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Assessing the Thrombogenic Potential of Heart Valve Prostheses: An Approach for a Standardized *In-Vitro* Method

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Abstract
Purpose—Thrombogenic complications are still a main issue in the general performance ofcardiovascular implants, especially heart valves. To date, the thrombogenic potential ofthose prostheses is pre-clinically assessed in time consuming animal studies withquestionable evidence.
Methods—In this study, we present a new in-vitro method to assess andcompare deficiencies of heart valve substitutes concerning their thrombogenicperformance and locate initial clot formation under physiological conditions usingporcine blood. Therefore, an athrombogenic pulse duplicator (THIA3) was developed that simulatesthe anatomic and hemodynamic conditions in the vicinity of the aortic valve. Validationof this tester was carried out with regard to hemodynamics, reproducibility and usingpositive and negative control valves and by comparison of clot locations with literaturedata from chronic animal trials with sheep using a St. Jude bileaflet valve.
Results—Validation of the tester showed quasi-physiological hemodynamics and reproducibleclot formation. Identical clot formations were found comparing findings in vitro with chronic animal trials.
Conclusion—The THIA 3 has proven its suitability for valid, reproducible evaluation of thrombogenicpotential of heart valves in a short period of time.

Keywords—Heart valve prostheses, Thrombosis, *In vitro* testing, Tester validation.

INTRODUCTION

Thrombogenic performance is a key issue for all implants in blood contact,[32] especially for mechanical heart valves.[11] Thromboembolic complications remain at levels of 2–4% in a patient year and together with anticoagulation related bleedings stand for 75% of all complications with heart valve prostheses, even under anticoagulation therapy.[10] According to Virchow's Triad thrombosis is influenced by surface (material and structure), flow regimen and the blood itself.[36,37] Despite deficiencies of current prostheses, there is still no validated, reproducible *in-vitro* method available to quantitatively assess the thrombogenic performance of heart valves under realistic hemodynamic conditions including the blood.

Due to the lack of standardized *in-vitro* test methods, general performance of newly developed prostheses is typically tested in time consuming *in-vivo* trials using large animals such as sheep. But their evidence especially with regard to thrombogenicity remains questionable.[9,28] Main difference towards the application in humans are the general hemodynamic conditions that have massive influence on the thrombogenic performance of the device. As a result, specific conclusions on the correlation of design and thrombosis formation are almost impossible.

In the past, several *in-vitro* test setups have been developed following different principles. Keggen and Martin established a milk clotting test, using enzyme activated milk, not blood.[20,29] One first *in-vitro* test setup using blood was the THIA (Thrombosis Tester Helmholtz Institute)[21] that had some principle deficiencies simulating physiologic hemodynamic condi-

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tions and had two valves in the test circuit interacting with each other.

The THIA2 overcomes these limitations and was specially developed for the use of human blood to compare different anticoagulants. Restrictions were mainly a limited operating range concerning Cardiac Output and pressure levels and an asymmetric flow field towards the valve, that were evaluated using computational fluid dynamics (CFD).[18,26,27]

Arjunon *et al*. developed another low volume heart valve tester specially designed for testing the activation of the coagulation cascade using 150 mL of fresh human blood. Main limitation of this tester is that a stiff titanium tube was used to mimic the aorta. This neglects the effect and influence of the flexible structures of the native aortic root.[1]

Bluestein *et al*. published successful studies on evaluating thrombogenic potential of heart valve prostheses and other devices. They focus on shear profiles of specific particle tracks out of CFD simulations and evaluate the activation of thrombocytes using their own developed dynamic couette-apparatus. Using this knowledge, locations inside the valve prone to thrombosis are forecasted.[7] Such results for different valve types were also compared with platelet activation states (PAS) measured in a blood loop using a pulsatile left ventricular assist device (LVAD).[6,8] This *in-vitro* approach misses the anatomic situation of the aortic root as well.

The aim of the current study was to develop and evaluate an *in-vitro* method (THIA3) that can deliver reproducible results to evaluate local clot formation in heart valve prostheses in a short period of time, with the ability to gain detailed insight into the flow field characteristics that might have caused clot formation during the test. These flow field characteristics can be evaluated in Computational Fluid Dynamic (CFD) studies with controlled boundary conditions (pressure, flow and valve motion) gained from the tester. Such methods enable researchers to gain better understanding in the field of flow-induced thrombus formation under complex flow conditions, and enable developers to evaluate and redesign valves for better performance.

