Platelet $P2Y_{12}$ Inhibitors Reduce Systemic Inflammation and Its Prothrombotic Effects in an Experimental Human Model

Mark R. Thomas, Samuel N. Outteridge, Ramzi A. Ajjan, Fladia Phoenix, Gurpreet K. Sangha, Rachael E. Faulkner, Rosemary Ecob, Heather M. Judge, Haroon Khan, Laura E. West, David H. Dockrell, Ian Sabroe, Robert F. Storey

**Objective**—Clinical studies suggest that platelet $P2Y_{12}$ inhibitors reduce mortality from sepsis, although the underlying mechanisms have not been clearly defined in vivo. We hypothesized that $P2Y_{12}$ inhibitors may improve survival from sepsis by suppressing systemic inflammation and its prothrombotic effects. We therefore determined whether clopidogrel and the novel, more potent $P2Y_{12}$ inhibitor, ticagrelor, modify these responses in an experimental human model.

**Approach and Results**—We randomized 30 healthy volunteers to ticagrelor (n=10), clopidogrel (n=10), or no antiplatelet medication (controls; n=10). We examined the effect of $P2Y_{12}$ inhibition on systemic inflammation, which was induced by intravenous injection of *Escherichia coli* endotoxin. Both $P2Y_{12}$ inhibitors significantly reduced platelet–monocyte aggregate formation and peak levels of major proinflammatory cytokines, including tumor necrosis factor $\alpha$, interleukin-6, and chemokine (C–C motif) ligand 2. In contrast to clopidogrel, ticagrelor also significantly reduced peak levels of IL-8 and growth colony-stimulating factor and increased peak levels of the anti-inflammatory cytokine IL-10. In addition, ticagrelor altered leukocyte trafficking. Both $P2Y_{12}$ inhibitors suppressed D-dimer generation and scanning electron microscopy revealed that ticagrelor also suppressed prothrombotic changes in fibrin clot ultrastructure.

**Conclusions**—Potent inhibition of multiple inflammatory and prothrombotic mechanisms by $P2Y_{12}$ inhibitors demonstrates critical importance of platelets as central orchestrators of systemic inflammation induced by bacterial endotoxin. This provides novel mechanistic insight into the lower mortality associated with $P2Y_{12}$ inhibitors in patients with sepsis in clinical studies. (Arterioscler Thromb Vasc Biol. 2015;35:2562–2570. DOI: 10.1161/ATVBAHA.115.306528.)

Key Words: antiplatelet agents • clopidogrel • fibrin • infection • sepsis • thrombosis • ticagrelor

Sepsis is one of the most devastating clinical syndromes in medicine, and severe sepsis still has a mortality rate of 20% to 30% and remains resistant to specific pharmacological therapy. It is well established that the antithrombotic effect of this is beneficial for patients with atherothrombosis, which has led to $P2Y_{12}$ medications becoming some of the most commonly prescribed medications worldwide. However, in addition, this inhibits the formation of platelet–leukocyte aggregates, which is primarily mediated by inhibition of platelet expression of the adhesion molecule P-selectin. A recent cohort study of 683,421 patients with sepsis has shown that current use of antiplatelet therapy is independently associated with a significant reduction in mortality from sepsis (odds ratio, 0.78; 95% confidence interval, 0.76–0.79; $P<0.001$). This also corresponds with previous observational studies that suggest that clopidogrel reduces mortality from sepsis. However, the mechanisms underpinning this reduction in mortality have not been clearly demonstrated in vivo because animal models of Platelet $P2Y_{12}$ inhibitors, such as clopidogrel and ticagrelor, inhibit a central ADP-mediated amplification pathway, and therefore, blunt a broad spectrum of platelet functions. It is well established that the antithrombotic effect of this is beneficial for patients with atherothrombosis, which has led to $P2Y_{12}$ medications becoming some of the most commonly prescribed medications worldwide. However, in addition, this inhibits the formation of platelet–leukocyte aggregates, which is primarily mediated by inhibition of platelet expression of the adhesion molecule P-selectin. A recent cohort study of 683,421 patients with sepsis has shown that current use of antiplatelet therapy is independently associated with a significant reduction in mortality from sepsis (odds ratio, 0.78; 95% confidence interval, 0.76–0.79; $P<0.001$). This also corresponds with previous observational studies that suggest that clopidogrel reduces mortality from sepsis. However, the mechanisms underpinning this reduction in mortality have not been clearly demonstrated in vivo because animal models of...
sepsis conflict about the immunomodulatory effects of clopidogrel, which may be species dependent.9–12

