

CLINICAL RESEARCH

Ticagrelor Reduces Thromboinflammatory Markers in Patients With Pneumonia



Travis R. Sexton, PhD,^a Guoying Zhang,^a Tracy E. Macaulay, PHARM.D,^a Leigh A. Callahan, MD,^b
Richard Charnigo, PhD,^c Olga A. Vsevolozhskaya, PhD,^c Zhenyu Li, PhD,^a Susan Smyth, MD, PhD^{a,d}

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CME/MOC Objective for This Article: Upon completion of this activity, the learner should be able to: 1) identify the potential role for treatment with the P2Y₁₂ inhibitor ticagrelor in settings outside of acute coronary syndrome;

2) examine the effect that ticagrelor has on systemic biomarkers of inflammation; and 3) discuss the mechanism of platelet inhibition with ticagrelor and the role of treatment in acute coronary syndrome

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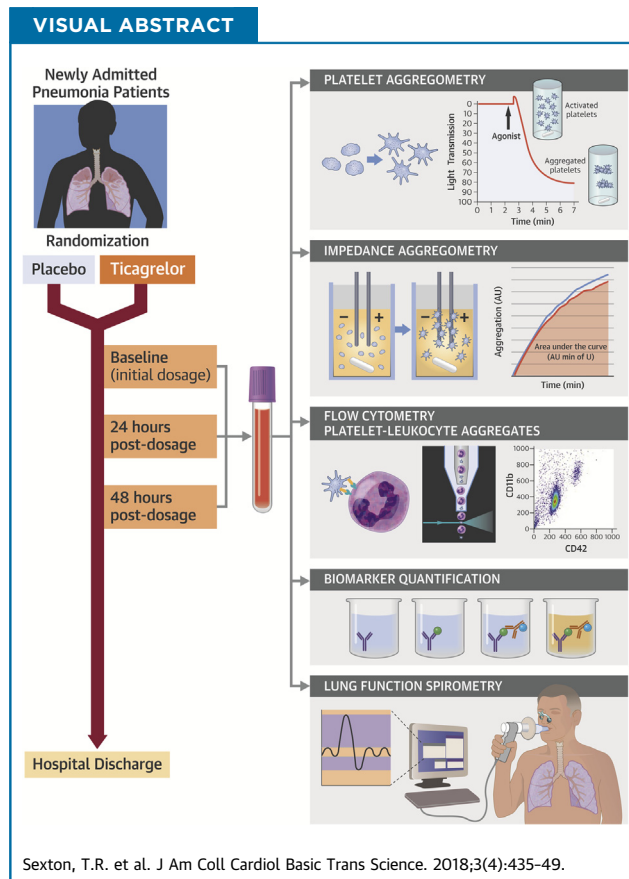
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From the ^aGill Heart and Vascular Institute, University of Kentucky, Lexington, Kentucky; ^bPulmonary, Critical Care & Sleep Medicine, University of Kentucky, Lexington, Kentucky; ^cDepartment of Biostatistics, College of Public Health, University of Kentucky, Lexington, Kentucky; and the ^dLexington VA Medical Center, Lexington, Kentucky. This work was supported by an investigator-initiated grant from AstraZeneca as well as an R01 from the National Institutes of Health National Heart, Lung, and Blood Institute HL123927. Drs. Smyth and Charnigo have been principal investigators and coinvestigators on AstraZeneca grants, including the one funding this work. Dr. Smyth has served on advisory boards for AstraZeneca. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

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HIGHLIGHTS

- As expected, ticagrelor reduced ex-vivo ADP-induced aggregation in patients with pneumonia compared with placebo.
- Ticagrelor reduced platelet-leukocyte interactions as well as plasma interleukin-6 within 24 h in patients with pneumonia compared with placebo.
- Ticagrelor acutely altered NETosis biomarkers, whereas placebo had no effect.
- Ticagrelor improved lung function and reduced need for supplemental oxygen in patients with pneumonia compared with placebo.

All authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the *JACC: Basic to Translational Science* [author instructions page](#).

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SUMMARY

Despite treatment advances for sepsis and pneumonia, significant improvements in outcome have not been realized. Antiplatelet therapy may improve outcome in pneumonia and sepsis. In this study, the authors show that ticagrelor reduced leukocytes with attached platelets as well as the inflammatory biomarker interleukin (IL)-6. Pneumonia patients receiving ticagrelor required less supplemental oxygen and lung function tests trended toward improvement. Disruption of the P2Y₁₂ receptor in a murine model protected against inflammatory response, lung permeability, and mortality. Results indicate a mechanistic link between platelets, leukocytes, and lung injury in settings of pneumonia and sepsis, and suggest possible therapeutic approaches to reduce complications. (Targeting Platelet-Leukocyte Aggregates in Pneumonia With Ticagrelor [XANTHIPPE]; NCT01883869) (J Am Coll Cardiol Basic Trans Science 2018;3:435-49) Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Sepsis is a complex syndrome of dysregulated host responses to infection that results in organ damage and carries a substantial risk of mortality. Pneumonia is a leading cause of sepsis and is the most common fatal infection acquired in hospitals. Despite advances in treatments, such as antibiotic therapy and intensive care, significant improvement in mortality rates remains elusive (1). Additionally, there is increasing awareness of the burden of cardiovascular complications in patients hospitalized with pneumonia (2-9). Novel treatment strategies in this high-risk patient population are needed.

In addition to an integral role in hemostasis, platelets contribute to inflammatory and immune responses. Platelets alter properties of endothelial cells and leukocytes, release soluble pro- and anti-inflammatory mediators, internalize microorganisms, and bind and sequester pathogens. Platelet activation and sequestration in pulmonary tissue is a key feature in inflammatory or infectious states such as sepsis and acute respiratory distress syndrome (10-12). Recent evidence suggests that antiplatelet therapy may improve outcomes in patients hospitalized with pneumonia. A retrospective, cohort study to evaluate the effects of antiplatelet therapy on the incidence and severity of community-acquired pneumonia (CAP) reported trends toward reduced instances of mechanical ventilation, mortality, and the composite endpoint of death in CAP inpatients (13). Similarly, an observational study of 224 hospitalized CAP patients showed lower use of intensive care unit care in patients receiving antiplatelet agents for at least 6 months compared with unmatched controls (14). A study of 615 patients consecutively admitted to medical and surgical intensive care units found that use of antiplatelet agents was associated with reductions in

mortality and attenuation in thrombocytopenia (15). More recently, a post hoc analysis on the PLATO (Platelet Inhibition and Patient Outcomes) trial, in which 18,624 patients presenting with acute coronary syndrome were randomized to receive standard of care with the adenosine diphosphate receptor P2Y₁₂ antagonist clopidogrel or ticagrelor, which provides stronger and more consistent P2Y₁₂ antagonism than clopidogrel, revealed that ticagrelor was associated with lower mortality in patients with subsequent pulmonary events or sepsis (16). In addition, ticagrelor was able to decrease the absolute risk of ischemic events compared with clopidogrel in patients enrolled in the PLATO trial that had a history of chronic obstructive pulmonary disease (COPD) (17).

