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Title Page

Manuscript Title: Effect of Bruton's Tyrosine Kinase inhibitors on platelet aggregation in patients with Acute Myocardial Infarction

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Abstract

Aims: Despite widespread use of dual antiplatelet therapy in acute myocardial infarction, there remains a residual risk of morbidity and mortality. Bruton's Tyrosine Kinase inhibitors have been found to inhibit platelet aggregation through the Glycoprotein VI collagen-mediated pathway. The Bruton's Tyrosine Kinase inhibitor, Ibrutinib is used in the management of haematological malignancies and another Bruton's Tyrosine Kinase inhibitor, ONO-4059 (also known as tirabrutinib), is in clinical development. This is an observational study to evaluate the effects of Ibrutinib and ONO-4059 on platelet aggregation after acute myocardial infarction.

Methods and Results: Twenty patients with a confirmed diagnosis of acute myocardial infarction were enrolled and blood samples obtained within 48 hours of hospital admission. All patients were on dual antiplatelet therapy; aspirin plus a P2Y₁₂ inhibitor (clopidogrel or ticagrelor). Blood samples were treated *ex vivo* with increasing concentrations of Ibrutinib (0, 0.5, 1, 2 μ M) and ONO-4059 (0, 0.2, 0.5, 1 μ M). Platelet aggregation was measured in response to collagen using a Multiplate analyser to estimate the area under the curve, with lower values indicating lower platelet aggregation. The median age was 63 years and 80% were male. The median area under the curve values for Ibrutinib concentrations 0 (control), 0.5, 1 and 2mmol/l were 18.5, 8 (P=0.0004), 4.5 (P<0.0001) and 2 (P<0.0001) units and for ONO-4059 concentrations 0 (control), 0.2, 0.5 and 1 μ M, median area under the curve values were 13, 12 (P=0.7), 6.5 (P=0.0001) and 5.5 (P=0.0004 compared to control).

Conclusion: The Bruton's Tyrosine Kinase inhibitors, Ibrutinib and ONO-4059, show further inhibition of platelet aggregation in blood samples from patients with acute myocardial infarction, receiving dual antiplatelet therapy in a dose dependent

manner. These results provide a rationale for Bruton's Tyrosine Kinase inhibitors to be tested as a potential new antiplatelet strategy for acute myocardial infarction.

Keywords: Ibrutinib; Acute myocardial infarction; Bruton's Tyrosine Kinase Inhibitor; Tyrosine Kinases; platelet aggregation

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1. Introduction

Dual antiplatelet therapy, with aspirin and a P2Y₁₂ inhibitor (commonly, clopidogrel or ticagrelor), is a cornerstone of the long-term management of acute myocardial infarction (MI). [1-3] Despite its widespread use, there is a 15% risk of death or recurrent events at 12 months, and 8% of acute MI patients are readmitted within one month of an acute episode. [4] This suggests there is scope for development of anti-platelet therapies which act via alternative pathways.

Ibrutinib is a Bruton's tyrosine kinase (BTK) inhibitor, which is licensed for the treatment of chronic lymphocytic leukaemia (CLL), Mantle Cell Lymphoma (MCL) and lymphocytic lymphoma and exerts its therapeutic effect by disrupting malignant B-cell function and survival. [5-8] With long-term follow up, more than half of patients on Ibrutinib experienced a bleeding event [9,10] and the pooled relative risk of the occurrence of any bleeding with Ibrutinib is 2.72. [11] The majority of bleeds are cutaneous in nature and there is a low occurrence of major bleeding. [8] *In-vitro* and *ex-vivo* studies have found Ibrutinib significantly inhibits platelet aggregation in response to collagen. [12-14] Ibrutinib irreversibly inhibits BTK and Tec, both of which are non-receptor tyrosine kinases involved in platelet aggregation, via the Glycoprotein VI (GPVI) collagen mediated pathway. [15,16] This pathway is not currently targeted in the management of acute MI.

Several other BTK inhibitors are in clinical development stages. [17] ONO-4059 (also known as tirabrutinib) is an irreversible selective inhibitor of BTK, which in a Phase I trial of 90 patients with relapsed/refractory B-cell malignancies had a lower incidence of associated toxicities compared to Ibrutinib. [18,19]

We hypothesise that the BTK inhibitors, Ibrutinib and ONO-4059, could have additional therapeutic antiplatelet activity in patients with acute MI. This study aimed to assess the *ex-vivo* effects of Ibrutinib and ONO-4059 on platelet aggregation in blood samples obtained from patients with acute MI treated with dual antiplatelet therapy.

2. Method

2.1. Study Design

Single-centre, observational, prospective study approved by a national accredited Research Ethics Committee and conducted according to the ethical guidelines of the Declaration of Helsinki and Good Clinical Practice. All patients provided written informed consent prior to participation.

