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Corresponding Author:	Max Wacker, M.D. Otto von Guericke Universitat Magdeburg Magdeburg, Saxony-Anhalt GERMANY
Corresponding Author's Institution:	Otto von Guericke Universitat Magdeburg
Corresponding Author E-Mail:	max.wacker@med.ovgu.de
Order of Authors:	Max Wacker, M.D.
	Ulf Betke
	Katrin Borucki
	Jörn Hülsmann
	George Awad
	Sam Varghese
	Maximilian Scherner
	Michael Hansen
	Jens Wippermann
	Priya Veluswamy
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TITLE:

An In Vitro Hemodynamic Loop Model to Investigate the Hemocytocompatibility and Host Cell Activation of Vascular Medical Devices

AUTHORS AND AFFILIATIONS:

Max Wacker[1], Ulf Betke[2], Katrin Borucki[3], Jörn Hülsmann[1], George Awad[1], Sam Varghese[1], Maximilian Scherner[1], Michael Hansen[4], Jens Wippermann[1], Priya Veluswamy[1]

[1]Department of Cardiothoracic Surgery, Otto-von-Guericke-University, Magdeburg, Germany
[2]Department of Mechanical Engineering, Institute for Materials and Joining Technology, Otto-von-Guericke-University, Magdeburg, Germany
[3]Institute of Clinical Chemistry and Pathobiochemistry, Otto-von-Guericke-University, Magdeburg, Germany
[4]Division of Cardiology and Angiology, Department of Internal Medicine, Otto-von-Guericke-University, Magdeburg, Germany

Corresponding author:
Max Wacker (max.wacker@med.ovgu.de)

Email addresses of co-authors:
Ulf Betke (ulf.betke@ovgu.de)
Katrin Borucki (katrin.borucki@med.ovgu.de)
Jörn Hülsmann (joern.huelsmann@med.ovgu.de)
George Awad (george.awad@med.ovgu.de)
Sam Varghese (sam.varghese@med.ovgu.de)
Maximilian Scherner (maximilian.scherner@med.ovgu.de)
Michael Hansen (michael.hansen@med.ovgu.de)
Jens Wippermann (jens.wippermann@med.ovgu.de)
Priya Veluswamy (priya.veluswamy@med.ovgu.de)

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host cell activation, hemocytocompatibility, bloodcompatibility, vascular medical device testing, biocompatibility, in vitro loop model

SUMMARY:

Presented here is a protocol for a standardized in vitro hemodynamic loop model. This model allows to test the hemocompatibility of perfusion tubes or vascular stents to be in accordance with ISO (International Organization for Standardization) standard 10993-4.

ABSTRACT:

In this study, the hemocompatibility of tubes with an inner diameter of 5 mm made of polyvinyl chloride (PVC) and coated with different bioactive conjugates was compared to uncoated PVC tubes, latex tubes, and a stent for intravascular application that was placed inside the PVC tubes. Evaluation of hemocompatibility was done using an in vitro hemodynamic loop model that is

recommended by the ISO standard 10993-4. The tubes were cut into segments of identical length and closed to form loops avoiding any gap at the splice, then filled with human blood and rotated in a water bath at 37 °C for 3 hours. Thereafter, the blood inside the tubes was collected for the analysis of whole blood cell count, hemolysis (free plasma hemoglobin), complement system (sC5b-9), coagulation system (fibrinopeptide A), and leukocyte activation (polymorphonuclear elastase, tumor necrosis factor and interleukin-6). Host cell activation was determined for platelet activation, leukocyte integrin status and monocyte platelet aggregates using flow cytometry. The effect of inaccurate loop closure was examined with x-ray microtomography and scanning electron microscopy, that showed thrombus formation at the splice. Latex tubes showed the strongest activation of both plasma and cellular components of the blood, indicating a poor hemocompatibility, followed by the stent group and uncoated PVC tubes. The coated PVC tubes did not show a significant decrease in platelet activation status, but showed an increased in complement and coagulation cascade compared to uncoated PVC tubes. The loop model itself did not lead to the activation of cells or soluble factors, and the hemolysis level was low. Therefore, the presented in vitro hemodynamic loop model avoids excessive activation of blood components by mechanical forces and serves as a method to investigate in vitro interactions between donor blood and vascular medical devices.

INTRODUCTION:

Hemocompatibility testing of medical devices is a crucial step in the development of new devices such as vascular stents or perfusion tubes for extracorporeal membrane oxygenation. Until today, animal models are considered as standard tools to finalize the procedure for testing the medical devices prior to its implementation in humans. Henceforth, it is necessary to find alternative in vitro models that further aid in minimizing investigations on animals. In this study, we, therefore, have explored a miniature in vitro hemodynamic loop model. The goal of this presented method is to test the in vitro blood compatibility of medical devices in accordance to the ISO 10993-4 standard.

The ISO 10993-4 standard describes standardized sets of clinical parameters to be investigated on blood specimen[1]. Briefly, these are thrombosis (platelet aggregation and count), coagulation (fibrinopeptide A, FPA), hematological analysis (whole blood cell count), hemolysis index (free plasma hemoglobin) and the complement system (terminal complement complex, sC5b9). However, additional markers, such as neutrophil polymorphonuclear elastase (PMN), interleukin 6 (IL-6) and tumor necrosis factor - alpha (TNF) reflecting the activation status of leukocytes can also be accounted for measurements. To determine and to quantify the circulating cell free proteins that are present in blood plasma, sandwich enzymatic linked immunosorbent assay (ELISA) represents a conventional and most reliable method[2,3]. Likewise, the phenotype and activation status of the host cells (e.g., leukocytes) can be quantified by detecting the cell surface expression of molecules by flow cytometry (FACS) that provides single cell suspension based readouts, where fluorescent labeled specific antibodies bind to the targeted cell surface molecules[4]. Scanning electron microscopy (SEM) is also recommended to determine thrombus formation on the tested material by the ISO 10993-4 standard[1]. This method can be complemented with X-ray microtomography (μCT), to perform structural analysis of the thrombus e.g., its thickness, size and localization in a 3D rendered image[5].

The rationale behind using this in vitro hemodynamic model is to screen for the best performing and compatible medical devices by understanding the basic physiological dynamics of blood components such as platelets, that are involved in the primary hemostasis or leukocytes and their interaction with different types of vascular devices. Such in vitro systems are highly demanded as they reduce the need for animal studies.

The here presented loop model fulfills these demands. This model was first described by A.B. Chandler in 1958 for the production of blood thrombi and is, therefore, also called Chandler Loop model[6]. Until now, this model has been used in a series of experiments and modifications to investigate the blood biocompatibility of medical devices[7-14]. It consists of polymer tubes, which are partly filled with blood and shaped into re-closable loops. These loops rotate in a temperature-controlled water bath to simulate vascular flow conditions with its hemorheological effects. Alternative methods such as pump driven models or models that use mechanical ball valves inside the loops to induce a blood flow inside the polymer tubes have already been described[15,16]. However, the overall advantage of the here presented method is that the mechanical force applied to the blood cells and proteins is low, avoiding hemolysis, and there is no contact between blood and connectors, that could possibly lead to flow turbulences and activation of blood components. The main activating factors inside the loop are the test material itself and the air that is trapped inside. This helps to minimize sources of measuring error and to deliver a high reproducibility, even if the blood-air interface can lead to protein denaturation[17]. It is also possible to investigate varieties of tubing materials and stent diameters without length or size restrictions thereby allowing the use of tubes of different length and inner diameter. Moreover, host hemocompatibilities on inaccurate loop closure and exposure to the uncoated tube surface are also possible to investigate. Other similar medical applications of this in vitro hemodynamic loop model is that it could also be used to study the interactions between immunotherapeutics (drugs) and blood components during either preclinical development or individual drug safety screening prior to first-in-man phase I clinical trial, or for the generation of thrombus material that can be used in further experiments[18-20].

This study describes a detailed protocol for testing the hemocompatibilities of perfusion tubes and/or stents. Here, the comparison between uncoated and coated PVC tubes (hepPVC: heparin coating, polyPVC: coating with an bioactive polymer). Lowered activation of platelets, but a higher activation of the coagulation system (FPA) were found for both coated tubes in comparison to the uncoated tubes. The hepPVC tubes used here are modified with covalently bound heparin to make them thromboresistant[21] and have already been employed in a loop model to optimize and characterize different parameters[22]. The polyPVC tubes used in this study are commercially available tubes used in clinical settings of extracorporeal blood perfusion and are coated with a heparin polymer to reduce their thrombogenicity[23]. Sometimes, in clinical applications even uncoated PVC tubes are used. Therefore, we included latex tubes as a positive control group that showed excessive activation of platelets, coagulation system, and soluble factors like IL-6, TNF and PMN elastase. Thrombus formation was noticed when inaccurate loop closure was simulated. This led to the activation of coagulation and complement system as well as leukocytes and platelets compared to the baseline conditions. Furthermore, blood contact to the here used stent material (bare metal nitinol stent, covered with carbon-impregnated

expanded polytetrafluoroethylene) led to higher platelet and leukocyte activation in terms of PMN elastase. Overall, the presented model did not induce hemolysis in any of the tested vascular devices as they were comparable to the baseline or static conditions, except for the latex tubes, where red blood cell (RBC) hemolysis was obvious. Moreover, these perfusion tubes can be examined either by imaging or by histology. Though histological evaluations might be feasible, we mainly focused on ELISA and flow cytometry to perform these experiments and thereby enabling the feasibilities of conducting experiments based on the here presented model for many laboratories. Thus, this method represents a feasible method to test the blood biocompatibility of vascular medical devices in accordance with the recommendations of the ISO 10993-4 standard. Furthermore, this method can be used whenever an interaction between blood and materials should be tested under flow conditions, mimicking the in vivo conditions.