Taken together, these observations suggest that antiplatelet therapy may improve outcomes in ambulatory patients with pneumonia, and potentially in sepsis. The mechanism for a beneficial effect could be multifactorial. Platelet activation is associated with worse cardiovascular outcomes in patients with pneumonia (2). Platelet activation results in adhesion, the expression of cell surface receptors, and the release of small molecules that can promote thrombosis and amplify the immune response. Specifically, platelet activation results in expression of P-selectin, increases platelet-leukocyte aggregates, and triggers the formation of neutrophil extracellular DNA nets. Platelet-leukocyte aggregates predict mortality in severe sepsis (18), and DNA nets associate with severity of sepsis and septic organ dysfunction (19). In animals, platelets and neutrophils accumulate in lungs in models of sepsis, during both bacterial and viral infection (20). Furthermore,

ABBREVIATIONS AND ACRONYMS

- ADP** = adenosine diphosphate
- CAP** = community-acquired pneumonia
- CI** = confidence interval
- COPD** = chronic obstructive pulmonary disease
- dsDNA** = doubled-stranded DNA
- HAP** = hospital-acquired pneumonia
- ELISA** = enzyme-linked immunosorbent assay
- FEV-1** = forced expiratory volume in 1 s
- IL** = interleukin
- IQR** = interquartile range
- K_{fc}** = capillary filtration coefficient
- LPS** = lipopolysaccharide
- LTA** = light transmission aggregometry
- MPO** = myeloperoxidase
- MVV** = maximum ventilation velocity
- NE** = neutrophil elastase
- NET** = neutrophil extracellular trap
- OR** = odds ratio
- PRP** = platelet-rich plasma
- TNF** = tumor necrosis factor
- TRAP** = thrombin receptor activating peptide
- WT** = wild-type

TABLE 1 Inclusion and Exclusion Criteria

| Inclusion criteria | |
|--|--|
| Men or nonpregnant women 18 yrs of age or older | |
| Subjects must be willing and able to give informed consent | |
| Diagnosis of CAP or HAP within 48 h of diagnosis or presentation to hospital | |
| Subjects must have new radiographic finding(s) consistent with pneumonia and at least 2 of the following signs: | |
| • Cough | |
| • Fever | |
| • Hypothermia | |
| • Purulent sputum production or respiratory secretion | |
| • Total WBC count >10,000/mm ³ , or >15% band forms, regardless of total peripheral white count; or leukopenia with total WBC <4,500/mm ³ | |
| • Auscultatory findings on pulmonary examination of rales and/or evidence of pulmonary consolidation (dullness on percussion, bronchial breath sounds, or egophony) | |
| • Hypoxemia | |
| • Increase in dyspnea and/or tachypnea (>20 breaths/min) | |
| Exclusion criteria | |
| Contraindication to ticagrelor (hypersensitivity of reaction to ticagrelor or another P2Y ₁₂ antagonist) | |
| Active or suspected major bleeding history | |
| Platelet count <100,000/mm ³ or International normalized ratio >1.5 | |
| Surgery within 30 days or anticipated major surgery | |
| Oral anticoagulant that cannot be stopped | |
| Inability or unwillingness of treating physician to reduce dose of aspirin to 100 mg | |
| Fibrinolytic therapy in the last 24 h | |
| Increased risk of bradycardic events—2nd or 3rd degree heart block, bradycardia induced syncope—unless pacemaker in place | |
| Underlying immunodeficiency (HIV, neutropenia, receiving immunomodulating reagents, active hematologic malignancy, functional or anatomic asplenia, and hypogammaglobulinemia) | |
| Concomitant therapy with CYP3A inducer; rifampin/rifampicin, phenytoin, carbamazepine | |
| Pregnancy or lactation | |
| Active treatment for cancer | |
| Acute, decompensated congestive heart failure | |
| Participation in another investigational drug or device study in the last 30 days | |
| Inability to administer enteric medication | |
| CAP = community-acquired pneumonia; HAP = hospital-acquired pneumonia; WBC = white blood count. | |

TABLE 2 Baseline Characteristics of XANTHIPPE Subjects

| | Placebo | Ticagrelor | p Value |
|--|-------------|-------------|---------|
| Demographics | | | |
| Men | 14 (47) | 13 (43) | 1.000 |
| Women | 16 (53) | 17 (57) | 1.000 |
| Age, yrs | 55.1 ± 16.0 | 56.6 ± 15.9 | 0.705 |
| Caucasian | 28 (93) | 29 (97) | 1.000 |
| African American | 2 (7) | 1 (3) | 1.000 |
| Medical history | | | |
| Hypertension | 15 (50) | 23 (77) | 0.060 |
| History of smoking | 21 (70) | 23 (77) | 0.771 |
| Diabetes | 9 (30) | 8 (27) | 1.000 |
| COPD | 17 (57) | 16 (53) | 1.000 |
| Medication on admission | | | |
| Aspirin | 7 (23) | 8 (27) | 1.000 |
| P2Y ₁₂ inhibitor | 4 (13) | 7 (23) | 0.506 |
| Antibiotics on enrollment | | | |
| Levofloxacin | 8 (27) | 14 (47) | 0.180 |
| Azithromycin | 7 (23) | 2 (7) | 0.146 |
| Ceftriaxone | 5 (17) | 5 (17) | 1.000 |
| Vancomycin | 8 (27) | 10 (33) | 0.779 |
| Levaquin | 6 (20) | 4 (13) | 0.731 |
| Cefepime | 3 (10) | 4 (13) | 1.000 |
| Penicillin | 1 (3) | 0 (0) | 1.000 |
| Zosyn | 3 (10) | 1 (3) | 0.612 |
| Doxycycline | 1 (3) | 2 (7) | 1.000 |
| Peracillin/tazobactam | 2 (7) | 1 (3) | 1.000 |
| Clindamycin | 1 (3) | 0 (0) | 1.000 |
| Metronidazole | 1 (3) | 3 (10) | 0.612 |
| Acyclovir | 0 (0) | 1 (3) | 1.000 |
| Aztreonam | 0 (0) | 1 (3) | 1.000 |
| Values are n (%) or mean ± SD. p Values for the qualitative variables were calculated using Fisher's exact test. p Values for quantitative variables were calculated with a 2-sample Student's t-test. | | | |
| COPD = chronic obstructive pulmonary disease. | | | |

METHODS AND MATERIALS

PATIENTS AND STUDY DESIGN. XANTHIPPE (Examining the effect of Ticagrelor on Platelet Activation, Platelet-Leukocyte Aggregates, and Acute Lung Injury in Pneumonia) is an investigator-designed and initiated trial (NCT01883869) was approved by the institutional review board at the University of Kentucky. All subjects provided written informed consent before participation. The XANTHIPPE trial enrolled patients admitted to the University of Kentucky hospitals with a diagnosis of pneumonia between March 26, 2014, and June 10, 2016. Patients were enrolled within 48 h of diagnosis of either CAP or hospital-acquired pneumonia (HAP), which was defined as pneumonia occurring in a patient with any hospital admission in the previous 3 months. Complete inclusion and exclusion criteria can be found in [Table 1](#). Demographics of all enrolled patients are presented in [Table 2](#). Patients that were taking a P2Y₁₂

antiplatelet therapy decreases platelet-leukocyte aggregates and reduces lung injury.

To explore the possibility that platelets underpin adverse events in pneumonia and sepsis and that potent antiplatelet therapy may attenuate the end-organ or cellular and tissue complications of a dysregulated immune response, we evaluated the effect of the direct-acting P2Y₁₂ antagonist ticagrelor on biomarkers of cardiovascular outcomes in a double-blinded, placebo-controlled randomized trial in patients diagnosed with pneumonia. We then performed mechanistic studies of ticagrelor on endotoxemia-associated mortality in mice. Our findings support a role for platelets as a link between adverse outcomes in pneumonia and sepsis, and provide mechanistic insight into potential beneficial actions of potent antiplatelet therapy in pneumonia.

antagonist upon enrollment were not included on the analysis of flow cytometry, platelet function, biomarker analysis, and spirometry.

The XANTHIPPE trial was a double-blind study in which subjects were randomized to placebo or ticagrelor (180-mg loading dose followed by 90 mg twice a day) for up to 7 days or until discharge from the hospital. Randomization and drug distribution were performed by the Investigational Drug Service at the University of Kentucky. Blood samples were collected at baseline just before administration of the loading dose of study medication and at approximately 24 and 48 h following the initial study dose. An additional blood sample was taken on day of discharge or day 7, whichever came first. Study follow-up occurred daily while admitted to the hospital and at 30 days after enrollment.

The primary efficacy endpoint of XANTHIPPE was the change in platelet-leukocyte aggregates (defined as the percentage of leukocytes with attached platelets) from baseline to 24 h following the initial dose of study medication. Secondary endpoints included change in platelet function, change in biomarkers associated with inflammation and thrombosis, and lung function as determined by bedside spirometry. Rates of serious adverse events and/or rehospitalization within 30 days of enrollment were determined by patient follow-up, by clinic or phone call, as well as by review of electronic medical records.