MATERIALS AND METHODS

Tester Setup

The THIA3 (Thrombosis tester of the Helmholtz Institute Aachen, version 3) was developed for comparison studies of at least two heart valves under the same hemodynamic conditions with the same blood for evaluating initial clot formation on heart valve surfaces. Pooling of blood from different species results in activation of the coagulation cascade. Therefore, the tester was designed to run with blood from only one blood batch of one individual limited to approximately 1 L that can safely been taken. Maximum volume for a single tester is limited to 450 mL. To enable a stroke volume of up to 100 mL without generating thrombogenic dead water areas and fulfill ISO 5840-1:2015[17] requirements concerning the distance between valve and downstream pressure measurement less volume was not feasible. Hydraulic design of the tester implements the ability to adjust various physiological and pathological hemodynamic conditions for aortic and pulmonary position.[24] The general test set up is shown in Fig. 1.

The tester consists of two chambers with single-use silicone inlays representing the ventricle and the aortic root with the tested heart valve in between. Both chambers are connected *via* a bypass parallel to the valve, to channel the fluid back from the aorta to the ventricle during diastole. A second valve inside the bypass was avoided to prevent any interference between this valve and the device under test.

The single-use inlays of the chambers are made of biocompatible silicone (Elastosil RTV 620, Wacker Chemie AG, Munich, Germany) and represent the blood contacting surface of the tester. They include the membranes at the ventricular and the aortic side that separate the blood from the driving circuit. To guarantee reasonable valve dynamics, physiologic flow field propagation around the valve is necessary.[4,5] Therefore, the aortic root geometry is generated with Bronstein-Semendjajev Epitrochoids for a tissue annulus diameter (TAD) of 25 mm, as proposed by Reul *et al*.[33] Linde *et al*. already showed the necessity for a physiologically compliant aortic root in this test setup by evaluating leaflet dynamics and hemolysis for different mechanical heart valves.[25] A compliance chamber around the silicone aortic root guarantees adjustable, physiologic compliance movement of this region. The geometry of the ventricular roof was designed using assumptions of von Wieting *et al*.,[38] while ventricle inlet and volume were optimized towards symmetricity using computational fluid dynamics (CFX, ANSYS Inc.) with a one-way coupled fluid-structure interaction (FSI) simulation including movement of the membranes. Simulation results were validated using the particle image velocimetry version of the tester.[24]

The whole tester is encapsulated by a climate chamber temperature-controlled to 37 °C.

The drive unit is connected *via* a compliance chamber behind the ventricular membrane. The hydraulic drive in combination with the compliance guarantees adjustable physiologic pressure gradients and pressure curves. Another compliance connected to

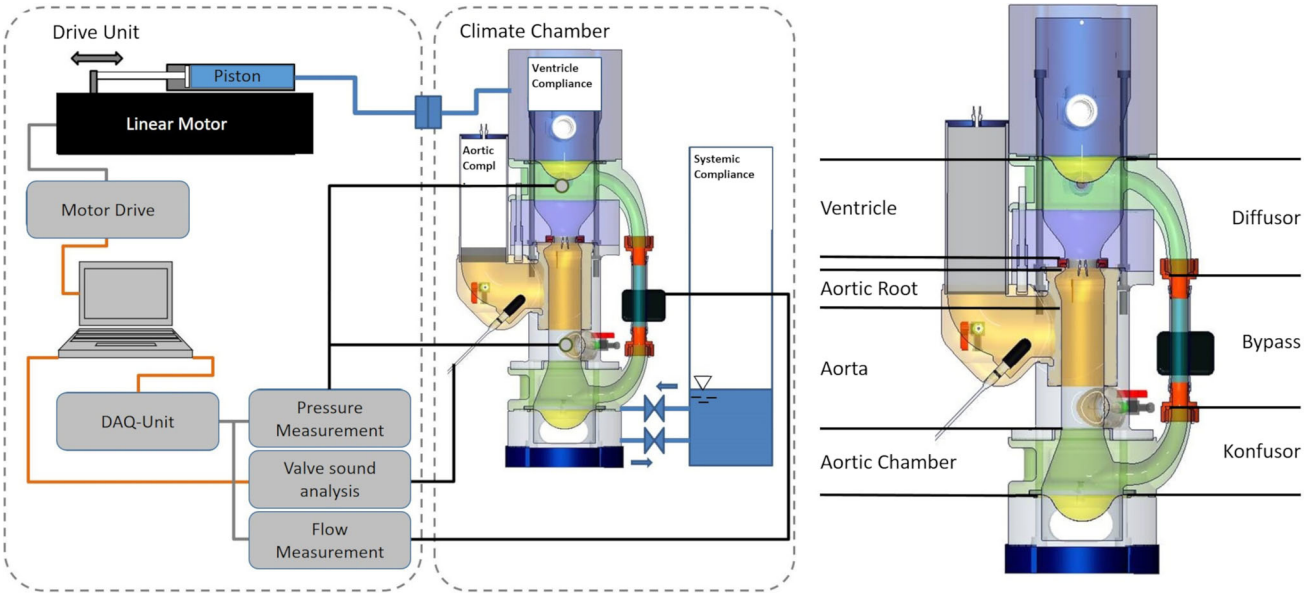

FIGURE 1. General tester setup of the THIA3 (left) and the test chamber (right) (based on Ref. 24).

