BJHaem
BRITISH JOURNAL OF HAEMATOLOGY

ORIGINAL PAPER

Cytoreductive treatment and association with platelet function and maturity in patients with essential thrombocythaemia

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Summary

Patients with essential thrombocythaemia (ET) have an increased risk of thrombo-embolic events, which may differ according to different cytoreductive drugs. We investigated the effect of cytoreductive treatment on platelet function and turnover in ET patients. Blood samples were obtained at 1 and 24 h after aspirin intake. Platelet function was evaluated by platelet aggregation and flow cytometry. Platelet turnover was assessed by immature platelet count, immature platelet fraction (IPF) and mean platelet volume (MPV). A total of 47 ET patients were included and grouped into 21 patients not receiving cytoreductive treatment, 15 patients receiving hydroxy-carbamide and 11 patients receiving pegylated interferon alpha (peg-IFN). Patients receiving peg-IFN had significantly higher IPF and MPV than the other ET groups. Patients not receiving cytoreductive treatment had significantly higher platelet aggregation 24 h after aspirin intake than the other ET groups (p-values from 0.03 to 0.0002). Patients receiving hydroxycarbamide had significantly higher expression of platelet granule makers, P-selectin and CD63, than patients receiving peg-IFN (p-values ≤0.003). Cytoreduction provides more consistent platelet inhibition compared with no cytoreductive treatment. Moreover, peg-IFN provides superior inhibition of platelet activation markers than hydroxycarbamide, which in part may explain differences in risk of thromboembolic events in ET patients.

KEYWORDS
essential thrombocythaemia, hydroxycarbamide, interferon-alpha, platelet activation, platelet function

INTRODUCTION

Essential thrombocythaemia (ET) is a myeloproliferative disorder characterized by increased platelet count and turn-over primarily due to high clonal thrombocytosis with a median survival of approximately 20 years.[1–3] Current risk stratification of ET patients is based on the estimated risk of future thromboembolic events.[1] This stratification divides ET patients into four groups ranging from very low risk to high risk, and is based on age, history of thrombosis and Janus kinase 2 (*JAK2*) mutation status.[1] The treatment algorithm in ET patients follows this stratification and aims to reduce the risk of thromboembolic events.[1,4] Hence, treatment with both anti-platelet drugs, primarily aspirin, and cytoreductive drugs, such as hydroxycarbamide or pegylated interferon alpha (peg-IFN), is recommended.[1] Studies have shown that in some ET patients, the effect of aspirin is reduced during the usual 24-h dosing interval.[5–8]

This observation may partly be explained by increased platelet turnover in ET patients, leading to the release of newly formed immature platelets unaffected by aspirin and thus manifesting as high on-treatment platelet reactivity.[7,8] In addition, it has been reported that the risk of thromboembolic events may differ between ET patients receiving different cytoreductive drugs, although the findings are inconsistent.[9,10] It remains unknown whether cytoreductive drugs alters platelet function and turnover differentially. In the present exploratory study, we aimed to investigate the effects of two commonly prescribed cytoreductive drugs on platelet function and platelet turnover in a cohort of ET patients treated with aspirin 75 mg daily.

METHODS

Study population and design

We performed an observational cohort study on ET patients older than 18 years diagnosed in accordance with the WHO criteria,[11] and treated with non-enteric coated aspirin 75 mg once daily. ET patients were excluded if they received any anti-thrombotic treatment other than aspirin. Patients were divided according to current cytoreduction therapy and the type of cytoreductive drug. This was an exploratory and hypothesis generating study using different methods for investigating various associations. The Central Denmark Region Committees in Biomedical Research Ethics (Reference number: 1–10–72-426-17) and the Danish Data Protection Agency (Journal number: 1–16–02-916-17) approved the study. Informed consent was obtained from all patients, and the study was conducted in accordance with the Helsinki-II Declaration.

Blood sampling

Blood sampling was obtained from an antecubital vein with a 21-gauge needle with minimum of stasis and standardized at 1 and 24 h after oral aspirin intake. Platelet count, haemoglobin, white blood cell count, platelet distribution width, mean platelet volume (MPV), immature platelet count (IPC) and immature platelet fraction (IPF) were assessed in whole blood anti-coagulated with ethylenediaminetetraacetic (EDTA) (Becton Dickinson Bioscience) using an automated haematological analyser (Sysmex XN-9000, Norderstedt). Plasma fibrinogen was measured using CS2100i (Sysmex). Creatinine, C-reactive protein and urate was analysed in lithium-heparin tubes (Becton Dickinson Bioscience) using Cobas 6000 (Roche).