FLOW CYTOMETRY. Platelet-leukocyte aggregates were determined by flow cytometry as previously described (21). Briefly, fresh whole blood collected in hirudin blood tubes (Diapharma, West Chester, Ohio) and incubated with the leukocyte-specific allophycocyanin (APC)-conjugated anti-CD11b/Mac-1 (BD Biosciences, San Jose, California) and platelet-specific fluorescein isothiocyanate (FITC)-conjugated anti-CD42b (BD Biosciences) for 15 min at room temperature while protected from light. Samples were fixed and red blood cells were lysed using FACS Lysing Solution (BD Biosciences). Flow cytometry was performed at the core facility at the University of Kentucky. Leukocytes (50,000/gate) were identified by the CD11b-positive signal compared with the isotype control. Platelet-leukocyte aggregates were identified by the subset of CD11b-positive cells that were also CD42b positive as compared with the isotype control. FlowJo software version 10 (TreeStar, Ashland, Oregon) was used to analyze samples. Patients that were taking a P2Y₁₂ antagonist upon enrollment were excluded from analysis.

PLATELET AGGREGATION AND PLASMA BIOMARKERS. Ex vivo platelet aggregation using platelet-rich

TABLE 3 Clinical Characteristics of XANTHIPPE Patients

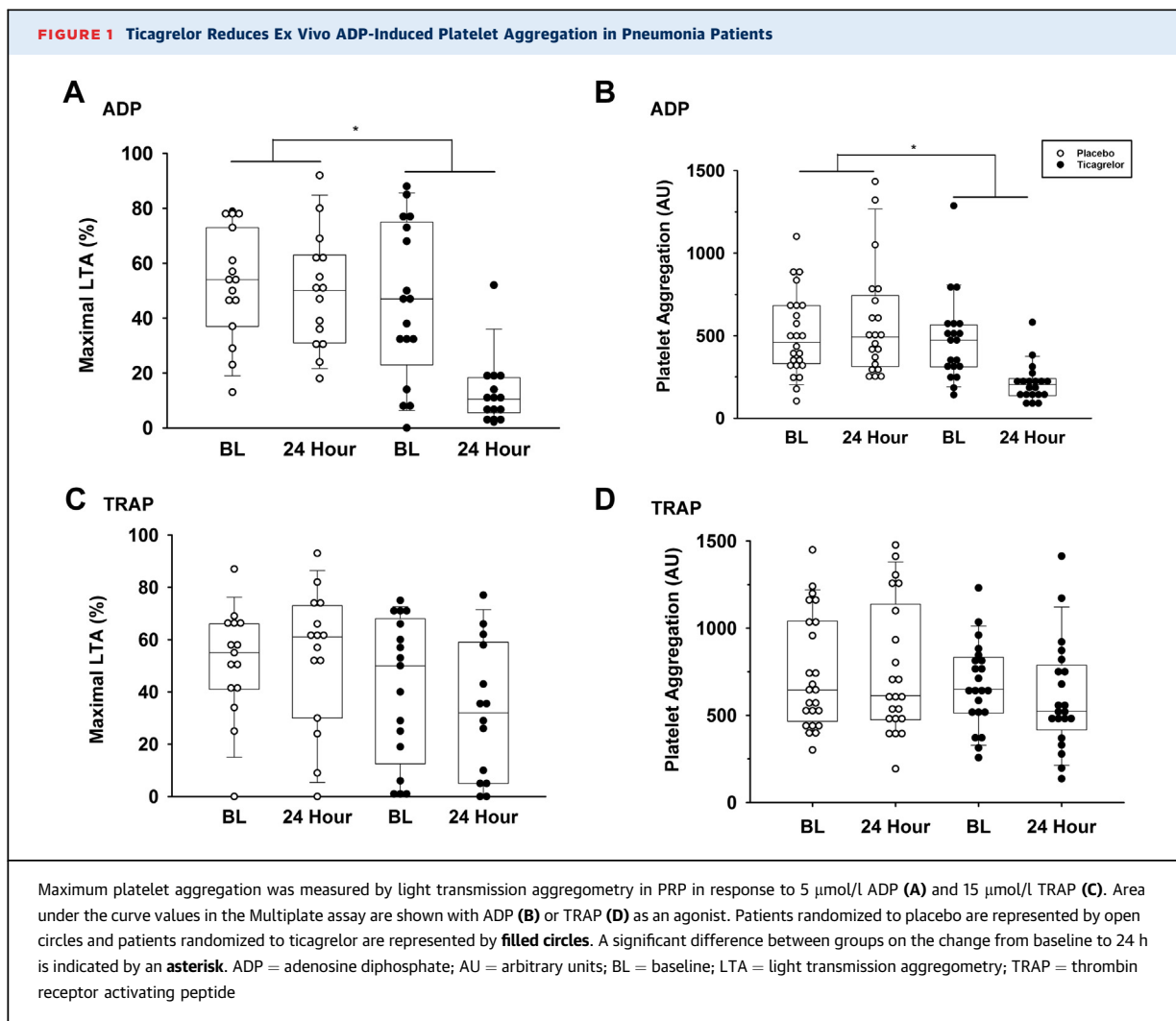
| | Placebo | Ticagrelor | p Value |
|-------------------------|--------------|--------------|---------|
| Pneumonia | | | |
| CAP | 21 (70) | 17 (57) | 0.422 |
| HAP | 9 (30) | 13 (43) | 0.422 |
| Blood counts | | | |
| Baseline platelet count | 248 ± 74 | 283 ± 85 | 0.094 |
| 24-h platelet count | 263 ± 94 | 291 ± 97 | 0.324 |
| Baseline WBC | 12.04 ± 5.18 | 12.13 ± 5.83 | 0.951 |
| 24-h WBC | 10.72 ± 5.10 | 12.91 ± 6.02 | 0.183 |

Values are n (%) or mean ± SD. p Values for the qualitative variables were calculated using Fisher's exact test. p Values for quantitative variables were calculated with a 2-sample Student's t-test.
 Abbreviations as in Table 1.

plasma (PRP) and whole blood was performed as previously described (22). Aggregation with PRP prepared from citrated blood was determined using a light transmission aggregometer (Chrono-log Corp., Havertown, Pennsylvania). PRP was incubated at 37°C for 2 min before treatment with thrombin receptor activating peptide (TRAP) or adenosine diphosphate (ADP) to final concentrations of 15 μmol/l and 5 μmol/l, respectively. Maximal aggregation and slope within the first 5 min were calculated using the AGGROLINK software (Chrono-log Corp.). For multiplate impedance analysis, blood was collected in hirudin tubes and incubated at 37°C for 3 min before the addition of agonist. The area under the curve after 6 min of agonist was recorded with the Multiplate system (Roche, Basel, Switzerland). Patients that were taking a P2Y₁₂ antagonist upon enrollment were excluded from platelet aggregation analysis.

Plasma for biomarker analysis was collected from citrate-theophylline-adenosine-dipyridamole (CTAD)-anticoagulated blood that was supplemented with ethylenediaminetetraacetic acid (EDTA) (final concentration of 10 mmol/l) before centrifugation at 3,000 g for 10 min. Plasma was aliquoted, flash frozen, and stored at -80°C. Tumor necrosis factor (TNF)-α, IL-6, IL-1β, IL-10, and sCD40L analytes were measured with a MAGPIX multiplex reader using a Milliplex MAP Kit (Millipore, Burlington, Massachusetts). Angiopoietin-1, angiopoietin-2, ELA-2, ENA-78, NAP-2, and platelet factor (PF)-4 analytes were measured using DuoSet enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, Minnesota). Patients taking a P2Y₁₂ antagonist upon enrollment were excluded from biomarker analysis.

Neutrophil extracellular traps (NET) were measured by ELISA using anti-myeloperoxidase (MPO) (part 842842 from R&D Systems) for capture and biotinylated anti-neutrophil elastase (NE) (Ab79962 from



Abcam, Cambridge, Massachusetts) for detection. MPO and NE are in complex with chromatin in NETs. Samples were calibrated to a serial dilution of serum and assigned arbitrary units based on detected signal. Quant-iT Picogreen kits (Thermo Fisher Scientific, Waltham, Massachusetts) were used to measure doubled-stranded DNA (dsDNA) in plasma.