the aortic membrane *via* a two-way resistance influencing systolic and diastolic pressures separately simulates the peripheral circulatory system of the body. To guarantee athrombogenicity of the test chambers, good washout without any stagnation area is necessary. Sample ports and pressure measurement ports are generally prone to thrombosis development. Pressure is measured across the silicone inlay and blood samples are taken *via* a modified plug that can be penetrated by a needle. Washout time for both chambers including all plugs *etc*. have been evaluated using the same CFD approach as described above using a two-phase flow model similar to Sonntag *et al.*[35] Washout time of lower than 10 s can be guaranteed. Linde already published these results elsewhere.[24]

Acquisition of Hydrodynamic Conditions

Ventricular and aortic pressure were measured using pressure sensors (DPT6000, CODAN pvb Medical GmbH, Lensahn, Germany). Flow through the valve was calculated by the sum of piston flow from the drive unit, volume compression inside the ventricular compliance and the flow through the bypass (SonoTT Clamp-On Transducer; Em-Tec GmbH, Finningen, Germany).

$$\dot{V}_{\text{valve}} = \dot{V}_{\text{Drive}} - \dot{V}_{\text{V,Compliance}} - \dot{V}_{\text{Bypass}}$$

To avoid misinterpretation of results it must be guaranteed that no thrombotic material embolizes inside the valve. Therefore, acoustic monitoring of valve opening and closing sound *via* a hydrophone implemented into the aortic root compliance chamber was established. Embolization of any sort is detected *via* changes in the opening or closing spectrum, respectively, and canceling of the test is possible.

Testing Procedure

The preliminary conditions of the blood including its anticoagulation are an important factor for testing success. Grabowski *et al.* showed that porcine as well as sheep thrombocytes have similar adhesion potential compared with human ones.[14] The intrinsic coagulation system is faster, and the extrinsic system is similar or slightly slower compared with human coagulation.[12,15] Porcine blood also complies with the ISO 10993-4:2017 as an alternative to human blood.[16] In this study, porcine blood from the cervical artery of slaughterhouse animals was used and immediately anticoagulated with low dose of low molecular weight heparin (LMWH; 1.3 IU/mL CLEXANE® multidose 100 mg/mL; sanofi aventis; Germany). LMWH has less influence on activation of thrombocytes compared with high molecular weight heparin, what can lead to embolizing thrombi.[34] Adding Protamine also has negative influence on the testing result, because it can reduce clot firmness[31] that also leads to embolization of thrombi, and it reduces shear and thrombin induced adhesion.[2,19,23] The air contact of the blood was kept to a minimum, to eliminate activation on this way.

Blood quality was evaluated before the beginning of the test using platelet count (Plt) and hematocrit (Hct) using a cell counter (CellTac; Nihon Kohden; Japan). Coagulation parameters were evaluated using activated clotting time (ACT/ Hemochrom Jr., ACT-LR,

Keller Medical GmbH; Germany), clotting time (CT), maximum clot firmness (MCF) and the clotting velocity (alpha) using full blood Thromboelastometry (TEM; RoTEG Pentapharm) of the extrinsic system (ExTEM). Base access was measured using a blood gas analyzer (ABL825 FLEX; Radiometer GmbH, Germany). Table 1 shows the used quality parameters with the starting and deviation values accepted for the test.

Hematocrit and platelet count are particularly important for flow-induced deposition of platelets. Reproducible platelet deposition is just possible in the presence of red blood cells and platelet concentrations above 5.000 Plt/μL.[3] To guarantee no particles inside the test fluid like micro thrombi or other tissue, blood was filtered during tester filling using a transfusion filter with a mesh width of 200 μm.

