
Suppl. Material and methods

Study design, subjects and study procedures.

This prospective randomized open-label, blinded endpoint (PROBE-design) study was performed according to the latest version of the declaration of Helsinki (2008), the Medical Research Involving Human Subjects Act (WMO) and Good Clinical Practice (GCP) and is registered at clinicaltrials.gov identifier NCT01978158.

After approval of the local ethics committee from the Radboud university medical centre, 45 males signed informed consent and were screened for eligibility. Forty healthy volunteers were included as they met all inclusion criteria and none of the exclusion criteria. Inclusion criteria were male sex, age 18-35 years and no known current medical or psychiatric diseases. Exclusion criteria were history, signs or symptoms of cardiovascular disease, chronic obstructive pulmonary disease or asthma, hemorrhagic diathesis or disorders associated with increased risk of bleeding, pathological bleeding, history of intracranial haemorrhage, history of dyspepsia, quantitative bleeding assessment tool (BAT) score > 31, immune deficiency, spontaneous vasovagal collapse, use of any medication, smoking, cardiac conduction abnormalities on electrocardiogram, hypertension (systolic blood pressure >160 mmHg or diastolic blood pressure >90 mmHg), hypotension (systolic blood pressure <100 mmHg or diastolic blood pressure < 50 mmHg), renal impairment (estimated glomerular filtration rate by Modification of Diet in Renal Disease formula < 60 ml/min), liver enzyme abnormalities (ALAT or ASAT > two times the upper limit of reference value), thrombocytopenia (thrombocytes < 150 x 10^9/L), anemia (hemoglobin < 8.0 mmol/L), febrile illness in the week before the endotoxin challenge, hypersensitivity to ticagrelor or any excipients and participation in another drug trial or donation for blood 3 months prior to, until 3 months after the planned endotoxin experiment.

Subjects were randomized to one of four study arms, i.e. ticagrelor and ASA (TA), clopidogrel and ASA (CA), placebo and ASA (PA) or placebo. All subjects received a loading dose of the allocated treatment on day one, and continued with maintenance doses during the seven days preceding the endotoxemia experimental day. Ticagrelor loading dose was 180mg, with a maintenance dose of 90 mg twice daily, clopidogrel loading dose was 300 mg, and with a maintenance dose of 90 mg daily, ASA loading dose was 320 mg, with a maintenance dose of 80 mg daily.
Subjects refrained from alcohol, nicotine and caffeine for 24 hours, and fasted overnight prior to the endotoxemia experiment. After admission to the intensive care research department, an arterial cannula (Angiocath; Becton Dickinson, USA) was placed in the radial artery and connected to a blood pressure monitoring system (Edward Lifesciences, Irvine, CA, USA) for blood pressure monitoring and blood withdrawal. A venous cannula provided access for intravenous hydration and endotoxin administration. Heart rate was monitored by a three-lead electrocardiogram and haemodynamic data was recorded from a Philips M50 monitor (Eindhoven, The Netherlands) with an interval of 30 seconds using an in-house developed system. Subjects were prehydrated with intravenous (i.v.) infusion of 1.5L glucose 2.5%/0.45% NaCl in one hour. Purified lipopolysaccharide (LPS, US Standard Escherichia Coli O:113 endotoxin) was obtained from the Pharmaceutical Development Section of the National Institutes of Health (Bethesda, MD, USA). The lyophilized powder was reconstituted in 5 mL saline 0.9% for injection and vortex mixed for 20 min after reconstitution. An i.v. bolus of LPS was administered at a dose of 1 ng/kg at time point 0 hours, continued by a continuous infusion at a dose of 1 ng/kg/h for 3 hours. Hydration was continued with a rate of 150 ml/h for 6 hours, and 75 ml/h for the rest of the experiment to ensure optimal hydration. Blood was drawn at several time points throughout the experiment. All samples were processed within 3 hours after collection. Temperature was measured using a tympanic thermometer (FirstTemp Genius 2; Covidien, Dublin, Ireland). Endotoxin induced flu-like symptoms (headache, nausea, shivering, muscle and back pain) were scored on a six-point scale (0 = no symptoms, 5 = worst ever experienced), resulting in a total symptom score of 0–25.