SPIROMETRY. Spirometry was performed following American Thoracic Society/European Respiratory Society guidelines (23) at the patient bedside using a CareFusion MicroLoop spirometer (Vyair Medical, Mettawa, Illinois). Relaxed spirometry, forced spirometry, and maximum ventilation velocity (MVV) were performed at baseline (before drug randomization), 24 h after dosage, and day of discharge on subjects that were able and willing to perform.

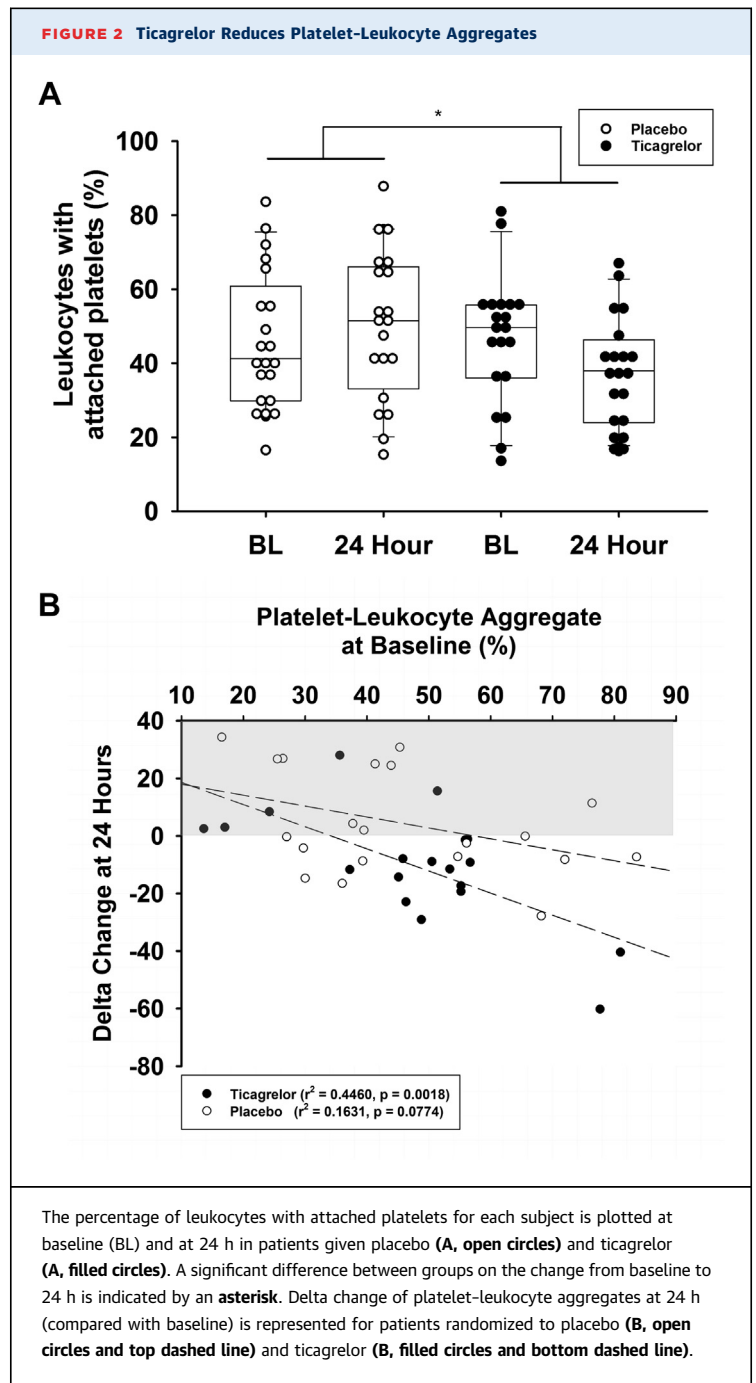
MOUSE STUDY. Mice deficient in $P2Y_{12}$ were generated as described previously (24). Littermate wild-type (WT) mice from heterozygous breeding were used as controls. The mice were bred and maintained in the University of Kentucky Animal Care Facility following institutional and National Institutes of Health guidelines after approval by the Animal Care Committee.

WT and $P2ry12^{-/-}$ male mice (8 to 10 weeks of age) were fed placebo, clopidogrel (25 mg/kg body weight per 24 h), or ticagrelor (10, 25, 50 mg/kg body weight, per 12 h) by oral gavage. Two h later, the animals were injected intraperitoneally with lipopolysaccharide (LPS) (0111:B4, 10 mg/kg in 200 μl , Sigma Aldrich, St. Louis, Missouri). Mice were assessed every 12 h for 5 days. Blood was collected at the indicated time points after LPS injection, and plasma was prepared by

centrifugation. TNF- α and IL-6 were measured by ELISA assay (eBioscience, San Diego, California).

DETERMINATION OF PULMONARY MICROVASCULAR PERMEABILITY. Lungs were isolated 6 h after injection of LPS and microvessel capillary filtration coefficient (K_{fc}) was measured as described previously (23). In brief, after 20-min equilibration perfusion to establish an isogravimetric condition, the outflow pressure was rapidly elevated by 10 cm of H₂O for 20 min. In response to the pressure increase, lung preparations gained weight due to net fluid accumulation. Lungs were dissected free of nonpulmonary tissue, and lung dry weight was determined. K_{fc} was calculated from the slope of the weight change normalized to the pressure change and lung dry weight.

STATISTICAL ANALYSES. Baseline and clinical characteristics in Tables 2 and 3 were compared between the 2 groups using Student's *t*-test and Fisher's exact test. For the repeated-measures data in the platelet aggregation, platelet-leukocyte aggregate assays, and for non-IL biomarkers (sCD40L, TNF- α , ENA-78, NAP-2, and PF-4), we fitted a linear mixed-effects model to compare groups at day 1, at day 2, and on change from day 1 to day 2. Logarithmic transformation was applied to some non-normally distributed data. For the IL-6, IL-10, and IL-1 β biomarkers, we compared groups at day 1, at day 2, and on change from day 1 to day 2 using Mann-Whitney rank sum tests. Linear regression was used to determine the relationship between baseline platelet-leukocyte aggregates and change at 24 h. For comparison of baseline biomarkers of treatment groups to healthy controls, a Mann-Whitney rank sum test was used. The MPO-NE assay measurements were non-normally distributed with some negative values so a logarithmic transformation was inappropriate. To contrast MPO-NE assay measurements between the ticagrelor group and the placebo group, we used non-parametric rank sum tests with a Bonferroni correction for multiple comparisons. The Picogreen assay measures were analyzed using the parametric mixed-modelling approach with unstructured covariance and a Tukey correction for multiple comparison. To assess the effects of ticagrelor on lung function, for each patient, we first computed differences between baseline and 24-h lung function measurements and then contrasted mean lung function differences between the ticagrelor group and the placebo group by a 2-sample Student's *t*-test with unequal variances. To evaluate the effects of P2Y₁₂ antagonists across doses as well as changes over time, appropriate contrasts were considered. For multiple group comparison with a



continuous response variable, we performed a 1-way analysis of variance. Relationships between mortality and P2Y₁₂ antagonists were analyzed using the log-rank test. To explore the role of P2Y₁₂ receptor in mice treated with either a placebo or with the P2Y₁₂ antagonists (ticagrelor or clopidogrel), Cox proportional hazards models were employed. To examine whether P2Y₁₂ antagonism (ticagrelor or clopidogrel) affects pulmonary microvascular permeability, K_{fc} were

| TABLE 4 Biomarkers | | | | | |
|--------------------|---------------------|---------------------|--------------------|--------------------|--------------------|
| | Healthy Plasma | Ticagrelor | | Placebo | |
| | | Day 1 | Day 2 | Day 1 | Day 2 |
| NAP-2 | 295 (228-333) | 291 (156-533) | 355 (213-676) | 148 (95-404) | 186 (115-337) |
| ENA-78 | 52.55 (40.42-64.38) | 48.9 (32.8-87.5) | 50.0 (39.8-102.5) | 39.4 (31.0-76.2) | 46.2 (35.7-73.6) |
| PF-4 | 273 (224-325) | 225 (119-339) | 232 (136-419) | 248 (121-360) | 291 (179-362) |
| IL-10 | 3.08 (1.81-4.17) | 3.76 (0.00-16.38) | 4.40 (1.70-16.35) | 4.67 (1.39-20.59) | 3.15 (0.00-35.28) |
| IL-6 | 0.82 (0.77-2.03) | 19.25 (3.20-42.52)* | 3.20 (1.80-14.19)* | 8.53 (0.99-17.74)* | 9.93 (2.00-37.33)† |
| TNF- α | 6.86 (4.66-8.08) | 8.02 (4.19-18.69) | 9.36 (5.76-23.02) | 9.98 (5.62-16.66) | 8.76 (6.40-21.44) |
| IL-1 β | 0.64 (0.42-0.86) | 0.60 (0.04-2.30) | 1.00 (0.28-2.44) | 0.62 (0.00-2.93) | 0.62 (0.00-7.54) |
| sCD40L | 57.2 (35.03-77.61) | 111.6 (50.0-255.4) | 140.2 (81.0-239.6) | 95.5 (53.5-211.5) | 142.3 (66.4-217.5) |

