Thrombosis in Myeloproliferative Neoplasms: A Single Center Experience of Using Whole Blood Platelet Aggregation Studies for Risk Assessment and Thromboprophylaxis

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Abstract
Thromboembolic complications are the most common causes of morbidity and mortality in patients with Philadelphia chromosome-negative myeloproliferative neoplasms (MPN); and prevention of these complications remains a significant clinical challenge. Effective thromboprophylaxis in MPN patients generally requires use of anti-platelet therapy, commonly aspirin; however, there are no standardized or universally accepted guidelines regarding the dose of aspirin. This study evaluates the usefulness of whole blood platelet aggregation (WBPA) studies to identify patients at risk for thrombosis and to achieve safe and effective long term thromboprophylaxis. One hundred and thirty-two consecutive patients were enrolled into this study. WBPA studies were performed at diagnosis in 125 patients to identify those with platelet hyperactivity (deemed to be at risk for thrombosis) and repeated 4 weeks after commencement of anti-platelet therapy to ascertain the efficacy. In patients with incomplete drug effect, treatment was revised and the study repeated until optimum effect was achieved. Results of the WBPA studies and anti-platelet therapy requirements were correlated with the underlying driver mutations and various international prognostic score of thrombosis for essential thrombocythemia (IPSET-Thrombosis) sub-groups. WBPA studies showed varying degrees of platelet hyperactivity in 115 patients. Based on these results, the patients were commenced on anti-platelet therapy comprising aspirin (dose ranging from 100mg twice or thrice weekly to 400mg daily) and clopidogrel (75mg daily) alone or in combination with aspirin or odorless garlic. None of the patients developed thrombosis during the follow up period ranging from 1-23 years (median 8yrs), while on the prescribed, individualized anti-platelet therapy. No significant differences were noted in terms of aspirin dose requirements between the JAK-2 positive and CALR or MPL positive patients, and, among the four IPSET-Thrombosis sub-groups. Patients with normal (9) or hypo (1) – activity were not given any anti-platelet therapy at diagnosis. Conclusion: Routine use of WBPA studies enables safe and effective risk-adapted thromboprohylaxis in MPN patients, irrespective of the underlying driver mutation and their risk predicted by the IPSET-thrombosis criteria.

Keywords
myeloproliferative neoplasms, thrombosis, risk assessment, thromboprophylaxis

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Introduction
Thrombosis, vascular and bleeding complications are the most common causes of morbidity and mortality in patients with the Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs) –ie, polycythemia vera, essential thrombocythemia and myelofibrosis.\textsuperscript{1-4} The risk of thrombosis is attributed to elevated cell counts, inflammatory cytokines that
are elaborated by MPN-specific driver mutations and host-specific inflammatory responses.5

Recently an International Prognostic Score of thrombosis for ET (IPSET-Thrombosis) has been proposed, to assess the thrombotic risk in individual patients with essential thrombocythaemia.6,7 IPSET-thrombosis identified four risk categories based on three adverse variables (thrombosis history, age >60 yrs and JAK-2 mutation: very low (no adverse features), low (presence of JAK-2), intermediate (age >60 yrs) and high (history of thrombosis or presence of both advanced age and JAK-2 mutation).

We have previously reported the usefulness of whole blood platelet aggregation (WBPA) studies in MPN patients to identify those at risk for thrombosis,8 and to optimize anti-platelet therapy in individual patients.9 Based on the results and our experience from these studies, we have used WBPA studies routinely on all new MPN patients referred to our center. This report, which also correlates the clinical course of patients with the underlying driver mutations and their risk predicted by the IPSET-Thrombosis criteria, describes our long term experience with this approach.

Materials and Methods

Patients

One hundred and thirty-two consecutive patients were entered into the study. A diagnosis of polycythemia vera, essential thrombocythaemia or myelofibrosis was made by standard criteria including bone marrow biopsy and/or molecular studies for JAK-2, CALR or MPL mutations.10 Two patients were diagnosed as MPN-unclassifiable (persistent, borderline blood cytosis without obvious cause, hypercellular marrow with morphological features of MPN and JAK-2 positivity) yet did not fulfill the criteria for a particular MPN. WBPA studies were performed at the time of diagnosis of MPN in all but seven patients (who were on oral anti-coagulant therapy for pre-existing atrial fibrillation).

