Platelet activation and multidrug resistance protein-4 expression in children and adolescents with different subtypes of primary thrombocythemia

The multidrug resistance protein-4 (MRP4), an ATP-binding cassette transporter, is involved in the efflux of several pharmacological and physiological compounds. MRP4 plays a role in modulating platelet function and influences platelet activation.[1] It has been identified as a modulator of the action of acetylsalicylic acid (ASA) in platelets. A high on-ASA residual platelet reactivity (HARPR) has been found in patients with MRP4 overexpression.[2] Furthermore, MRP4 overexpression induced by drugs mediates a hyperreactive platelet phenotype.[3] Recent studies have demonstrated that ASA also modulates various microRNA[4,5] and gene expression, such as that of PPARα, resulting in platelet MRP4 overexpression.[6] MRP4 knockout mice have reduced platelet function, delayed arterial thrombosis and prolonged bleeding time.[7] In patients who had undergone a surgical bypass procedure, MRP4 overexpression was found to have a role in reducing the effect of ASA and, at the same time, ASA induced platelet MRP4 upregulation.[8] In most patients with essential thrombocythemia (ET), ASA has been reported to incompletely inhibit platelet thromboxane A2.[9] Based on our experience, we suggested that the subtypes of primary thrombocythemia (PT) in children are different from those found in adults with ET: 40% had *JAK2*(V617F) or CALR-mutated ET, 36% had a *MPL*(S505A) mutation and received a diagnosis of hereditary thrombocytosis (HT), while 24% had *JAK2*, *CALR* and *MPL* wildtype ET.[10,11] Moreover, thrombotic events, frequent in adult ET and rare in pediatric PT, have been observed in HT children with the *MPL*(S505A) mutation during treatment with ASA.[12] This study was designed to evaluate and correlate MRP4 expression and platelet function in children and adolescents aged <20 years at diagnosis with different subtypes of PT.

MRP4 protein and platelet aggregation were evaluated in 30 healthy volunteers (HV) and in 41 PT patients (males: 18; females 23; median age at diagnosis: 14.5 years; range: 3 months-20 years). Of the 41 PT patients, 10 had the *JAK2*(V617F) mutation, 6 harbored CALR mutations, 10 carried a *MPL*(S505A) mutation and 15 were *JAK2, CALR* and *MPL* wildtype. Patients were grouped as follows: patients with *JAK2*(V617F) and *CALR* mutations (n=16); patients who had *JAK2, CALR* and *MPL* wildtype genes, defined as having triple-negative ET(n=15); patients with a *MPL*(S505A) mutation,