Values are median (interquartile range) in pg/mL. *Day 1 value is significantly different from healthy plasma (Mann-Whitney rank sum test). †Significant difference between the 2 treatment groups in the change from baseline to 24 h (linear mixed modeling or Mann-Whitney rank sum test).

contrasted using 1-way analysis of variance with a Tukey adjustment for multiple comparison. Statistical analysis was executed on SAS versions 9.3 and 9.4 (SAS Institute, Cary, North Carolina), SigmaPlot version 13.0 (Systat Software, San Jose, California) and R version 3.2.3 (R Project for Statistical Computing, Vienna, Austria). Graphs in figures were generated in SigmaPlot version 13.0.

RESULTS

TICAGRELOR MODULATES INFLAMMATORY RESPONSE IN PATIENTS HOSPITALIZED WITH PNEUMONIA. To evaluate the effect of the direct acting P2Y₁₂ antagonist ticagrelor in pneumonia, we randomized patients hospitalized within 48 h of diagnosis to receive placebo or ticagrelor for up to 7 days. **Table 2** lists demographic and baseline characteristics of patients enrolled in the XANTHIPPE trial. The average age was 55.1 ± 16.0 years in the placebo group (53% female) and 56.6 ± 15.9 years in the ticagrelor group (57% female). There were no significant differences in history of hypertension, smoking history, diabetes, and COPD between the 2 groups. There was no significant difference between the 2 groups with regard to aspirin use before enrollment. There were no significant differences in antibiotic treatment at the time of enrollment between the 2 groups. Twenty-one subjects were diagnosed with CAP in the placebo arm (70%) and 17 subjects in the ticagrelor arm (57%) (**Table 3**). At 30 days, there were a total of 7 rehospitalizations (4 in the placebo group and 3 in the ticagrelor group) and 3 deaths (3 in the placebo group and 0 in the ticagrelor group). No major bleeding occurred in any of the patients.

No significant differences in platelet or white blood cell counts were observed in the 2 groups at baseline or at 24 h after intervention (**Table 3**). At baseline,

maximum ADP- and TRAP-induced platelet aggregation were similar in the 2 groups (**Figure 1**). At 24 h, patients randomized to ticagrelor had lower ADP-induced aggregation as measured by light transmission aggregometry [LTA] (**Figure 1A**) and multiple electrode impedance assay (**Figure 1B**). No significant differences were observed between the groups at 24 h in aggregation induced by the PAR1 agonist TRAP by LTA (**Figure 1C**) or multiple electrode impedance assay (**Figure 1D**). Consistent with the effects of ticagrelor, the change in ADP-induced aggregation from baseline to 24 h was significantly different between subjects assigned to placebo and ticagrelor ($p = 0.0065$ [LTA] and $p < 0.0001$ [impedance assay]). No significant difference was noted for TRAP-induced aggregation ($p = 0.1283$ [LTA] and $p = 0.2937$ [impedance assay]).

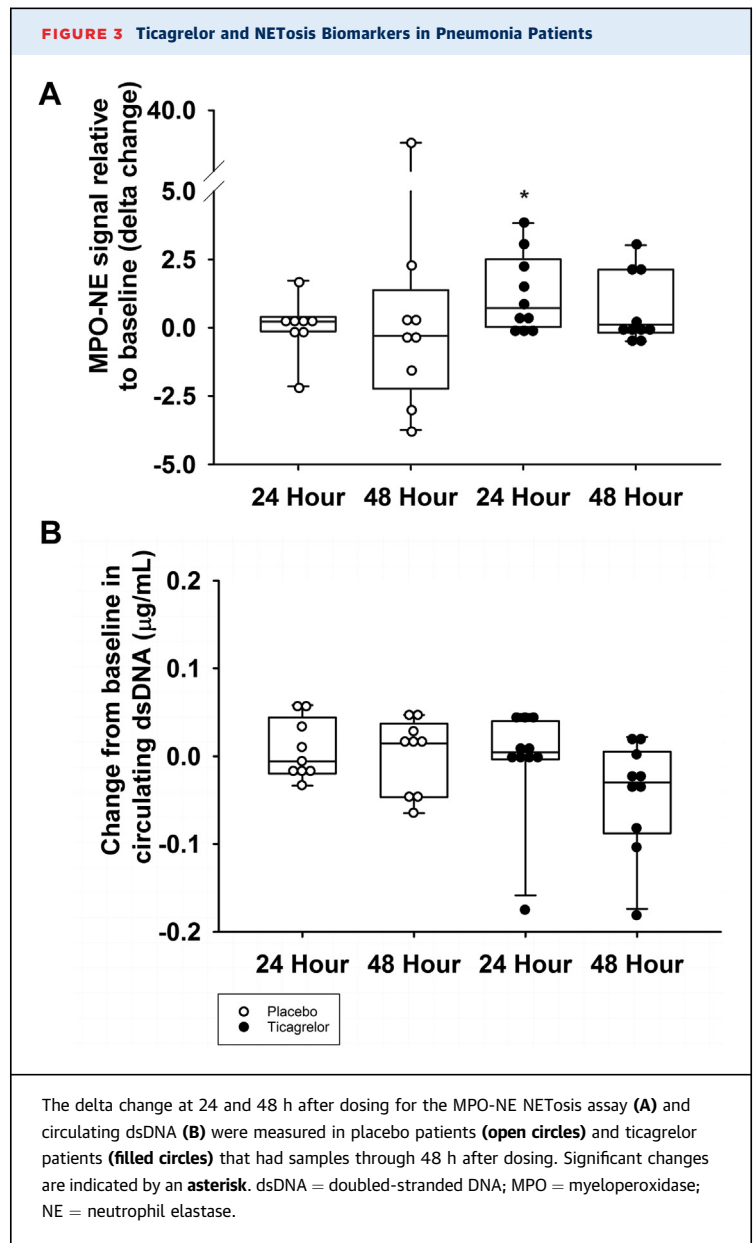
Local inflammatory events mediated by leukocytes and platelets have been proposed to underlie acute lung injury, and circulating platelet-leukocyte heterotypic cell aggregates increase in animal models of lung injury (25). Therefore, we determined whether ticagrelor altered platelet-leukocyte aggregates in patients with pneumonia. No difference in platelet-leukocyte aggregates in placebo and ticagrelor groups was present at baseline (**Figure 2A**). Between baseline and 24 h after drug administration, a significant difference in the change in heterotypic cell aggregates was observed in the placebo and ticagrelor groups ($p = 0.0244$). The percentage of leukocytes with attached platelets declined from a median of 49.65% (interquartile range [IQR]: 36.00% to 55.73%) at baseline to 37.90% (IQR: 23.93% to 46.35%) at 24 h after ticagrelor; whereas in subjects randomized to placebo, the median percentage of leukocytes with attached platelets was 41.30% (IQR: 31.35% to 60.85%) at baseline and increased to 52.20% (IQR: 40.40% to 66.30%) at 24 h. Interestingly, the magnitude of the response to ticagrelor depended on the

percentage of leukocytes with attached platelets at baseline, with larger responses to ticagrelor (Figure 2B) observed in individuals with more platelet-leukocyte aggregates at baseline.