2.2. Subject Selection and Recruitment

Patients with acute MI were recruited between March and July 2016. Inclusion criteria were individuals aged >18 years with a clinical diagnosis of acute MI with elevated serum troponin, with or without electrocardiogram (ECG) changes of myocardial ischaemia (+/-ST elevation). Exclusion criteria were participation in another research study and difficult venepuncture.

2.3. Blood sample collection and analysis

A single venous blood sample was collected in a Hirudin vacutainer (Roche, UK) between 24 and 48 hours of hospital admission. Blood samples were immediately analysed. Blood samples were treated with 0.5, 1 and 2 μ M of Ibrutinib and with 0.2,

0.5, 1 μ M of ONO-4059. A control sample (0 μ M) was also tested alongside each inhibitor. The concentrations used were determined through preliminary investigation carried out in healthy volunteers. Accuracy of platelet aggregation is subject to time delays between collection and analysis, therefore as only 4 samples could be analysed at one time, a 1-hour incubation period was used for Ibrutinib and 30 minutes for ONO-4059. All concentrations of Ibrutinib were analysed simultaneously for each patient, as were all concentrations of ONO-4059. Ibrutinib and ONO-4059 were purchased from Selleck Chemicals (USA). They were dissolved in dimethyl sulfoxide (DMSO) and diluted in phosphate buffered saline (PBS). Platelet aggregation in response to 20 μ l of collagen (Roche, UK), which equates to a final concentration of 3.2 μ g/mL, was measured using a multiplate analyser (Roche, UK) in accordance with manufacturer's guidelines. As platelets bind to the multiplate sensors, there is an increase of impedance and this is transformed into an Aggregation Unit. The aggregation unit is then plotted against time. The overall platelet activity is measured by calculating the area under the curve (AUC).

2.4. Statistical analysis

This was an observational pilot study, so no formal sample size calculations were undertaken. Baseline characteristics are reported as percentages of the study population. Normally distributed baseline characteristics are reported as means with standard deviations. Non-normally distributed baseline characteristics are reported as medians with interquartile ranges. For each concentration of Ibrutinib and ONO-4059 the median AUC with interquartile ranges is given. Lower AUC values indicate less platelet aggregation. Comparisons between control (0 μ M) and samples treated with Ibrutinib and ONO-4059 were done with Wilcoxon signed rank test as the data

were not normally distributed. A p -value of $p < 0.05$ was considered to be statistically significant.

3. Results

22 participants were enrolled and two participants were excluded due to non-MI diagnosis at discharge. Of the 20 participants included in the final analysis, 6 patients did not have blood samples treated with ONO-4059, as early in the study the doses were adjusted for this inhibitor.

Baseline characteristics are shown in Table 1. The mean age of participants was 63.2 ± 11.4 years and 80% of participants were male. All patients received dual antiplatelet therapy, with aspirin (300mg loading dose and 75mg maintenance) and a P2Y12 inhibitor (30% received clopidogrel and 75% ticagrelor; three patients received both Clopidogrel and Ticagrelor whilst in hospital; Clopidogrel was given as a loading dose and subsequently switched to Ticagrelor whilst in hospital). Participants also received a beta-blocker, angiotensin-converting enzyme inhibitor/angiotensin II receptor blocker (ACEi/ARB) and a statin. Three patients received GPIIIa/IIb inhibitors during admission.

Table 1: Baseline Characteristics

<u>Characteristic</u>	n (%) (n=20)
Age (years, mean \pm SD)	63.2 \pm 11.4
Male	16 (80)
Current smoker	5 (25)

Family history of CAD (n=19)	12 (63)
Diabetes	1 (5)
Hypertension	7 (35)
Hypercholesterolaemia	9 (45)
History of CAD	5 (25)
NSTEMI	14 (70)
STEMI	6 (30)
Inpatient coronary angiography	18 (90)
Site of infarct	
Anterior	5 (25)
Inferior	5 (25)
Anterolateral	2 (10)
Indeterminate	5 (25)
Inferior Posterior	1 (5)
Lateral	2 (10)
Medication given during hospitalisation	
Aspirin	20 (100)
Clopidogrel	6 (30)
Ticagrelor	15 (75)
Beta-blocker	15 (75)
ACEi	16 (80)
ARB	2 (10)
Diuretics	1 (5)
Aldosterone	1 (5)

Glycoprotein IIb/IIIa inhibitors	3 (15)
Statin	19 (95)
Peak Troponin I level (ng/L) median (IQR)	1101.5 (275 – 7112.75)
Platelet count (10⁹/L) median (IQR)	242 (199.5 - 287.25)

ACEi = angiotensin converting enzyme inhibitor, ARB = angiotensin receptor blocker, CAD = coronary artery disease, IQR = interquartile range, NSTEMI = Non ST-elevation myocardial infarction, SD = standard deviation, STEMI = ST-elevation myocardial infarction

For Ibrutinib concentrations of 0, 0.5, 1, 2 μ M, the median (IQR) AUC for platelet aggregation were 18.5 (7 – 29.25), 8 (2.5 – 18.75), 4.5 (1 – 15.75) and 2 (0 – 10.5) (P value of 0.0004, <0.0001 and <0.0001 respectively compared to control: Figure 1). For ONO-4059 concentrations of 0, 0.2, 0.5, 1 μ M, the mean (IQR) platelet aggregation was 13 (7 – 23.5), 12 (8.75 – 22.25), 6.5 (2.75 – 14.25), 5.5 (2.5 – 9.25) (p values 0.7, 0.0001 and 0.0004 respectively compared to control: Figure 2).

