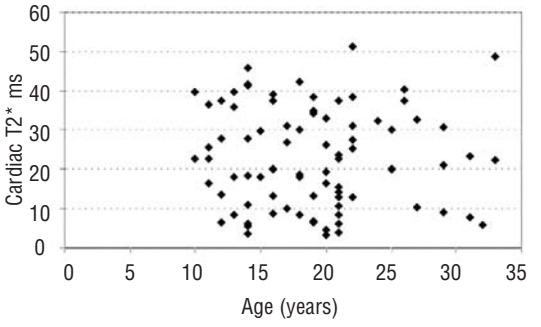

Figure 1. Correlation between cardiac T2* and age.

years had cardiac T2* lower than 10 ms. This could be due to sub-optimal chelation therapy but this is unlikely in view of their reasonable serum ferritin levels. The possibility of a genetic component for the susceptibility of cardiac iron loading in some populations should also be considered. A polymorphism of the glutathione S-transferase gene (*GSTM1* null genotype) has been associated with decreased signal intensity ratios on CMR in 41 Taiwanese patients.[11] However, our analysis of 81 Omani patients in this study found no correlation between null genotypes of either *GSTM1* or *GSTT1*.

Finally, adjusting chelation in heavily iron loaded patients, in particular increasing deferiprone dose, has resulted in a marked improvement in cardiac siderosis. The most severely affected patients (cardiac T2* lower or equal than 10 ms) showed a significant improvement from a mean of 7.3 ms±2.2 at baseline (range 3.4-10.2 ms) to 9.4 ms±3.6 (range 4.8-18.9 ms) at 18 months follow-up ($p<0.005$).

The availability of T2* MR at our institution has had a significant impact on patient management. All patients with substantial cardiac siderosis (T2* lower than 15 ms) (except one who had had deferiprone-induced agranulocytosis) have had combination therapy,[12] with optimization of deferiprone dose from 75 mg/kg/day to 90-100 mg/kg/day, in addition to deferoxamine ×3-5 weeks if serum ferritin was greater than 500 ng/mL. T2* CMR is a powerful tool in assessing cardiac siderosis and our results have allowed us to focus on those patients who are at most risk.

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Characterization of 35 new cases with four different *MPL*W515 mutations and essential thrombocytosis or primary myelofibrosis

Recently, mutations of *MPL*, the gene coding for the thrombopoetin receptor, were demonstrated in ~5% of cases of primary myelofibrosis (PMF) and in ~1% of all cases of essential thrombocytosis (ET).[1,2] They represent gain-of-function mutations that confer constitutive activation of the JAK-STAT pathway like *JAK2V*617F.[1,2] Two different amino acid exchanges of codon W515 resulting in a tryptophane to leucine (W515L) or lysine (W515K) were described. So far, W515 mutations have been found in ET and PMF, but were never detected in polycythemia vera (PV). Most cases had wild type *JAK2*V617.[1,3] To evaluate the *MPL*W515 mutations as markers for routine diagnostics of *JAK2*V617 unmutated myeloproliferative neoplasms (MPN), we performed analyses for *MPL*W515 mutations in a total of 869 selected MPN patients (399 males; 470 females; 12.2–90.3 years; median 60.5 years) from January 2006

Table 1. Summary of patients characteristics.