Sample Collection

Venous blood was collected using a light tourniquet through 21-gauge needles into vacuette tubes (Becton Dickinson). A 4 ml EDTA tube was collected first, followed by four 3.5 ml 3.2% trisodium citrate tubes, collected using a loose tourniquet. The EDTA sample was used for determining the platelet count (Sysmex, Roche) and the citrate tubes were used for the WBPA studies using the lumi-aggregometer (Chronolog).

Unless being tested for the efficacy of prescribed anti-platelet medication, the patients were required to avoid any aspirin or NSAID agents for 10 days prior to testing, no garlic or other herbal remedies for 2 days, and no alcohol for 24 h. Testing on all samples was completed within 3 h of collection.

Whole Blood Platelet Aggregation Studies8,9

Whole blood platelet lumi-aggregometry was performed using the Chronolog Lumi-aggregometer (Model 700). Platelet aggregation was measured as an increase in impedance across an electrode and dense granule release was determined by luminescence using 100µl firefly luciferin-luciferase (DKSH, Australia). The citrate tubes were mixed by gentle inversion and then diluted with saline into a ratio of 1:1, 450µl citrate whole blood with 450µl saline into nine plastic cuvettes. A further cuvette was prepared containing 900µl of citrated whole blood for investigation of spontaneous aggregation. Disposable cuvettes were added to each cuvette. The tubes were capped and warmed at 37°C for 3 min prior to testing.

At commencement of testing, a standard amount of ATP (2 nM) was added to the first cuvette and the luminescence calculated. To the second cuvette, 1U/ml thrombin was added and the luminescence measured over 6 min. Simultaneous measurements of release and impedance were then measured on the next cuvettes over 6 min after agonist addition. In each case, the cuvettes were warmed for 3 min and impedance electrodes placed in the cuvette and calibration checked. The agonists used were 5 µl ADP, 10µl ADP, 2 µg/ml collagen, 0.5 mM/ml arachidonic acid, 0.2 mg/ml ristocetin and 1 mg/ml ristocetin (DKSH, Australia). Measurement of luminescence was not required for ristocetin. Platelet hyperactivity was determined as an increase in impedance or release with one or more agonists above the reference range.

Spontaneous aggregation was assessed in the final diluted cuvette and the cuvette containing the undiluted sample. Each cuvette was placed in the aggregometry wells for 15 min. Spontaneous platelet aggregation was present if the impedance increased by more than 1 Ohm over the 15 min.9

The reference range was established by testing 20 volunteers (Table 1) and compared with ranges established from previous whole blood aggregometry.8

WBPA studies were repeated four weeks after commencement of anti-platelet therapy.9 In patients with incomplete drug effect, treatment was revised and the study repeated until optimum effect was achieved. Aspirin dosage was considered adequate (ie, therapeutic) if the response to ristocetin gave a plot showing aggregation and disaggregation and if the response to arachidonic acid showed inhibition. A reduction in response to ADP was considered to reflect adequacy of clopidogrel therapy. Response to garlic was considered optimal when the overall platelet function corrected from hyperactivity to normal or hypo-activity. Combination of anti-platelet therapies was deemed therapeutic if there was correction from hyperactive to normal or hypo-active levels of aggregation and dense granule release; additionally, in patients on aspirin as one of the agents, adequate response included inhibition of aggregation with arachidonic acid.

Anti-Platelet Therapy

After documentation of platelet hyperactivity, patients were commenced on anti-platelet therapy, comprising mostly aspirin. Patients with a borderline abnormal result with one of the agonists and/or spontaneous platelet aggregation (Grade I platelet hyper-activity) were commenced on aspirin 100mg
Table 1. Reference Interval.

| AGONIST                        | IMPEDANCE (OHMS) | RELEASE (Nm) |
|--------------------------------|------------------|--------------|
|                                | RANGE | HYPO | HYPER | RANGE | HYPO | HYPER |
| COLLAGEN 2 µg/ml               | 16–40 | <15  | >40   | 0.4–2.0 | <0.4 | >2.0  |
| ADP 5 µM                       | 10–32 | <9   | >33   | 0.1–2.0 | <0.1 | >2.0  |
| ARACHIDONIC ACID 0.5mM         | 16–40 | <15  | >40   | 0.5–2.0 | <0.5 | >2.0  |
| RISTOCETIN 1 mg/ml             | <5    | <5   | >35   |        |      |       |

Low result associated with von Willebrand’s Disease

Platelet hyperactivity: Increase in impedance or release with one or more agonists above the reference range.

