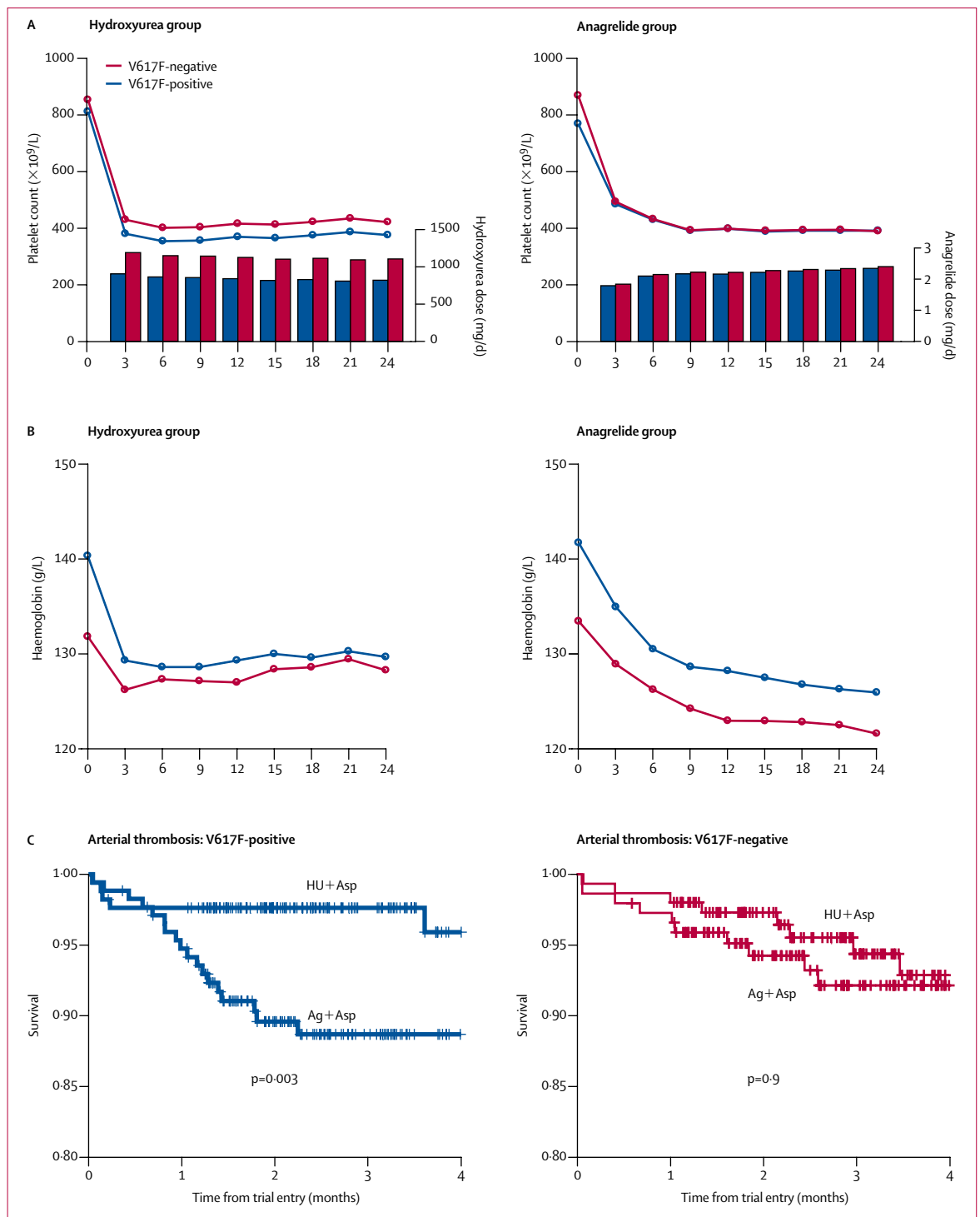
Data are number of events. \*Assessed by Pearson's  $\chi^2$  test with Yates' continuity correction for events preceding diagnosis and log-rank test for events after trial entry. †Lower limb arterial embolus (2); upper limb arterial thrombosis. ‡Pericardial (2), urinary (2), post-operative and obstetric

**Table 3: Major thrombotic, haemorrhagic, and transformation events after trial entry and in the year before diagnosis**

there were increased numbers of venous thromboses after trial entry, although the increases were not significant. Pulmonary emboli and uncommon sites for thrombosis (retinal vein, cerebral sinus, and splanchnic veins) were more noticeable in V617F-positive patients than in V617F-negative patients, as they are in patients with polycythaemia vera.