Platelet aggregation

Whole blood was collected in hirudin tubes (Roche) followed by 30 min of resting. Platelet aggregation was analysed within 2 h using the multiplate analyser (Roche).[12] To induce platelet aggregation, arachidonic acid (AA) (ASPItest, 0.5 mM), adenosine diphosphate (ADPtest 6.5, µM), and thrombin-receptor-activating-peptide (TRAP, 32 µM) were used as agonists. Platelet aggregation was quantified as area under the curve (AUC, aggregation units [AU] × minutes). If AUC by each of the two electrodes pairs varied more than 20% from the mean, measurements were repeated.

Platelet activation

Blood was collected in sodium citrate 3.2% tubes (Terumo Europe) followed by 1 h of resting. Preparation and fixation of samples was completed within 2 h. To assess platelet activation, the expression of the following activation-dependent markers on the platelet surface was measured by flow cytometry: bound fibrinogen, P-selectin (CD62p) and CD63 using a combination of the following fluorescence-labelled antibodies: anti-fibrinogen-FITC (polyclonal chicken, Diapensia HB), CD62p-APC (P-selectin, clone, Psel.KO2.3, eBioscience), CD63-PECy7 (GP53, clone H5c6, Becton Dickinson Bioscience) and CD42b-PE (GPIb, clone HIP1, eBioscience). All fluorescence-labelled antibodies were diluted in HEPES-buffer [NaCl 137 mM, KCl 2.7 mM, MgCl2 1 mM, 4-(2-hydroxyethyl)-1-piper-azineethanesulfonic acid (HEPES)] and titrated to saturating concentrations. Platelets were activated with the following agonists: ADP (10.7 mM, Sigma-Aldrich), TRAP (10 mM, JPT, Berlin Germany), collagen-related peptide (1.5 µg/ml, University of Cambridge, UK) and AA (0.58 mM, Sigma-Aldrich). The flow cytometry analysis was performed employing a NAVIOS flow cytometer (Beckman Coulter). For each sample, 10 000 platelets were assessed and identified with the antibody CD42b, followed by exclusion of platelet–platelet aggregates. To determine the percentage of positive platelets, gates where set to include 1%–2% positive events for bound fibrinogen and CD63 and 0.1%–0.2% for P-selectin on the negative control as previously optimized using single stained platelets and matching isotype controls.[13] Additional settings followed the MIFlowCyt guideline[14] including compensation, analysis and signal characteristics as previously described.[13,15] Quality control of fluorescence and particle size was performed daily according to the manufacturer's instructions using the Flow-Check Pro and Flow-set Pro (Beckman Coulter). Platelet measurements were expressed as median fluorescence intensity (MFI) and as percentage (%-gated) of marker positive platelets. A preactivation below 15% was considered acceptable.[13]

Thromboxane B_2

Collected blood was allowed to clot for 1 h at 37°C. Samples were then centrifuged for 10 min at 2600 g and stored at −80°C until analysis. Serum thromboxane B2 (TXB2) was measured in duplicates by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (Cayman Chemical). Samples were reanalysed with

appropriate dilutions if the results were outside the standard curve. Furthermore, samples were reanalysed if the duplicate measurements varied more than 20%.

Statistical analysis

The distribution of all data was evaluated by Q-Q plots and histograms. Continuous data were described as mean ± standard deviation (SD) when data were normally distributed and, if not, as median and interquartile range (IQR). Categorical data were described by percentages. We performed univariate linear regression to identify the continuous effect of independent determinants on platelet function. In the univariate linear regression analysis, a p-value < 0.10 was considered statistically significant using F-statistics. We performed multivariate regression using a parsimonious approach thereby only including significant predictors in the multivariate model. Further removal of included predictors from the multivariate regression model worsens the model fit defined as a significant reduction in the adjusted R-squared value. The multivariate regression analysis was not performed, if only one predictor was significant in the multivariate model. Variance inflation factor (VIF) was calculated, and only predictors with a VIF < 2 was included in the final multivariate regression model. Differences were analysed using an unpaired Mann–Whitney test for non-normally distributed data for continuous variables and Fisher's exact test for categorical variables. Paired analyses were performed using Wilcoxon signed rank test. Correlation analyses were performed with Spearman's ρ for data not following normal distribution. All tests of significance were two-tailed, with a probability value of $p < 0.05$. All statistics was performed in RStudio (Integrated Development for R., PBC) and GraphPad Prism 6 (GraphPad Software Inc.).

RESULTS

A total of 48 ET patients were included in the study. As only one ET patient received anagrelide as cytoreduction, this patient was excluded for further analysis. Table 1 shows baseline characteristics of the remaining 47 ET patients grouped according to current cytoreduction status: 21 patients did not receive cytoreductive treatment, 15 patients were treated with hydroxycarbamide and 11 patients received peg-IFN.