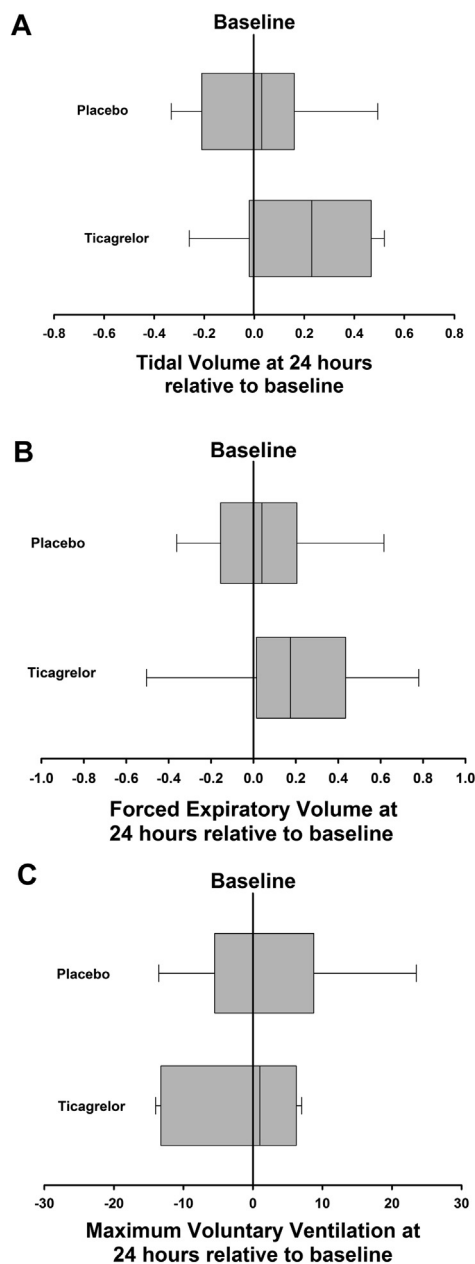
The effects of ticagrelor on other markers of platelet activation and systemic plasma biomarkers of inflammation were examined next. sCD40 ligand is released from the platelet membrane during activation. Baseline plasma sCD40L levels in both the placebo and ticagrelor groups were similar to levels in healthy controls and increased at 24 h irrespective of randomization arm (Table 4). Of a series of inflammatory biomarkers, including IL-6, TNF- α , IL-1 β , IL-10, NAP-2, and ENA-78, only IL-6 was above normal levels at baseline in the 2 groups. From baseline to 24 h, IL-6 levels changed significantly differently in the placebo and ticagrelor groups ($p = 0.0226$). In subjects randomized to ticagrelor, median plasma IL-6 levels declined by 83% from 19.25 pg/ml at baseline to 3.20 pg/ml at 24 h. No decrease was observed in the placebo group, which had plasma IL-6 levels of 8.53 pg/ml at baseline and 9.93 pg/ml at 24 h after randomization.

Platelets may also contribute to the formation of NETs, protein-DNA complexes released from neutrophils that contain MPO and NE in complex with chromatin. NETs facilitate pathogen clearance; however, their presence has also been associated with sepsis and sepsis organ dysfunction. We therefore measured the levels of MPO-NE complexes as a marker of NETosis in XANTHIPPE patients up to 48 h after placebo or ticagrelor. Somewhat surprisingly, relative to the baseline values, a significant increase in MPO-NE complexes was observed at 24 h ($p = 0.0410$) in the ticagrelor group. No significant change occurred in the placebo group (Figure 3A). Circulating ds-DNA quantified by the PicoGreen assay (Thermo Fisher Scientific), did not significantly change at 24 h after treatment in either group (Figure 3B), although a near-significant reduction in dsDNA occurred in the ticagrelor group at 48 h after therapy ($p = 0.0511$).

The effects of ticagrelor on lung function and supplemental oxygen requirements were assessed, respectively, by bedside spirometry on patients willing and able to perform the test and by patient records. In patients not receiving a P2Y₁₂ antagonist at baseline, 10 of the patients randomized to ticagrelor and 13 randomized to placebo were able to perform spirometry tests at both baseline and 24 h. In patients receiving ticagrelor, the mean tidal volume increased by 0.205 l from baseline to 24 h, whereas the change in the placebo group was only 0.008 l (Figure 4A). The ticagrelor group also displayed a



mean increase of 0.185 l in forced expiratory volume in 1 s (FEV-1) at 24 h, as compared with a mean 0.057-l change in placebo patients (Figure 4B). Finally, MVV decreased on an average by 1.51 l/min at 24 h in ticagrelor patients, whereas MVV in the placebo group increased by 2.31 l/min (Figure 4C). However, none of the differences between ticagrelor-treated patients and the placebo group were statistically significant (change in tidal volume p value = 0.187, change in FEV-1 $p = 0.387$, change in MVV $p = 0.447$). Ticagrelor patients did, however, have a significant reduction away from supplemental oxygen (odds ratio [OR]: 1.08, 95% confidence interval [CI]: 1.01 to

FIGURE 4 Ticagrelor and Lung Function Within 24 h in Pneumonia Patients

Lung function was tested in subjects willing and able to undergo spirometry testing. Data were collected at baseline and at 24 h. Changes at 24 h from baseline (midline in all 3 graphs) are shown for tidal volume (A), FEV-1 (B), and MVV (C). Units reported are liters for tidal volume and FEV-1 (A, B) and liters per minute for MVV (C). Data from patients randomized to placebo are shown in the **open boxes**, whereas patients randomized to ticagrelor are represented in the **shaded boxes**. Each **box** represents the interquartile range (25% to 75%), whereas the **whiskers** represent the full range of the data. The **vertical line in each interquartile range box** represents the median value (note that the median for the placebo group in C overlaps with the midline). FEV-1 = forced expiratory volume in 1 s; MVV = maximum ventilation velocity.

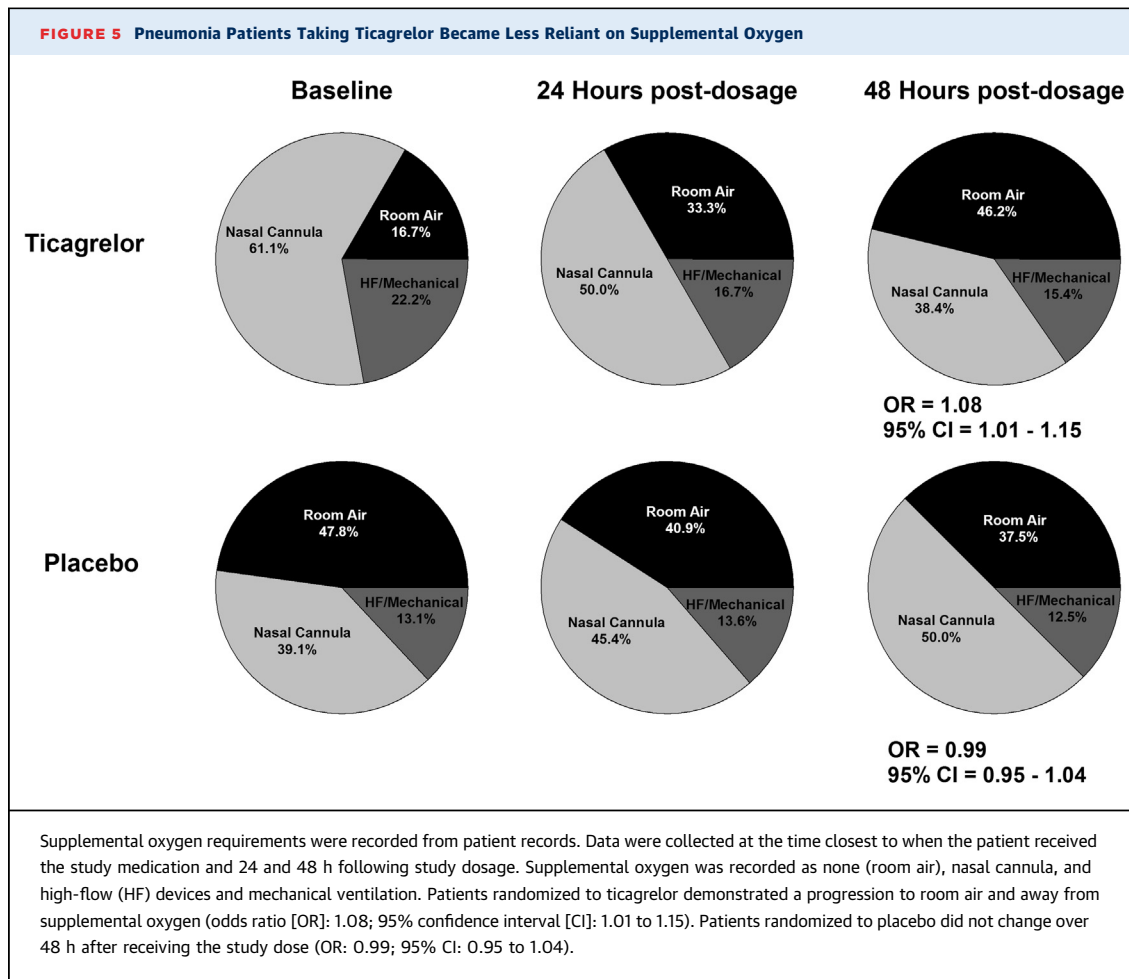
1.15) compared with no change in placebo patients (OR: 0.99; 95% CI: 0.95 to 1.04) (Figure 5).