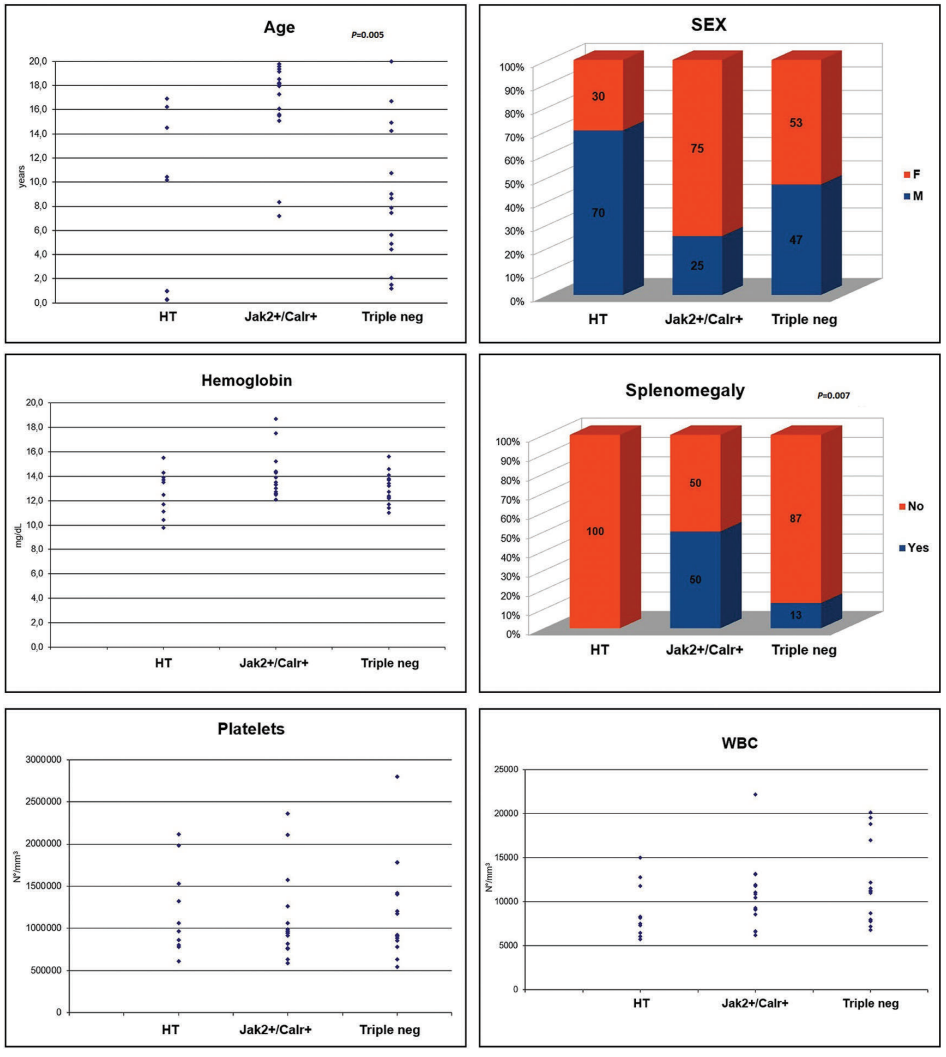

Figure 1. Characteristics of patients grouped according to genotype. Age, sex, splenomegaly, hemoglobin, white blood cell count, and platelet count in patients grouped according to genotype. HT: patients with the *MPL*(S505A)-mutation; *JAK2*+/CALR+: patients with a *JAK2*(V617F) or *CALR* mutation; triple-neg= patients with wildtype *MPL*, *JAK* and *CALR* genes; HT: hereditary thrombocytosis; WBC: white blood cells.