N.	Diagnosis	Sex	Age of onset (y)	Age at examination (y)	Sample	Mutation subtype	Ratio (MPL^{mut}/MPL^{wt})	Karyotype	WBC ($\times10^9$/L)	Hb (g/L)	Platelets ($\times10^9$/L)	Previous therapy
1	ET	F	51	51	PB	W515A	0.2	46,XX	8.6	11.9	743	None
2	ET	F	89	89	PB	W515K	Homozygous	46,XX	8.0	10.7	420	No
3	ET	F	80	81	PB	W515K	Homozygous	n.a.	3.2	10.4	589	No
4	ET	F	63	71	PB	W515K	Homozygous	n.a.	7.1	13.6	1042	Anagrelide
5	ET	M	81	81	PB	W515K	0.2	n.a.	5.0	12.8	100	No
6	ET	F	43	45	PB	W515L	0.8	n.a.	9.8	13.4	848	No
7	ET	F	77	77	BM	W515L	0.1	46,XY	6.4	9.4	1100	No
8	ET	M	78	78	PB	W515L	0.5	n.a.	7.8	12.2	648	Cumarine
9	ET	M	64	59	BM	W515L	0.3	46,XY	5.8	14.6	672	Anagrelide
10	ET	M	72	72	BM	W515L	0.3	46,XY	6.4	12.7	1222	n.a.
11	ET	F	52	59	PB	W515L	0.3	46,XX	6.7	8.7	767	HU, anagrelide
12	ET	F	47	47	PB	W515L	0.1	n.a.	6.7	11	660	No
13	ET	F	63	63	PB	W515L	0.2	n.a.	5.5	12.7	637	No
14	ET	F	78	78	BM	W515L	0.2	46,XX	3.7	9.3	1500	No
15	ET	M	77	77	PB	W515L	0.3	n.a.	11.2	9.5	460	n.a.
16	ET	F	70	70	PB	W515L	0.3	n.a.	8.2	11.9	726	Cumarine
17	ET	F	75	75	BM	W515K	0.2	46,XX,del(5)(q14q34), del(13)(q12q22)	10.6	10.7	908	ASS
18	ET	M	81	81	PB	W515L	0.2	n.a.	9.4	12.4	750	No
19	ET	F	67	67	PB	W515L	0.5	n.a.	6.3	12.4	787	No
20	ET	M	n.a.	83	BM	W515K	0.8	n.a.	5.4	9.8	320	n.a.
21	ET	F	n.a.	47	PB	W515K	Homozygous	n.a.	6.7	13.1	725	n.a.
22	ET*	M	30	39	PB	W515K	Homozygous	n.a.	13.5	14.7	873	HU, anagrelide
23	ET*	F	67	72	PB	W515K	0.1	n.a.	7.56	12.3	624	ASS
24	ET*	F	66	72	PB	W515L	Homozygous	46,XX	5.4	11.2	175	Steroids, ASS, HU
25	ET*	M	57	64	PB	W515L	Homozygous	n.a.	11.9	10.6	119	n.a.
26	ET*	M	58	60	PB	W515L	0.4	n.a.	2.8	8.0	66	Thalidomide, anagrelide, EPO
27	PMF	M	64	66	PB	W515K	Homozygous	n.a.	5.7	10.8	212	No
28	PMF	F	71	71	PB	W515K	Homozygous	n.a.	5.4	11.2	120	No
29	PMF	F	77	79	BM	W515L	0.4	46,XX	5.2	5.6	90	Steroids
30	PMF	M	73	76	PB	W515L	0.2	n.a.	32.3	13.1	90	ASS
31	PMF	M	61	63	PB	W515K	Homozygous	n.a.	3.7	6.1	221	Supportive therapy
32	PMF	M	63	71	PB	W515K	Homozygous	n.a.	5.5	10.6	400	No
33	PMF	M	n.a.	72	PB	W515R	Homozygous	n.a.	8.2	11.9	726	n.a.
34	s-AML after PMF	M	73	76	PB	W515L	Homozygous	46,XX	61.0	8.0	22	n.a.
35	CMML-1	F	82	82	BM	W515L	0.5	46,XX	14.000	12.5	896	No

*y: years; PB: peripheral blood, BM: bone marrow, HU: hydroxyurea, ET: essential thrombocytosis, PMF: primary myelofibrosis; WBC: white blood counts; ASS: acetylsalicylic acid; EPO: erythropoietin. *accompanying mild bone marrow fibrosis; n.a.: not analyzed.*

to December 2007. The patients seleted presented ET or suspected ET due to high thrombocyte counts (n=356), or PMF (n=193). In addition, 269 unclassified MPN and 51 PV were analyzed. There was a strong selection towards ET patients with *JAK2*V617 wild type (324/356) as we were mainly interested in further genetic characterization of this subgroup. Only 32 *JAK2*V617F mutated ET and 89 *JAK2*V617F mutated PMF were investigated to look for potential double mutations. Analysis for the *MPL*W515 mutation status was performed on peripheral blood (519 samples) or bone marrow (350 samples) by a melting curve based LightCycler assay with primers spanning W515 as previously described.[4] Cases with altered melting curve patterns were further analyzed by sequencing (Figure 1). Sensitivity of the assay was estimated by a limiting dilution assay (cDNA with homozygous *MPL*W515K in *MPL*W515wt cDNA) and was at least 5% (*Online Supplementary Figure 1*). Analysis for *JAK2*V617F was performed as previously described.[5] Cases were further evaluated by cytomorphology, cytochemistry, histopathology, and cytogenetics/FISH. The classification of disorders followed WHO criteria.[6]

In total, 35 *MPL*W515 mutations were detected in the 869 selected patients. A detailed description of these