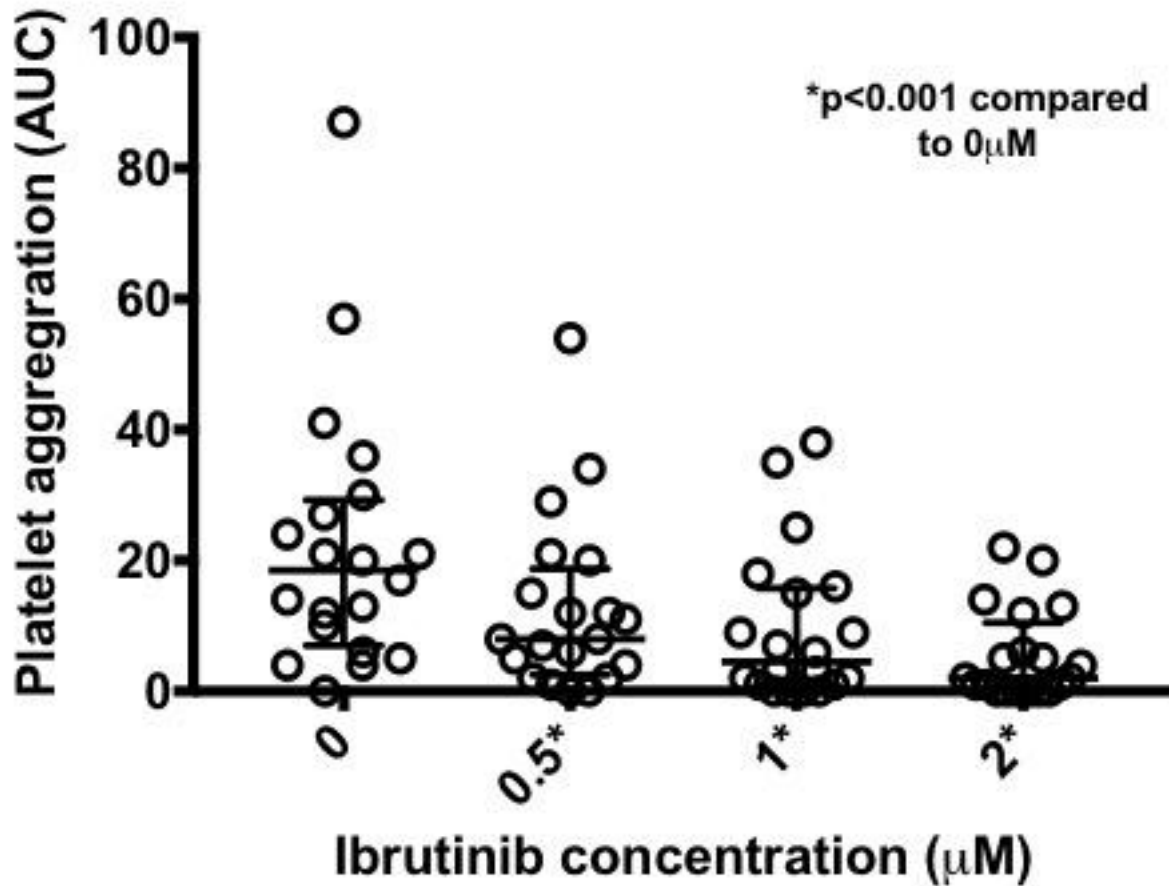


Figure 1: Platelet aggregation was measured using a Multiplate analyser (collagen agonist concentration 3.2 µg/mL) with ex-vivo addition of increasing doses of Ibrutinib in blood samples from patients with acute myocardial infarction treated with dual anti-platelet therapy (n=20) Each circle represents individual data points, with horizontal lines representing median values, with lower and upper quartiles. Wilcoxon signed rank test was carried out to compare 0µM (control) with 0.5, 0.1 and 0.2µM of Ibrutinib.