P2Y₁₂ RECEPTOR FUNCTION MODULATES RESPONSE IN ACUTE INFLAMMATORY PRECLINICAL MODELS.

Our results indicate that ticagrelor has effects on systemic biomarkers of inflammation in patients with pneumonia. To explore the role of P2Y₁₂ receptor function in an acute inflammatory model, mice were treated with placebo, ticagrelor, or clopidogrel before intraperitoneal LPS (10 mg/kg). Within hours of LPS administration, plasma inflammatory and anti-inflammatory cytokine levels increased. At 1 h in WT mice, plasma TNF- α levels were approximately 20-fold higher than baseline (Figure 6A). Ticagrelor treatment at all doses reduced TNF- α at 1 h, but this decrease was statistically significant only for 25 mg/kg dose ($p < 0.01$). Over longer times (2 to 8 h), ticagrelor had no significant effect on reducing TNF- α levels (Figure 6A). Ticagrelor elicited an increase in IL-10 levels after LPS. At higher doses (25 and 50 mg/kg), but not the lower dose (10 mg/kg), ticagrelor-treated mice had increased IL-10 at all time points compared with controls ($p < 0.05$ for all treatment doses and times, except ticagrelor dose 50 mg/kg at 4 h; $p = 0.06$) (Figure 6B). Clopidogrel had no significant effect on TNF- α , but IL-10 levels were elevated at 2 h and 8 h ($p < 0.05$ for both time points).

In comparison to WT controls, mice lacking the P2Y₁₂ receptor (*P2ry12*^{-/-}) were protected from death from endotoxemia ($p < 0.001$) (Figure 6C). Ticagrelor, but not clopidogrel, conferred protection in WT animals ($p < 0.05$) (Figure 6D) without an additive effect in the *P2ry12*^{-/-} background.

Endotoxemia causes an inflammatory reaction, associated with disruption in endothelial barrier function, enhanced permeability, and the development of tissue edema. In the lung, this can manifest as acute lung injury. To examine whether P2Y₁₂ antagonism affects pulmonary microvascular permeability, K_{fc} was measured in isolated perfused lung preparations. Lungs from mice pre-treated with ticagrelor at higher doses (25 and 50 mg/kg) displayed lower K_{fc} after LPS treatment ($p < 0.05$) (Figure 6E), suggesting that ticagrelor reduced LPS-induced pulmonary capillary permeability. Conversely, ticagrelor at a lower dose (10 mg/kg) and clopidogrel did not yield a statistically significant reduction in K_{fc} levels. Taken together, our findings in a preclinical model indicate that ticagrelor can attenuate inflammation and may protect against inflammation-associated acute lung permeability, which could have a benefit in patients with pneumonia.

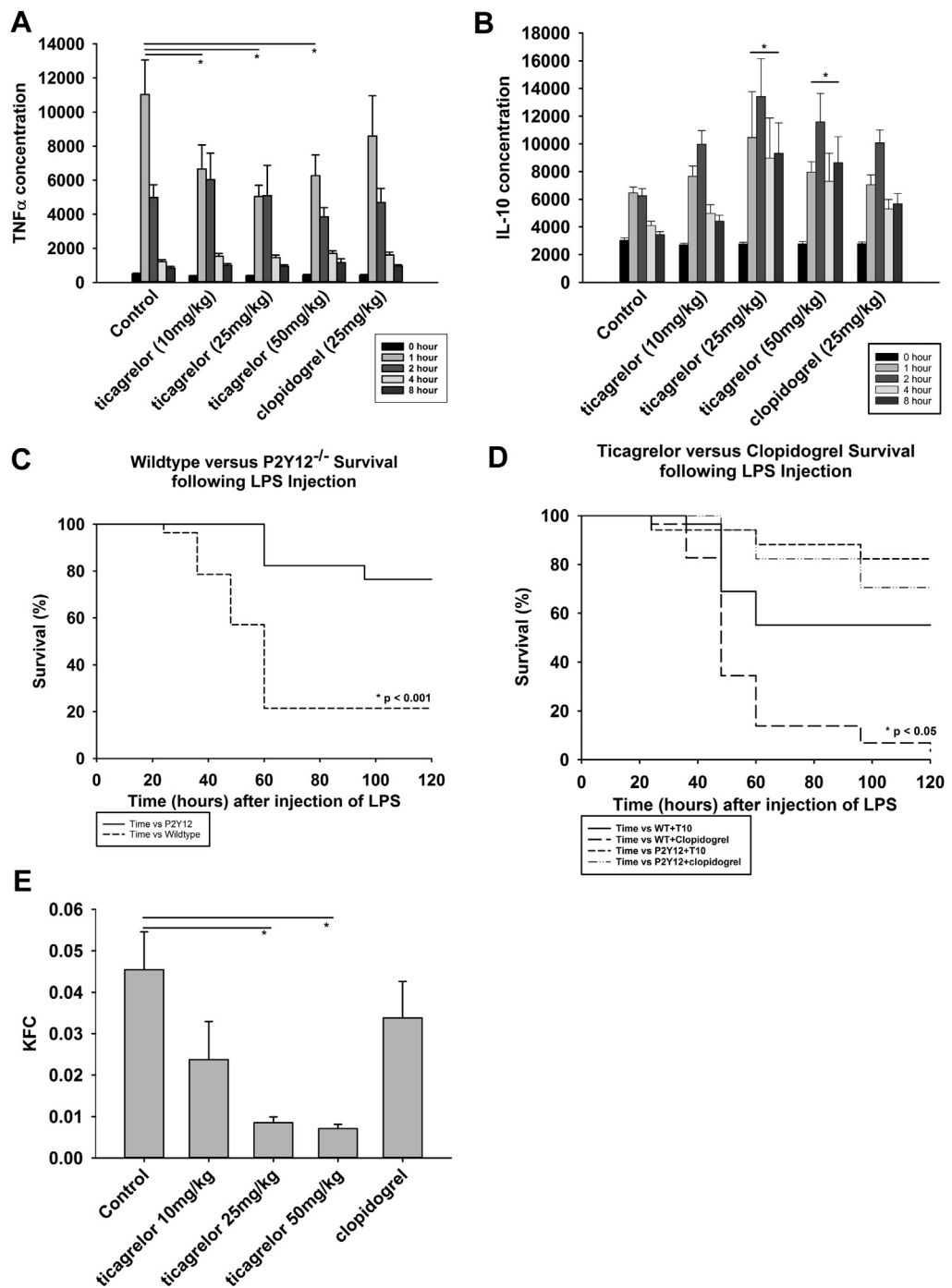


DISCUSSION

Pneumonia is one of the most common causes of death from infectious diseases worldwide. Left untreated, it can lead to serious complications such as acute lung injury and sepsis. Sepsis, a complex and poorly understood condition, carries a high mortality rate, and current treatments are generally supportive. Here, we identify the platelet P2Y₁₂ receptor as a potential target to prevent the complications of sepsis and for reducing lung injury, and suggest that the P2Y₁₂ antagonist ticagrelor may be of clinical benefit in the treatment of pneumonia. Taken together, these findings support a novel mechanism to prevent life-threatening complications of pneumonia and may explain the mortality benefit observed in patients randomized to ticagrelor in the PLATO trial (26).