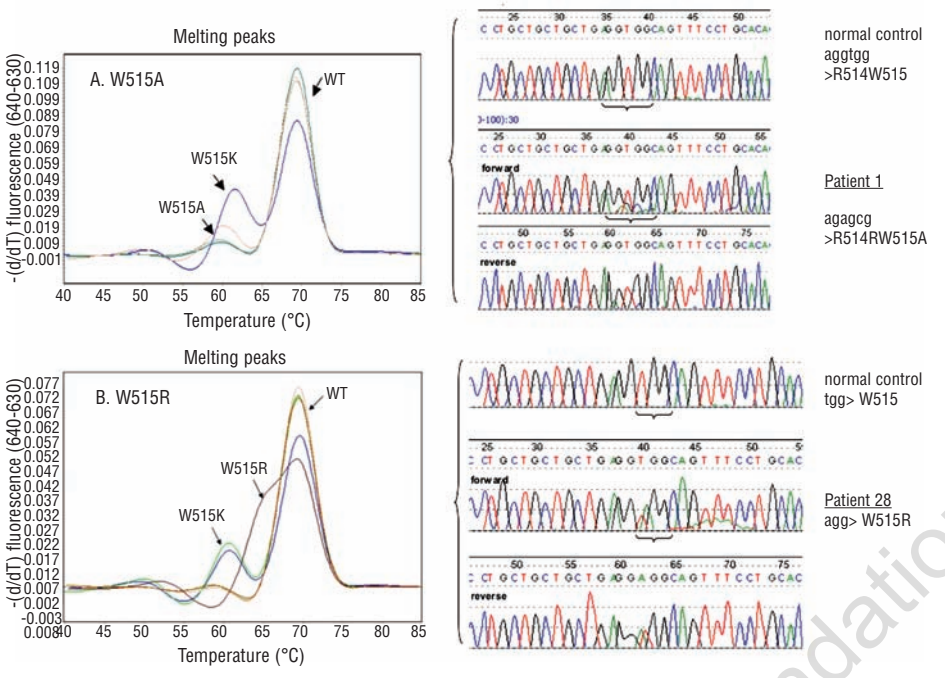

Figure 1. (A) **The novel *MPL*W515A mutation in case 1. This case revealed an approximately 0.2 mutation to wild type (wt) level. On the left the melting curve assay that detects the mutation. On the right further characterization by sequencing compared to a wt allele.** (B) **The novel MPLW515R mutation in case 28. On the left the melting curve assay that detects the mutation. On the right further characterization by sequencing compared to a wt allele. In addition to the W515R mutation this case has a silent mutation at AA position R515 due to g>a exchange.**

*MPL*W515 mutated patients is given in Table 1. In the total cohort with *JAK2*V617wt ET, any *MPL*W515 mutation was detected in 19 out of 324 patients (5.9%). In 104 *JAK2*V617wt PMF, a total of 10 *MPL*W515 mutations was detected (9.6%). In contrast, in the *JAK2*V617F mutated cases (32 ET; 89 PMF), no *MPL*W515 mutation was detected. Sequencing of the 35 *MPL*W515 mutations revealed four different *MPL*W515 subtypes: 20 patients had a W515L mutation, 13 a W515K, one case showed a so far not described W515A mutation leading to a tryptophane to alanine exchange, and another case a novel W515R associated to a tryptophane to arginine exchange (Figure 1). Although the functional relevance of these two new mutations still has to be evaluated, it has to be hypothesized that the replacement of a large amino acid by a smaller one probably alters the protein structure in both novel mutation subtypes.

Mutation/wild type ratios of greater than 1.0 indicated that at least some cells in the respective patient showed loss of the wildtype allele (LOH). Such high mutation ratios were detected in 13/35 (37%) mutated cases. Eight of these cases with high ratios were at advanced stage with a disease history of 2-9 years. Mutation ratios greater than 1% for the W515 were more frequent in PMF/s-AML following PMF (6/8 cases; 75%) when compared to ET (7/26; 27%) (*Online Supplementary Table S1a*). The mutation/wild type ratios in the remaining 22 patients were between 0.1 and 0.9% (median: 0.3%) (*Online Supplementary Table S1b*). Based on the applied method, cells with LOH could not be excluded in these *low level* cases because unapparent homozygousity based on dilution of homozygous cells with wild type cells may be present. However, the low level cases at least had less cells with LOH and thus the ratios may be important. This allows the hypothesis that higher proportions of W515 mutated alleles in total could indicate progression of disease. High mutation ratios were more frequent in the W515K (9/13; 69%) than in W515L (3/20; 15%) (p=0.034) corresponding to the results of Vannucchi *et al.*[7] and Beer *et al.*[8] Thus, the W515K mutation seems more often associated with loss of the wildtype allele. Karyotypes were available in 12 *MPL*W515 mutated cases. Eleven had a normal karyotype; one case had a del(5)(q14q34) and a del(13)(q12q22). It is remarkable that at least at the microscopic level, no LOH of chromosome 1p, where MPL is located, was detectable. This issue has to be investigated with more sophisticated techniques like SNP-array analyses.