PROTOCOL:

This study was approved by the Ethics Committee of the medical faculty of the University Hospital Magdeburg (application number 88/18) and the subjects provided written informed consent prior to the blood drawing procedure.

1. Heparin stock preparation and blood sampling

1.1. Calculate the amount of blood needed for the entire experiments.

NOTE: Almost, 5 mL of heparinized blood is required for each vascular device in duplicates (loops). Likewise, 5 mL of blood is required for each baseline and static condition which is kept aside at room temperature.

1.2. Prepare a heparin stock solution at the concentration of 100 IU/mL by diluting the unfractionated heparin (e.g., Rotexmedia) in deionized water. Use 150 µL of this solution for the heparinization of 10 mL fresh blood.

1.3. Calculate the amount of blood as well as plasma that are needed for whole blood cell count, ELISA and FACS assays.

NOTE: The measurements represented in this study are obtained from two loops running in parallel (one as duplicate) with a total blood volume of 10 mL.

1.4. Based on the required amount of blood, fill 10 mL syringes with 150 µL of heparin stock solution to prevent blood coagulation with a final heparin concentration of 1.5 IU/mL blood.

1.5. Draw blood using a butterfly (size: 21 G). Fill the syringes very gently to avoid hemolysis or cell activation due to excessive vacuum.

NOTE: Permission from the local ethical committee should be obtained before the commencement of the experiments. Obtain informed consent from each human blood donor. Ensure that the blood donor is healthy and does not take any medication, especially no

antiplatelet agents or non-steroidal anti-inflammatory drugs for at least 10 days prior to the experiments. Of note, same donor is preferred when comparing different types of vascular devices for the first time as presented in this manuscript. To further evaluate the inter-individual differences, the protocol can be repeated with different donors.

1.6. Collect blood in a glass beaker, avoid excessive agitation.

2. In vitro hemodynamic loop assembly

2.1. Fill the water bath until the water level reaches up to the center of the rotation unit. Set water temperature to 37 °C.

2.2. Loop assembly for testing different tubing materials (polyPVC, hepPVC, PVC and latex)

2.2.1. Cut two 50 cm long pieces of each tube material (inner diameter 5 mm) with the tube cutter. Ensure that the cutting surface is flat, as this is particularly important for perfect closure for the loops with small diameters so that blood flows without any distortion on the fitting edge.

NOTE: This entire protocol utilizes the tubes with an inner diameter of 5 mm.

2.2.2. To ease the handling and to avoid post-sample processing delay after rotation, run only four loops (2 materials in duplicates) in parallel.

2.2.3. To generate a loop shape, plug the open endings of the tubes into a short piece of silicon tube fitting the outer diameter of the investigative tube.

2.2.4. Use the polycarbonate tension bands to ensure proper closing of the loops. When used for the first time, adjust the length of the bands to fit the outer diameter of the loop by cutting the tension band with scissors in required lengths and secure it with a 3 mm torque wrench.

2.2.5. Carefully tighten the locking screw of the tension band connector under inspection of the tube endings. Adjust the closing force so that no gap remains between the tube endings. If the locking screw is totally tightened and the tension of the polycarbonate band seems too low to close the gap between the tube endings, open the locking the system and cut a few mm of the tension band. Repeat this, until accurate loop closure is achieved **(Figure 1A)**.

2.2.6. If the tubing material is very soft and tends to slip inside the tension bands, fix the loop with electrical tape to the tension band **(Figure 1B,C)**.

2.2.7. Prepare an additional loop of PVC for temperature control with the same dimensions as the test loops.

2.2.8. Secure the loops in the loop cradle of the rotation unit outside the water bath. Thereafter, attach the loop cradle to the rotation unit inside the water bath **(Figure 1E)**.

2.2.9. Dismantle the tension band partly from the loops and unplug one end of each tube to open the loop.

2.2.10. Gently fill each loop with 5 mL of blood with a 5 mL serological pipette. Mix blood gently twice in the glass beaker by slowly pipetting up and down before loading into the loops.

2.2.11. Take the disposable thermometer and place it inside the temperature control loop. If the thermometer is too large, cut it with scissors to fit smaller tubes. Fill the loop with 5 mL of deionized water at room temperature.

2.2.12. Close the loops and ensure whether the loops, the tension bands and the rack are properly fitted.

2.2.13. Set rotation speed to 30 rounds per minute (rpm) and rotate for 3 h.

2.3. Loop assembly for testing the impact of improper loop closure (gap)

2.3.1. Prepare four loops (polyPVC) as described in 2.2.

2.3.2. Close two of the loops properly with the tension bands and avoid any gap between the tube endings.

2.3.3. For the other two loops plug the open endings into the bigger tube as described in 2.2.3, but leave a gap of 1-2 mm in between the loop endings. Do not use the tension bands for these loops **(Figure 1D)**.

2.3.4. Prepare one temperature control loop as described in 2.2.7 and 2.2.11.

2.3.5. Fill all loops with blood as described in 2.2.10.

2.3.6. Set the rotation speed to 30 rpm and rotate for 3 h.

2.4. Loop assembly for stent testing

2.4.1. Prepare four loops as described in 2.2 and following.

NOTE: To assess the blood biocompatibility of stents, the tubing material itself should be tested to be biocompatible in order to prevent masking of cell activation. If no data exists, the tube material itself can be tested as described in 2.2. Furthermore, the diameter range within the stent can be applied (see manufacturer's instructions) and should fit the inner diameter of the tube material.

2.4.2. Open two of the loops and take the tube out of the tension band system.

2.4.3. Insert the stent into the middle of the tube as per the manufacturer's instructions.

2.4.4. Use the other two loops without stent as control. Fill all loops with blood as described in 2.2.10.

2.4.5. Set rotation speed to 30 rpm and rotate for 3 h.

2.5. Temperature control

2.5.1. At any time during duration and when rotation is stopped, the temperature of the blood inside the loops is indicated by the thermometer inside the temperature control loop. To read off the temperature, stop the rotation and immediately read off the temperature indicated by the thermometer.

3. Blood sample processing

3.1. After rotation, let the loops stand in the rack for 2 min in an upward position to let the blood accumulate at the bottom of the loops, avoiding any spilling while opening the loops.

3.2. Inspect the blood left for static conditions: If this blood is coagulated, an improper heparinization is ultimately suspected. In this case, repeat the experiment preferably also with another blood donor.

3.3. Carefully take the loops out of the rack. Open the connectors and let the blood flow into a 10 mL glass beaker.

3.4. Pool the blood from the tubes that are run in duplicates.

3.5. Draw fresh blood for baseline analysis from the same donor as described in 1.5 and 1.6.

3.6. Collection of sodium citrate blood

3.6.1. For the collection of sodium citrate blood, fill four 1.5 mL tubes, each with 111 µL sodium citrate solution collected from sodium citrate tubes, to generate a final concentration of 3.2% of sodium citrate.

3.6.2. Fill each tube with 1 mL of blood from the glass beakers as described in 1.6 and 3.3.

3.6.3. Keep the blood at room temperature and use the this blood for FACS analyses as described in 12.

NOTE: To change the length of the loops for different experimental setups, properly plan aliquots based on the amount of measurements to be made. It may be necessary to establish all required

measurements with fresh blood prior to the real experiments to ensure the volume of blood in the loops is enough for all measurements.

3.7. Collection of ethylenediaminetetraacetic acid (EDTA) blood and plasma samples.

3.7.1. From each glass beaker with blood (see 1.6 and 3.3) transfer 4.5 ml of blood into one 5 ml EDTA tube. Fill two EDTA tubes from each 10 ml glass beaker with blood.

3.7.2. Gently mix the blood and keep it on ice.

3.7.3. Transfer 2 mL of blood into a 2 mL locking centrifugation tube and use this blood for blood cell count and free hemoglobin measurement as described in 5. And 6.

3.7.4. Centrifuge the remaining blood at 3,500 *x g* for 20 min.

3.7.5. Carefully collect the plasma in the supernatant in 500 µL aliquots and immediately freeze at -80 °C.

4. Scanning electron microscopy and µCT images

4.1. After blood sample processing, rinse the emptied loops prepared as described in 2.3 with 10 mL of phosphate-buffered saline (PBS) each.

4.2. Carefully cut off a 1 cm long sample from each end of each tube with a scalpel.

4.3. Incubate sample overnight at 4 °C in a 2% glutaraldehyde solution.

CAUTION: Inhalation of aldehyde vapors can cause nasal symptoms such as a runny nose ore persistent stuffiness and airway irritation, and contact with skin causes dermatitis. Aldehydes should be handled in a fume-hood while wearing gloves, a protective gown and safety goggles.

4.4. Rinse the sample (3 times) with PBS.

4.5. Prepare a 1% solution of osmium tetroxide (OsO_4) in deionized water and incubate sample for 15 min at room temperature.

CAUTION: OsO_4 is a strong oxidizing agent, it can be reduced by exposure to light. To avoid the reduction during preparation, store OsO_4 in a brown glass bottle. OsO_4 is extremely volatile, its fumes are toxic to eyes, nose and throat. Always work under a fume-hood and use gloves and protecting clothing, ensuring that no part of the body is exposed to OsO_4. Contact your institution's guidelines regarding handling of waste disposal and storage. In general, OsO_4 is storable for several months, but it needs special glass bottles with Teflon liner and a desiccator, because OsO_4 can discolor internal surfaces and contents of the refrigerator in the presence of leaking fumes.