Ticagrelor, but not clopidogrel, attenuated the inflammatory response to LPS in mice by blunting the increase in TNF- α and elevating IL-10 levels. The

elevation of IL-10 exclusively occurred at 25 and 50 mg/kg doses of ticagrelor and not at the lower 10 mg/kg dose indicating a threshold concentration of ticagrelor must be achieved in order to elicit an anti-inflammatory effect. Furthermore, there seems to be a ceiling to IL-10 increase because we did not observe higher levels in the 50 mg/kg dose compared with the 25 mg/kg dose. Moreover, animals pretreated with ticagrelor or lacking P2Y₁₂ receptors were more likely to survive endotoxemia. Inflammation and infections have been associated with platelet activation and platelet-leukocyte heterotypic aggregate formation. Ticagrelor administration to patients with pneumonia reduced platelet-leukocyte aggregates in circulation and lowered IL-6 levels, consistent with an anti-inflammatory effect. Given that platelet-leukocyte aggregates serve as a sensitive *in vivo* marker of platelet activation, and increase from a mean of approximately 19% to 34% in patients presenting with acute myocardial

FIGURE 6 Ticagrelor Reduces Inflammation, Increases Lung Function, and Protects Against Mortality in a Murine Sepsis Model

The concentrations of TNF- α (A) and IL-10 (B) at 0 h up to 8 h post-LPS injection are graphed for control mice and mice treated with ticagrelor or clopidogrel (A and B, reported as mean with bar indicating SE). Significant difference over the time course compared with the control is indicated by an asterisk in A and B. Survival curves of wild-type (WT) mice and P2y12^{-/-} mice injected with LPS are plotted in C, whereas D shows survival curves of WT and P2y12^{-/-} mice treated with clopidogrel or ticagrelor (T10) before LPS injection. Significance between mutant and WT (A) and P2Y₁₂ antagonist treated and untreated (D) is indicated by an asterisk. Capillary filtration coefficients (K_{FC}) are graphed for control mice and mice treated with ticagrelor or clopidogrel (E, reported as mean with bar indicating SE). Significant differences between treatment group and control are indicated by an asterisk. IL = interleukin; LPS = lipopolysaccharide; TNF = tumor necrosis factor.

infarction, the differences observed with ticagrelor likely reflect a meaningful effect of platelet activation in pneumonia. These anti-inflammatory effects of P2Y₁₂ antagonism are consistent with those observed in a rat model of LPS-induced sepsis in which pre-treatment with clopidogrel reduced IL-6 and TNF- α (27), as well as a mouse model of sepsis and acute lung injury in which clopidogrel-treated and P2Y₁₂-null mice were resistant to sepsis-induced lung injury (28). These anti-inflammatory effects of P2Y₁₂ are also consistent with retrospective clinical studies in which anti-platelet drugs had a favorable effect on patients with organ dysfunction (such as sepsis) and CAP (14,15,29). However, in all of those studies, there was no benefit of clopidogrel on mortality. Here, we demonstrate, not only an anti-inflammatory effect of ticagrelor apparently equal to or greater than that of clopidogrel, but also improvement on survival in LPS-treated mice. As a potent, direct-acting P2Y₁₂ antagonist, ticagrelor may be particularly well suited to reduce adverse events mediated by platelets in the setting of inflammation. In the setting of acute coronary syndrome, patients taking clopidogrel have high on-treatment platelet reactivity that can be overcome with ticagrelor (30,31). Although not yet reported, a similar phenomenon may occur in other inflammatory settings such as pneumonia and sepsis. Additionally, by inhibiting the adenosine transport ENT1, ticagrelor may have adenosine-mediated effects that could promote vasodilation, platelet inhibition, and modulate inflammation (32). Although work in our lab has traditionally focused on anti-inflammatory effects by reducing platelet-leukocyte interactions, there is some evidence from translational work that these platelet-leukocyte interactions can be beneficial. In a study by Tunjungputri et al. (33), pro- and anti-inflammatory effects of ticagrelor were observed in blood exposed to Pam3CSK4 (TLR2-mediated) and LPS (TLR4-mediated), respectively. Interestingly, in a study investigating the effectiveness of the P2Y₁₂ antagonist prasugrel in reducing coagulation and inflammation in a human LPS model, prasugrel had no effect on platelet-leukocyte aggregation (34). Thus, it is possible that ticagrelor has unique effects on platelet-leukocyte interactions.

During sepsis, endothelial dysfunction leads to systemic capillary leak and multiple-organ failure. The pulmonary microvessels are especially prone to

disruptions in integrity, and they may also be affected in pneumonia-type infections. Sequestration of platelets with leukocytes in pulmonary tissue is a key feature in inflammatory or infectious states (35). Activated platelets alone or neutrophil-platelet aggregates may occlude small pulmonary vessels to perpetuate local inflammation or line the endothelium to contribute to leakage of plasma and cellular components across a normally impermeable barrier into the alveolar and interstitial spaces of the lung. Through these and other mechanisms, platelet-leukocyte interactions can disrupt lung endothelial barrier function in sepsis and acute respiratory distress syndrome (11,36). Our results suggest that potent P2Y₁₂ antagonism with ticagrelor can interrupt key molecular signaling pathways responsible for lung permeability.

In this work, we have shown both murine and clinical measures that suggest improved lung function during sepsis and pneumonia following treatment with ticagrelor. Furthermore, we observed a baseline-dependent effect of ticagrelor on circulating platelet-leukocyte aggregates following ticagrelor treatment in pneumonia patients, although this observation could be due, in part, to regression to the mean. Patients with higher baseline platelet-leukocyte aggregates also had greater reductions in platelet-leukocyte aggregates following ticagrelor treatment. These reductions in platelet-leukocyte aggregates could possibly explain the improvements observed in lung function in both our animal and human models.

STUDY LIMITATIONS. The XANTHIPPE study was a prospective study designed to target pneumonia patients. Due to a combination of pneumonia patients having the capacity to consent as well as exclusion criteria, the recruited population was less sick than we had anticipated. We also acknowledge that cecal ligation and puncture model of sepsis may carry more clinical relevance than the endotoxin model that was employed; however, the inflammatory response to sepsis and the anti-inflammatory effects of ticagrelor are likely similar between the 2 models.

ADDRESS FOR CORRESPONDENCE: Dr. Susan S. Smyth, Gill Heart and Vascular Institute, 741 South Limestone, University of Kentucky, BBSRB B345, Lexington, Kentucky 40536. E-mail: ssmyt2@uky.edu.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Ticagrelor is commonly used in patients with acute coronary syndrome. This work indicates a potential use for ticagrelor to target inflammation and improve lung function in the setting of pneumonia. The findings suggest the possibility that ticagrelor could prevent complications of sepsis, although larger-scale clinical studies are required.

TRANSLATIONAL OUTLOOK: Over the last decade, it has become clear that platelets play vital roles that

extend well beyond hemostasis. In this work, the authors demonstrate that targeting the platelet P2Y₁₂ receptor reduces platelet-leukocyte interactions, alters inflammatory biomarkers, and can improve lung function in the context of pneumonia and in experimental models of sepsis. Preclinical models should provide additional mechanistic insight into the benefits of antiplatelet therapy beyond preventing the complications of atherosclerotic disease.

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KEY WORDS inflammation, leukocytes, platelets, pneumonia, sepsis

APPENDIX For supplemental figures, please see the online version of this paper.



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