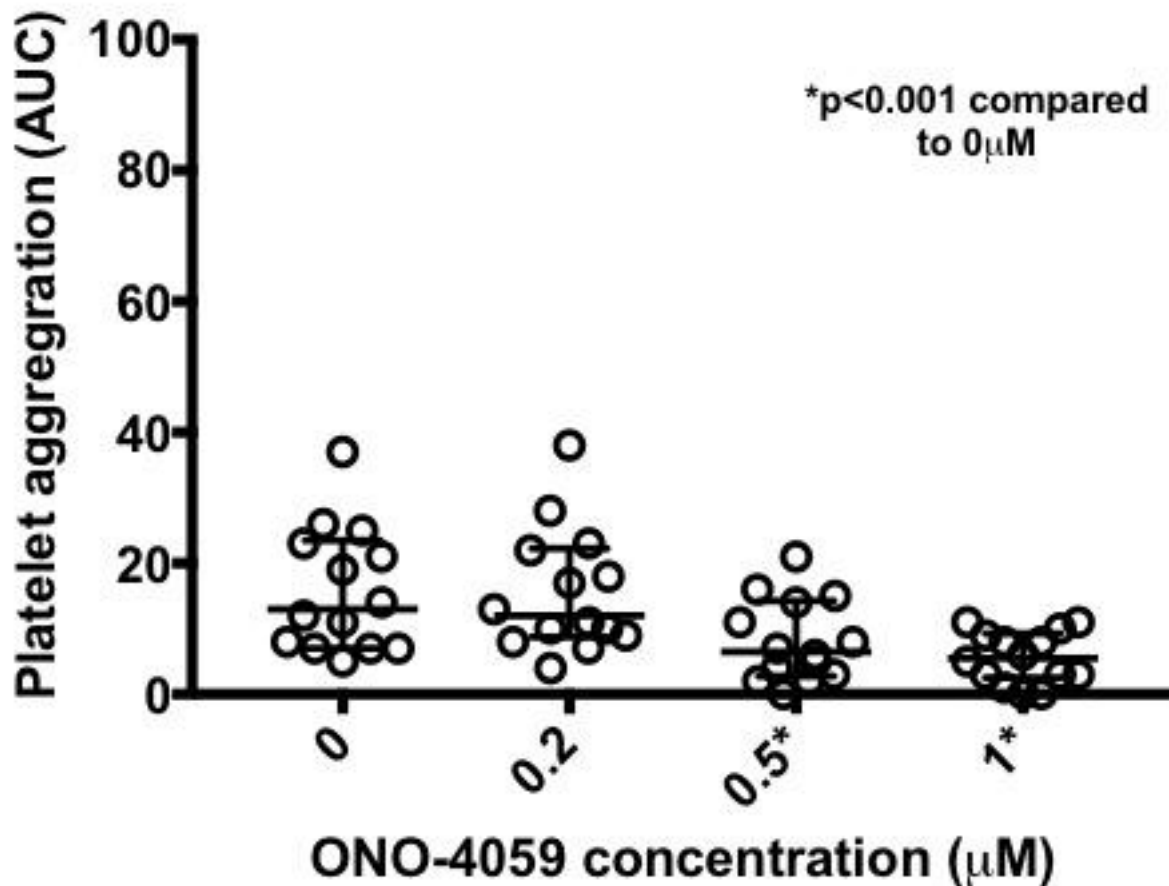


Figure 2: Platelet aggregation was measured using a Multiplate analyser (collagen agonist concentration 3.2 µg/mL) with ex-vivo addition of increasing doses of ONO-4059 in blood samples from patients with acute myocardial infarction treated with dual anti-platelet therapy (n=14) Each circle represents individual data points, with horizontal lines representing median values, with lower and upper quartiles. Wilcoxon signed rank test was carried out to compare 0µM (control) with 0.2, 0.5 and 1µM of ONO-4059.

4. Discussion

The BTK inhibitors Ibrutinib and ONO-4059 provide dose dependent inhibition of platelet aggregation in *ex-vivo* samples from patients with acute MI already treated with dual antiplatelet therapy.

Rushworth et al. [14] analysed platelet aggregation in blood samples obtained from patients with CLL/MCL treated with Ibrutinib to investigate the *in-vitro* effect of Ibrutinib on platelet function. Platelet aggregation in response to collagen was significantly inhibited while platelet aggregation in response to ADP was also inhibited, but to a lesser extent. Platelet aggregation in response to Arachidonic Acid and Thrombin Related-activated Peptide (TRAP) was not inhibited. Levade et al. [12] incubated healthy donor blood with Ibrutinib and demonstrated that Ibrutinib inhibits collagen induced platelet aggregation in a dose dependent manner with higher doses being associated with lower maximum platelet aggregation. Kamel et al. [13] and Levade et al. [12] demonstrated that platelet aggregation in response to collagen was inhibited in patients receiving Ibrutinib for CLL/MCL, but not in response to ADP, Arachidonic acid, adrenaline, ristocetin, and U46619 (thromboxane A₂). Patients with CLL/MCL with bleeding complications on Ibrutinib were found to have significantly lower platelet aggregation compared to those without evidence of bleeding. [13] More recently, Nicolson and colleagues report that inhibition of BTK kinase actually causes only partial inhibition of GPVI signalling in platelets and they provide evidence that BTK supports GPVI signalling by functioning as an adapter protein as well as a kinase. [20] Taken together, these findings support the hypothesised role of BTK in GPVI collagen mediated pathway in platelets and our

findings provide further evidence of this in patients with acute MI receiving dual antiplatelet therapy.