Platelet production and turnover

The ET patients receiving no cytoreductive treatment had significantly higher platelet count than ET patients receiving either hydroxycarbamide or peg-IFN (Table 1). Furthermore, ET patients on hydroxycarbamide treatment had significantly higher platelet count than ET patients receiving peg-IFN (Table 1). ET patients treated with hydroxycarbamide had significantly lower IPC than ET patients not receiving cytoreductive treatment (Table 1). ET patients on peg-IFN treatment had significantly higher IPF and MPV than both ET patients not receiving cytoreductive treatment and ET patients in hydroxycarbamide (Table 1). No other statistically significant differences were observed (Table 1).

Platelet aggregation

ET patients receiving hydroxycarbamide had significantly higher platelet aggregation using AA as agonist at 1 h after aspirin intake than both ET patients not receiving cytoreductive treatment (571 [IQR: 438–818] vs. 409 [IQR: 259–546] AU × minutes, $p = 0.046$) and ET patients on peg-IFN treatment (571 [IQR: 438–818] vs. 280 [IQR: 229–502] AU × minutes, $p = 0.04$) (Figure 1B). No significant differences in platelet aggregation using ADP or TRAP as agonist at 1 h after aspirin intake were observed (Figure 1).

At 24 h after aspirin intake, ET patients not receiving cytoreductive treatment had significantly higher platelet aggregation, than ET patients receiving either hydroxycarbamide or peg-IFN regardless of the agonist used (Figure 1). Patients on hydroxycarbamide treatment had significantly higher platelet aggregation using TRAP as agonist than ET patients receiving peg-IFN treatment (1301 [IQR: 1109–1379] vs. 1111 [IQR: 932–1226] AU × minutes, $p = 0.047$) (Figure 1C). No other significant differences were observed (Figure 1).

ET patients not receiving cytoreductive treatment had significantly higher platelet aggregation at 24 h after aspirin intake regardless of used agonist than ET patients receiving hydroxycarbamide treatment and in peg-IFN treated patients (p-values 0.03–0.0002) (Table 2).

Platelet activation

ET patients receiving hydroxycarbamide had significantly higher MFI expression of CD63 at 1 h after aspirin intake than ET patients on peg-IFN treatment (Table 3), whereas no significant difference in MFI expression of CD63 was observed between ET patients on hydroxycarbamide and ET patients not receiving cytoreductive treatment (Table 3). ET patients on hydroxycarbamide had significantly higher MFI expression of P-selectin at 1 h after aspirin intake than both ET patients not receiving cytoreductive treatment and ET patients in peg-IFN treatment (Table 3). No consistent significant differences in MFI expression of bound fibrinogen at 1 h after aspirin intake or %-gated expression at one 1 h after aspirin intake despite measured activation marker were observed (Table 3).

No consistent significant differences in expression of platelet activation markers were observed at 24 h aspirin intake between ET patients according to cytoreduction therapy (Table 4).

TABLE 1 Baseline characteristics of patients with essential thrombocythaemia according to cytoreductive treatment

	No therapy $n = 21$	Hydroxycarbamide $n = 15$	Peg-IFN $(n = 11)$	Cytoreduction vs. No therapy	Hydroxycarbamide vs. Peg-IFN	[a]Reference interval
Demographics						
Age, years	59 (51;71)	70 (67;73)	50 (45;65)	$p = 0.23$	$p = 0.002$	
Male sex, n (%)	8 (38)	5 (33)	7 (63)	$p = 0.77$	$p = 0.23$	
Risk factors						
Body mass index, kg/m²	23 (21;26)	25 (21;27)	25 (23;28)	$p = 0.62$	$p = 0.65$	
Current smokers, n (%)	3 (14)	1 (7)	0 (0)	$p = 0.31$	$p = 1.0$	
Medical history						
Time from diagnosis, years	3 (2;5)	4 (3;8)	5 (3;7)	$p = 0.02$	$p = 0.91$	
Previous thromboembolic event, n (%)	0 (0)	1 (7)	3 (27)	$p = 0.12$	$p = 0.28$	
Microcirculatory disturbances, n (%)	5 (24)	4 (27)	3 (27)	$p = 0.99$	$p = 0.99$	
JAK2 mutation, n (%)	16 (76)	9 (60)	6 (55)	$p = 0.23$	$p = 0.99$	
CALR mutation, n (%)	5 (24)	3 (20)	4 (36)	$p = 1.0$	$p = 0.41$	
MPL mutation or triple negative, n (%)	0 (0)	3 (20)	1 (9)	$p = 0.12$	$p = 0.61$	
Biochemistry and haematology						
Haemoglobin, mmol/l	8.7 (8.4;9.4)	8.5 (7.7;8.7)	8.4 (7.3;9)	$p = 0.03$	$p = 0.82$	7.3–10.5
Leucocyte count, $\times 10^9$/l	6.8 (6.3;7.9)	5.0 (4.6;5.9)	3.9 (3.4;6.1)	$p < 0.0001$	$p = 0.30$	3.3–10.5
Platelet count, $\times 10^9$/l	606 (534;898)	502 (438;596)	371 (265;409)	$p < 0.0001$	$p = 0.002$	145–400
Immature platelet count, $\times 10^9$/l	21.5 (11.8;32.2)	13.7 (8.5;20.1)	16.4 (11.5;30.4)	$p < 0.10$	$p = 0.31$	2.5–16.6
Immature platelet fraction, %	3.5 (2.0;4.7)	3 (1.7;4)	4.6 (4.2;7.3)	$p = 0.38$	$p = 0.001$	1.1–6.1
Mean platelet volume, fl	9.7 (9.1;10.4)	9.5 (9;10)	10.9 (10.2;11.2)	$p = 0.30$	$p = 0.0004$	6.5–11.9
Platelet distribution width, %	10.8 (10;12.3)	9.9 (9.3;11.7)	13.3 (11.4;13.9)	$p = 0.99$	$p = 0.0004$	8.3–56.6
Creatinine, µmol/l	71 (61;81)	65 (56;84)	64 (60;74)	$p = 0.35$	$p = 0.99$	45–105
C-reactive protein, mg/l	<8.0	<8.0	<8.0	$p = 0.24$	$p = 0.24$	<8.0
Urate, mmol/l	0.29 (0.27;0.37)	0.31 (0.26;0.34)	0.36 (0.32;0.38)	$p = 0.24$	$p = 0.02$	0.15–0.48
Fibrinogen, µmol/l	8.1 (7.4;9.4)	8.2 (7.6;9.9)	8.2 (7.6;10.3)	$p = 0.41$	$p = 0.89$	5.0–12.9