**Cytokine measurement**

Ethylendiaminetetraacetic-(EDTA) anticoagulated blood was immediately centrifuged after withdrawal at 2000 g, 4°C for 10 minutes. The obtained plasma was stored at -80°C until further analysis. Plasma concentration of the cytokines Tumor Necrosis Factor (TNF)α, Interleukin (IL)-6, IL-8, IL-10 and IL-1 receptor antagonist (IL-1RA) and the chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β and monocyte chemoattractant protein-1 (MCP-1), also known as C-C motif chemokine ligand 2 (CCL2), were determined batchwise in anticoagulated plasma by a multiplex immunoassay assay according to the manufacturer’s instructions (Bio-Plex, BioRad, Hercules, USA and Milliplex, Millipore, Billerica, USA).
**Plasma adenosine**

Plasma adenosine levels were determined using an in-house developed method as described previously. Blood was drawn with a special syringe system that immediately mixed the blood with a solution that consisted of pharmacological blockers of adenosine formation, transport and degradation at the tip of the syringe in a 1:1 ratio. Haematocrit values in the blocking solution-blood mixture and in whole blood were determined to correct for dilution. The mixture was centrifuged at 2000 g, 4°C for 10 minutes and stored at -80°C until further analysis.

The blocker solution consisted of 40 μM dipyridamole (adenosine transport inhibitor), 10 μM erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) (adenosine deaminase inhibitor), 10 μM iodosotubericidine (ITU) (adenosine kinase inhibitor), 13.2 mM Na2EDTA (disodium ethylenediamine tetraacetate) (inhibits release from platelets and acts as a 5’-nucleotidase inhibitor), 118 mM NaCl, and 5 mM KCl. Plasma adenosine concentrations were determined by high performance liquid chromatography (HPLC). In brief, 36 volumes of plasma were mixed with 1 volume of perchloric acid (70%) and 20 volumes of 0.5M trioctylamine in chloroform, followed by centrifugation (3 min, 13400rpm, RT). Four volumes of supernatant were mixed with 1 volume of chloroacetaldehyde (6x diluted in 1M acetate buffer, pH 4.5) followed by derivatization (60 min, 500rpm, 70 °C). Chloroform was added (3.3 volumes), the mixture was centrifuged (3 min, 13400 rpm, RT), the supernatant was transferred to a HPLC vial and injected. Adenosine was separated by HPLC system (Thermo Scientific, Rockford, USA) using a Polaris C18-A column (150 x 4.6 mm) with gradient elution using eluent A (50 mM NH₄H₂PO₄, 5 mM sodium 1-hexanesulfonate monohydrate [pH 3.0], and 2% MeOH) and eluent B (H₂O: ACN: THF; 50:49:1). The retention time was approximately 10 min. Quantification was based on peak areas of the samples and reference standards measured with fluorescence (excitation: 280 nm; emission: 420 nm).

**Assessment of P2Y12 inhibition**

Specific effectiveness of P2Y₁₂ inhibition was determined by the level of phosphorylation of vasodilator-stimulated phosphoprotein (VASP) in citrated blood (3.2% sodium citrate, Vacutainer, Becton Dickinson) whole blood using an ELISA-based assay (CY-QUANT VASP/P2Y12, BioCytex, Marseille, France) according to manufacturer’s instructions. This test monitors the responsiveness to P2Y₁₂ inhibitors, and P2Y₁₂ inhibition is expressed as platelet reactivity index (PRI%), a value < 50% is considered as adequate platelet inhibition.
**Platelet activation and responsiveness**