In the study by Kamel et al., the majority of patients received 420mg of Ibrutinib daily. [13] Pharmacokinetic and pharmacodynamics studies suggest *in-vivo* plasma concentration of 0.4 μ M is achieved with a once daily dose of 560mg; [21, 22] a 420mg dose of Ibrutinib is likely to have a lower plasma concentration. Our results show inhibition of platelet aggregation with Ibrutinib at a concentration of 0.5 μ M which could be achieved with current therapeutic doses. The exact doses to be tested in potential future studies in acute MI remain to be determined.

Ibrutinib acts via BTK and Tec expressed in platelets, both of which are non-receptor tyrosine kinases belonging to the Tec family kinases (TFK). [15, 16] Inhibition of BTK and Tec interferes with the downstream signalling of the GPVI collagen mediated pathway. Platelet adhesion, an initial step in thrombus formation, occurs when GPVI binds to exposed collagen. This leads to PI3K dependent phosphorylation of BTK and Tec. These then in turn activate PLC γ 2. [15, 23] Ibrutinib treated platelets have reduced phosphorylation of PLC γ 2 and autophosphorylation of BTK. [12] Ibrutinib therapy also affects other pathways involved with platelet aggregation, including platelet activation by von Willebrand factor (vWF), reducing platelet adhesion onto vWF with high shear rate. [12] Additionally, BTK is essential in GPIb dependent thrombus formation [24] and is involved in activation of platelets via C-type lectin-like receptor-2 (CLEC-2). [25] BTK and Tec also appear to be involved in the later stages of thrombus formation via the fibrinogen receptor integrin α IIb β 3, which leads to phosphorylation of the two tyrosine kinases. [26,27]

Animal models of GPVI-deficiency have demonstrated potential protection against platelet activation, adhesion and thrombus formation at sites of vascular injury. [28-30] Increased GPVI expression has been linked to patients with acute MI, which may suggest GPVI inhibition could be beneficial. [31] Studies targeting GPVI are underway in stable coronary artery disease. [32,33]

Collagen exposed on atherosclerotic plaques is morphologically different to collagen within physiological tissue. [34,35] Busygina et al. explored the *in-vitro* and *ex-vivo* effects of Ibrutinib and ONO-4059 on platelet aggregation in response to atherosclerotic plaque material and native collagen fibres, in static and high shear arterial flow conditions. Both BTK inhibitors inhibited platelet aggregation in response to plaque derived collagen and native collagen under static conditions, however with high shear arterial flow platelet aggregation was impaired with plaque material, but not with native collagen fibres. [36] This suggests that Ibrutinib and ONO-4059 may be advantageous in atherosclerotic disease by inhibiting thrombus formation on atherosclerotic plaques while sparing physiological platelet function.

4.1. Limitations

This was an exploratory *ex-vivo* study with a small sample size, therefore the results cannot be extrapolated to the use of these agents in a clinical setting. This study cannot provide any reliable information on safety aspects of BTK inhibitors, including risk of bleeding, (or other reported side effects such as thrombocytopenia, neutropenia and increased risk of infection) given in the context of acute MI where other antithrombotic treatments are also given. All these potential side effects would need to be carefully assessed before BTK inhibitors could be used as a treatment

acute MI. In this study we only measured platelet aggregation in response to collagen, whereas BTK inhibitors may have other mechanisms of inhibition particularly in the context of acute MI and routinely administered antiplatelet therapy, which could be explored in future studies. Measurement of platelet aggregation is subject to a number of influences that need to be carefully controlled and results can be affected by factors such as smoking on the day of testing, shaking or mixing of blood and the time between obtaining sample and analysis. Samples were transported and analysed as soon as possible and the estimated average time from obtaining blood samples to analysis was 70 minutes. This was in keeping with recommendations for collection, transport and analysis for measurement of platelet aggregation. [37]

4.2. Conclusion

We have demonstrated that the BTK inhibitors Ibrutinib and ONO-4059 show dose dependent inhibition of platelet aggregation, at physiologically relevant concentrations, in blood samples taken from patients with acute MI on dual antiplatelet therapy. These preliminary observations support further exploration of BTK inhibitors as potential therapeutic antiplatelet agents for coronary artery disease through the GPVI collagen pathway, which is not utilised in current anti-platelet regimens.