The rates of major haemorrhage and overall survival after trial entry between V617F-positive and V617F-negative patients did not differ. There were also no differences in the rates of transformation to either myelofibrosis or to acute myeloid leukaemia/ myelodysplasia, although numbers in this last category were small. However, it is striking that all six patients who transformed to polycythaemia vera were V617F-positive ( $p=0.01$ ).

The response of blood counts to treatment and the doses needed were analysed with mixed effects models<sup>22</sup> for 640 high-risk patients in the MRC PT-1 study for whom *JAK2* genotype data were available. To control for potential confounding effects, age, sex, blood counts at



**Figure 3: Platelet count, haemoglobin, and survival free of arterial thrombosis according to mutation status and time from trial entry**  
 Estimated population means for platelet count and medication dose (A) and haemoglobin (B) in patients randomised to hydroxyurea (left) and anagrelide (right); and (C) Survival free of arterial thrombosis in patients with high-risk ET.

trial entry, and whether or not the patient was newly diagnosed at trial entry, were entered as covariates.

Effect sizes and significance rates were much the same for intention-to-treat analysis and in analyses restricted to

patients remaining on allocated treatment. Here we present intention-to-treat analyses. In the hydroxyurea group, V617F-positive patients had a persistently lower platelet count than V617F-negative individuals (mean

corrected difference,  $-39.7 \times 10^9/L$ ; standard error  $12.5 \times 10^9/L$ ), an effect not seen for patients randomly assigned to anagrelide ( $11.0 \times 10^9/L$ ;  $12.5 \times 10^9/L$ ;  $p=0.004$  for drug-mutation interaction) (figure 3, A). Furthermore, V617F-positive patients on hydroxyurea required lower doses to control their platelet count than V617F-negative patients ( $-210$  mg daily;  $37$  mg daily;  $p<0.0001$ ), whereas no analogous difference was seen in patients receiving anagrelide ( $0.16$  mg daily;  $0.15$  mg daily;  $p=0.3$ ) (figure 3, A). Thus, compared with mutation-negative patients, V617F-positive patients were especially sensitive to hydroxyurea, with lower platelet counts despite receiving lower doses of drug, a pattern not seen with anagrelide.

Haemoglobin concentrations and white cell counts were consistent with the idea that V617F-positive patients are more sensitive to hydroxyurea. Patients who were positive for the V617F mutation had significantly higher haemoglobin concentrations at trial entry compared with those without the mutation. However, in those randomly assigned to hydroxyurea this difference was subsequently reduced ( $-0.7$  g/L;  $1.2$  g/L) (figure 3, B), whereas it was maintained in those allocated anagrelide ( $2.3$  g/L;  $1.2$  g/L;  $p=0.04$  for drug-mutation interaction). Similarly, initial differences in white cell count were subsequently reduced in those on hydroxyurea ( $0.1 \times 10^9/L$ ;  $0.2 \times 10^9$ ) but not anagrelide ( $1.1 \times 10^9/L$ ;  $0.2 \times 10^9$ ;  $p=0.0003$  for drug-mutation interaction).

The PT-1 trial showed that high-risk patients randomly assigned to anagrelide plus aspirin had higher rates of arterial thrombosis, major haemorrhage, and myelofibrotic transformation, but lower rates of venous thrombosis, compared with those in the hydroxyurea group.<sup>17</sup> We assessed whether these outcomes were affected by *JAK2* status in 640 high-risk patients.

No significant interaction was noted between allocated treatment and mutation status for major haemorrhage, myelofibrotic transformation, or venous thrombosis, although the small numbers of events means that an interaction cannot be excluded with certainty. By contrast, V617F-positive patients randomised to anagrelide had higher rates of arterial thrombosis than those randomised to hydroxyurea (19 vs five patients;  $p=0.003$ ; figure 3, C), whereas for V617F-negative patients there were equal numbers of arterial thromboses in the two groups (ten patients in each group;  $p=0.9$ ). Comparison of these results showed a significant interaction between allocated treatment and mutation status for arterial thrombosis ( $p=0.04$  for interaction).