Ticagrelor is a novel P2Y12 inhibitor, which causes more potent and consistent P2Y12 inhibition than clopidogrel13 and also weakly inhibits cellular uptake of adenosine.14 In the PLATElet inhibition and patient Outcomes (PLATO) study of >180,000 patients with acute coronary syndromes, ticagrelor reduced all-cause mortality compared with clopidogrel (hazard ratio, 0.78; P<0.001), which was out of proportion to its incremental cardiovascular benefit.15 Intriguingly, ticagrelor was associated with lower mortality related to infection (hazard ratio, 0.67; P<0.05)16,17 and fewer deaths after sepsis and pulmonary infections than clopidogrel.18

We therefore sought to determine the mechanistic impact of P2Y12 inhibitors on pathophysiological processes that are central to sepsis responses in humans. We hypothesized that P2Y12 inhibitors may reduce mortality from sepsis by suppressing systemic inflammation and its prothrombotic effects, mediated by inhibition of platelet–leukocyte interactions. We hypothesized that the more potent P2Y12 inhibitor, ticagrelor, suppresses these responses more potently than clopidogrel. To test these hypotheses in humans, we used a well-established model of systemic inflammation, which involves intravenous injection of Escherichia coli endotoxin (LPS) into healthy volunteers.19 The particular strength of this unique model is that it allows direct assessment of dynamic cellular and molecular pathways that are also major mediators of the pathophysiology of sepsis in humans.

**Figure 1.** Levels of proinflammatory cytokines tumor necrosis factor-α (TNF-α; A), interleukin (IL)-6 (B), chemokine (C–C motif) ligand 2 (CCL2; C), growth colony-stimulating factor (G-CSF; D), IL-8 (E), IL-10 (F), and high-sensitivity C-reactive protein (hsCRP; G) before and after 1 week of antiplatelet treatment and after lipopolysaccharide (LPS) administration (t=0 hours). Data expressed as mean±SEM (n=10 in each group). The overall effect of LPS and the effect of ticagrelor and clopidogrel (both compared with control at each time point) determined using 2-way ANOVA with Dunnett correction for multiple comparisons for the cytokines (*P<0.05, **P<0.01, and ***P<0.001). For hsCRP, the effect of ticagrelor and clopidogrel compared with control was determined using ANOVA of area under the curve.
**Materials and Methods**

We randomized 30 healthy volunteers to ticagrelor (n=10), clopidogrel (n=10), or no antiplatelet medication (controls; n=10) to determine their effect on systemic inflammation, which was induced by intravenous injection of *E coli* endotoxin (2 ng/kg LPS) using a well-established method. Additional details on the Materials and Methods are available in the online-only Data Supplement.

**Results**

Baseline characteristics were comparable in all of the treatment groups (Table I in the online-only Data Supplement). Thirty healthy volunteers underwent LPS administration (see CONSORT [Consolidated Standards of Reporting Trials] flowchart [Figure I] in the online-only supplement). To avoid any possibility of administering intravenous *E coli* LPS to a pregnant female, volunteers were only included if they were not of child-bearing potential; no eligible female subjects volunteered and so all recruited volunteers were male. Compliance was assessed from a diary and pill-count and all subjects were >90% compliant. After LPS administration, all subjects developed anticipated flu-like symptoms and signs of sepsis that peaked at 90 to 180 minutes and resolved within 6 hours (Table II in the online-only Data Supplement). There were no unexpected adverse reactions.

Both ticagrelor and clopidogrel reduce peak levels of interleukin (IL)-6, tumor necrosis factor α (TNF-α), and chemokine (C–C motif) ligand 2 (CCL2), whereas ticagrelor additionally reduces peak levels of IL-8 and growth colony-stimulating factor and increases peak levels of IL-10.