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7. Declarations of Interest: none

ACCEPTED MANUSCRIPT

8. References

1. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK, Clopidogrel in Unstable Angina to Prevent Recurrent Events Trial Investigators. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. *N Engl J Med*. 2001 Aug 16;**345**(7):494-502.
2. Wiviott SD, Braunwald E, McCabe CH, Montalescot G, Ruzyllo W, Gottlieb S, Neumann FJ, Ardissino D, De Servi S, Murphy SA, Riesmeyer J, Weerakkody G, Gibson CM, Antman EM; TRITON-TIMI 38 Investigators. Prasugrel versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med*. 2007 Nov 15;**357**(20):2001-2015.
3. Wallentin L, Becker RC, Budaj A, Cannon CP, Emanuelsson H, Held C, Horrow J, Husted S, James S, Katus H, Mahaffey KW, Scirica BM, Skene A, Steg PG, Storey RF, Harrington RA; PLATO Investigators, Freij A, Thorsén M. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med*. 2009 Sep 10;**361**(11):1045-1057.
4. Fox KA, Dabbous OH, Goldberg RJ, Pieper KS, Eagle KA, Van de Werf F, Avezum A, Goodman SG, Flather MD, Anderson FA Jr, Granger CB. Prediction of risk of death and myocardial infarction in the six months after presentation with acute coronary syndrome: prospective multinational observational study (GRACE). *BMJ*. 2006 Nov 25;**333**(7578):1091.
5. Chanan-Khan A, Cramer P, Demirkan F, Fraser G, Silva RS, Grosicki S, Pristupa A, Janssens A, Mayer J, Bartlett NL, Dilhuydy MS, Pylypenko H, Loscertales J, Avigdor A, Rule S, Villa D, Samoilova O, Panagiotidis P, Goy A, Mato A, Pavlovsky MA, Karlsson C, Mahler M, Salman M, Sun S, Phelps C, Balasubramanian S, Howes A, Hallek M; HELIOS investigators. Ibrutinib combined with bendamustine and

rituximab compared with placebo, bendamustine, and rituximab for previously treated chronic lymphocytic leukaemia or small lymphocytic lymphoma (HELIOS): a randomised, double-blind, phase 3 study. *Lancet Oncol.* 2016 Feb;**17**(2):200-211.

6. Dreyling M, Jurczak W, Jerkeman M, Silva RS, Rusconi C, Trneny M, Offner F, Caballero D, Joao C, Witzens-Harig M, Hess G, Bence-Bruckler I, Cho SG, Bothos J, Goldberg JD, Enny C, Traina S, Balasubramanian S, Bandyopadhyay N, Sun S, Vermeulen J, Rizo A, Rule S. Ibrutinib versus temsirolimus in patients with relapsed or refractory mantle-cell lymphoma: an international, randomised, open-label, phase 3 study. *Lancet.* 2016 Feb 20;**387**(10020):770-778.

7. Burger JA, Tedeschi A, Barr PM, Robak T, Owen C, Ghia P, Bairey O, Hillmen P, Bartlett NL, Li J, Simpson D, Grosicki S, Devereux S, McCarthy H, Coutre S, Quach H, Gaidano G, Maslyak Z, Stevens DA, Janssens A, Offner F, Mayer J, O'Dwyer M, Hellmann A, Schuh A, Siddiqi T, Polliack A, Tam CS, Suri D, Cheng M, Clow F, Styles L, James DF, Kipps TJ; RESONATE-2 Investigators. Ibrutinib as Initial Therapy for Patients with Chronic Lymphocytic Leukemia. *N Engl J Med.* 2015 Dec 17;**373**(25):2425-2437.

8. Byrd JC, Brown JR, O'Brien S, Barrientos JC, Kay NE, Reddy NM, Coutre S, Tam CS, Mulligan SP, Jaeger U, Devereux S, Barr PM, Furman RR, Kipps TJ, Cymbalista F, Pocock C, Thornton P, Caligaris-Cappio F, Robak T, Delgado J, Schuster SJ, Montillo M, Schuh A, de Vos S, Gill D, Bloor A, Dearden C, Moreno C, Jones JJ, Chu AD, Fardis M, McGreivy J, Clow F, James DF, Hillmen P; RESONATE Investigators. Ibrutinib versus ofatumumab in previously treated chronic lymphoid leukemia. *N Engl J Med.* 2014 Jul 17;**371**(3):213-223.

9. Wang ML, Blum KA, Martin P, Goy A, Auer R, Kahl BS, Jurczak W, Advani RH, Romaguera JE, Williams ME, Barrientos JC, Chmielowska E, Radford J,