4.6. Take out samples, transfer them in a new 15 mL centrifuge tubes and rinse samples 3 times with PBS.

4.7. Prepare a series of ethanol with varying concentrations (25%, 50%, 75%, 95%, 100%)

4.8. Dehydrate samples in ethanol: incubate for 20 min each in 25%, 50%, 75% and 90% and 30 min in 100%.

4.9. Take samples out of 100% ethanol and let it air dry overnight at room temperature.

4.10. Examine samples in the scanning electron microscope at an acceleration voltage of 5 kV and with the X-ray µCT scanner.

5. Blood cell count

5.1. Take 2 mL of the EDTA blood obtained as described in 3.7.3

5.2. Insert the tube into the automated hematology analyzer and follow the manufacturer's instructions.

6. Measurement of free hemoglobin (fHb) in plasma

6.1. Thaw one plasma sample from each condition obtained as described in 3.7.5. Store on ice after thawing.

NOTE: Always thaw frozen plasma samples in a water bath at 37 °C and immediately transfer to ice, containing some water, to lower the temperature. This is important to avoid activation of blood components during thawing.

6.2. Use the fHb reagent and follow the manufacturer's instructions. Avoid contamination after opening and protect the reagent from direct light (sun, UV light).

6.3. Use a pipetting scheme (see manufacturer's instructions) with 1:5 dilution.

6.4. Add 1000 µL of the hemoglobin reagent in a 1.6 mL semi-micro cuvette. Use this cuvette to determine the blank value.

6.5. Add 1000 µL of the hemoglobin reagent and 250 µL of the plasma sample in another semi-micro cuvette.

6.6. Mix the contents in both cuvettes by flushing the pipette thoroughly by repeatedly filling with reaction mixture, and incubate at least 3 min at room temperature.

6.7. Determine the extinction (E) of the sample against fHb reagent as blank reagent. Calculate the fHb-concentration (mmol/l) of the sample: E_{540}-E_{680} x 0.452.

7. Measurement of FPA

7.1. Thaw a plasma sample from each condition obtained as described in 3.7.5 and store on ice after thawing.

7.2. Use the FPA Elisa kit and follow the manufacturer's instructions.

8. Measurement of sC5b9

8.1. Thaw a plasma sample from each condition obtained as described in 3.7.5 and store on ice after thawing.

8.2. Use the sC5b-9 ELISA kit and follow the manufacturer's instructions.

9. Measurement of PMN

9.1. Thaw a plasma sample from each condition obtained as described in 3.7.5 and store on ice after thawing.

9.2. Use the PMN-Elastase ELISA kit and follow the manufacturer's instructions.

10. Measurement of TNF

10.1. Microplates must be coated one day before running the ELISA. For coating, add 100 µL of capture antibody solution to all wells, seal plate and incubate overnight at 2 °C-8 °C.

10.2. Thaw one plasma sample from each condition obtained as described in 3.7.5. Store on ice after thawing.

10.3. Use the TNF ELISA kit and follow the manufacturer's instructions.

11. Measurement of IL-6

11.1. Microplates must be coated with capture antibodies one day prior to the experiment. For coating, add 100 µL of capture antibody solution to all wells of the microplate provided with the set, seal plate and incubate overnight at 4 °C

11.2. Thaw a plasma sample from each condition obtained as described in 3.7.5. and store on ice after thawing.

11.3. Use the IL-6 ELISA kit and follow the manufacturer's instructions..

12. FACS analyses

12.1. Prepare 500 mL of FACS buffer by adding 10 mL of fetal calf serum and 2 mL of EDTA solution (0.5 M) to 488 mL of 1x PBS. The FACS buffer can be stored for 4 weeks at 4 °C.

12.2. Use 100 µL of the sodium citrate blood (see 3.6.3) for each staining procedure (monocyte platelet aggregates (MPA), platelet activation (PA), leukocyte integrins (LI)).

12.3. Pipette 100 µL of blood from each sample into a 5 mL FACS tube. From each sample, prepare 3 tubes for antibody staining and label with MPA (monocyte platelet aggregates) panel, PA (platelet aggregates) panel and LI (leukocyte integrin) panel.

12.4 Mix the rest of the 4 samples into one 5 mL FACS tube. Use this mix for unstained and fluorescence minus one (FMO) controls.

12.5. Prepare 6 FACS tubes by pipetting 100 µL of the mixed sample blood into each tube. Label 3 tubes as unstained and 3 tubes as FMO-CD41, FMO-CD62P and FMO-CD162.

12.6. Add 100 µL of 4% paraformaldehyde solution and incubate for 15 min in the dark at room temperature.

CAUTION: Paraformaldehyde is toxic to skin and eyes. It can cause serious pulmonary irritation when inhaled and can lead to lung damage after prolonged exposure. Furthermore, it is classified as a carcinogen and a reproductive toxin. Always wear gloves and safety glasses and work under the fume hood.

12.7. Add 1 mL of wash buffer to each tube and centrifuge at 139 *x g* for 5 min. Discard the supernatant and repeat wash step two times.

12.8. For red blood cell (RBC) lysis, add 1 mL of 1x buffer RBC lysis buffer (dilute 10x RBC lysis buffer in deionized water), mix by slowly pipetting up and down and incubate for 5 min at RT in the dark.

12.9. Prepare compensation beads for single stains. To 1 mL of FACS buffer add 4 drops of each positive and negative beads. Vortex thorough and add 100 µL of beads solution to one 5 mL FACS tube.

12.10. Prepare 4 tubes with beads and label as CD14-FITC, CD41-BV421, CD45-APC and CD62P-PE. Add 1 mL of FACS buffer to each tube and centrifuge at 139 x g for 5 min. Discard supernatant and keep on ice.

12.11. After RBC lysis, add 1 mL of FACS buffer to each tube and wash as described in 12.7. Discard supernatant.

12.12. Prepare antibody cocktails:

12.12.1. MPA panel: Add 4 µL of CD45-APC, 4 µL of CD14-FITC and 4 µL of CD41-BV421 to 388 µL of FACS buffer.

12.12.2. PA panel: Add 1.6 µL of CD41-BV421 and 12 µL of CD62P-PE to 386.4 µL of FACS buffer.

12.12.3. LI panel: Add 4 µL of CD45-APC and 8 µL of CD162-BV421 to 388 µL of FACS buffer. Keep on ice.

12.13. Prepare single stains: Add 0.5 µL to 499.5 µL of FACS buffer each for CD14-FITC, CD41-BV421 and CD45-APC. For CD62P-PE add 0.3 µL to 499.7 µL of FACS buffer. Keep on ice.

12.14. Prepare FMO controls: (i) MPA panel (FMO-CD41): Add 1 µL CD14-FITC antibody and 1 µL of CD45-APC antibody to 98 µL FACS buffer. (ii) PA panel (FMO-CD62P): Add 0.4 µL of CD41-BV421 to 99.6 µL of FACS buffer. (iii) LI panel (FMO-CD162): Add 1 µL of CD45-APC to 99 µL of FACS buffer. Keep FMO controls on ice.

12.15. Vortex antibody cocktails and add 100 µL of each antibody cocktail as prepared in 12.12 to each of the respective labeled tubes (MPA, PA and LI panel) as described in 12.3 and mix gently by pipetting up and down. Keep on RT in the dark.

12.16. Vortex single stain antibody dilutions and add 100 µL of the single stain antibody dilutions as prepared in 12.13. to each of the respective labeled tubes with beads as described in 12.7 and mix gently by pipetting up and down. Keep on RT in the dark.

12.17. Vortex FMO antibody cocktails and add 100 µL of each FMO-1 antibody cocktail as prepared in 12.14. to each of the respective labeled tubes (FMO controls) as described in 12.5. and mix gently by pipetting up and down. Keep at RT in the dark.

12.18. Incubate all tubes with antibody staining for 30 min at RT in the dark.

12.19 Take the three tubes for unstained control (see 12.4) and resuspend pellet in 250 µL of FACS buffer. Keep on ice.

12.20. After incubation time, wash all tubes except unstained controls one time with FACS buffer as described in 12.7. Resuspend pellet in in 250 µL of FACS buffer and acquire data on the flow cytometer.

12.21. Use unstained cells for negative settings and compensation mouse beads for positive settings in FACS software. Further, use FMO to control the gating strategy, where FMO-CD41 is used in MPA panel, FMO-CD62P is used in PA panel and FMO-CD162 is used in LI panel.

12.22. Acquire almost 0.5 x 10^6 - 0.25 x 10^6 events per tube for FACS. Save data and analyze with an analysis software (version 9.9.6).

REPRESENTATIVE RESULTS:

All presented data, except FACS plots, were analyzed with a statistics software. The FACS plots were analyzed using flow cytometry software.

The analysis of whole blood cell count did not show any significant differences with respect to erythrocytes between all tested conditions **(Figure 2)**. But, platelets and leukocytes were drastically reduced in the latex group, indicating a very poor biocompatibility of latex. This is further underlined by increased levels of free hemoglobin in the latex group, indicating the fact that except for the latex group, none of the other vascular devices or conditions led to extensive hemolysis (**Figure 2)**. Further, the coated PVC tubes, polyPVC and hepPVC, as well as the tested stent did not lead to thrombosis by means of platelet and leukocyte loss, while latex exhibited the highest platelet and leukocyte loss, followed by uncoated PVC tubes that showed a decreased trend.

While all the tested vascular devices led to increased activation of the coagulation system (FPA) and complement component (sC5b-9), the hepPVC loops exhibited a trend for decreased levels of FPA and sC5b-9 when compared specifically to polyPVC loops (**Figure 3)**. Interestingly, uncoated PVC and Gap loops showed lower levels of FPA compared to polyPVC, though not reaching the level of statistical significance. Nevertheless, latex loops exhibited significantly increased levels of FPA when compared to baseline and static conditions.