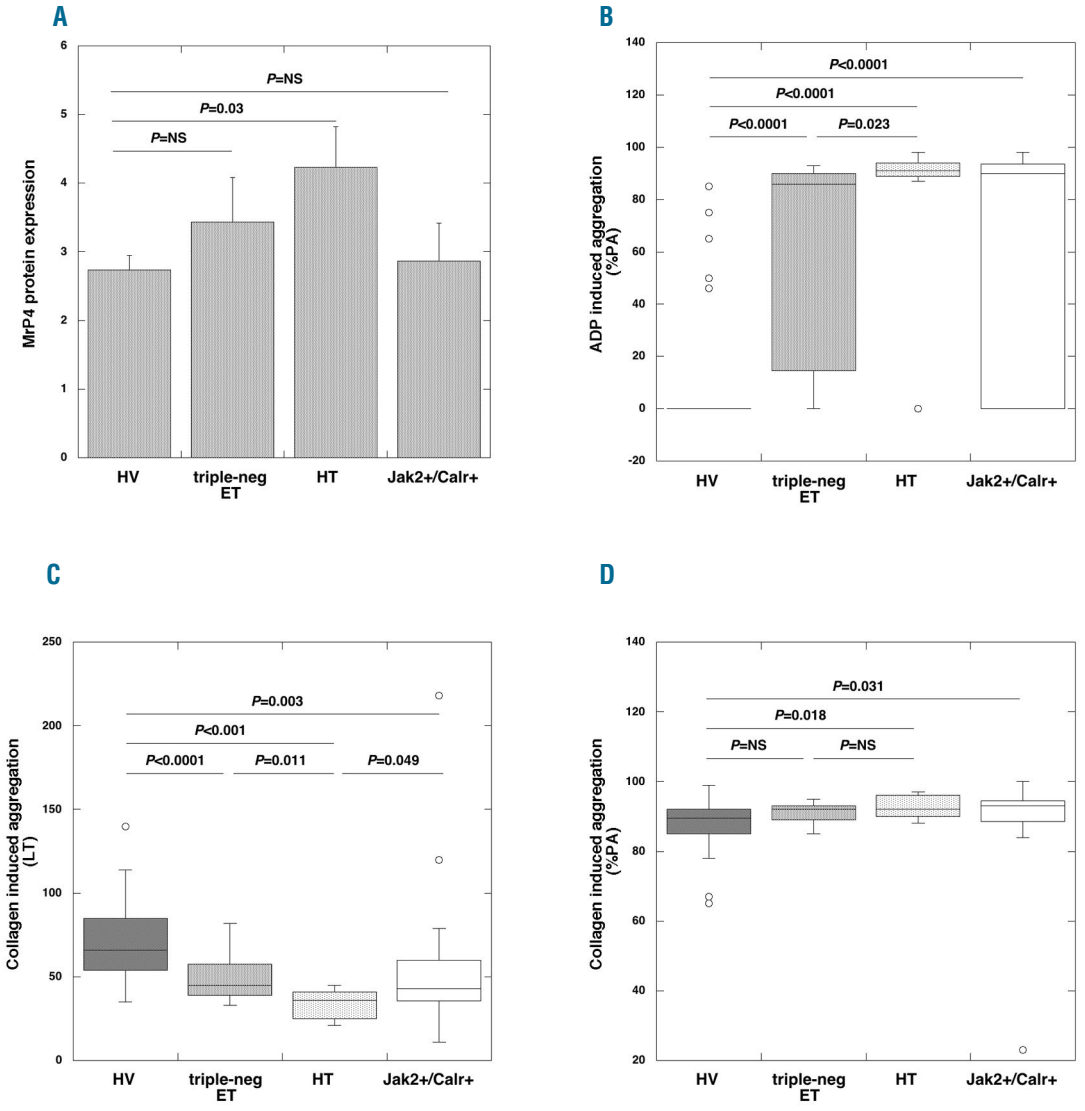

Figure 2. MRP4 protein expression and platelets aggregation. (A) Densitometric analysis results are reported as the ratio between the samples and a standard sample obtained from MRP4-transfected cells (HEK 293) (0.2 μg/μL of protein). Data were normalized to those of actin expression and are reported as mean ± the standard error of mean. (B) Box plot of platelet aggregation induced by ADP (0.8 μM). Platelet aggregation is reported as a percentage measured 4 min after adding ADP in all patients. (C, D) Box plots of platelet aggregation induced by collagen (1 μg/mL). Platelet aggregation is reported as the lag-time (C) and percentage measured 4 min (D) after the addition of collagen. HV: healthy volunteers; triple-neg ET: patients with wildtype *MPL*, *JAK* and *CALR* genes; HT: patients with the *MPL*(S505A)-mutation; JAK2+/CALR+: patients with a *JAK2*(V617F) or *CALR* mutation; LT: lag time; %PA: percentage platelet aggregation; NS: not significant: HT: hereditary thrombocytosis; ET: essential thrombocythemia.

defined as having HT (n=10). Platelet preparations were processed as previously described.[6] MRP4 platelet protein expression was evaluated by western blot according to Massimi et al.13 The densitometric analysis was carried out using the National Institutes of Health Image Ganalyzer program and the results are reported as the ratio between the samples and a standard sample obtained from MRP4-transfected cells (HEK293) (0.2 μg/μL of protein), as previously described.[3] Platelet aggregation was evaluated in platelet-rich plasma in a four-channel aggregometer (AggRam, Helena Laboratories, Beaumont, TX, USA) according to Temperilli *et al.*[14] The results are reported as the percentage of aggregation (%PA) observed after 4 min of stimulation with ADP (0.8 μM) and collagen (1 μg/mL) (Helena Laboratories, Beaumont, TX, USA). Platelet aggregation results are shown as a box plot graph. Statistical analyses were performed using the Mann-Whitney U-test, the Kruskal Wallis test, the χ^2 test, Wilcoxon test or Student *t* test, as appropriate. A *P* value <0.05 was considered statistically significant. This study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Ethics Committee. Informed written consent was obtained from each patient and/or parents and from the volunteers.