We assessed systemic inflammation in response to LPS administration by measuring the release of major proinflammatory cytokines, and we determined the modulatory effect of P2Y12 inhibitors. Plasma levels of IL-6, TNF-α, IL-8, CCL2, growth colony stimulating factor, and high-sensitivity C-reactive protein (hsCRP) significantly increased after LPS administration (all *P*<0.001; Figure 1). Compared with control, both P2Y12 inhibitors had a marked effect on the proinflammatory cytokine response, reducing peak levels of TNF-α (66% reduction [*P*<0.001] and 60% reduction [*P*<0.001], respectively; Figure 1A), IL-6 (47% reduction [*P*<0.001] and 28% reduction [*P*=0.001], respectively; Figure 1B), and CCL2 (38% reduction [*P*<0.001] and 19% reduction [*P*=0.049], respectively; Figure 1C). In addition, ticagrelor, but not clopidogrel, significantly reduced peak levels of growth colony-stimulating factor (51% reduction; *P*<0.001; Figure 1D) and IL-8 (29% reduction; *P*=0.001; Figure 1E) compared with control. Ticagrelor, but not clopidogrel, also significantly increased peak levels of the anti-inflammatory cytokine IL-10 compared with control (54% increase; *P*=0.02; Figure 1F). Neither drug significantly modified the hsCRP response (Figure 1G).

**Ticagrelor Inhibits LPS-Induced Platelet–Monocyte Aggregate Formation**

Formation of platelet–leukocyte aggregates (defined as leukocyte expression of the platelet marker CD42a) amplifies leukocyte release of proinflammatory cytokines. We therefore investigated whether this is a mechanism by which P2Y12 inhibitors reduce systemic inflammation. Ticagrelor significantly reduced formation of platelet–monocyte aggregates compared with control (21% versus 36%; *P*<0.001) that occurred 6 hours after LPS administration (Figure 2A). Clopidogrel also significantly reduced the formation of platelet–monocyte aggregates compared with control (23% versus 36%; *P*=0.04; Figure 2A). A similar pattern of effect of LPS and modulation by the antiplatelet medications was seen in...
platelet–neutrophil aggregate formation, but the effects on platelet–neutrophil aggregate formation were not statistically significant (Figure 2B). Platelet P-selectin expression did not significantly change after LPS administration (Figure 2C).

Inhibition of platelet P2Y12 ADP receptors was also assessed by measuring platelet aggregation, platelet–leukocyte aggregate formation and platelet P-selectin expression in response to ADP added ex vivo. Ticagrelor and clopidogrel inhibited ADP-induced platelet aggregation, platelet–monocyte aggregate formation, platelet–neutrophil aggregate formation, and platelet P-selectin expression compared with control at all time points (all $P<0.001$; Figure 2). After randomized treatment, platelet aggregation responses after 5 minutes exposure to ADP (final platelet aggregation response) were 2±1%, 14±6%, and 70±11% in the ticagrelor, clopidogrel, and control groups, respectively (Figure II in the online-only supplement). Final platelet aggregation responses did not significantly change after LPS administration in any of the treatment groups (all $P>0.05$).

**Ticagrelor Increases Neutrophil Counts and Alters Monocyte Dynamics During Systemic Inflammation**

Because the formation of platelet–leukocyte aggregates facilitates adhesion of leukocytes to the endothelium and subsequent extravasation, we investigated whether inhibition of these processes by P2Y12 inhibitors affects leukocyte trafficking. Ticagrelor potentiated the increase in neutrophil count, which was significantly higher than controls 2 to 4 hours after LPS administration ($P<0.05$; Figure 3B) and may have been because of inhibition of nonspecific sequestration of neutrophils. Clopidogrel did not have a significant effect (Figure 3B). Similarly, subjects receiving ticagrelor showed altered monocyte dynamics. Transient monocyte sequestration was observed after LPS administration in all volunteers, but recovery from this was significantly greater in the ticagrelor and clopidogrel groups (Figure 3C). Neither P2Y12 inhibitor significantly affected the decrease in platelet count that occurred after LPS administration (Figure 3D).

**LPS Induces Prothrombotic Changes in the Fibrin Network that are Attenuated by Ticagrelor**

The development of a stable fibrin clot represents the critical final stage of thrombosis and has the potential to be modified by systemic inflammation. Turbidimetric assays of individual samples showed that fibrin clot maximum absorbance (a measure of clot density) and lysis area (a complex measure that assesses both clot formation and lysis) increased after LPS administration ($P<0.001$; Figure 4A and 4B). Ticagrelor