Valves were tested under physiological aortic conditions with 120/80 mmHg and a beat rate of 70 bpm. Blood samples were taken hourly and compared with the samples taken from a blood bag stored inside the climate chamber at the beginning and the end of the test. For blood sample analyses, plasmatic coagulation parameters (INR, aPTT, Fibrinogen using MC10, Merlin Medical, Germany) and RoTEM parameters (CT, MCF, alpha using RoTEM, Pentapharm) were measured using two different methods: ExTEM and HepNaTEM. ExTEM is a standard procedure evaluating the extrinsic coagulation system. HepNaTEM is a custom custom made analysis using Heparinase for anticoagulation elimination without any further activator. This allows for analysis of the consumption of coagulation factors on the one hand (ExTEM) and the activation of coagulation on the other hand (HepNaTEM). After the experiment was stopped, tester surfaces were evaluated and blood was filtered again to detect potential embolized thrombi. Mechanical valves in this study were cleaned after every test using Descocleaner (Dr, Schumacher GmbH, Malsfeld, Germany) and isopropanol in an ultrasonic bath (Emmi-20HC, EMAG AG, Germany) and manual procedures using cotton patches. Cleaning procedure was evaluated under a light microscope (VHX-600, Keyence Inc., Japan).

TABLE 1. Blood quality parameters for heart valve thrombosis testing.

Parameter	Start	Deviation
Platelet count	270 × $10^3/\mu$L	± 50 × $10^3/\mu$L
Hematocrit	37%	± 4%
ACT	210 s	± 20 s
CT (Extem)	67 s	± 8 s
MCF (Extem)	50 mm	± 10 mm
cBase (B, ox)	− 5 mmol/l	± 5 mmol/

Validation of the test setup was carried out through the following three stages:

(1) The hydrodynamic validation was carried out by comparing the THIA3 to a validated reference pulse duplicator using water-glycerol solution.
(2) A positive and a negative reference were tested (n = 6) for 3 h using the protocol described above. As negative reference, a St. Jude Medical (SJM) bileaflet valve was chosen (TAD = 25 mm). As positive reference, a polycarbonate urethane (PCU) valve was custom-designed with several potentially thrombogenic features such as high surface roughness and stagnation areas implemented (Fig. 2).
(3) Clot findings from the St. Jude Medical bileaflet valve tested with the proposed protocol were compared with findings from animal trials. Valves were implanted in the pulmonary position of sheep without anticoagulation from second week postoperatively until valve dysfunction was detected on fluoroscopy by Meuris *et al.*[30]

RESULTS

Validation Step 1: Pulse Duplicator Comparison

Hemodynamics of the THIA3 were compared to a reference pulse duplicator[22] that complies with hydrodynamic requirements of the ISO 5840:2009. Figure 3 shows raw pressure and flow measurement data with a time resolution of 1 kHz.

The main difference between both testers is the ventricular pressure curve during diastole. The reference curve drops to zero immediately after valve closure. The THIA3 drops slower with an almost linear decrease to zero shortly before systole. In general, waveforms in the THIA3 show more disturbances due

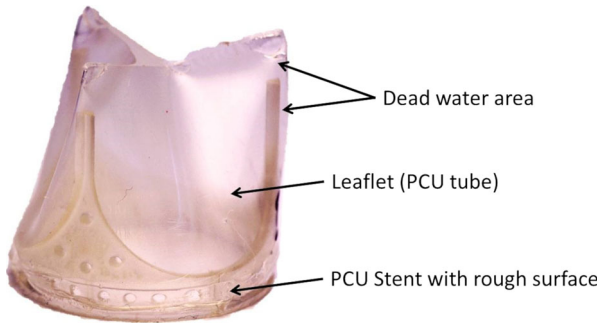

FIGURE 2. Custom designed PCU valve with thrombogenic features (based on Ref. 24).