Platelet activation and responsiveness was assessed in citrated (3.2% sodium citrate, Vacutainer, Becton Dickinson) whole blood using flow cytometry within 3 hours. Whole blood (5µL) was added to a mix of hepes-buffered saline, saturating concentrations of FITC-labeled anti-fibrinogen (DAKO, Heverlee Belgium), PE-labeled anti-CD62p (anti-P-selectin; BD Biosciences, San Jose, CA, USA) and PC7-labeled anti-CD61 (anti-glycoprotein IIIa; Beckman Coulter, Brea, CA, USA). Platelet reactivity was assessed by *ex vivo* stimulation with either platelet agonist adenosine diphosphate (ADP; 3.4 and 125 µM; Sigma-Aldrich, Zwijndrecht, The Netherlands) or cross-linked collagen-related-peptide (CRP-XL; 39ng/L or 625ng/L). CRP-XL was a generous gift from Prof. dr. R. Farndale (Cambridge, UK) and was prepared as described previously. After incubation for 20 minutes at room temperature a 500µL fixative solution (0.4% formaldehyde) was added. Samples were directly measured on a FC500 flow cytometer (Beckman Coulter) and data were analyzed using Kaluza® software (Beckman Coulter). Platelets were gated on forward-scatter (FS), side-scatter (SS) and CD61 positivity. Subsequently expression of CD62p, a measure of degranulation, and fibrinogen after ADP stimulation were expressed as median fluorescence intensity (MFI).

**Leukocyte and platelet counts**

Analyses of leukocyte counts, differentiation and thrombocyte counts were performed in EDTA anticoagulated blood using routine analysis methods also used for patient samples (flow cytometric analysis on a Sysmex XE-5000).

**Statistical analysis**

Data are expressed as a mean ± SEM or as median [interquartile range], according to their distribution. Data were tested for normality using the Shapiro-Wilk test. To evaluate the effect of platelet inhibitors on cytokine responses, PRI and activity and responsiveness, repeated measures two-way ANOVAs (interaction term of group and time) were performed between specific pairs of groups: 1. To evaluate the effects of ASA, placebo-ASA was compared to placebo 2. To evaluate the effect of clopidogrel or ticagrelor added to ASA, placebo-ASA was compared to clopidogrel-ASA and ticagrelor-ASA. 3. In case of an effect of ticagrelor or clopidogrel added to ASA, a comparison of clopidogrel-ASA with ticagrelor-ASA was made to assess for differences between these P2Y_{12} inhibitors. Baseline values of prostaglandins, plasma adenosine and VASP-P platelet reactivity index were compared with Students t-test or Mann-Whitney test. Differences in changes over time were analyzed with Friedman test with post-hoc Dunn’s Multiple Comparison Test (thromboxane B and...
VASP-P) or Wilcoxon signed rank test (adenosine). Haemodynamic parameters, temperature, symptom scores, platelet and leukocyte counts were analyzed for differences between all four groups using a repeated measures two-way ANOVA (interaction term of group and time). Correlation between platelet responsiveness and plasma cytokine levels were calculated using Pearson’s correlation. All statistical analyses were performed using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA, USA). A two-sided p value <0.05 was considered statistically significant.

**Suppl. Figure 1.**

Gating strategy for platelet-monocyte and platelet-neutrophil complex formation in a representative sample. Monocytes (blue) were selected based on size (forward-scatter; FS), granularity (side-scatter; SS) and expression of CD16, CD14 and HLA-DR as shown in the upper scattergrams. Neutrophils were gated based on size (FS), granularity (SS) CD14, CD16 and HLA-DR as shown in the lower scattergrams. The markers were chosen to distinguish neutrophils and monocytes during experimental human endotoxemia. Platelet interaction was assessed using the % of CD61-positive events shown in the right overlay graph. An example of the different populations of monocytes (Blue) and neutrophils (green) are shown left in the scattergram. Kaluza Analysis 1.5 (Beckman Coulter, Brea, CA, USA) was used for analysis.
Suppl. Figure 2.

CONSORT Flow chart of study inclusion and follow up.
Suppl. Figure 3.

Correlations between tumor necrosis factor (TNF)α (panel A and B) and interleukin-6 (panel C and D) and platelet reactivity expressed as P-selectin expression upon stimulation with high doses of adenosine diphosphate (ADP) (Panel A and C) or collagen related peptide (CRP-XL) at time of endotoxin administration. Cytokine production is expressed as the area under the concentration-time curve (ng/ml*h). Pearson’s correlation coefficients were not significant. ASA: acetylsalicylic acid.
Correlations between tumor necrosis factor (TNF)α (panel A and B) and interleukin-6 (panel C and D) and platelet monocyte complex (PMC) formation and platelet neutrophil complex (PNC) formation at 4h after start of endotoxin administration. Cytokine production is expressed as the area under the concentration-time curve (ng/ml*h). Pearson’s correlation coefficients were not significant. ASA: acetylsalicylic acid.
Suppl. Figure 5.