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**Figure 3.** Leukocyte (A), neutrophil (B), monocyte (C), and platelet (D) counts before and after 1 week of antiplatelet treatment and after lipopolysaccharide (LPS) administration (t=0 hours). Data expressed as mean±SEM (n=10 in each group). The overall effect of LPS and the effect of ticagrelor and clopidogrel (both compared with control at each time point) were determined using 2-way ANOVA with Dunnett correction for multiple comparisons (*$P<0.05$, **$P<0.01$, and ***$P<0.001$).
significantly reduced the rise in maximum absorbance after LPS administration compared with control (percentage increase from baseline of 33% versus 109%; \( P = 0.02 \); Figure 4A). Similarly, ticagrelor also reduced the increase in lysis from baseline of 46% versus 147%; \( P = 0.01 \); Figures 5 and 6). These changes have been shown to increase clot stability and confer resistance to fibrinolysis, whereas clopidogrel had a similar less potent effect that was not statistically significant (Figure 4A and 4B). Clopidogrel had a similar, less potent effect that was not statistically significant. LPS administration resulted in more compact clot formation (Figure 5), shown by a significant increase in fibrin clot density \( (P = 0.02) \) and a decrease in fibrin fiber diameter \( (P = 0.01) \); Figures 5 and 6). These changes have been shown to increase clot stability and confer resistance to fibrinolysis, both of which contribute to a prothrombotic state. Ticagrelor significantly reduced LPS-induced changes in fiber density and fiber diameter (Figure 6), whereas clopidogrel had a similar less potent effect that was not statistically significant. LPS induced a marked increase in D-dimer (Figure 4C), which peaked at 4 hours \( (P < 0.001) \). Ticagrelor significantly reduced peak levels of D-dimer by 48% compared with control \( (P < 0.001) \) and clopidogrel significantly inhibited peak levels by 19% compared with control \( (P = 0.01) \).

Discussion

Sepsis is a devastating syndrome for which therapeutic options remain limited. In addition to the immediate collateral host tissue damage and mortality caused by sepsis, there is a 20-fold increase in risk of myocardial infarction and stroke after sepsis by mechanisms that are not fully understood. Data from clinical studies of platelet P2Y\(_{12}\) inhibitors suggest that their use improves mortality from sepsis. However, animal models of sepsis have conflicted about the immunomodulatory effect of platelet P2Y\(_{12}\) inhibition on sepsis responses, which may be species dependent. We therefore sought to determine the mechanistic impact of P2Y\(_{12}\) inhibitors on key molecular and cellular pathways that are central to sepsis responses in humans. We investigated the effects of P2Y\(_{12}\) inhibitors on leukocyte responses, interactions with platelets that may govern such interactions, and with activation of the coagulation system. Our data point to a substantial modulatory effect of ticagrelor in particular and place the regulation of platelet activation at the heart of systemic inflammation induced by LPS in humans.

This study demonstrates marked suppression of response to bacterial endotoxemia by platelet P2Y\(_{12}\) inhibitors. Both P2Y\(_{12}\) inhibitors potently reduced peak levels of D-dimer and major proinflammatory cytokines, including IL-6, TNF-\(\alpha\), and CCL2. In contrast to clopidogrel, ticagrelor also significantly reduced peak levels of IL-8 and growth colony-stimulating factor and increased the peak level of IL-10 compared with control. In addition, ticagrelor reduced platelet--leukocyte aggregate formation, altered leukocyte trafficking, and suppressed prothrombotic changes in fibrin clot ultrastructure. Because these changes in fibrin clot structure have been shown to shift the hemostatic balance toward thrombosis, this represents a novel mechanism by which ticagrelor inhibits the prothrombotic consequences of systemic inflammation.

