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

1. Hooper JMW, Stuijver DJF, Orme SM, van Zaane B, Hess K, Gerdes VE, et al. Thyroid Dysfunction and Fibrin Network Structure: A Mechanism for Increased Thrombotic Risk in Hyperthyroid Individuals. J Clin Endocrinol Metab. 2012 May;97(5):1463–73.