Hematologic features at diagnosis (platelets, white blood cells and hemoglobin concentration) were similar in the different groups of patients, whereas splenomegaly was more frequently found in *JAK2*(V617F)- and *CALR*-mutated patients (*P*=0.019). Moreover, *JAK2*(V617F)-mutated patients were significantly older than the other patients (*P*=0.005) (Figure 1).

MRP4 protein expression was significantly upregulated in HT patients (4.23±0.59) compared to that found in HV (P=0.03). This upregulation was more evident, but not significantly different, in triple-negative ET patients (3.43±0.64) than in HV (2.74±0.20). MRP4 protein expression was similar in *JAK2*(V617F)/*CALR*-mutated ET patients and in HV (2.86±0.54 and 2.74±0.20, respectively) (Figure 2A). Regarding platelet function, patients with HT showed a statistically significantly greater response to ADP 0.8 μM (median: 91 %PA; range: 0-98 %PA) compared to triple-negative patients (median: 86 %PA; range: 0-93 %PA) (P=0.023). The response to ADP was greater, albeit not significantly different, in HT than in *JAK2*(V617F)/*CALR*-mutated patients (median: 90 %PA; range: 0-98 %PA) (Figure 2B). All patients (triple-negative ET, *JAK2*(V617F)/*CALR*-mutated ET and HT) showed significantly greater aggregation compared to HV (P<0.0001). A significantly shorter lag-phase in response to collagen (1 μg/mL) was observed in HT (median: 36 s; range: 21-45) compared to triple-negative and *JAK2*(V617F)/*CALR*-mutated ET patients (median: 45 s; range: 33-82, P=0.011 and median: 43 s; range: 11-218, P=0.049, respectively). Compared to the lag-phase in response to collagen in HV (median: 66 s; range: 35-140), the lag-phase was significantly shorter in HT (P<0.001) triple-negative ET (P<0.001) and *JAK2*(V617F)/*CALR*-mutated ET (P=0.003) patients (Figure 2C). A significant enhancement of aggregation was evident when comparing HT patients (median: 92 %PA; range: 88-97 %PA) to HV (median: 89,5 %PA; range: 65-99 %PA) (P=0.018), and *JAK2*(V617F)/*CALR*-mutated patients (median: 93 %PA; range: 23-100 %PA) to HV (P=0.031). No significant differences were observed between HT, triple-negative ET (median: 92 %PA; range: 85-95 %PA) and *JAK2*(V617F)/*CALR*-mutated patients, probably due to the high variability and a lower number of patients analyzed (Figure 2D). To our knowledge, this is the first study providing evidence that both children and adolescents with MPL(S505A)-mutated HT show higher platelet reactivity compared to patients with ET. In addition, platelet reactivity correlates with MRP4 protein overexpression. This is in accordance with our previous study which demonstrated that platelets with MRP4 overexpression induced by drugs are more reactive to the agonist.[2] A recent study showed that individuals positive for human immunodeficiency virus show a hyperreactive platelet phenotype due to increased levels of platelet MRP4 expression.[15] Another study demonstrated that the abnormal megakaryopoiesis that characterizes ET accounts for a shorter-lasting antiplatelet effect of low-dose ASA through a faster renewal of platelet cyclooxygenase-1. It has been suggested that the impaired platelet inhibition can be corrected by modulating the time interval of administration rather than the dose of ASA.[9] We previously reported that an increased expression of MRP4 in platelets contributes to HARPR and that inhibition of MRP4-mediated transport reduces HARPR in patients on chronic ASA treatment.[2] Based on the results of this study, we can hypothesize that MRP4 overexpression may also have a role in reducing ASA activity in PT. This evidence helps to shed light onto the thrombotic events observed in HT *MPL*(S505A) patients despite treatment with ASA.

In conclusion, this study shows that children and adolescents with *MPL*(S505A) HT have greater platelet reactivity compared to patients with ET. Analysis of MRP4 expression levels in human platelets may be a useful biomarker in order to identify patients less sensitive to ASA treatment. We hypothesize that the addition of MRP4 inhibitors could enhance the effects of ASA. Based on these data, a cooperative study testing the use of MRP4 inhibitors in combination with ASA will be planned, with the aim of confirming our results and hypothesis.

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