In accordance with the whole blood cell counts, latex loops exhibited highest levels of TNF, IL-6 and PMN elastase **(Figure 4)**, reaching the level of statistical significance when compared to rest of the groups in terms of TNF and IL-6 **(Figure 4A,B)**, whereas to static and baseline conditions in terms of PMN elastase **(Figure 4C)**. These results indicate the potent activation of leukocytes by latex. The baseline levels of activation markers were always comparable to static conditions, indicating a proper heparinization of the blood.

Interestingly, it was shown that platelet and leukocyte counts for gap induced loops were only slightly reduced with moderate activation of the coagulatory system (FPA) and leukocytes (PMN elastase), though improper loop closure with resulting flow turbulences and blood contact to the uncoated, rough cutting surface led to macroscopically visible clots at the splice **(Figure 1F)**. The clots and its distribution over the whole splice surface were evident with µCT and SEM images, while no clot was found when the loops were closed with the external closing device leaving no gap between the loop endings **(Figure 5)**.

Flow cytometric analysis of host blood cells that were stained with platelet specific markers, CD41 and platelet activation marker CD62P, are shown in **Figure 6A,B**. Here, the latex tubes exhibited exceedingly high median fluorescence intensity (MFI) for CD62P on blood platelets, followed by stent, whereas heparin coated polyPVC tubes exhibited minimal activation of platelets depicting anti-thrombogenic property of polyPVC tubes. Furthermore, leukocytes were classified based on

the CD45 and SSC (side scatter) based granularity into (i) granulocytes; (ii) monocytes and (iii) lymphocytes **(Figure 7)**, and the expression of $CD162^+$ integrin was detected on each subpopulation of leukocytes that are known to interact with the CD62P on platelets[24]. It was noticed that the integrin expressions were drastically reduced on granulocytes and lymphocytes in latex loops. This result was in line with lowered levels of total frequencies of leukocytes in the latex loops **(Figure 2)**. In general, the integrin levels were higher among monocytes when compared to granulocytes and lymphocytes, indicating the likelihood for the monocyte interaction with activated platelets. In this regard, monocyte platelet aggregates were also evaluated by staining the blood cells with CD14 (as monocyte marker) and CD41 (as platelet marker) and ultimately to identify double positive cells i.e. $CD14^+CD41^+$MPA **(Figure 8)**. Here, we noticed that the stent group exhibited the highest levels of CD41 expression on the MPA, followed by the latex group, indicating an increased tendency to form MPA, despite the reduced frequency of monocyte (<1 %) in the latex loops.

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of the in vitro hemodynamic loop model and its modifications. (**A**) Loop for the gap experiment with external loop closing system, leaving no gap at the splice. (**B**) Loop made of polyPVC coated PVC tube and stent inside (arrow). (**C**) Loop made of latex tube. (**D**) Loop for the gap experiment without the external loop closing system leaving a gap between the tube endings (arrow). (**E**) Loops placed in the loop cradle inside the water bath and filled with blood. (**F**) Thrombus resulting in a gap at the splice (arrow) after rotation.

Figure 2: Results for blood cell count and plasma hemoglobin. (**A**) Erythrocytes count. (**B**) Platelets count. (**F**) Leukocytes count. (**D**) Free plasma hemoglobin. The results indicate the poor biocompatibility of latex, leading to excessive hemolysis. Data are presented as mean value; error bars indicate SEM. n=1.

Figure 3: Results for activation of the coagulation and complement system. (**A**) Coagulation system activation, measured by levels of Fibrinopeptide A (FPA) (**B**) Complement system activation, measured by levels of sC5b-9. While latex tubes evoked significant elevated levels of the FPA, the complement activation was strong for all tested materials. Data are presented as mean value, error bars indicate SEM. *p<0.05, n=1.

Figure 4: Leukocyte activation markers. (**A**) Tumor necrosis factor alpha (TNF). (**B**) Interleukin 6 (IL-6) (**C**) PMN Elastase**.** The results indicate increased activation of leukocytes due to elevated levels of the analyzed markers, followed by stent loops, that only led to increased levels for PMN Elastase but not TNF or IL-6. Data are presented as mean value, error bars indicate SEM. *p<0.5; **p<0.01, n=1.

Figure 5: Imaging of the splice of the loops. (**A**) µ-computer tomography (µCT) of loops with improper closing (gap). The red areas indicate thrombus material. (**B**) Rendering of the luminal side of the tube. The rectangular selection indicates the area for scanning electron microscopy (SEM) **(C).** (**D**) µCT of loops with external loop closing device and no gap at the splice, and (**E**) rendering and view of the luminal surface. No thrombus material was found. (**F**) SEM image of

the rectangular selection in (**E**). No thrombus material was found on the cutting surface.

Figure 6: FACS plot for platelet activation (CD62P). (**A**) Representative FACS plot (basic condition) showing the blood $CD41^+$ platelets. (**B**) Graph showing the platelet activation status reflected by the mean fluorescence intensity (MFI) of the different types of vascular devices in comparison to the static RT and baseline conditions. The data bars present data from single measurements.

Figure 7: FACS plot for leukocyte integrin (CD162). (**A**) Representative FACS plot (basic condition) showing the blood $CD45^+$ leukocytes and subgroups (**B**) Graph showing the leukocyte $CD162^+$ integrin mean fluorescence intensity (MFI) of the different types of vascular devices in comparison to the static and baseline conditions. The data bars present data from single measurements.

Figure 8: FACS plot for platelet monocyte aggregates (CD41/CD14). (**A**) Representative FACS plot (basic condition) showing the gating for blood monocytes ($CD45^+/CD14^+$), platelets ($CD41^+$) and monocyte platelet aggregates ($CD41^+/CD14^+$) (**B**) Graph showing the $CD41^+$ mean fluorescence intensity (MFI) on monocyte platelet aggregates for the various vascular devices compared to the static and baseline conditions. The data bars present data from single measurements.

DISCUSSION:

This study has shown that the presented in vitro hemodynamic loop model offers a reliable method for testing the in vitro blood compatibility of medical devices in accordance to the ISO 10993-4 standard.

Critical steps in the protocol include the drawing of blood and filling the tubes with blood, where excessive vacuum or agitation should be avoided to prevent the blood components from activation by the handling procedure. Furthermore, it is very important to immediately freeze the plasma samples and keep them on ice after thawing, as the complement and coagulation system activation can be tampered by keeping the samples on room temperature for a longer time.

Since this model has both merits and demerits when compared to other in vitro models, several factors have to be taken into account while designing the experiments.

First, the loops can be varied in length and diameter to fit various experimental setups. In case the setup includes contrasting tubes of varying inner diameters, it should be kept in mind that the differences in diameter will result in different shear forces, thereby affecting the coagulation and complement cascade[7]. Second, the rotation speed was set to 30 rpm in this experiment. This will result in a blood flow of approximately 25 cm/s, which is comparable to the blood flow velocity in human coronary artery bypass grafts[25]. The strain rate, generated by the rotation of the loops, is the major parameter that will initiate biochemical cascades of blood components, including cells and cell-free proteins. But as blood is a non-Newtonian fluid, the strain rate will also be influenced by the tube curvature, respectively the length of the tubes that are closed to loops[10]. Whenever the rotation speed or loop size is changed, it is important to consider that the

correlation between strain rate and rotation speed is not linear. The correlation between the rotation speed and strain rate is not sufficiently examined until today and further studies are required to investigate these particular parameters[10,26,27]. However, based on a model for laminar boundary layer, the given tube diameter of 5 mm and the rotation speed of 25 cm/s, a rough estimation of the wall shear stress (WSS) would indicate values between 2.20-22.00 pascal for a distance of 1,00-0,01 mm to the wall of the tube when the blood density is estimated to be 1060 $kg*m^{-3}$ and the kinetical viscosity is set to 0.0025 $pascal*s$[28,29]. Interestingly, also a more detailed computational analysis of flow dynamics in the curvature of human coronary arteries showed WSS values ranging from 11.33 to 16.77 pascal at roughly comparable parameters for the velocity, density and viscosity of the blood[30].

Beside this limitation, the presented loop model is a-pressure less system, that does not mimic the intravascular blood pressure ratios of the human vascular system.

Next important limitation is that the blood is in contact with air inside the loops, which brings additional interferences. Such a blood-air contact is impacted by two parameters, which includes the gas permeability of the tubes and the retainment of air inside the loops while filling them with blood. Every tube material possesses a certain gas permeability that can lead to significant changes in gas concentrations inside the tubes. While some authors state that the resultant effect of the gas permeability on activation of blood components remains unclear[31], it is known that the function of the blood coagulators is highly sensitive to a pH-shift, that may be caused by CO_2 diffusion[32-34]. Here, we have tested the biocompatibility of blood perfusion tubes under indoor air conditions, comparable to clinical scenarios of extracorporeal blood perfusion. For future improvements of the presented model, incubation of the whole model in a CO_2 incubator and performing blood pH validation before and after incubation might be useful to further standardize this model.

Also, the blood-air interface inside the loops can lead to activation of plasma proteins and cell fractions of the blood[35,36]. The roller pump driven devices without air inside the tubes may avoid the issue of blood-air interface, but they certainly induce damage to blood cells with significant elevated levels of hemoglobin compared to the here presented loop model, and the hemoglobin in plasma can interfere with the sensitivity of tested analytes in ELISA[16]. In this study we have shown that the hemolytic effect of the loop model itself remains minimal while using biocompatible materials such as heparin coated PVC tubes. Thus, the model is, on the one hand, not causing excessive cell damage compared to pump driven models, but on the other hand inducing plasma proteins due to blood air contact. Of note, van Oeveren et al. developed a ball-valve based loop model avoiding air inside the loops[16]. This promising alternative to the here presented loop model may overcome the problem of the blood-air interface, however, compared to the model presented here, platelet adhesion is still higher for the ball-valve based loop model.