## Discussion

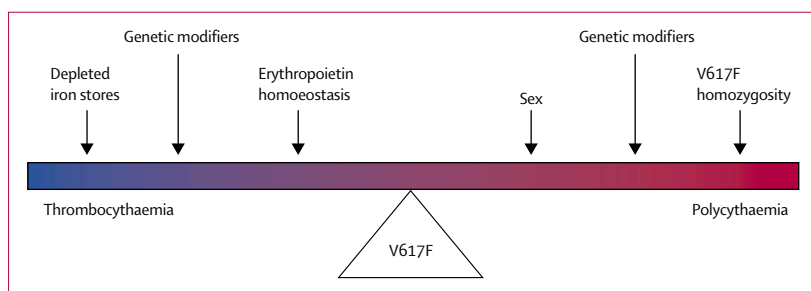
Our analysis of patients with essential thrombocythaemia enrolled in three prospective studies has shown that the *JAK2* V617F mutation unequivocally divides the disease into two subtypes, with the V617F-positive group showing phenotypic similarities to polycythaemia vera. The combined cohort provides a

unique resource for studying essential thrombocythaemia, especially in view of its large size, centralised review of endpoints, and comprehensive follow-up. Moreover, the participation of a large number of secondary and tertiary centres, together with the inclusion of patients in all risk categories, suggest that the results are of general relevance.

Although *JAK2* mutation status defines two subgroups of essential thrombocythaemia, predicting an individual's *JAK2* status based on routine clinical and laboratory features would be difficult. This observation might explain why the two subtypes have not been recognised previously, and probably indicates the multiple homeostatic mechanisms that maintain the steady-state number of mature blood cells within a narrow range. Compared with mutation-positive patients, those without the mutation tend to have a more pronounced and isolated thrombocytosis, with less active erythropoiesis and granulopoiesis. V617F-negative individuals do nonetheless have features characteristic of a myeloproliferative disorder, including cytogenetic abnormalities, hypercellular bone marrow with abnormal megakaryocyte morphology, *PRV1* overexpression, growth of erythropoietin-independent erythroid colonies, and a risk of myelofibrotic or leukaemic transformation.

Two other groups have reported the presence of erythropoietin-independent erythroid colonies in V617F-negative patients with myeloproliferative disorders,<sup>15,24</sup> and one group has shown that two-thirds of V617F-negative patients with essential thrombocythaemia have clonal granulopoiesis.<sup>24</sup> The molecular pathogenesis of V617F-negative essential thrombocythaemia remains obscure and might well be heterogeneous. Importantly, our results indicate that megakaryocyte-restricted mutation of *JAK2* is not a frequent occurrence in patients without the mutation in their peripheral blood granulocytes. Moreover, we have previously reported that 19 V617F-negative patients with essential thrombocythaemia did not have somatic mutations in genes encoding other JAK family members (Janus kinase 1, Janus kinase 3, Tyrosine kinase 2), Signal transducer and activator of transcription 5A (*STAT5A*), or 5B (*STAT5B*).<sup>25</sup>

V617F-positive patients had increased haemoglobin concentration, neutrophil counts, bone-marrow erythropoiesis, and granulopoiesis, together with more venous thromboses, especially in unusual anatomical sites, compared with patients without the mutation. These are all features of polycythaemia vera.<sup>26</sup> Moreover, most patients with polycythaemia vera are V617F-positive by allele-specific PCR,<sup>12</sup> and all six essential thrombocythaemia patients in our cohort who subsequently transformed to polycythaemia vera were V617F-positive. Our results therefore imply that V617F-positive thrombocythaemia and polycythaemia could be better viewed as a continuum, and not as two distinct entities (figure 4).



**Figure 4: Continuum model for V617F-positive thrombocythaemia and polycythaemia**  
The position of the arrows does not denote the relative strength of the various modifiers.