(A) Mean arterial pressure (MAP), (B) heart rate, (C) temperature and (D) symptom score during endotoxin-elicited systemic inflammation. Endotoxin administration started at t=0 hours and continued until t=3 hours. Data are expressed as mean and SEM. MAP decreased over time, with a concurrent increase in heart rate and temperature (1-way ANOVA in P group, p<0.0001 for all parameters). There was a small difference in MAP between groups (2-way ANOVA p=0.04), although post-hoc analysis did not point out what the discrepant group or time point was. There was no effect of study treatment on heart rate, temperature and symptom score. P: placebo, PA: placebo and acetylsalicylic acid, TA: ticagrelor and acetylsalicylic acid, CA: clopidogrel and acetylsalicylic acid.
Suppl. Figure 6. 

(A) platelet-, (B) neutrophil-, (C) monocyte-, and (D) lymphocyte counts during endotoxin-elicited systemic inflammation. Endotoxin administration started at t=0 hours and continued until t=3 hours. Data are expressed in box plots with minimal and maximal observed values. Platelets, monocytes and lymphocytes decreased temporarily due to endotoxemia (1-way ANOVA in P group, p<0.0001 for all parameters), without any effect of study treatment on cell counts. P: placebo, PA: placebo and acetylsalicylic acid, TA: ticagrelor and acetylsalicylic acid, CA: clopidogrel and acetylsalicylic acid.
<table>
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<th>Condition</th>
<th>Patient group</th>
<th>Study type</th>
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<th>Effect</th>
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<td>Sepsis and systemic inflammatory syndrome</td>
<td>(1) ICU patients with severe sepsis or septic shock</td>
<td>Retrospective cohort study</td>
<td>979</td>
<td>ASA vs no ASA</td>
<td>Logistic regression</td>
<td>In-hospital mortality</td>
<td>adjusted odds ratio 0.57 (0.39-0.83)</td>
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<td>(2) Surgical ICU patients with sepsis</td>
<td>Retrospective cohort study</td>
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<td>ASA vs no ASA</td>
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<td>In-hospital mortality</td>
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<td>(3) ICU patients with first time sepsis or SIRS</td>
<td>Retrospective cohort study</td>
<td>5523</td>
<td>ASA vs no ASA</td>
<td>Logistic regression (total cohort)</td>
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<td>Logistic regression: absolute risk reduction of 6.2% (3.5-9.5%)</td>
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<td>Logistic regression and case-control design (n=372748)</td>
<td>Mortality</td>
<td>Logistic regression: adjusted odds ratio 0.82 (0.81-0.83)</td>
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<td>(5) Patients with pneumonia and septic shock</td>
<td>Prospective observational study</td>
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<td>Hazard ratio 0.25 (0.08-0.82)</td>
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<td>(6) ICU patients with sepsis</td>
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<td>972</td>
<td>Antiplatelet therapy vs no antiplatelet therapy†</td>
<td>Propensity analysis (n=300)</td>
<td>30-day mortality</td>
<td>Hazard ratio 1.21 (0.79-1.84)</td>
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<td>Pneumonia</td>
<td>(7) Patients with community acquired pneumonia</td>
<td>Prospective observational study</td>
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<td>ASA vs no ASA</td>
<td>Cox regression and propensity analysis</td>
<td>30-day mortality</td>
<td>Regression: Hazard ratio 0.43 (0.25-0.75)</td>
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<td>(8) Patients with community acquired pneumonia</td>
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<td>224</td>
<td>Anti-platelet drugs vs no anti-platelet drugs‡</td>
<td>Logistic regression</td>
<td>Need for ICU admission</td>
<td>Adjusted odds ratio cohort: 0.32 (0.1-1.0) age matched: 0.19 (0.04-0.87)</td>
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Aspirin, clopidogrel and ticlopidine, Acetylsalicylic acid, clopidogrel, dipyridamole.
Suppl. References