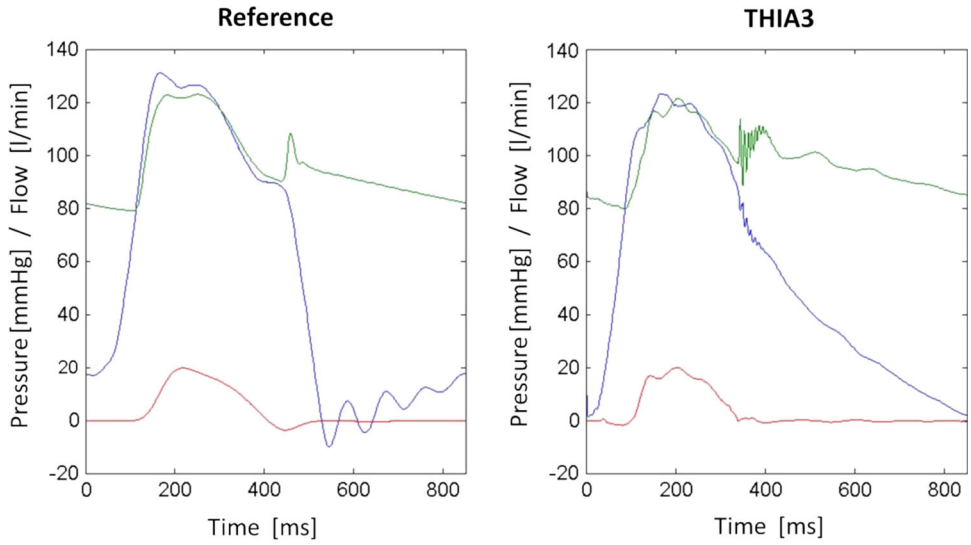

FIGURE 3. Comparison of pressure (ventricle: blue; aorta: green) and flow (red) wave forms of a reference pulse duplicator with the THIA3 for a cardiac output of 5 L/min [(based on Ref. 24).

to the direct incompressible measurement, visible in the valve closing signal. High frequency fluctuations in the THIA3 pressure signal are related to resonance effects from the aortic root compliance. The absence of rebounding and cavitation could be proven in another study.[33] Long-term tests over 24 h showed that no drift in pressure and flow were apparent.[24] Blood sampling of up to 50 mL had no influence on pressure distribution and level. Due to the lack of a second valve inside the bypass, a backflow of 1 L/min appears during systole under physiological aortic conditions. This results to a backflow volume of 5 mL.

Flow field in front and behind the valve has been evaluated using particle image velocimetry under physiologic conditions (120/80 mmHg; 5 L/min). Figure 4 shows the velocity distribution in the center plane between the SJM's leaflets rotational axes at peak systole.

It shows a symmetric flow distribution in front of the valve. Downstream of the valve characteristic vortices building up inside the bulbs of the aortic root that were described as enhancing valve closure.[13] Maximum flow velocity was 0.89 m/s.

Validation Step 2: In-Vitro Blood Test

The second validation step was carried out using the PCU and the SJM valve. The tester surfaces were observed to be completely clean from any thrombogenic adhesions, as also the surfaces of the SJM. The PCU valve showed major thrombus formation in the predicted locations (Fig. 5). Histological evaluations showed high concentrations of fibrin (red area) and platelets (bright networks) with captured erythrocytes (orange circles), here shown exemplarily for one of the

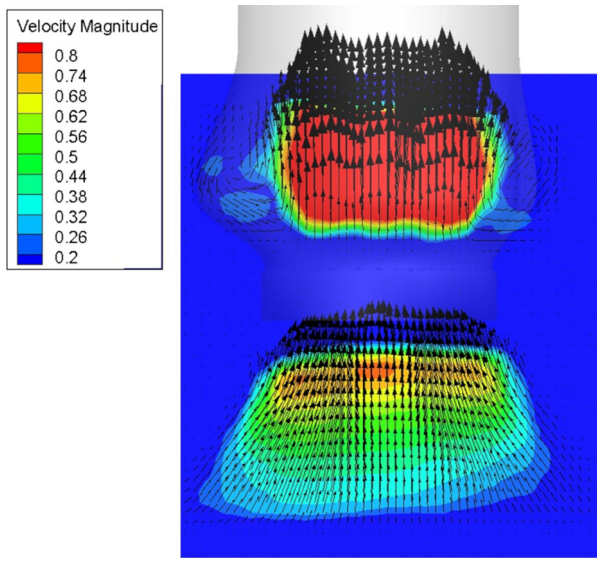

FIGURE 4. Velocity distribution in front and behind a SJM valve inside the THIA3.

found thrombus.This was also reflected by a strong decrease of remaining platelets ($\Delta PLT = -180 \times 103/\mu L$) in blood with the PCU valve, while concentrations did not change for the SJM ($\Delta PLT = 17 \times 103/\mu L$). Figure 6 shows the progress of platelet concentration with standard deviation over testing procedure for both valves.