Note: Values are medians (interquartile range) unless otherwise indicated. Differences were analysed with unpaired Mann–Whitney test for continuous variables and with Fisher's exact test for categorical variables.

Abbreviations: CALR, calreticulin; CRP, C-reactive protein; JAK2, Janus kinase 2; peg-IFN, pegylated interferon alpha (pegasys).

[a] Reference interval is combined for men and female.
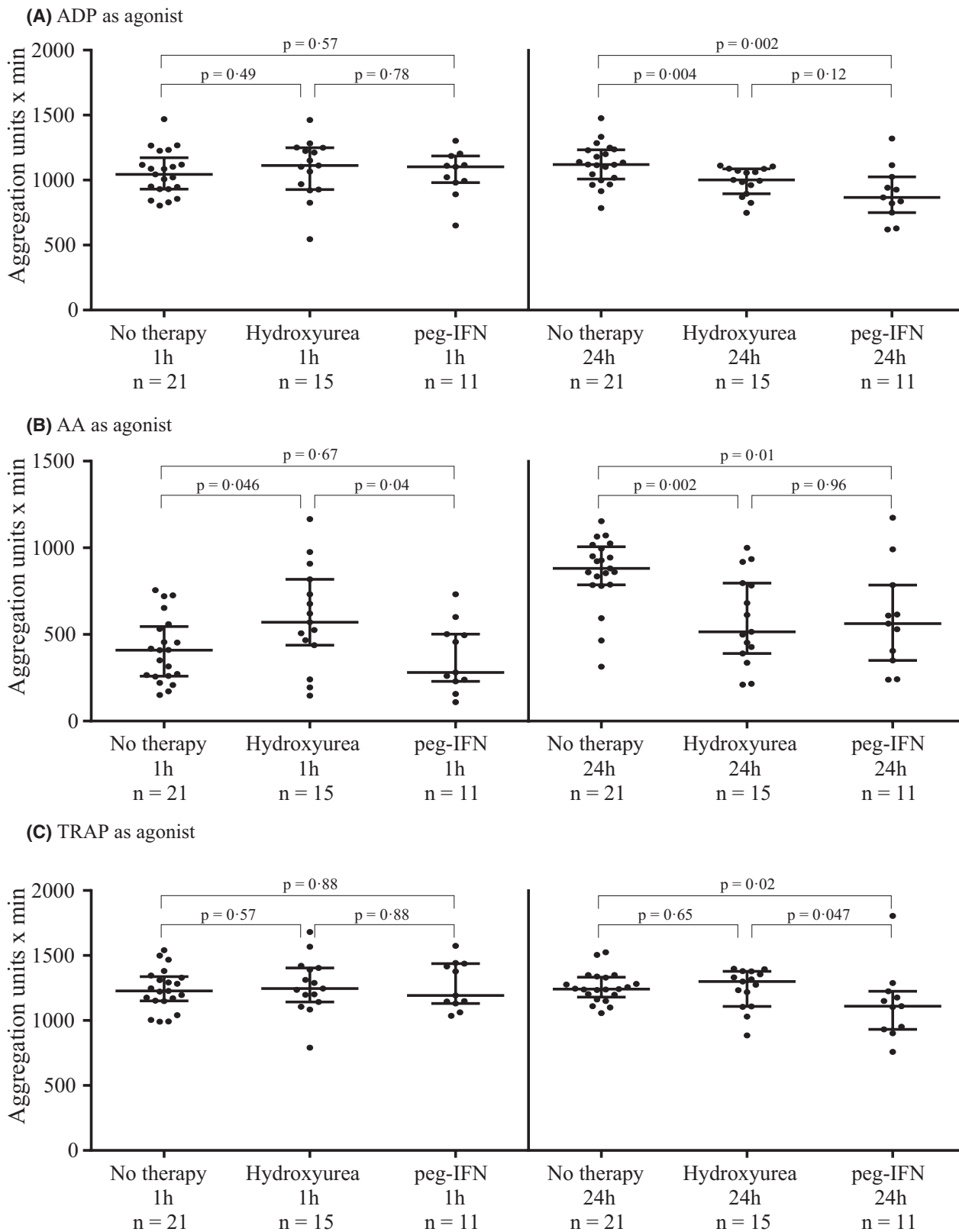
(A) ADP as agonist