- Stilgenbauer S, Dreyling M, Jdrzejczak WW, Johnson P, Spurgeon SE, Zhang L, Baher L, Cheng M, Lee D, Beaupre DM, Rule S. Long-term follow-up of MCL patients treated with single-agent ibrutinib: updated safety and efficacy results. *Blood*. 2015 Aug 6;**126**(6):739-745.
10. Byrd JC, Furman RR, Coutre SE, Burger JA, Blum KA, Coleman M, Wierda WG, Jones JA, Zhao W, Heerema NA, Johnson AJ, Shaw Y, Bilotti E, Zhou C, James DF, O'Brien S. Three-year follow-up of treatment-naïve and previously treated patients with CLL and SLL receiving single-agent ibrutinib. *Blood*. 2015 Apr 16;**125**(16):2497-2506
11. Caron F, Leong DP, Hillis C, Fraser G, Siegal D. Current understanding of bleeding with ibrutinib use: a systematic review and meta-analysis. *Blood Adv*. 2017 May 9;**1**(12):772-778
12. Levade M, David E, Garcia C, Laurent PA, Cadot S, Michallet AS, Bordet JC, Tam C, Sié P, Ysebaert L, Payrastre B. Ibrutinib treatment affects collagen and von Willebrand factor-dependent platelet functions. *Blood*. 2014 Dec 18;**124**(26):3991-3995.
13. Kamel S, Horton L, Ysebaert L, Levade M, Burbury K, Tan S, Cole-Sinclair M, Reynolds J, Filshie R, Schischka S, Khot A, Sandhu S, Keating MJ, Nandurkar H, Tam CS. Ibrutinib inhibits collagen-mediated but not ADP-mediated platelet aggregation. *Leukemia*. 2015 Apr;**29**(4):783-787.
14. Rushworth SA, MacEwan DJ, Bowles KM. Ibrutinib in relapsed chronic lymphocytic leukemia. *N Engl J Med*. 2013 Sep 26;**369**(13):1277-1278.
15. Quek LS, Bolen J, Watson SP. A role for Bruton's tyrosine kinase (Btk) in platelet activation by collagen. *Curr Biol*. 1998 Oct 8;**8**(20):1137-1140.

16. Oda A, Ikeda Y, Ochs HD, Druker BJ, Ozaki K, Handa M, Ariga T, Sakiyama Y, Witte ON, Wahl MI. Rapid tyrosine phosphorylation and activation of Bruton's tyrosine/Tec kinases in platelets induced by collagen binding or CD32 cross-linking. *Blood*. 2000 Mar 1;**95**(5):1663-1670.
17. Thompson PA, Burger JA. Bruton's tyrosine kinase inhibitors: first and second generation agents for patients with Chronic Lymphocytic Leukemia (CLL). *Expert Opin Investig Drugs*. 2018 Jan;**27**(1):31-42.
18. Walter HS, Rule SA, Dyer MJ, Karlin L, Jones C, Cazin B, Quittet P, Shah N, Hutchinson CV, Honda H, Duffy K, Birkett J, Jamieson V, Courtenay-Luck N, Yoshizawa T, Sharpe J, Ohno T, Abe S, Nishimura A, Cartron G, Morschhauser F, Fegan C, Salles G. A phase 1 clinical trial of the selective BTK inhibitor ONO/GS-4059 in relapsed and refractory mature B-cell malignancies. *Blood* 2016 Jan 28;**127**(4):411-419.
19. Walter HS, Jayne S, Rule SA, Cartron G, Morschhauser F, Macip S, Karlin L, Jones C, Herbaux C, Quittet P, Shah N, Hutchinson CV, Fegan C, Yang Y, Mitra S, Salles G, Dyer MJS. Long-term follow-up of patients with CLL treated with the selective Bruton's tyrosine kinase inhibitor ONO/GS-4059. *Blood*. 2017 May 18;**129**(20):2808-2810.
20. Nicolson PLR, Hughes CE, Watson S, Nock SH, Hardy AT, Watson CN, Montague SJ, Malcor JD, Thomas MR, Pollitt AY, Tomlinson MG, Pratt G, Watson SP. Inhibition of Btk by Btk-specific concentrations of ibrutinib and acalabrutinib delays but does not block platelet aggregation to GPVI. *Haematologica*. 2018 Jul;haematol.2018.193391 [Epub ahead of print]
21. Advani RH, Buggy JJ, Sharman JP, Smith SM, Boyd TE, Grant B, Kolibaba KS, Furman RR, Rodriguez S, Chang BY, Sukbuntherng J, Izumi R, Hamdy A, Hedrick