(PO) twice weekly; and those with borderline abnormal results with two agonists (Grade II platelet hyperactivity) were commenced on aspirin 100mg thrice weekly. Patients with clear evidence of platelet hyperactivity (Grade III) were commenced on aspirin 100mg daily. Clopidogrel and odorless garlic were used in patients with history of previous aspirin intolerance. Therapeutic modifications were made if the patient developed intolerance or side effects attributable to the anti-platelet drug or when the repeat WBPA studies (performed after four weeks of therapy) showed persistent features of hyperactivity, suggestive of inadequate or incomplete drug effect.

Results

Table 2 summarizes the clinical details of the 132 patients. Twenty-four of the 27 PRV patients have been treated with hydroxyurea (Hu) 20-30mg/Kg twice or thrice weekly,11 two patients who were initially treated with Hu are now on long-acting α-Interferon (α-IFN) weekly because of increasing dosage requirement. One patient who was initially treated with Hu, is currently on additional ruxolitinib because of transformation to MF. Thirty-five of the 98 ET patients have not received any cyto-reductive therapy. Sixty-one patients have been treated with Hu; One patient who was initially treated with Hu, is currently on long-acting α-IFN because of increasing dosage requirement. One patient is on a combination of Hu and ruxolitinib because of transformation to MF. All five patients with MF are on Hu therapy,11 while the two MPN-U patients are not on any cyto-reductive therapy.

One hundred and fifteen patients with documented platelet hyperactivity were commenced on anti-platelet therapy. Seven of the 132 patients (ET-5, MF-2) were on oral anti-coagulant therapy for atrial fibrillation at diagnosis; WBPA studies were not performed on these patients, nor anti-platelet therapy commenced at diagnosis. Ten of 132 patients who had normal platelet function (PRV-2, ET-6, MF-1) or hypofunction (PRV-1) were not commenced on any anti-platelet therapy.

Table 3 shows details of anti-platelet therapy (at diagnosis) in the 115 of 132 patients, correlated to their IPSET-Thrombosis sub-group and the underlying driver mutations; for the sake of completion details of the other 17 patients who had normal or hypo-active platelet function (10) or were on long-term anti-coagulant therapy (7) are also shown. The mean weekly aspirin dosage for the four IPSET-Thrombosis sub-groups were: VL – 1222mg, L – 1098mg, Int – 733mg and High – 1064mg. The mean weekly aspirin dose for patients who were JAK-2 positive and those with CALR or MPL mutation were 1077mg and 960mg, respectively.

The dosage of aspirin was reduced because of development of an easy bruising tendency in three patients (PV – 1: 300mg to 200mg/d; ET – 2: 400mg/d to 200mg/d; 200mg/d to 100mg/d). None of the other 112 patients on anti-platelet therapy manifested any bleeding complications. One patient who had normal platelet function at diagnosis presented with troublesome migraine headaches about six years later; repeat studies showed borderline platelet hyperactivity and she was commenced on aspirin 100mg thrice weekly, with a good response.

Ten patients (PRV-3, ET-7) developed AF during the follow-up period and were changed over from anti-platelet to anti-coagulant therapy. However, anti-platelet therapy was re-introduced subsequently because of recurrent superficial thrombophlebitis (ET-5) or erythromelalgia (ET-2). Anti-platelet therapy was also added to anti-coagulant therapy in one MF patient because of recurrent transient cerebral ischemic attacks.

None of the 115 patients who were commenced on anti-platelet therapy after documentation of platelet hyperactivity at diagnosis developed any arterial or venous thrombosis while on therapy. However, eight patients (PV – 2, ET – 6) developed clinical thrombotic events when off anti-platelet therapy (poor compliance – 2, on non-haematological medical advice – 6); cannula-induced upper limb venous thrombosis – 2; superficial thrombophlebitis – 1; lower limb DVT – 2; pulmonary embolism – 2; transient cerebral ischemic attack – 1). Recom mencement of anti-platelet therapy has precluded recurrence of any further thrombotic events.