Inhibition of platelet--leukocyte aggregate formation demonstrates a mechanism by which platelet P2Y\(_{12}\) inhibition reduced systemic inflammation because the formation of platelet--monocyte aggregates amplifies monocyte release of proinflammatory cytokines, including TNF-\(\alpha\), CCL2, and IL-8. Levels of residual platelet P2Y\(_{12}\) reactivity before endotoxin administration significantly correlated with subsequent inflammatory and prothrombotic responses (Appendix in the online-only Data Supplement), suggesting that the responses were P2Y\(_{12}\) mediated. Ticagrelor and clopidogrel belong to different chemical classes, inhibit platelet P2Y\(_{12}\) receptors by different mechanisms, and do not have any structural similarities or shared metabolites. From a pharmacological perspective, shared non-P2Y\(_{12}\)-mediated effects are, therefore, unlikely. P2Y\(_{12}\) receptors were originally identified to be almost exclusive to platelets, although they have now also been identified on a limited number of other cell types. The extent to which leukocytes express P2Y\(_{12}\) still remains...
unclear, particularly as platelets often contaminate isolated leukocyte preparations. In mice, dendritic cells express P2Y₁₂, which seems to mediate the secretion of certain cytokines, such as IL-12. This offers an additional mechanism by which P2Y₁₂ may mediate inflammatory responses, although it has not been established whether dendritic cells function in the same way in humans. Recent studies have demonstrated that vascular smooth muscle cells also express P2Y₁₂, which mediates CCL2 release. This may have contributed to the modulatory effect of P2Y₁₂ inhibition on inflammation, although it has been shown that clopidogrel does not inhibit vascular smooth muscle cells P2Y₁₂ in rats, possibly because of the potential for nucleated cells to regenerate P2Y₁₂. Ticagrelor is also a weak inhibitor of cellular uptake of adenosine, which increases extracellular levels of adenosine. Because adenosine can increase macrophage production of IL-10, which is associated with a reduction in TNF-α and IL-6, this offers a further mechanism by which ticagrelor may modify systemic inflammation.

LPS administration induced platelet–leukocyte aggregate formation, which was inhibited by ticagrelor in particular. Platelet–monocyte aggregate formation potentiates monocyte release of proinflammatory cytokines, including TNF-α, CCL2, and IL-8, mediated by nuclear factor-κB. In addition, platelet–neutrophil aggregates have been implicated in the pathophysiology of acute lung injury. Therefore, greater inhibition of the formation of platelet–leukocyte aggregates represents a mechanism by which ticagrelor may have conferred greater protection against excessive innate immune activation during sepsis compared with clopidogrel in the PLATO study. Our data show that LPS administration induced an initial neutropenia and monocytopenia, which is held to be because of rapid sequestration of leukocytes to the endothelium. This was then followed by a rapid increase

Figure 5. Representative electron microscope images of fibrin clots formed from plasma ex vivo in each treatment group immediately before and 6 hours after lipopolysaccharide (LPS) administration. Clots were prepared in duplicate and 4 photographs were taken of each clot at each time point. In the control group, there is an increase in fibrin network density after LPS, whereas in the clopidogrel and ticagrelor groups, this is not apparent.
in neutrophil count because of bone marrow mobilization. It is notable that neutrophil counts were consistently higher in the ticagrelor group compared with the control group, despite lower overall levels of proinflammatory cytokines. This was apparent from early time points onwards, which suggests that this was because of inhibition of leukocyte sequestration, rather than increased bone marrow mobilization, which would tend to have a more delayed effect. It is logical that inhibition of platelet–leukocyte aggregate formation reduces leukocyte sequestration, as formation of platelet–leukocyte aggregates upregulates leukocyte expression of adhesion molecules and facilitates adhesion to the endothelium.30

In our study, and in other studies, platelet expressed P-selectin was not increased at 30 minutes or 6 hours after LPS administration. This is likely to be because of the transient nature of platelet expression of P-selectin, which is shed by degranulated platelets, although they continue to function normally and aggregate.31 It has been asserted that the formation of platelet–monocyte aggregates is, therefore, a more reliable marker of platelet activation in vivo.32 The precise cause of thrombocytopenia related to systemic inflammation has still not been clarified and the relative contribution of platelet activation is unclear. Neither ticagrelor nor clopidogrel significantly attenuated the LPS-induced reduction in platelet count. The findings of our study, therefore, suggest that thrombocytopenia is not entirely mediated by platelet activation or the formation of platelet–monocyte aggregates, as P2Y12 inhibitors inhibit these processes. The formation of platelet–neutrophil aggregates, or other processes where platelet P2Y12 has a less prominent role, may therefore have a greater contribution toward thrombocytopenia.

