Supplementary material to Mani et al. “Ex vivo effects of low-dose rivaroxaban on specific coagulation assays and coagulation factor activities in patients under real life conditions” (Thromb Haemost 2013; 109.1)

METHODS

HPLC-MS/MS
The 160 plasma samples of the 40 patients were sent to Bayer Pharma AG after pseudonymisation. The rivaroxaban concentrations of the plasma samples were validated by HPLC–MS/MS at Bayer Pharma AG, Wuppertal, Germany, according to Rohde et al. [12]. Precipitation of the plasma proteins with methanol containing the internal standard was followed by centrifugation; the plasma supernatants were injected directly onto the HPLC–MS/MS system. Exact concentrations could be measured between 0.5 and 500 ng/ml. The lower limit of quantification (LLOQ) of the method was defined as the lowest concentration of rivaroxaban that could be quantitatively determined with acceptable precision and accuracy. The LLOQ was assessed at 0.5 ng/ml.

Clotting factor activities
The exogenous activities of factors II, V, VII and X were determined in a modified prothrombin time (PT)-test in combination with their respective coagulation factor-deficient plasma. The Innovin® PT-test was used with deficient plasma, Standard Human Plasma, Control Plasma N and Control Plasma P from Siemens Healthcare Diagnostics, Marburg, Germany.

The endogenous factor activities of factors VIII, IX, XI and XII were determined by means of a modified activated partial thromboplastin time (aPTT)-test in combination with their respective coagulation factor deficient plasma. The SynthASil® aPTT-test was used with HemosIL®-deficient plasma and a factor diluent from Instrumentation Laboratory, Lexington, MA, USA.

The Berichrom® Factor XIII assay is manufactured for the chromogenic determination of Factor XIII activity by Siemens Healthcare Diagnostics, Marburg, Germany. In this
test system factor XIII of the plasma sample is converted by the action of thrombin into factor XIIIa. Factor XIIIa cross-links with a specific peptide substrate to glycine ethyl ester and releases ammonia, which is measured in a parallel enzymatic reaction.

Protein S and protein C activities
Functional protein S activity was measured using the clotting assay STA Protein S from Diagnostica Stago, Asnières sur Seine, France. Protein S, the cofactor of activated protein C, enhances the anticoagulant effect and can be measured by the prolongation of the clotting time in samples that are enriched with factor Va. The free protein S antigen concentration was determined using the HemosIL® Free Protein S reagent (Instrumentation Laboratory, Lexington, MA, USA), in which diluted plasma was incubated with latex polystyrene particles adsorbed with a monoclonal antibody against human protein S.

Protein C activity was determined using the chromogenic assay HemosIL® Protein C (Instrumentation Laboratory), in which diluted plasma is incubated with Protein C activator solution. The change in optical density at 405 nm is recorded after adding a chromogenic substrate that is specific for activated protein C.

D-dimer
The STA LIATEST D-DI (Diagnostica Stago, Asnières sur Seine, France) was developed for the quantitative determination of D-dimer, a plasmin-resistant antigenic determinant produced during degradation of a fibrin clot by plasmin. The immuno-turbidimetric test system contains a bovine albumin-stabilised suspension of latex particles coated with D-dimer-specific monoclonal mouse antibodies.

Heparin-platelet factor 4 antibodies
The diagnosis of heparin-induced thrombocytopenia (HIT) is based on clinical criteria using a scoring system and is confirmed by in vitro demonstration of heparin-platelet factor 4 (HPF4) antibodies using functional immunoassays. In this study, HPF4 antibodies were detected using an enzyme-linked immunosorbent assay (ELISA) Asserachrom® HPIA from Diagnostica Stago, Asnières sur Seine, France. This assay utilises plastic microwells precoated with HPF4 complexes, which capture anti-HPF4 antibodies from patient plasma. Added peroxidase-labelled anti-human IgG, IgA, and
IgM antibodies bind to available antigenic determinants of the immobilised antibodies. A coloured reaction is formed and measured with a photometer.

Anti-cardiolipin antibodies
Detection of antibodies against cardiolipin as an important criterion for the diagnosis of antiphospholipid syndrome (APS) and determination of IgG or IgM isotypes were performed using ELISA kits (Orgentec-Diagnostika, Mainz, Germany). Microplates were coated with highly purified cardiolipin and saturated with purified human beta-2-glycoprotein I as a co-factor.

von Willebrand factor
Ristocetin-cofactor activity was evaluated using the BC vWF reagent (Siemens Healthcare Diagnostics, Marburg, Germany) containing stabilised platelets, ristocetin and EDTA in lyophilised form. In the presence of ristocetin, the vWF in the plasma sample causes agglutination of stabilised platelets. This agglutination process reduces the turbidity, which is detected by the coagulation analyser as a change in optical density.
The vWF antigen concentration was determined using the vWF Ag* test kit (Siemens Healthcare Diagnostics, Marburg, Germany). Samples containing vWF antigen are aggregated with small polystyrene particles to which specific polyclonal antibodies against vWF have been attached. The aggregation is detected by the change in optical density.