With regard to the static control, it is of note that glass itself has been shown to be a potent activator of the coagulatory system[37]. However, in the presented setup, incubation in a glass beaker (static control) did not lead to excessive host cell activation or activation of the coagulatory system compared to the baseline levels directly after drawing the blood. In

conclusion, it might be helpful to use for example polypropylene tubes, if the static control shows high levels of activation.

Regardless of whether it is a loop based or a pump-driven model, these in vitro models completely lack the authentic biological interactions that are mainly contributed by an intact endothelium, which is an ideal blood contacting surface. The rationale behind this issue is more evident when a medical device like a stent is being tested, which might impart different outcomes, in terms of activation and plasma proteins, during its interaction with blood components in the presence of endothelium. This declares to be a major drawback of all discussed in vitro systems mimicking the circulatory system. Hence, to overcome this issue, new microfluidic systems that are completely covered with endothelium are gaining immense interest, but nevertheless in comparison to the loop model presented here, they are still limited to accommodate smaller blood volumes and minimal flow rates[38,39]

Thus, we conclude that the Chandler Loop model remains to be a robust model for conducting standardized tests on the blood biocompatibility of vascular medical devices in the field of cardiovascular research.

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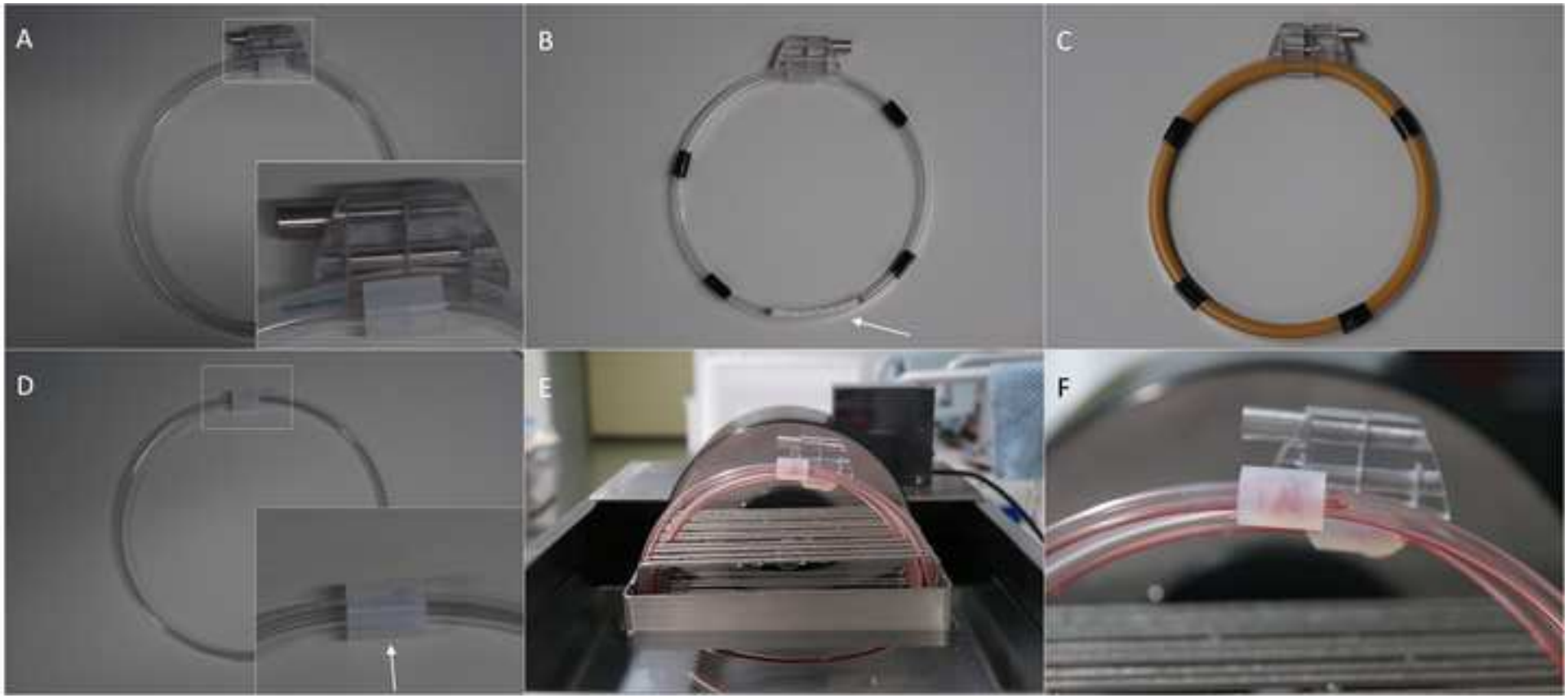

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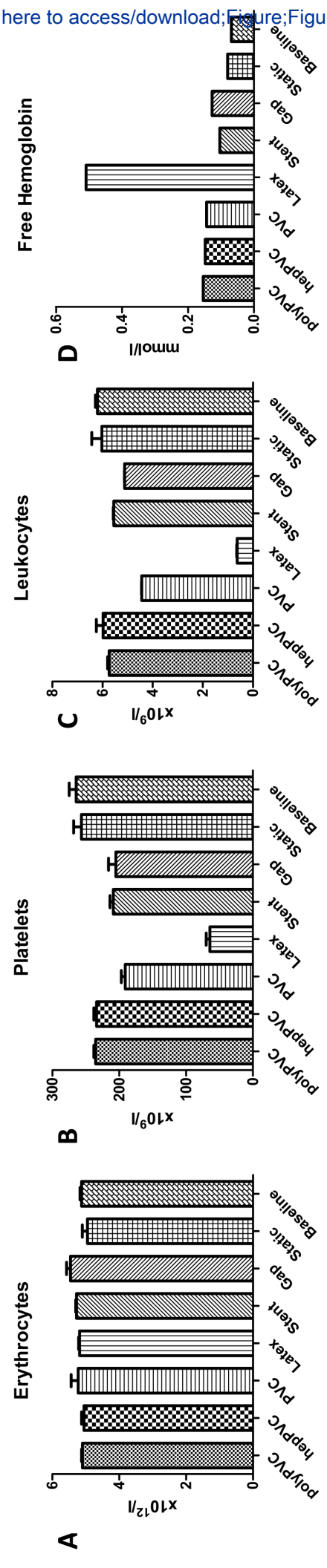

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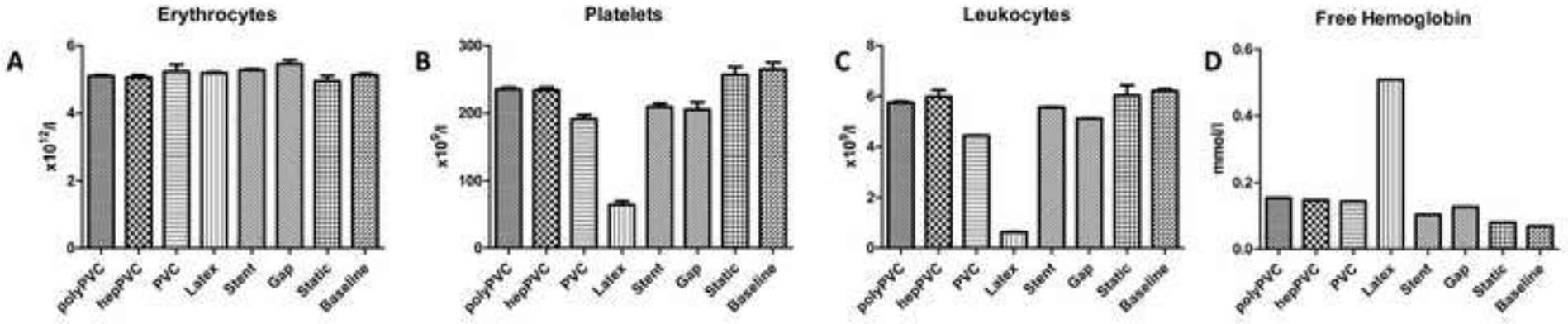

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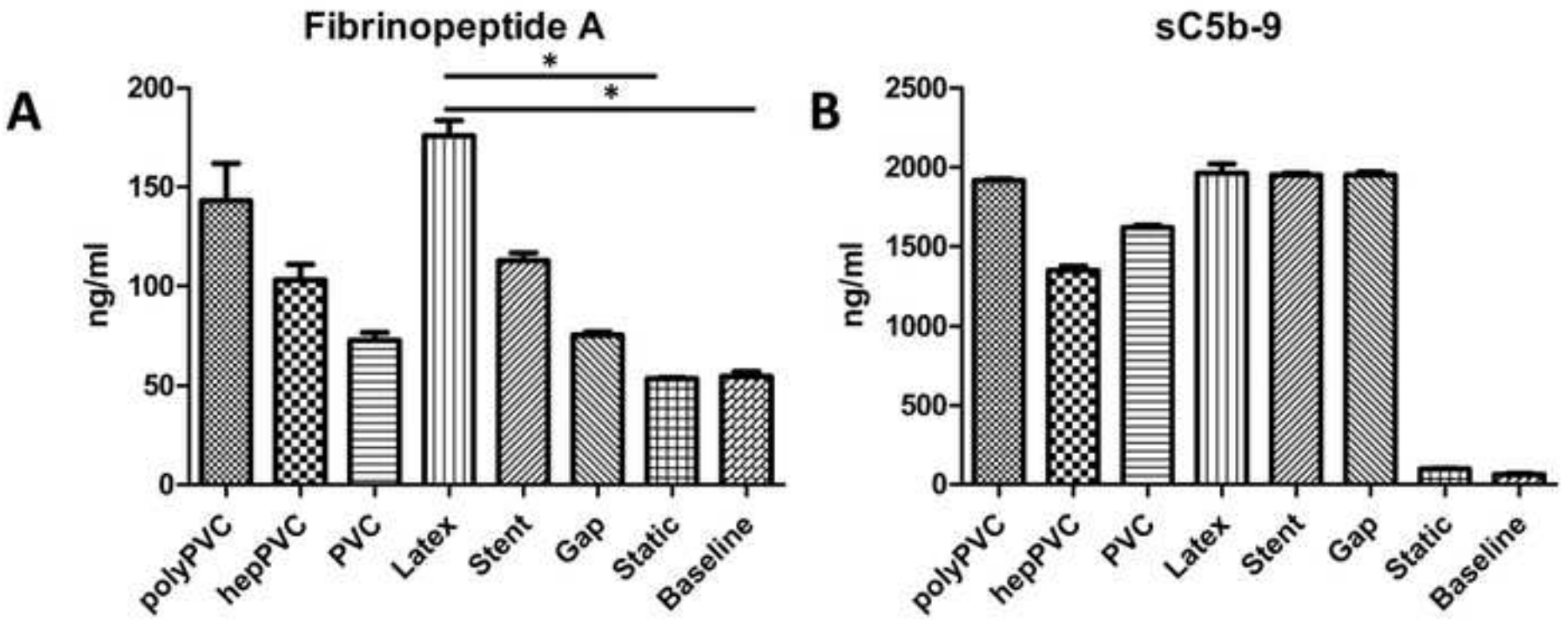