Our study provides many novel insights into potential mechanisms for increased risk of atherothrombotic events after bacteremia and sepsis.23 Although a prothrombotic state is well recognized in sepsis,31 the underlying mechanisms are incompletely understood and the relative role of platelets has not been well defined. This study demonstrates that exposure to bacterial LPS directly causes prothrombotic changes in the fibrin network that increase clot stability and confer resistance to fibrinolysis, both of which shift the hemostatic balance toward thrombosis.32 Platelet P2Y12 inhibitors inhibited these prothrombotic effects of LPS and our results suggest that this may have been because of a reduction in levels of proinflammatory cytokines because TNF-α, in particular, has been shown to be a potent activator of the coagulation system in vivo.14 The greater overall effect of ticagrelor on these pathways compared with clopidogrel suggests a mechanism, by which ticagrelor reduced cardiovascular death after infection in the PLATO study. The combined effect of ticagrelor, in particular, on leukocyte production of cytokines, leukocyte sequestration, platelet–leukocyte aggregate formation, and subsequent changes in fibrin clot ultrastructure point to a substantial role for platelets in orchestrating the innate immune response to LPS. This suggests potential for timed platelet P2Y12 inhibition in patients with infection to modify the risk of sepsis and associated thrombotic complications. In this study and in observational clinical studies, subjects were already taking P2Y12 inhibitors at the onset of systemic inflammation and sepsis, respectively. However, no studies have established the effect of administration of P2Y12 inhibitors to patients with established sepsis. The greater infectious and inflammatory burden of established sepsis presents a different balance of risks and benefits and it is critical that any studies that investigate these medications in the context of sepsis carefully address the optimal timing of administration. In sepsis, excess fibrin deposition and impaired anticoagulant mechanisms lead to exhaustion of the coagulation cascade, causing coagulopathy and bleeding.1 Although antiplatelet medications normally exacerbate bleeding, this may therefore be counterbalanced by attenuating the prothrombotic state that drives the development of coagulopathy during sepsis. In support of this, antiplatelet medications are not associated with excess bleeding in patients with sepsis in observational studies.7 However, it is important to recognize that patients with established coagulopathy may have their antiplatelet medications discontinued in these studies and are less likely to benefit from these agents.

Ticagrelor and clopidogrel are some of the most commonly prescribed medications worldwide because of their established benefit in the management of atherothrombosis. The results of this study, therefore, have potentially important clinical implications for millions of patients who are currently treated with these medications. In addition, there is great interest in the use of specific immunomodulatory therapy for the
treatment of acute coronary syndromes. The results of our study elucidate the background anti-inflammatory effects of medications that are already used for acute coronary syndromes. This is crucial information for determining the most appropriate inflammatory targets in the design of novel treatment strategies.

A limitation of this study is that clopidogrel has a less potent inhibitory effect in patient populations compared with its effects in healthy volunteers. This is not the case for ticagrelor that has a consistently potent effect in patient populations. Therefore, the results of this study may actually underestimate the additional efficacy of ticagrelor compared with clopidogrel in suppressing systemic inflammation in patients. Although this study demonstrates the key cellular and molecular pathways by which platelet P2Y12 inhibitors could reduce mortality from sepsis, further randomized human studies are needed to determine whether this improves outcomes in patients.

In conclusion, this study demonstrates that clopidogrel and ticagrelor have a marked effect on multiple critical mechanisms involved in the pathophysiology of sepsis. This suggests a promising line of investigation for novel applications of P2Y12 inhibitors in a syndrome that has proved elusive to almost all previous pharmacological strategies. The greater overall effect of ticagrelor compared with clopidogrel also provides critical mechanistic insight into the lower mortality after sepsis observed in the ticagrelor group compared with the clopidogrel group in the PLATO study.

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2570

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The main findings of this study were that platelet P2Y<sub>12</sub> inhibitors, such as ticagrelor and clopidogrel, potently suppress systemic inflammation and its prothrombotic effects in an experimental human model. This provides critical mechanistic insight into the findings of clinical studies that have shown that treatment with platelet P2Y<sub>12</sub> inhibitors is associated with a reduced mortality from sepsis. This suggests the potential for novel strategies for the treatment of sepsis, which has a high mortality and has remained elusive to almost all other therapeutic approaches. This study demonstrates that platelet P2Y<sub>12</sub> inhibitors reduce systemic inflammation induced by bacterial endotoxin in vivo. Ticagrelor and clopidogrel are some of the most commonly prescribed medications worldwide because of their established benefit in the treatment of acute coronary syndromes and atherothrombosis. The findings of this study are, therefore, important for cardiologists and the millions of patients worldwide who are already treated with these medications for atherothrombosis.
Platelet P2Y\textsubscript{12} Inhibitors Reduce Systemic Inflammation and Its Prothrombotic Effects in an Experimental Human Model

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Supplemental Material

Platelet P2Y₁₂ inhibitors reduce systemic inflammation and its prothrombotic effects in an experimental human model