- E, Fowler NH. Bruton tyrosine kinase inhibitor ibrutinib (PCI-32765) has significant activity in patients with relapsed/refractory B-cell malignancies. *J Clin Oncol*. 2013 Jan 1;**31**(1):88-94.
22. Cheng S, Ma J, Guo A, Lu P, Leonard JP, Coleman M, Liu M, Buggy JJ, Furman RR, Wang YL. BTK inhibition targets in vivo CLL proliferation through its effects on B-cell receptor signaling activity. *Leukemia*. 2014 Mar;**28**(3):649-657.
23. Atkinson BT, Ellmeier W, Watson SP. Tec regulates platelet activation by GPVI in the absence of Btk. *Blood*. 2003 Nov 15;**102**(10):3592-3599.
24. Liu J, Fitzgerald ME, Berndt MC, Jackson CW, Gartner TK. Bruton tyrosine kinase is essential for botrocetin/VWF-induced signaling and GPIIb-dependent thrombus formation in vivo. *Blood*. 2006 Oct 15;**108**(8):2596-2603.
25. Manne BK, Badolia R, Dangelmaier C, Eble JA, Ellmeier W, Kahn M, Kunapuli SP. Distinct pathways regulate Syk protein activation downstream of immune tyrosine activation motif (ITAM) and hemITAM receptors in platelets. *J Biol Chem*. 2015 May 1;**290**(18):11557-11568.
26. Laffargue M, Ragab-Thomas JM, Ragab A, Tuech J, Missy K, Monnereau L, Blank U, Plantavid M, Payrastre B, Raynal P, Chap H. Phosphoinositide 3-kinase and integrin signalling are involved in activation of Bruton tyrosine kinase in thrombin-stimulated platelets. *FEBS Lett*. 1999 Jan 22;**443**(1):66-70.
27. Bye AP, Unsworth AJ, Vaiyapuri S, Stainer AR, Fry MJ, Gibbins JM. Ibrutinib Inhibits Platelet Integrin $\alpha\text{IIb}\beta\text{3}$ Outside-In Signaling and Thrombus Stability But Not Adhesion to Collagen. *Arterioscler Thromb Vasc Biol*. 2015 Nov;**35**(11):2326-35.
28. Massberg S, Gawaz M, Grüner S, Schulte V, Konrad I, Zohlnhöfer D, Heinzmann U, Nieswandt B. A crucial role of glycoprotein VI for platelet recruitment to the injured arterial wall in vivo. *J Exp Med*. 2003 Jan 6;**197**(1):41-49.

29. Bender M, Hagedorn I, Nieswandt B. Genetic and antibody-induced glycoprotein VI deficiency equally protects mice from mechanically and FeCl₃ -induced thrombosis. *J Thromb Haemost.* 2011 Jul;**9**(7):1423-1426.
30. Bultmann A, Li Z, Wagner S, Peluso M, Schönberger T, Weis C, Konrad I, Stellos K, Massberg S, Nieswandt B, Gawaz M, Ungerer M, Münch G. Impact of glycoprotein VI and platelet adhesion on atherosclerosis--a possible role of fibronectin. *J Mol Cell Cardiol.* 2010 Sep;**49**(3):532-542.
31. Bigalke B, Haap M, Stellos K, Geisler T, Seizer P, Kremmer E, Overkamp D, Gawaz M. Platelet glycoprotein VI (GPVI) for early identification of acute coronary syndrome in patients with chest pain. *Thromb Res.* 2010 May;**125**(5):e184-189.
32. ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US) 2000 Feb 29 – Identifier: NCT03312855 Intracoronary Stenting and Antithrombotic Regimen: Lesion Platelet Adhesion as Selective Target of Endovenous Revacept (ISAR-PLASTER).; 2017 Oct 18 [cited 2018 Oct 16] Available from: <https://clinicaltrials.gov/ct2/show/NCT03312855?term=NCT03312855&rank=1>
33. ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US) 2000 Feb 29 – Identifier: NCT01645306 Revacept in Symptomatic Carotid Stenosis (Revacept/CS/02); 2012 Jul 20 [cited 2018 Oct 16] Available from: <https://clinicaltrials.gov/ct2/show/NCT01645306?term=NCT01645306&rank=1>
34. Schulz C, Penz S, Hoffmann C, Langer H, Gillitzer A, Schneider S, Brandl R, Seidl S, Massberg S, Pichler B, Kremmer E, Stellos K, Schönberger T, Siess W, Gawaz M. Platelet GPVI binds to collagenous structures in the core region of human atheromatous plaque and is critical for atheroprogession in vivo. *Basic Res Cardiol.* 2008 Jul;**103**(4):356-367.

35. Penz S, Reiningger AJ, Brandl R, Goyal P, Rabie T, Bernlochner I, Rother E, Goetz C, Engelmann B, Smethurst PA, Ouwehand WH, Farndale R, Nieswandt B, Siess W. Human atheromatous plaques stimulate thrombus formation by activating platelet glycoprotein VI. *FASEB J*. 2005 Jun;**19**(8):898-909.
36. Busygina K, Jamasbi J, Seiler T, Deckmyn H, Weber C, Brandl R, Lorenz R, Siess W. Oral Bruton tyrosine kinase inhibitors selectively block atherosclerotic plaque-triggered thrombus formation. *Blood*. 2018 Jun 14;**131**(24):2605-2616.
37. Harrison P, Mackie I, Mumford A, Briggs C, Liesner R, Winter M, Machin S; British Committee for Standards in Haematology.. Guidelines for the laboratory investigation of heritable disorders of platelet function. *Br J Haematol*. 2011 Oct;**155**(1):30-44.

Highlights

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Effect of Bruton's Tyrosine Kinase inhibitors on platelet aggregation in patients with Acute Myocardial Infarction

- Bruton's tyrosine kinase inhibitors affect the glycoprotein VI collagen pathway
- They add further platelet inhibition in blood samples after myocardial infarction
- This provides a rationale for clinical testing as potential new antiplatelet agents

ACCEPTED MANUSCRIPT