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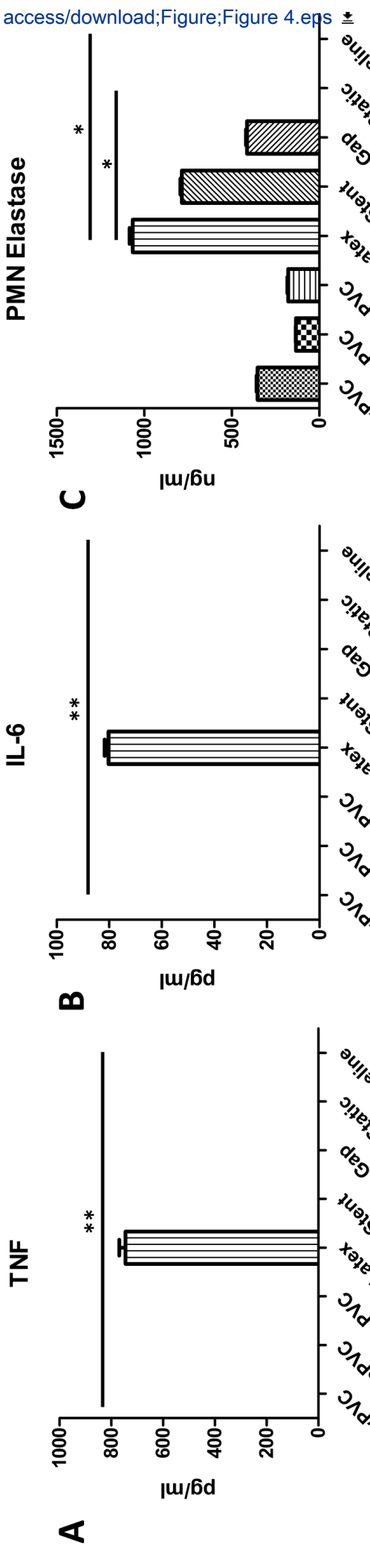

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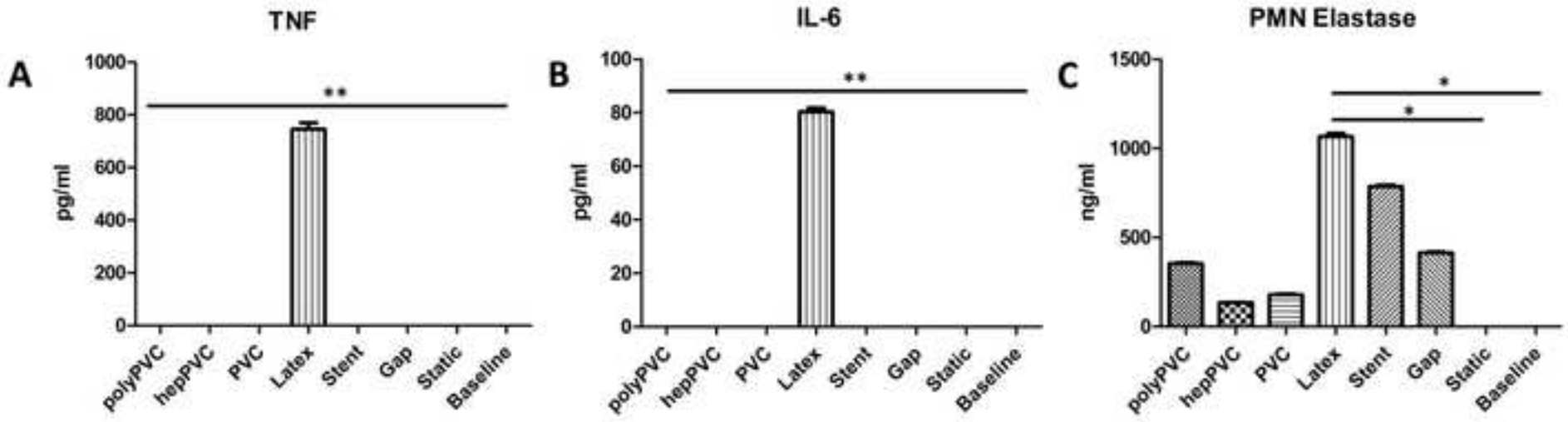

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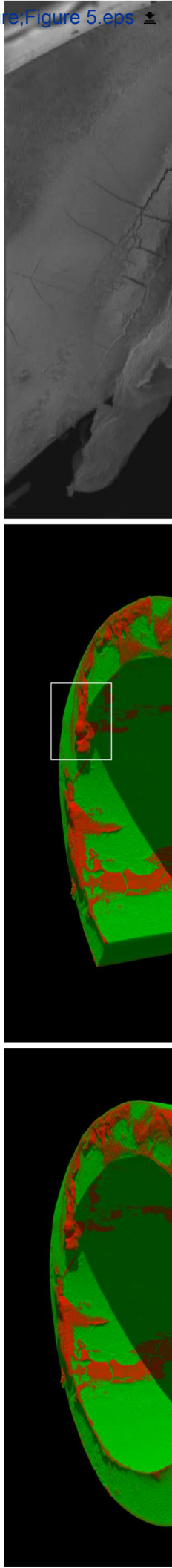

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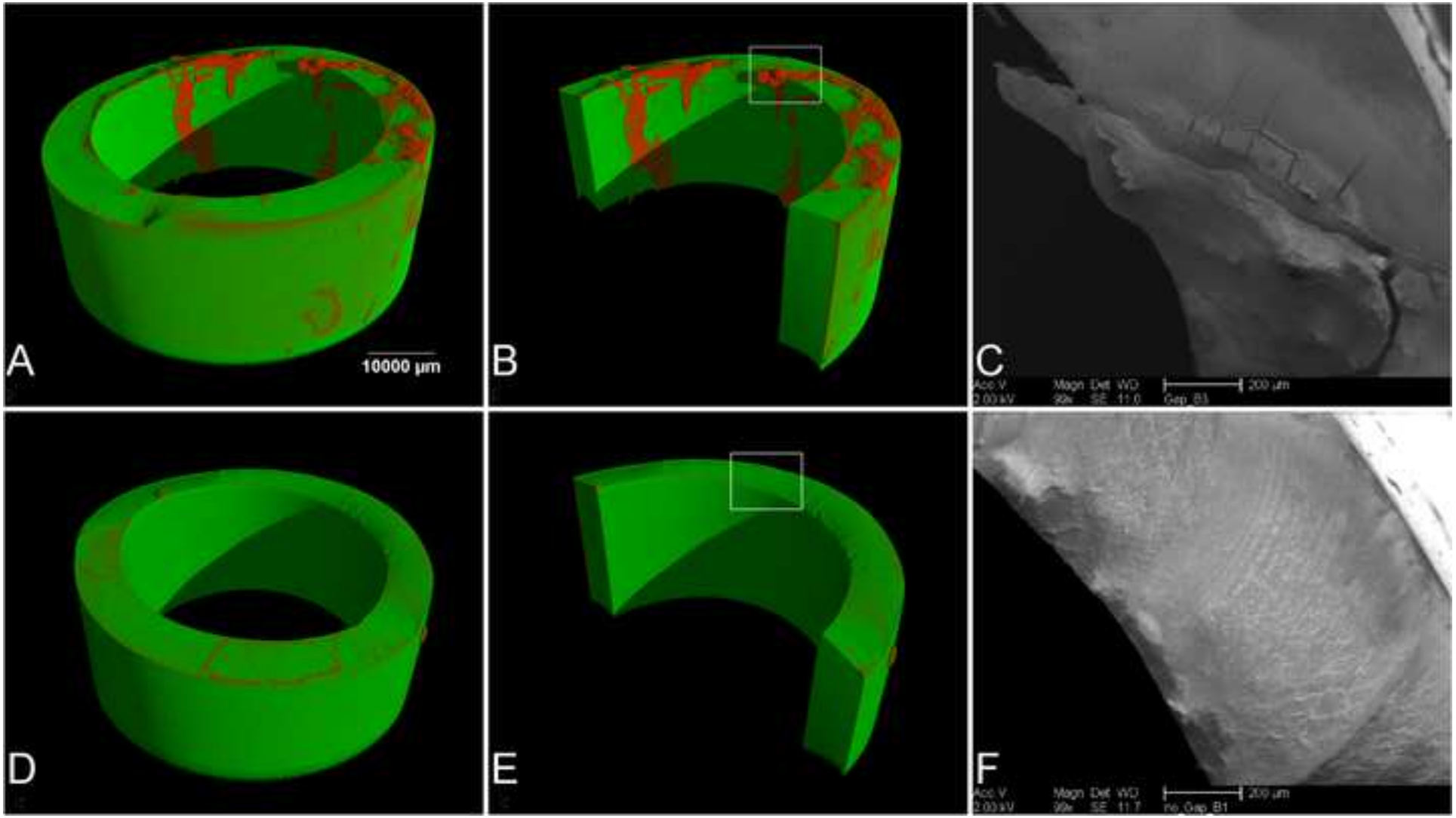