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Supplemental Results

Residual Platelet P2Y12 Reactivity Prior to LPS Correlates with Subsequent Peak Levels of TNFα and D-dimer

TNFα was the first pro-inflammatory cytokine to increase, consistent with its central role in the pathophysiology of systemic inflammation and its prothrombotic effects. Each of the following indices of platelet reactivity to ADP pre-LPS correlated with subsequent peak levels of TNFα after LPS administration: platelet-monocyte aggregate formation (r=0.55; p=0.002; Fig III in the online supplement), final platelet aggregation (r=0.54; p=0.002) and platelet P-selectin expression (r=0.42; p=0.023). Peak levels of TNFα correlated with peak levels of D-dimer (r=0.60; p<0.001) and fibrin clot maximum absorbance (r=0.48; p=0.01).
### Supplemental Table

#### Table I Baseline and treatment characteristics

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<tr>
<td>Duration of clopidogrel treatment – days (SEM)</td>
<td>0 (0)</td>
<td>7.1 (0.1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Duration of ticagrelor treatment – days (SEM)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>6.7 (0.3)</td>
</tr>
</tbody>
</table>

There were no significant differences in baseline characteristics between groups.
Table II Hemodynamic parameters before treatment, after treatment and 3 hours after LPS administration

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Before LPS</th>
<th>3 Hours After LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>72±4</td>
<td>70±2</td>
<td>94±4*</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>70±5</td>
<td>68±4</td>
<td>94±4*</td>
</tr>
<tr>
<td>Ticagrelor</td>
<td>74±2</td>
<td>71±3</td>
<td>93±3*</td>
</tr>
<tr>
<td>Mean arterial blood pressure (mm Hg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>87±2</td>
<td>88±3</td>
<td>84±2</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>89±3</td>
<td>88±3</td>
<td>82±3</td>
</tr>
<tr>
<td>Ticagrelor</td>
<td>87±3</td>
<td>87±3</td>
<td>80±2</td>
</tr>
<tr>
<td>Temperature °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>36.1±0.1</td>
<td>35.9±0.1</td>
<td>37.9±0.2*</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>36.0±0.1</td>
<td>36.0±0.1</td>
<td>38.1±0.2*</td>
</tr>
<tr>
<td>Ticagrelor</td>
<td>36.0±0.1</td>
<td>36.0±0.1</td>
<td>37.7±0.2*</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. * = P<0.05 compared to value before LPS administration.
Supplemental figures

Figure I. CONSORT flow diagram presenting the enrolment, intervention allocation, follow-up and data analysis with number of subjects for each group.
Figure II. Final platelet aggregation response measured after 1 week of ticagrelor, clopidogrel or control. Data expressed as mean ± SEM (n=10 in each group). Effect of ticagrelor and clopidogrel compared to control using ANOVA.

Figure III. Correlation between residual platelet reactivity after 1 week of randomized treatment (as demonstrated by ADP-induced platelet-monocyte aggregate formation) and subsequent TNFα response. Correlation determined by Pearson correlation coefficient.
Materials and Methods

Study population

This prospective, randomized, open-label study was approved by the Sheffield Research Ethics Committee (UK) and the Medicines and Healthcare products Regulatory Agency (UK) and was conducted in accordance with Good Clinical Practice guidelines. Subjects provided written informed consent. Inclusion criteria were age more than 18 years with no significant medical issues, no regular use of medication and willingness to abstain from consuming caffeine (an adenosine receptor antagonist). Exclusion criteria included any clinically significant abnormality detected on screening (medical history, physical examination, ECG and routine blood tests), recent blood donation or vaccination, a history of alcohol or drug abuse or a contraindication to study medication. The study was registered at http://www.clinicaltrials.gov (unique identifier NCT01846559).

Experimental protocol

Volunteers were randomized to receive one week of ticagrelor 90 mg twice daily (n=10), clopidogrel 75 mg once daily (n=10) or no antiplatelet medication (controls; n=10). Ticagrelor and clopidogrel-treated subjects received loading doses of 180 mg and 300 mg respectively. One venous cannula was inserted into an antecubital vein in each arm. One cannula was used for blood sampling and the other for administration of LPS and intravenous fluid (250 ml 0.9% saline over 30 minutes prior to LPS administration, then 500 ml 0.9% saline over 4 hours after LPS administration). 2 ng/kg E. coli O:113 LPS (Clinical Center Reference Endotoxin, National Institutes of Health, Bethesda, MD) was administered over 1 minute at t = 0 hours. Venous blood samples were collected at baseline (prior to any randomized medication), prior to LPS administration and at the following time points after LPS administration: 5, 15 and 30 minutes and 1, 1.5, 2, 4, 6 and 24 hours. All laboratory measurements were performed by staff blinded to treatment allocation.