This viewpoint suggests that, in patients at the thrombocythaemia end of the continuum, the effects of the V617F mutation on erythropoiesis are constrained by physiological mechanisms, including erythropoietin suppression and depleted iron stores, or by genetic modifiers, either acquired or constitutional. Acquisition of homozygosity for the V617F mutation could favour development of a polycythaemic phenotype, since homozygosity for mutant *JAK2* occurs in roughly 30% of patients with polycythaemia vera, but is rare in essential thrombocythaemia.<sup>12–15</sup> Sex could influence presentation of V617F-positive disease, since polycythaemia vera is more common in men,<sup>27,28</sup> whereas V617F-positive thrombocythaemia is more common in women (table 1). Other genetic modifiers probably affect disease phenotype in a manner analogous to polygenic predisposition to many common diseases, a notion that might also account for the variability in platelet count, white cell count, and marrow fibrosis in V617F-positive patients.

Other workers report<sup>24</sup> that V617F-positive patients have higher haemoglobin concentrations than V617F-negative patients. Another group has reported that V617F-positive patients develop more complications than those without the mutation, although this analysis grouped together patients with polycythaemia vera, essential thrombocythaemia, and idiopathic myelofibrosis, all of which have different prognoses and frequencies of the V617F mutation.<sup>15</sup> Some researchers have postulated that mutation of *JAK2* might be a secondary event, arising after the development of a myeloproliferative disorder.<sup>15</sup> However, our data did not show any significant differences between V617F-positive and V617F-negative patients with essential thrombocythaemia in disease duration or in the frequency of features associated with advanced disease, such as splenomegaly, abnormal cytogenetics, and myelofibrotic or leukaemic transformation. Moreover, transplantation experiments show that expression of mutant *JAK2* in bone marrow cells is sufficient to cause short-latency erythrocytosis in mice.<sup>13</sup> These findings suggest an alternative model in which mutation-positive and mutation-negative essential thrombocythaemia represent distinct disorders, with the *JAK2* mutation sufficient for a myeloproliferative phenotype.

*JAK2* status was also associated with distinct responses to treatment. Patients with essential thrombocythaemia who were V617F-positive required substantially lower doses of hydroxyurea and yet had greater reductions in platelet counts, white cell counts, and haemoglobin concentration than did V617F-negative patients. No such effect was seen in patients receiving anagrelide. Furthermore, although subgroup analysis should be interpreted with caution,<sup>29</sup> a reduced prevalence of arterial thrombosis in V617F-positive patients receiving hydroxyurea compared with anagrelide accords well with the significantly increased sensitivity of V617F-positive patients to hydroxyurea.

Our results do not alter the conclusions of the PT-1 trial,<sup>17</sup> but they do suggest that V617F-positive patients gain particular benefit from hydroxyurea compared with anagrelide. The data presented here show that essential thrombocythaemia can be divided into two distinct subgroups with different presentation, complications, and response to therapy. Our results also show that V617F-positive essential thrombocythaemia and polycythaemia vera share multiple features, and suggest that the two disorders form a continuum, an idea with major implications for the classification, diagnosis, and management of these myeloproliferative disorders.

#### Contributors

P J Campbell and L M Scott equally participated in the design and interpretation of the experiments, genotype analysis, and writing of the paper. G Buck coordinated the day-to-day running and data entry of the three trials. P J Campbell did the statistical analyses under the guidance of K Wheatley, who also oversaw the running of the trials. C L East co-ordinated the DNA sample and consent form collection and clinical event validation. J T Marsden and A Duffy did the erythropoietin and ferritin assays, and E M Boyd and A J Bench did the *PRV1* expression assays. M A Scott and L M Scott set up and analysed the erythropoietin-independent erythroid colony assays respectively. G S Vassiliou purified and analysed the platelet cDNA. D W Milligan, J T Reilly and S R Smith participated in the acquisition and submission of patient data and samples. W N Erber, D Bareford and B S Wilkins were the three haematopathologists who reviewed the trephine biopsies. C N Harrison and A R Green are the trial co-ordinators for PT-1 and the intermediate and low-risk studies. A R Green planned, directed, and co-ordinated the research and revised the manuscript. All authors have reviewed the manuscript.

#### Conflict of interest statement

J T Reilly, D Bareford, C N Harrison, and A R Green have received consulting fees from Shire Pharmaceuticals. C N Harrison has also received research support and lecture fees from Shire.

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