Discussion

Our experience from this longitudinal study has confirmed the usefulness of WBPA studies to assess the thrombosis risk in individual patients with MPN and to optimize the anti-platelet therapy to achieve effective long-term thromboprophylaxis. Documentation of normal or hypo-active platelet function...
negates the need for anti-platelet therapy and tailoring the dose of the anti-platelet drug minimizes the risk of treatment-related clinical bleeding, commonly seen in MPN patients. This study also highlights three other clinical management issues: i) the occurrence of change in platelet activity in some patients, necessitating either dosage reduction of anti-platelet agent (because of decreasing platelet hyperactivity) or, commencement of anti-platelet therapy (because of development of platelet hyperactivity); ii) the need to consider closely monitored treatment related bleeding complications in others; and, iii) individualized anti-platelet therapy negates the need for twice daily dose of aspirin for thromboprophylaxis.

Both genomic alteration and acquired factors such as systemic inflammation are thought to be determinants of thrombotic risk in MPN patients. JAK-2 mutation-positive patients have been shown to have higher levels of in-vivo platelet activation markers. However, no significant correlation has been found between the degree of thrombocytosis and thrombosis; and, none of the platelet defects has been shown to be causal for thrombosis.

On the other hand, platelet activation has been shown to lead to platelet/leucocyte/endothelial interaction and secretion of inflammatory mediators, highlighting the role of platelets as inflammatory sentinels in the prothrombotic scenario in MPN patients. “Platelet hyperactivity, which can be identified by WBPA studies, seems to be the marker and reliable predictor of the prothrombotic tendency.”

Effective thromboprophylaxis in MPN patients generally requires use of anti-platelet therapy (commonly aspirin) and cyto-reduction therapy in those with significant blood cytosis. However, there are no standardized or universally accepted guidelines regarding the dose of aspirin or the choice of cyto-reductive therapy. The aspirin dose regimen, based on large clinical studies, has changed from 100mg daily to 100mg two or three times a day. Recently, the routine use of aspirin has even been abandoned in low-risk ET patients because of increased incidence of bleeding episodes. These diverse recommendations and practices highlight the limitation of the “one-size-fits-all” approach leading to sub-optimal thromboprophylaxis in some patients and treatment related bleeding complications in others. Our approach of using WBPA studies at diagnosis to identify patients at risk and tailored, individualized aspirin dosage has made the treatment effective as well as safe. We have also used the WBPA studies to ascertain the efficacy of therapy in patients treated with clopidogrel and/or garlic because of aspirin intolerance; and, re-evaluate and re-calibrate the anti-platelet therapy in patients who develop symptoms suggestive of change of platelet function.

Hydroxyurea (Hu), given at the dose of 20-30mg/Kg twice or thrice weekly, has been our first-line therapy in all MPN patients who require cyto-reductive therapy. This regimen has been very effective and well tolerated, achieving a sustained response over many years. Our experience differs from those described by Dom et al (who reported normalization of blood counts mostly only in the first months of therapy), Tefferi et al (marked oscillation of platelets) and Tauscher et al (marked oscillation of blood counts). However, we note that these authors have used a daily dosage schedule of Hu therapy (500mg – 1500mg/d, 1000mg/d and 500 – 3500mg/d, respectively) and the treatment was de-escalated or discontinued because of drug-related toxicity. In contrast, most of our patients have remained on intermittent Hu therapy for the long-term without any problems. We hope our report will generate

### Table 2. Summary of Clinical Details of Patients

| MPN | Patients | Age | Driver Mutation | Platelet Function | Follow-up |
|-----|----------|-----|-----------------|-------------------|-----------|
|     | No | M:F | Range (yrs) | Median (yrs) | JAK-2 | CALR | MPL | Hyper | Normal | Hypo | Range (yrs) | Median (yrs) |
| PRV | 27 | 15:12 | 35–90 | 68 | 26 | - | - | 24 | 2 | 1 | 1–19+ | 9 |
| ET  | 98 | 36:62 | 17–91 | 68 | 70 | 20 | 2 | 87 | 6 | - | 1–23+ | 7 |
| MF  | 5 | 5:0 | 61–86 | 75 | 4 | 1 | - | 2 | 1 | - | 1–8+ | 6 |
| MPN-U | 2 | 0:2 | 50–56 | 53 | 2 | - | - | 2 | - | - | 1–3+ | 2 |
| ALL | 132 | 56:76 | 17–91 | 53 | 102 | 21 | 2 | 115 | 9 | 1 | 1–23+ | 8 |