Cell count, immunoassays, D-dimer and platelet aggregation

Blood was collected into EDTA anticoagulant tubes prior to cell counting using an automated Sysmex cell counter (XN-9000, Sysmex, Milton Keynes, UK). Blood samples for isolation of plasma were collected into tubes containing trisodium citrate dihydrate (3.13% w/v), centrifuged immediately at 1,500 g for 10 minutes and the supernatant stored at -80°C. Plasma levels of cytokines were measured by cytometric bead array using standardised kits (BD™ Cytometric Bead Array, Becton Dickinson [BD], Oxford, UK). High-sensitivity C-reactive protein (hsCRP) was measured using a Siemens BN II Nephelometer (Siemens, UK). D-dimer was measured by a Sysmex 2100i (Sysmex, UK) using the INNOVANCE D-dimer assay. Final platelet aggregation responses after 5 minutes exposure to 30 μM ADP were assessed in platelet rich plasma using a PAP-8E optical aggregometer (BioData, Horsham, PA).

Flow cytometry

Platelet P-selectin was measured by flow cytometry: 40 μl of citrate-anticoagulated whole blood was added to a combination of saline or ADP (final concentration 30 μM), APC-conjugated CD61 (104316, BioLegend, London, UK) and PE-Cy5-conjugated CD62P (551142, BD, UK) and incubated in the dark for 20 minutes. Platelets were gated on morphological characteristics and expression of CD61 and median fluorescence of CD62P was used to determine platelet P-selectin expression. Flow cytometry was also used to determine platelet-leukocyte aggregate formation: 480 μl of citrate-anticoagulated whole blood was added to saline or ADP (final concentration 30 μM) and stirred for 10 minutes.
Two ml diluted FACSLyse solution (BD, UK) was then added to 180 µl of blood to simultaneously lyse erythrocytes and fix the leukocytes. This was centrifuged at 300 g for 5 minutes and the pellet was resuspended in 100 µl PBS + 10% bovine serum albumin. This suspension was then stained with PE-conjugated CD14 (555398, BD, UK) and FITC-conjugated CD42a (558818, BD, UK). Monocytes were gated based on morphological characteristics and expression of CD14. Neutrophils were gated based on morphological characteristics and exclusion of monocytes. Platelet-leukocyte aggregate formation was determined by monocyte or neutrophil median fluorescence of the platelet marker CD42a. Samples were processed for analysis by flow cytometry immediately after blood was sampled. Samples from all treatment groups were sampled within the same time frame. All flow cytometric analysis was performed with an Accuri C6 multi-color flow cytometer (BD, UK).

**Fibrin clot structure**

Fibrin clot characteristics were studied in each individual at 4 time points using a validated high-throughput turbidimetric assay, as previously described (1). To further visualise fibrin networks, fibrin clots were prepared from pooled plasma of 10 volunteers from each treatment group, as previously described (1). Fibrin clot structure was assessed using scanning electron microscopy. All clots were prepared in duplicate at 4 different time points and photographed at x5,000, x10,000 and x30,000 magnifications in 4 different areas using a field-emission scanning electron microscope (Quinta 200F FEG ESEM, FEI company, Netherlands). In each of these photographs, fiber diameter (n=40) was determined using image analysis software (ImageJ 1.48; National Institutes of Health, USA). Fibrin network density was also determined using ImageJ, by converting all images to binary using a fixed threshold and calculating the percentage of white pixels. To exclude bias, all clots were viewed by 2 operators blinded to the type of sample.

**Statistical analysis**

An independent statistician designed the statistical analysis plan prior to the commencement of the study. Area under the curve of hsCRP over a 24-hour period following LPS administration was compared between treatment groups using ANOVA. More complex variables, such as WBC, were compared using repeated measures two-way ANOVA with Dunnett’s correction for multiple comparisons. Correlation between variables was determined by Pearson correlation coefficient. P<0.05 was considered to be statistically significant. Analyses were performed using SPSS 21 (Chicago, Illinois) and GraphPad Prism 6 (San Diego, CA). Data are presented as mean ± SEM.
References