(B) AA as agonist

(C) TRAP as agonist

FIGURE 1 Differences in platelet aggregation at 1 and 24 h samples after ingestion of 75 mg non-enteric-coated aspirin according to current cytoreductive treatment

TABLE 2 Platelet aggregation, comparing 1 and 24h samples after ingestion of 75 mg non-enteric-coated aspirin within the same treatment group and difference comparing samples divided by current cytoreductive treatment in patients with essential thrombocythaemia

	No therapy (A) n = 21				Hydroxyurea (B) n = 15				Peg-IFN (C) n = 11				***p-values		
	1 h	24 h	*% diff	**p-value	1 h	24 h	*% diff	**p-value	1 h	24 h	*% diff	**p-value	A vs. B	A vs. C	B vs. C
ADP as agonist															
	1044 931–1173	1121 1009–1234	7	0.23	1113 927–1249	1003 895–1088	−10	0.15	1102 981–1185	867 750–1026	−21	0.054	0.004	0.007	0.68
AA as agonist															
	409 259–546	882 787–1007	116	<0.0001	571 438–818	516 390–797	−11	0.93	280 229–502	563 351–786	101	0.32	0.0002	0.04	0.28
TRAP as agonist															
	1227 1151–1338	1242 1181–1335	1	0.57	1245 1143–1404	1301 1109–1379	4	0.80	1192 1130–1437	1111 932–1226	−7	0.08	0.78	0.03	0.14

Note: Values are aggregation units × minutes and indicated as medians and interquartile range. *Percentage difference calculated as (24h sample vs. 1 h sample) divided by 1 h sample, **p-value of the difference within the same groups are calculated using the Wilcoxon matched-pairs signed rank test, ***p-value of differences between groups are calculated using the Mann Whitney test.

Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; peg-IFN, pegylated-interferon; TRAP, thrombin-receptor-activating-peptide; 1 h, 1 hour sample; 24 h, 24 hour sample.

Predictors of platelet aggregation, serum thromboxane and platelet activation markers

Univariate regression analyses for predicting platelet aggregation and TXB_2 measurements are shown in Table S1. Statistically significant univariate predictors (*n* ranging from 2 to 4) were included in multivariate regression analyses, as shown in Table S2. Platelet count was the most consistent independent predictor of platelet aggregation, whereas age were independent predictors of TXB_2 levels.

Univariate regression analyses for predicting the expression of activation-dependent markers on the platelet using flow cytometry are shown in Table S3–S5. No variables were consistent independent predictors of bound fibrinogen, CD63 or P-selectin expression in the multivariate regression analysis (data not shown).

Differences in platelet indices and platelet function markers based on mutation type

TRAP-induced expression of activation dependent platelet surface markers measured with flow cytometry differed between ET patients with JAK2 mutation (*n* = 31) and ET patients with calreticulin (CALR) mutation (*n* = 12) independently of cytoreduction treatment (Figure S1). No significant differences were observed between these treatment groups regarding platelet activation markers, platelet count, IPC, IPF, MPV, TXB_2 or platelet aggregation (all *p*-values >0.05, data not shown). Four patients had either MPL mutation or triple negative mutation status and were excluded from the abovementioned analysis.

DISCUSSION

We included 47 ET patients and investigated the effect of two commonly prescribed cytoreductive drugs on platelet function and turnover.

The main finding of our study is that ET patients treated with hydroxycarbamide had significantly higher expression of activation-dependent platelet granule markers than ET patients receiving peg-IFN treatment. This finding cannot solely be explained by inherent differences between treatment groups, as age or platelet count are not predictors for activation-dependent platelet granule markers.