**PRV**: polycythemia vera; **ET**: essential thrombocythemia; **MF**: myelofibrosis; **MPN-U**: unclassifiable.

### Table 3. Anti-platelet Therapy Correlated with IPSET-Thrombosis and Driver Mutations

| Anti-platelet therapy at diagnosis | IPSET-Thrombosis (No. of patients) | Driver Mutation (No. of patients) |
|-----------------------------------|------------------------------------|----------------------------------|
|                                   | VL | L | Int | High | JAK-2 | CALR | MPL |
| 100 mg x 2–3/wk                   | 2  | 3 | 2  | 6    | 9    | 4    | 1   |
| 100 mg daily                      | 2  | 10| 11 | 28   | 39   | 8    | -   |
| Aspirin 200 mg daily              | 2  | 6 | 2  | 14   | 19   | 3    | 2   |
| 300 mg daily                      | 3  | 3 | -  | 10   | 14   | 3    | -   |
| 400 mg daily                      | -  | - | -  | -    | I    | -    | -   |
| Clopidogrel                       | -  | - | 2  | 3    | 2    | 1    | -   |
| Aspirin + Clopidogrel             | -  | - | -  | 3    | 3    | -    | -   |
| Clopidogrel + garlic              | 1  | - | -  | 1    | 1    | -    | -   |
| Normal/Hypo Platelet              | -  | - | -  | -    | 8    | 1    | -   |
| Function                          | -  | - | -  | -    | -    | -    | -   |
| Oral Anticoagulant Rx            | -  | 1 | 1  | 5    | 6    | -    | -   |

**VL**: very low risk; **L**: low risk; **Int**: intermediate risk; **High**: high risk.
interest and discussion about the appropriate use of Hu in MPN patients, leading to a large, multi-center, randomized study in the future.

“JAK-2V617F mutation is now recognized as a definite risk factor for thrombosis in MPN patients.” 7,17 Although patients with this mutation are known to display higher levels of in-vivo platelet activation markers,25,26 there has been no clinical study to date to correlate the aspirin dosage requirements in these patients in comparison to those with CALR or MPL mutations. Our present study (Table 3) has shown no significant difference in the three driver mutation groups. Around 50% of patients in each group achieved optimum anti-platelet effect at the aspirin dose of 100mg/d or less; while, one in seven patients in the JAK-2 or the CALR sub-group required aspirin 300mg/d or more. The long-term response to anti-platelet therapy was also the same in all three. driver mutation sub-groups – ie, no thrombotic events while the patients remained on the prescribed, individualized dose. We also note that 8 of 102 JAK-2 positive patients and 1 of 21 CALR positive patients did not require any anti-platelet therapy at diagnosis (cf normal platelet function).

Barbui et al6 and Haider et al7 have proposed a scoring system (IPSET-Thrombosis) to assess the risk of thrombosis in individual ET patients, to enable a risk-adapted therapy approach. However, the IPSET-Thrombosis score has not been prospectively validated. In the present study, we have correlated the WBPA results and the anti-platelet therapy requirements with the IPSET-Thrombosis score in all MPN patients–Table 3. Ten of the 14 patients in the “very low” risk group and 23 of the 24 patients in the “low risk” group had documented platelet hyper activity and were given anti-platelet therapy for thromboprophylaxis. Approximately 1 in 2 or 3 patients in the “very-low”, “low” and “high” risk sub-groups required aspirin 100mg/d or less, while 1 in 5, 6 or 8 patients, respectively, required aspirin 300mg/d or more to achieve optimum anti-platelet therapy. Thirteen of the 18 patients in the “intermediate” group required aspirin 100mg/d or less; none required 300mg/d. Finally, we note that six of the 76 patients in the “high-risk” group, who had normal platelet function, have been managed without any anti-platelet therapy for 6-22 yrs (mean 12.5 yrs). These results suggest that the risk-adapted therapy approach advocated by Barbui et al6 and Haider et al,7 is probably best served by using WBPA studies routinely in all newly diagnosed MPN patients. To the best of our knowledge, the potential utility of WBPA studies to tailor the anti-platelet therapy in individual MPN patients has not been reported previously. The limitation of this study is that it emanates from a single center, utilizing a test which is not in routine use in most haematology laboratories.

**Conclusion**

Routine use of WBPA studies enables safe and effective risk-adapted thromboprophylaxis in MPN patients irrespective of the underlying driver mutations and their risk predicted by the IPSET-Thrombosis criteria. The utility of this approach deserves, as well as requires, validation in larger studies from major tertiary referral centers.

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