Changes in RoTEM parameters showed similar results. ExTEM reflected consumption of coagulation parameters with increasing CT ($\Delta CT = 154$ s), and reduced MCF ($\Delta MCF = -47$ mm) and alpha ($\Delta\alpha = -54°$) for the PCU- valve and unchanged values for the SJM (Fig. 7). HepNaTEM parameters reflect activation of the coagulation system (including

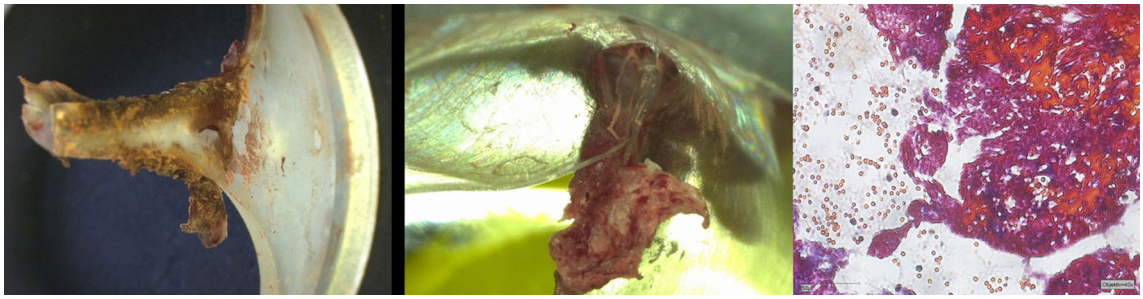

FIGURE 5. Thrombosis formation on the PCU-valve surface (left: Rough Stent surface; middle: dead water area between stent and leaflet; right: Ladewig staining of a thrombus) (based on Ref. 24).

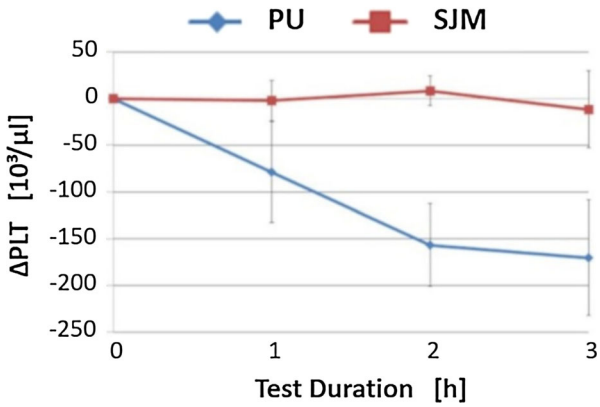

FIGURE 6. Change of platelet concentration in the blood from the start for the PCU and the SJM-valve (based on Ref. 24).

platelets). Changes in CT were unspecific with high standard deviation showing a decrease reflecting activation. MCF and alpha showed increasing values similar for both valves in the first two hours reflecting activation. At the end of the tests, both values dropped for the PCU valve due to the massive consumption of coagulation parameters and platelets.

Validation Step 3: In-Vivo Comparison

For the third validation step, a St. Jude Medical (SJM) bileaflet valve was tested six times for four hours. The resulting adhesions were then compared to thrombosis that occurred after *in-vivo* trials in sheep with valves implanted into pulmonary position using no anticoagulation carried out by Meuris *et al.* Exemplary pictures are shown in Fig. 8.

Both views from upstream and from downstream show similar adhesions in the hinge area. The amount of adhesion is fewer on the valves tested *in vitro*. The left picture of Fig. 8 shows adhesions at the Thumbnail region of the St. Jude Medical valve produced *in vitro*. Ellis *et al.* proposed this region as prone to thrombosis, because of a recirculation area[13] that we also found in CFD simulations.[24] The locations of the findings were reproducible throughout all the carried out tests.

The hinge regions of the tested valves were also examined using SEM (Scanning electron microscopy). Figure 9 shows activated, aggregated platelets with fibrin strands with trapped erythrocytes.

No thrombotic material was found on any of the tester surfaces after the tests, and no embolized thrombi were found during blood filtration and histological examination of the blood after test ending.

DISCUSSION

The new *in-vitro* test setup has proven its suitability for the evaluation of the thrombotic potential of heart valve prostheses in the aortic position. It opens many options to evaluate valve performance under physiological and pathological hydrodynamic (e.g. hyper-, hypotension) and anatomic conditions (e.g. malformed aortic root), as well as different valve positions (aortic, mitral, pulmonary and tricuspid position). Anatomic simulation of the valve annulus and its vicinity also enables realistic testing of transcatheter and other heart valve prostheses that have different anchoring mechanisms inside the aortic root. These structures are often in the middle of the blood flow and thrombogenic effects seem obvious.