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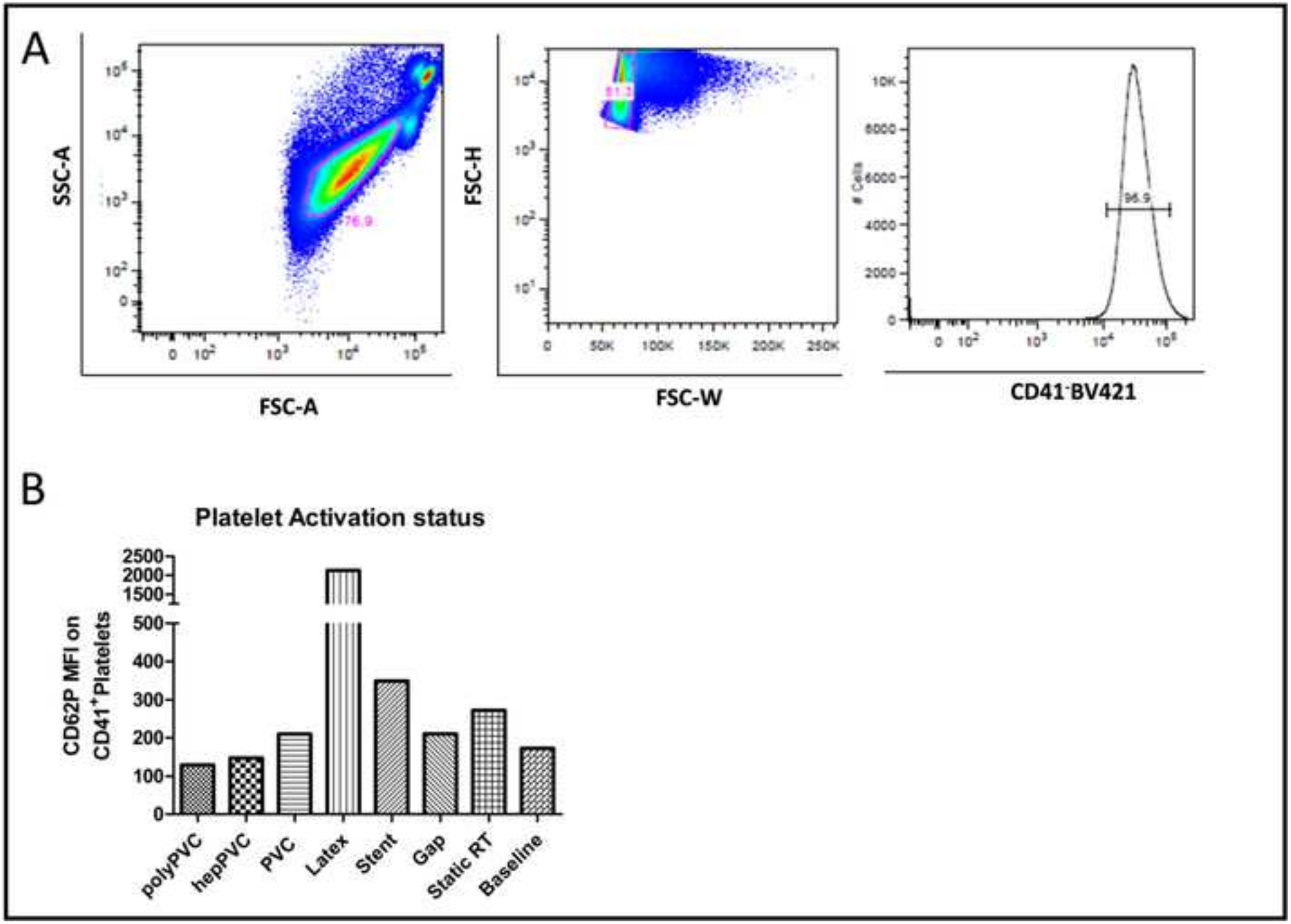

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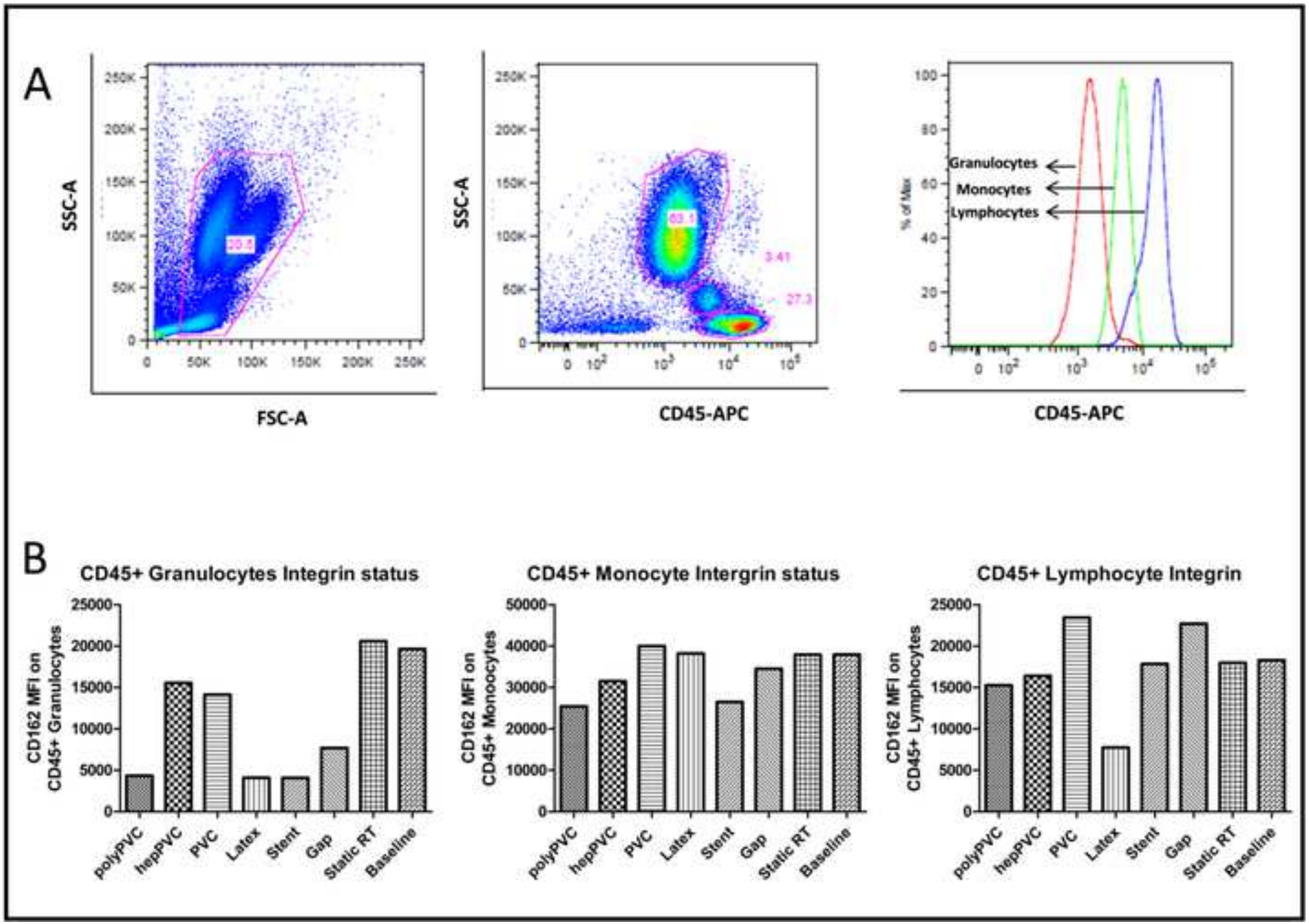