Currently, it is well-established that cytoreduction in ET patients protects against thromboembolic events.[1,16] Our study suggests that a more consistent platelet inhibition throughout the usual 24-h aspirin dosing interval in ET patients treated with cytoreduction may be one of the mechanisms for this underlying protective effect. However, it remains unknown if the risk of thromboembolic events differs between ET patients treated with different cytoreductive drugs. Some studies indicate that the risk may differ,[9,10,17,18] whereas a recent phase 3 study found no differences.[19] However, the study was not powered to detect a difference in the rate of thromboembolic events between

TABLE 3 Expression of platelet activation markers using flow cytometry at 1 h after aspirin intake in patients with essential thrombocythaemia divided by cytoreductive treatment in no therapy (n = 21), hydroxycarbamide (n = 15) and pegylated interferon (n = 11).

Agonist	Median fluorescence intensity						% gated					
	No therapy A	Hydroxy-carbamide B	Peg-IFN C	p-value A vs. B	p-value A vs. C	p-value B vs. C	No therapy A	Hydroxy-carbamide B	Peg-IFN C	p-value A vs. B	p-value A vs. C	p-value B vs. C
Bound fib												
Col	7.0 (5.2;9.0)	6.8 (5.9;7.9)	6.7 (5.5;7.5)	0.66	0.55	0.39	87.4 (73.7;93.3)	91.1 (83.8;93.9)	91.3 (83.4;93.9)	0.31	0.73	0.80
ADP	4.7 (3.3;5.8)	4.4 (3.8;4.9)	4.6 (3.4;5.2)	0.70	0.70	0.57	73.4 (63.8;78.9)	74.9 (71.6;78.1)	76.6 (66.5;81.2)	0.37	0.33	0.54
TRAP	4.7 (3.7;6.5)	4.9 (4.2;6.1)	4.2 (3.8;5.8)	0.62	0.65	0.30	86.4 (63.9;90.7)	85.9 (84.8;89.6)	83.9 (80.9;89.7)	0.47	0.53	0.65
AA	12.3 (9.2;13.6)	10.5 (8.4;12.0)	11.4 (9.0;13.3)	0.25	0.79	0.65	96.7 (94.0;97.7)	95.2 (93.0;97.9)	96.3 (92.4;98.4)	0.66	0.97	0.96
CD63												
Col	28.0 (21.4;32.9)	27.1 (24.7;31.2)	15.2 (11.8;17.9)	0.67	**0.0005**	**<0.0001**	68.5 (47.8;78.5)	74.1 (66.7;81.1)	76.9 (61.3;82.3)	0.17	0.31	0.65
ADP	18.2 (15.6;21.3)	16.3 (15.5;20.7)	8.7 (8.2;11.7)	0.72	**<0.0001**	**<0.0001**	38.0 (26.9;45.5)	37.9 (33.9;43.2)	43.6 (33.6;49.7)	0.55	0.09	0.18
TRAP	28.0 (21.4;30.3)	28.0 (25.2;30.7)	15.3 (13.5;17.6)	0.40	**0.0003**	**<0.0001**	69.1 (43.7;80.6)	76.7 (69.8;80.7)	81.3 (72.1;82.6)	0.19	**0.03**	0.16
AA	22.2 (17.8;25.5)	20.3 (19.2;26.3)	9.5 (8.7;13.5)	0.97	**<0.0001**	**<0.0001**	60.4 (54.1;64.0)	63.3 (53.6;66.9)	58.8 (54.2;65.7)	0.39	0.85	0.72
P-selectin												
Col	38.1 (27.8;50.6)	54.1 (44.1;59.7)	37.7 (26.8;46.0)	**0.008**	0.73	**0.003**	94.4 (88.3;97.8)	96.7 (96.3;97.8)	97.0 (94.2;97.8)	0.24	0.59	0.87
ADP	22.0 (17.7;58.6)	24.7 (23.6;27.3)	20.1 (17.9;21.8)	**0.01**	0.17	**0.0001**	94.6 (91.9;95.7)	94.8 (93.1;96.7)	95.5 (91.9;96.7)	0.61	0.38	0.76
TRAP	39.1 (26.9;58.5)	59.2 (51.7;65.9)	43.2 (35.4;45.2)	**0.01**	0.66	**0.0006**	97.6 (90.4;98.5)	98.2 (97.5;99.0)	98.0 (96.9;98.9)	**0.04**	0.10	0.81
AA	33.9 (27.2;40.2)	41.7 (33.6;48.1)	27.3 (23.2;36.1)	**0.01**	0.14	**0.003**	97.1 (96.6;98.0)	97.6 (97.1;98.3)	97.0 (96.2;99.0)	0.41	0.81	0.61

Note: Medians and interquartile range are presented. Statistically significant p-values are highlighted in bold.

Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; bound fib, bound fibrinogen; col, collagen-related peptide; peg-IFN, pegylated interferon; TRAP, thrombin-receptor-activating-peptide.

TABLE 4 Expression of platelet activation markers using flow cytometry at 24 h after aspirin intake in patients with essential thrombocythaemia divided by cytoreductive treatment in no therapy (n = 21), hydroxycarbamide (n = 15) and pegylated interferon (n = 11).

Median fluorescence intensity

Agonist	No therapy A	Hydroxy-carbamide B	Peg-IFN C	p-value A vs. B	p-value A vs. C	p-value B vs. C
Bound fib						
Col	7.8 5.8;9.2	7.2 6.5;8.4	6.1 5.5;7.5	0.81	0.24	0.17
ADP	4.9 3.8;5.5	4.9 3.7;5.9	4.3 3.4;5.1	0.70	0.47	0.48
TRAP	5.8 4.6;6.8	4.9 4.2;5.9	4.2 4.1;4.7	0.07	**0.01**	0.31
AA	11.9 9.0;13.4	10.4 9.0;11.9	11.4 9.7;13.8	0.24	0.96	0.34
CD63						
Col	24.5 17.3;30.6	29.1 16.2;33.6	22.8 12.4;26.6	0.25	0.27	0.08
ADP	15.5 12.6;17.5	18.7 12.3;21.0	15.3 8.4;21.4	0.22	0.90	0.26
TRAP	26.1 19.6;30.6	28.0 16.2;29.6	22.6 12.6;27.81	0.99	0.16	0.16
AA	18.7 13.5;22.8	19.9 13.5;27.1	18.5 9.7;24.5	0.54	0.95	0.64
P-selectin						
Col	47.2 35.4;56.9	49.4 38.8;60.2	37.1 21.8;44.0	0.84	0.10	0.08
ADP	24.3 20.4;26.1	24.3 19.9;28.3	22.8 16.4;28.0	0.73	0.90	0.77
TRAP	53.6 42.1;65.6	45.6 40.2;61.9	36.4 33.0;55.1	0.23	**0.04**	0.25
AA	39.1 28.0;42.8	31.5 28.3;38.4	35.1 27.8;44.4	0.13	0.84	0.66

%-gated

Agonist	No therapy A	Hydroxy-carbamide B	Peg-IFN C	p-value A vs. B	p-value A vs. C	p-value B vs. C
Bound fib						
Col	93.0 82.8;94.8	92.9 85.6;94.6	81.8 60.0;91.1	0.97	**0.01**	**0.002**
ADP	75.9 70.2;81.3	78.8 75.9;81.5	72.4 61.8;81.7	0.16	0.56	0.15
TRAP	89.0 86.0;92.4	88.2 79.8;90.2	80.3 68.2;86.0	0.18	**0.001**	0.09
AA	96.5 93.7;97.8	96.3 93.0;97.4	96.5 94.6;97.4	0.47	0.94	0.51
CD63						
Col	77.4 64.4;81.0	74.6 60.7;80.8	57.9 43.1;74.6	0.95	**0.02**	**0.02**
ADP	39.7 34.5;47.4	41.6 33.5;45.7	40.6 26.3;47.3	0.85	0.37	0.36
TRAP	80.0 58.8;80.8	77.0 58.8;79.7	64.0 52.6;79.7	0.14	**0.004**	0.36
AA	64.6 59.7;70.2	55.9 51.4;64.6	62.3 59.8;65.5	**0.02**	0.37	0.26
P-selectin						
Col	97.0 95.1;97.9	96.9 52.2;98.1	94.3 78.9;97.3	0.80	0.08	**0.01**
ADP	95.1 92.9;96.2	95.5 94.7;97.0	96.1 92.5;97.1	0.09	0.36	0.97
TRAP	97.9 97.6;98.7	98.2 96.0;99.0	97.5 93.6;98.8	0.83	0.37	0.85
AA	97.2 96.5;97.9	97.3 96.8;98.0	98.5 97.5;98.8	0.94	**0.03**	0.05

Note: Medians and interquartile range are presented. Statistically significant *p*-values are highlighted in bold.

Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; bound fib, bound fibrinogen; col, collagen-related peptide; peg-IFN, pegylated interferon; TRAP, thrombin-receptor-activating-peptide.

hydroxycarbamide and peg-IFN treated high-risk ET patients.[19] It has been reported that peg-IFN is an effective treatment in ET patients refractory or intolerant to hydroxycarbamide therapy.[20] While no international consensus exists, younger ET patients (<40 years old) tend to receive peg-IFN rather than hydroxycarbamide as first line cytoreduction due to a theoretical increased risk of leukaemic transformation related to hydroxycarbamide.[21–23] In this cohort, the allocation to cytoreductive treatment was based on clinical risk assessment using age above 60 years and additional cardiovascular risk factors for selection. Thus, ET patients not receiving cytoreductive treatment were younger. The choice of cytoreductive drug was decided by the treating physician considering patient preferences, but with a strong trend towards younger patients receiving peg-IFN and older patients getting hydroxycarbamide. In line with this, we found that ET patients receiving peg-IFN treatment were younger than ET patients receiving hydroxycarbamide, which might confound comparisons of treatment groups. However, it remains unknown whether the possible difference in the risk of thromboembolic events according to treatment with different cytoreductive drugs can be explained exclusively by this age variance, or if different cytoreductive drugs alter platelet function and turnover to a different extent. As age was not a consistent predictor of platelet function in our regression analyses, our findings suggest that hydroxycarbamide and peg-IFN alter platelet function in different ways. Interestingly, we found that while there were no consistent significant differences in platelet aggregation and in the expression of bound fibrinogen, ET patients receiving hydroxycarbamide had significantly higher expression of platelet granule markers than ET patients treated with peg-IFN.

ET patients have an accelerated platelet turnover leading to a higher proportion of newly produced immature platelets.[7] These immature platelets are known to be more reactive than mature platelets explained by the ability to produce proteins important for platelet function.[24,25] However, in the present study, we found that ET patients in peg-IFN treatment had significantly higher levels of platelet turnover markers (IPF and MPV) than ET patients in hydroxycarbamide treatment, whereas the opposite was the case for platelet activation markers. No significant difference was observed in the absolute number of immature platelets. Differences in platelet turnover between ET patients treated with different cytoreductive drugs may therefore not solely explain the observed differences in risk of thromboembolic events.

The observed differences in platelet indices in ET patients according to different cytoreductive drugs may partly reflect their different mechanisms of action. In ET patients, hydroxycarbamide exerts its effect by inhibiting ribonucleotide diphosphate reductase activity leading to cell death.[26] Hydroxycarbamide thereby reduces the platelet count as well as levels of other blood cells.[26] The assumed relevant mechanism of actions of peg-IFN in ET patients includes an antiproliferative effect on megakaryocytes and a reduction in platelet half-life.[27] We found that peg-IFN was more effective in reducing the platelet count than hydroxycarbamide, while no significant difference in IPC were observed. Furthermore, the observed increased level of IPF and MPV in peg-IFN

treated ET patients compared with hydroxycarbamide treated ET patients may reflect the ability of peg-IFN to effectively reduce platelet count without a corresponding increase in platelet production. In addition, the difference in platelet markers between treatment groups likely also reflects the diverse influence on megakaryopoiesis by hydroxycarbamide and peg-IFN and the fact that cytoreduction may not reverse all abnormal platelet markers.[28] In addition, a recent study found that longer treatment with peg-IFN was more effective in normalizing levels of blood cells and reducing driver mutation burden, while hydroxycarbamide produced more histopathologic responses.[19] Our study shows for the first time that hydroxycarbamide and peg-IFN lead to alterations in activation-dependent granule markers in platelets. The mechanism of action of these drugs is still not fully understood.

The strengths of the present study were the inclusion of strictly WHO-defined ET patients and the use of several methods and agonists to examine platelet function and maturity. In addition, the anti-thrombotic therapy was strictly standardized. However, some limitations have to be considered. First, based on the observational exploratory nature of the study, caution is required when comparing findings between the no treatment, hydroxycarbamide and peg-IFN treatment groups in the study. Also, even if the total number of included ET patients was reasonably large, dividing patients into groups leads to a reduction in power. Information on other concomitant pharmacological treatment including dugs that may influence platelet function was unfortunately not available. Furthermore, our study was not powered to assess clinical events.

In conclusion, treatment with cytoreductive drugs in ET patients provides a more consistent inhibition of platelet function throughout the day compared with ET patients without cytoreduction. ET patients treated with hydroxycarbamide have an increased expression of activation-dependent granule markers compared with ET patients receiving peg-IFN. It remains to be explored if the observed difference in platelet function is reflected in the risk of thromboembolic events.

AUTHOR CONTRIBUTIONS

All authors designed the research study; Oliver Buchhave Pedersen performed the research; all authors analysed the data and contributed to writing the manuscript.

CONFLICT OF INTEREST

None related to this manuscript. The authors report the following general conflicts: Erik Lerkevang Grove has received speaker honoraria or consultancy fees from Alexion Pharma, AstraZeneca, Bayer, Boehringer Ingelheim, Bristol-Myers Squibb, Lundbeck Pharma, Pfizer, MSD, Mundipharma,

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