The THIA3's ability to generate reproducible hydrodynamics around heart valves enables a good correlation with numeric flow field simulations (CFD). With the incorporated option to evaluate flow field through the heart valve under identical conditions using particle image velocimetry (PIV), even validation of CFD studies can be carried out with high correlation. In combination with reasonable CFD studies evaluating the flow field inside the valves, this tester is a powerful tool to optimize heart valves towards a better thrombogenic performance. Good correlation between specific flow fields and found thrombosis in the test could already been shown for the SJM and a trileaflet valve (Triflo; Triflo Inc.). For both mechanical valves also significant difference in the amount of thrombus adhering on the valve could be proven.[24]

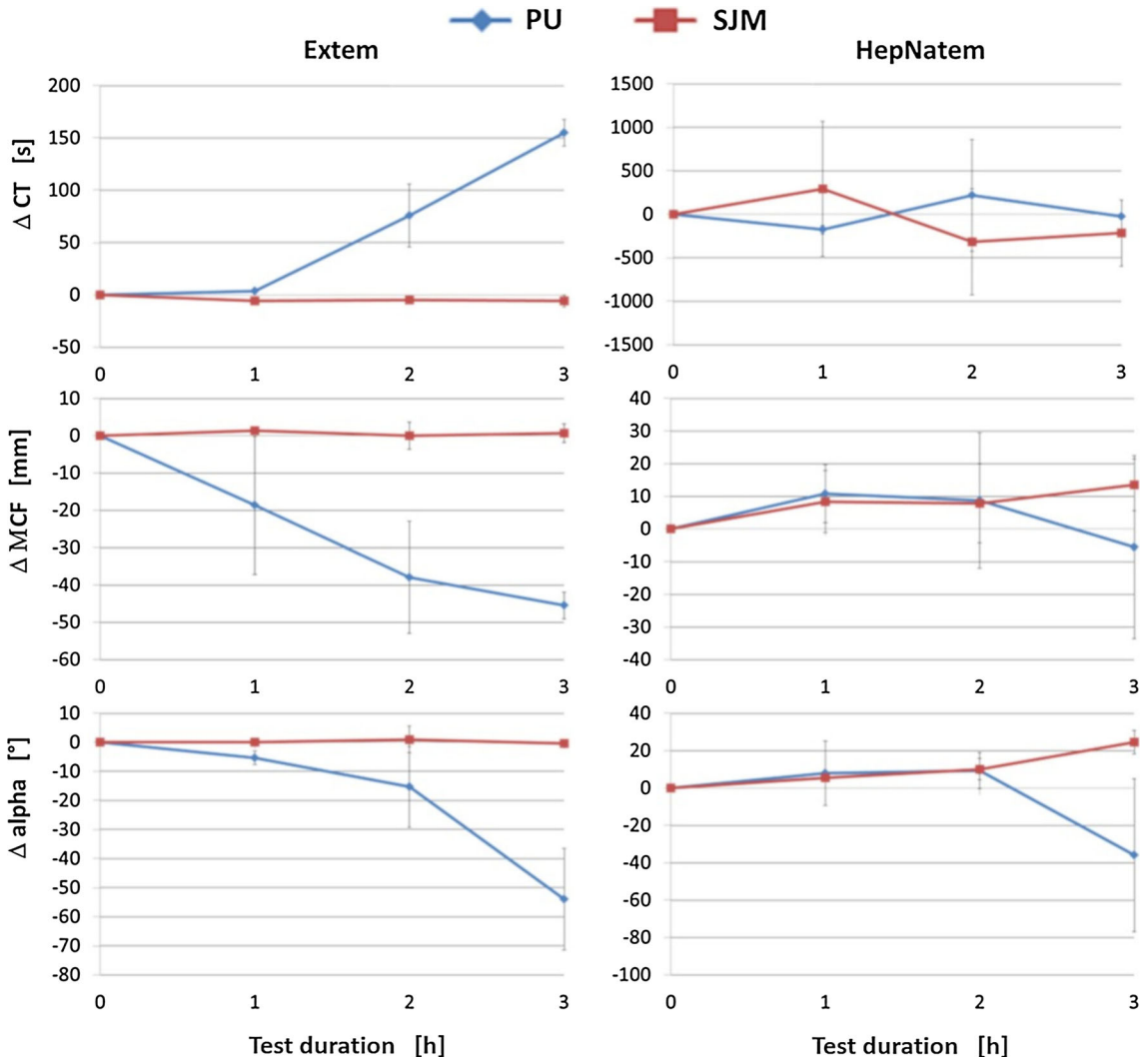

FIGURE 7. CT, MCF and alpha measured using Extem and HepNatem for the PU and the SJM valve tested in the THIA3 (based on Ref. 24).