The frequency of the *MPL*W515 mutation in our cohort corresponded to previous studies with 5.3% in the *JAK2*V617wt ET and 9.6% in *JAK2*V617wt PMF.[1,2] In contrast to previous findings, in our small cohort of 121 *JAK2*V617F mutated patients with ET and PMF there was no case with an *MPL*W515, whereas others found such a coexistence in up to 22% of *MPL* mutated MF cases.[1,3,9]

Finally, the W515 mutations have so far been identified in ET and PMF only.[1] Based on the new potential of the *MPL*W515 mutation in diagnostics, here one case (n. 35, Table 1) which had previously been classified as CMML probably has to be reclassified as ET due to thrombocytosis and the W515L mutation. As mutation analysis for MPLW515 mutations is easy and fast to perform, this case is a good example of how the respective mutation is now of potential help in routine diagnostics to reclassify suspected myeloproliferative diseases and discriminate them from reactive disorders.

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*JAK2*V617F mutational status and allele burden have little influence on clinical phenotype and prognosis in patients with post-polycythemia vera and post-essential thrombocythemia myelofibrosis

The *JAK2*V617F mutational status and mutated allele burden were evaluated in 65 patients with post-polycythemia vera or post-essential thrombocythemia myelofibrosis (PPV/PET-MF). All PPV-MF patients harbored the mutation as compared to 27% of PET-MF. The V617F allele burden was higher in PPV- than in PET-MF (72% *vs.* 50%); 78% of the patients had greater than 50% V617F allele burden, supporting an adverse role of highest allele burden for MF transformation. In cases of PET-MF, no meaningful difference between JAK2V617F mutated and unmutated patients could be ascertained. Patients in the highest quartile of V617F allele burden were significantly older and had higher leukocyte and $CD34^+$ cell count in peripheral blood than those in lower quartiles. There were 8 patients developing acute myeloid leukemia, who were equally distributed among *JAK2*V617F mutated or unmutated patients. We conclude that presence and burden of *JAK2*V617F mutation provide little clinically relevant information in patients with PPV/PET-MF.

Discovery of acquired recurrent molecular abnormalities in JAK2 (*JAK2*V617F mutation in exon 14 or mutations, insertions, deletions in exon 12) or MPL (mostly MPLW515L/K) has improved the diagnostic approach to the *classic* Philadelphia chromosome-negative chronic myeloproliferative disorders, defined as myeloproliferative neoplasms (MPNs) in the upcoming revised WHO classification.[1] Virtually all patients with polycythemia vera (PV) have a mutation in JAK2, which is represented by the V617F allele in greater than 95% of cases; frequency of *JAK2*V617F mutation is 60-70% in patients with essential thrombocythemia (ET) or primary myelofibrosis (PMF), while 10% of PMF patients[2] and up to 8% of *JAK2*V617F unmutated ET[3,4] patients harbor MPLW515L/K mutation. Presence of these molecular abnormalities constitutes a major diagnostic criteria in the 2008 WHO classification.[1] A number of studies have addressed the relevance of V617F mutational status and of mutated allele burden on hematologic characteristics and clinical presentation. In patients with PV or ET, the amount of V617F allele measured in granulocytes was found to be positively associated with hemoglobin level and leukocyte count and inversely with platelet count; furthermore, the higher the mutational burden, the higher the risk of presenting aquagenic pruritus, of developing splenomegaly or suffering from cardiovascular events, and of requiring cytotoxic therapy.[5] In PMF, results have been conflicting. An association of *JAK2*V617F mutational status with poorer overall survival was reported by Campbell *et al.*,[6] but this has not been confirmed by others.[7,8] A greater risk of developing large splenomegaly and leukemia was found in *JAK2*V617F mutated PMF patients included in a large study,[8] while conversely a better overall and leukemia-free survival for patients presenting the highest V617F allele burden was reported in an analysis from the Mayo Clinic.[9]