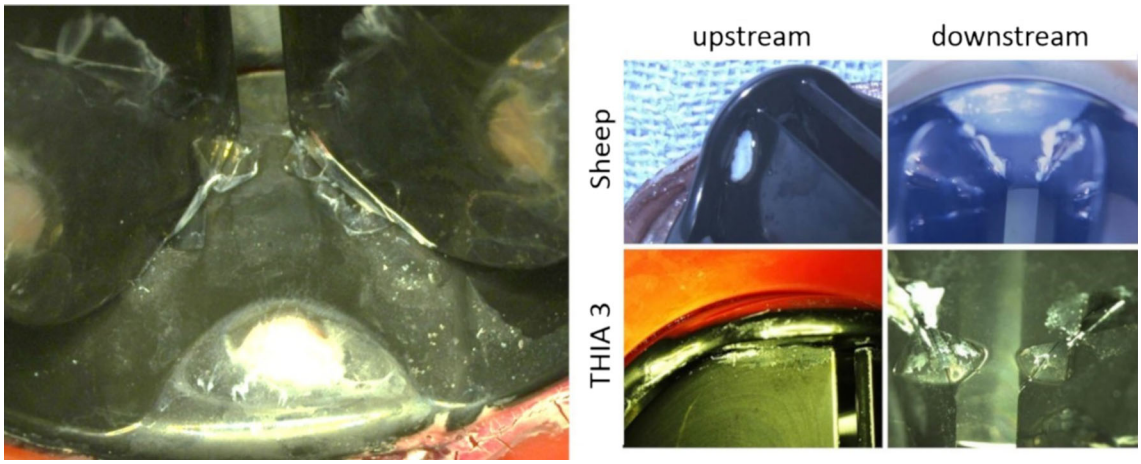

FIGURE 8. Initial clot formation in the thumb nail region of the SJM on the left side. Comparison of *in-vivo* and *in-vitro* findings showing similar locations of initial clot formation on the right side (based on Ref. 24).

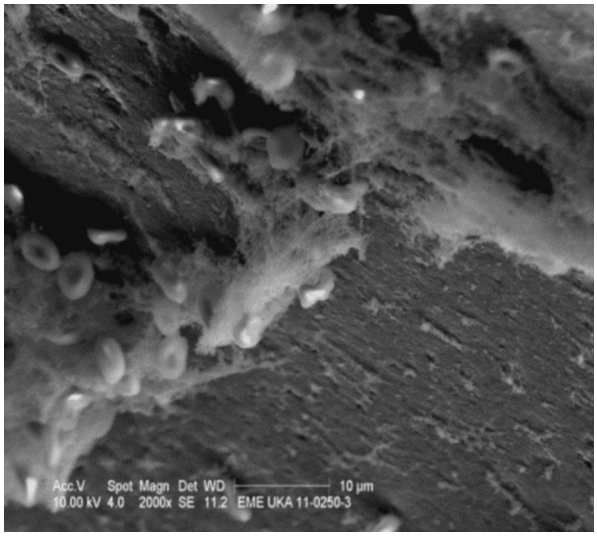

FIGURE 9. Picture from SEM showing the bearing region of the SJM valve with adhesions of fibrin strands, thrombocytes and some erythrocytes (based on Ref. 24).

A limitation of this testing procedure is the blood itself. Porcine blood is similar, but not fully equal to human blood regarding its thrombogenic behavior. However, it reflects a worst-case scenario, especially in combination with the pre-activation of the slaughterhouse blood. Therewith, a relatively high variance of the testing results occurs with regard especially to the coagulation parameters. Thus, a higher number of test cycles might be necessary to gain statistically significant results for the coagulation activation. Thrombus locations seem independent, as they occurred at the same spots with comparable amounts in every test. Regarding the low volume of 450 mL of the tester, it is also possible to use fresh human blood for deeper investigations. Another limitation that had to be accepted to facilitate this low volume, is the lower pressure drop during the beginning of diastole, that influences the leakage flow during this time frame. The exact influence on thrombus formation are in focus for future studies.

The used anticoagulant (LMWH) showed the most reproducible results. Preliminary tests with High Molecular Weight Heparine (HMWH) showed high rates of embolizing thrombi filtrating the blood after testing procedure.[24] Citrate also fulfills these requirements, but eliminates the chance to evaluate parameters like PF4. Another limitation is the flow field towards the valve. It is a necessary simplification of the natural flow field inside the heart in order to increase repeatability and to enable good correlation with CFD simulations of the tested valve.

Despite these limitations, this developed method enables a fast and reproducible assessment of the thrombogenic potential of a heart valve prostheses. This enables heart valve developers to improve implant design without long-lasting, expensive animal trials that may even produce uncertain results in many cases.

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest. This article does not contain any studies with animals performed by any of the authors.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors. Presented animal data were taken from earlier studies.

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