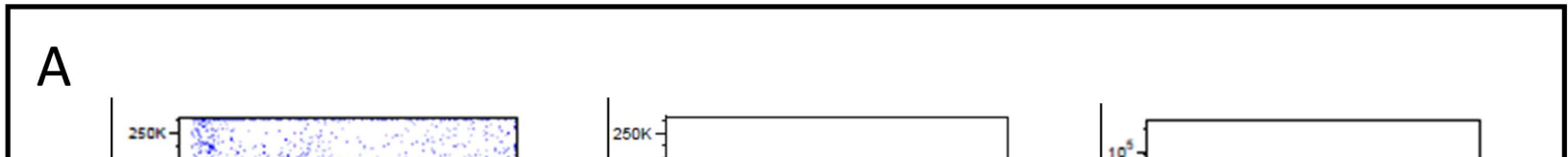

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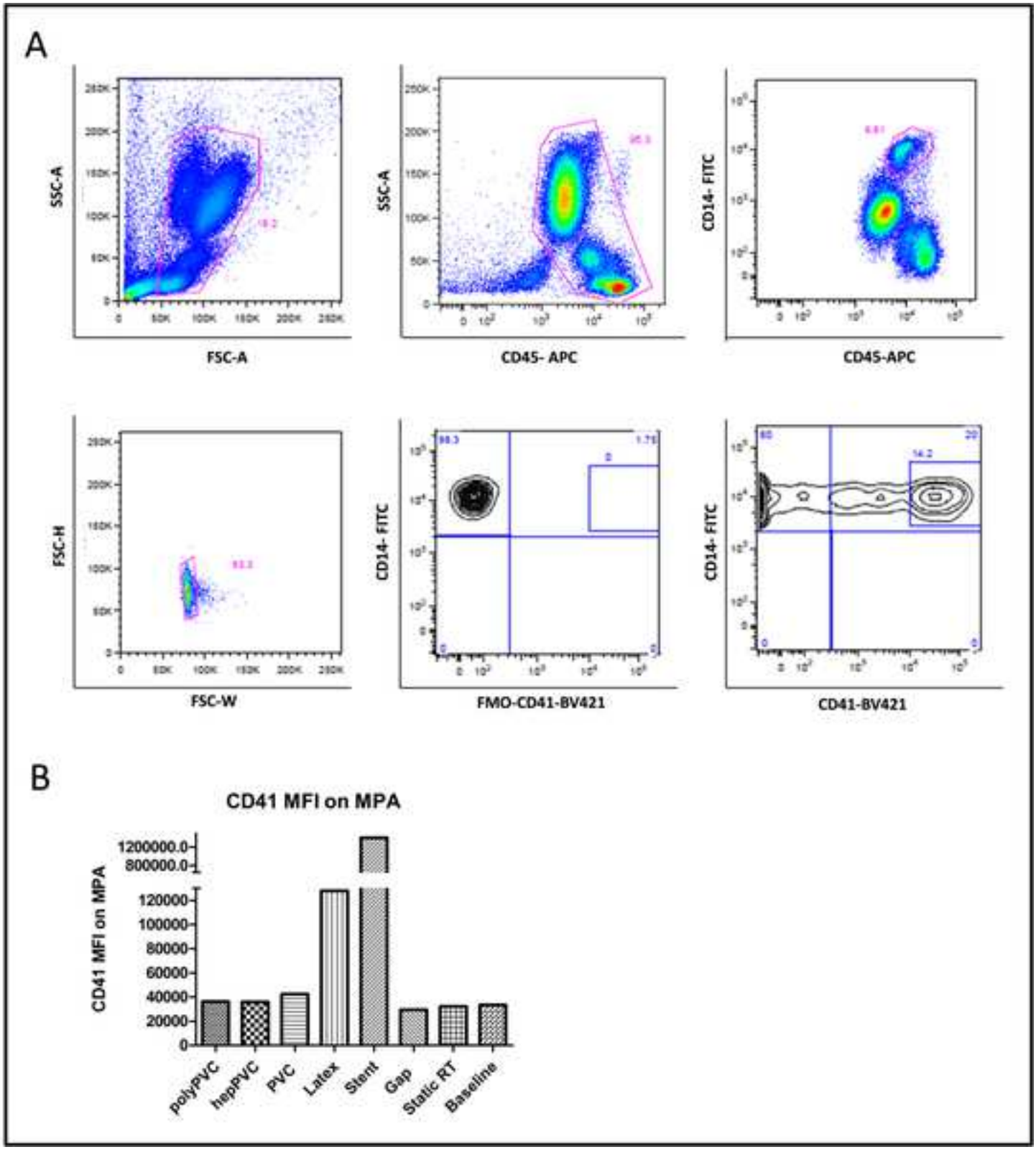


Name of Material/ Equipment

5 ml tube, K3 EDTA
Anti-Mouse Ig, κ/Negative Control Compensation Particles Set
APC anti-human CD45 Antibody
BD LSR Fortessa II cell analyzer
BD Vacutainer Citrate Tubes
BD Vacutainer one-use holder
BD Vacutainer Safety-Lok butterfly canula 21 G
Beaker glass ROTILABO short 10 ml
Beaker glass ROTILABO short 50 ml
Brilliant Violet 421 anti-human CD162 Antibody
Brilliant Violet 421 anti-human CD41 Antibody
Centrifuge ROTINA 420 | 420 R
Centrifuge tubes, 50 ml
CHC Super modified, 5mm PVC tubing
Circular Precision Cutter
Closing Unit (complete with tension bands)
Electric tape Scotch Super 33+
ELISA MAX Deluxe Set Human IL-6
ELISA MAX Deluxe Set Human TNF-a
Eppendorf Pipette Research plus, single channel, inkl. epT.I.P.S. box, 0,1 – 2,5 µL, gray
Eppendorf Pipette Research plus, single channel, inkl. epT.I.P.S. box, 0,5 – 10 µL, gray
Eppendorf Pipette Research plus, single channel, inkl. epT.I.P.S. box, 10 – 100 µL, yellow
Eppendorf Pipette Research plus, single channel, inkl. epT.I.P.S. box, 100 – 1,000 µL, blue
Eppendorf Pipette Research plus, single channel, inkl. epT.I.P.S. box, 20 – 200 µL, yellow
Eppendorf Pipette Research plus, single channel, inkl. epT.I.P.S. sample bag, 0,5 – 5 mL, violet
Ethylenediaminetetraacetic acid solution
FACS tubes polystyrene 5.0 ml round bottom
Fetal bovine serum Gold Plus
FITC anti-human CD14 Antibody
Fluency plus stent 13.5 x 60 mm
Free Hemoglobin fHb Reagent
Gibco PBS Tablets
Gloves Vasco Nitril white L
Gloves Vasco Nitril white M
Glutaraldehyde 25% aequous solution
Heparin, 25.000 IE in 5 ml
Human Fibrinopeptide A (FPA) ELISA Kit
Kodan tincture forte colourless
Latex tube, ID 5 mm
Loop Stand
Medimex venous tourniquet classic
Microplate reader Infinite 200 Pro M Plex
Microplate shaker PMS-1000i
Nalgene Metric non-phthalate PVC tubing, ID 5 mm
NexTemp (Standard) Single-Use Clinical Thermometer
Nunc MaxiSorp ELISA Plates, uncoated
Osmium tetroxide solution

Paraformaldehyde Solution, 4% in PBS
PE anti-human CD16Antibody
PE anti-human CD62P (P-Selectin) Antibody
Pipette controller, pipetus
Pipette tips epT.I.P.S. 0.2 - 5 ml
Pipette tips epT.I.P.S. standard 0,1 - 10µl
Pipette tips epT.I.P.S. standard 2 - 200µl
Pipette tips epT.I.P.S. standard 50 - 1000µl blue
PMN (Neutrophil) Elastase Human ELISA Kit
Probe stand ROTILABO combi
Rack for rotation unit (12 slots 3/8 '' with variable slot width)
RBC Lysis Buffer (10X)
Reagent reservoirs
Rotation Unit
Safe-Lock micro test tubes 0.5 ml
Safe-Lock micro test tubes 1.5 ml
sc5b9 Human ELISA KIT
Scalpel no 10
Scanning electron microscope XL30 ESEM-FEG
Screw top bottle ROTILABO Clear glass, 1000 ml, GL 45
Screw top bottle ROTILABO Clear glass, 500 ml, GL 45
Semi-micro cuvette 1.6 ml
Serological pipette 10.0 ml
Serological pipette, 25.0 ml
Serological pipette, 5.0 ml
Silicon tube, inner diameter 8 mm, outer diameter 12 mm
Sprout mini centrifuge
Stop Solution for TMB Substrate
Swabs, sterile
Syringe, 10 ml
Temperature controlled water basin
tert-Butanol, 99.5%, extra pure, ACROS Organics
TMB Substrate Set
Trillium PVC tube, 5 mm ID
Tween 20
UV-Vis Spektrometer Lambda 2
Vornado Mini Vortexer
XN-3000 workstation blood analyzer
µ-CT Phoenix Nanotom S

Company	Catalog Number
Sarstedt	32332
Becton Dickinson BioSciences	552843
BioLegend	368512
Becton Dickinson	647465
Becton Dickinson	369714
Becton Dickinson	364815
Becton Dickinson	367282
Carl Roth GmbH + Co. KG	X686.1
Carl Roth GmbH + Co. KG	X688.1
BioLegend	328808
BioLegend	303730
Hettich Zentrifugen	4701 | 4706
Greiner Bio-One GmbH	227261
Corline Sweden	1807-148
ebo kunze industriedesign, Neuffen, Germany	CLS 007-20
ebo kunze industriedesign, Neuffen, Germany	CLS 008-20
VWR	MMMA331933
BioLegend	430504
BioLegend	430204
Eppendorf AG	3123000012
Eppendorf AG	3123000020
Eppendorf AG	3123000047
Eppendorf AG	3123000063
Eppendorf AG	3123000055
Eppendorf AG	3123000071
Sigma-Aldrich	03690-100ML
Corning BV	352052
Bio-Sell	FBS.GP.0500
BioLegend	367116
Angiomed GmbH & Co	FVM14060
Bioanalytics GmbH	004001-0250
Thermo Fisher Scientific	18912014
B. Braun Deutschland GmbH & Co.KG	9208437
B. Braun Deutschland GmbH & Co.KG	9208429
Sigma Aldrich	G6257-100ML
Rotexmedica, Trittau, Germany	PZN 3862340
Hölzel Diagnostika	abx253234
Schülke & Mayr GmbH	104012
Laborhandel24 GmbH	305 0507
ebo kunze industriedesign, Neuffen, Germany	CLS 009-20
ROESER Medical GmbH	310005
Tecan	TEC006418I
VWR	444-0041
VWR	NALG8703-0508
Medical Indicators	2112-20
BioLegend	423501
Fisher Scientific	10256970

Thermo Fisher Scientific	AAJ19943K2
BioLegend	302008
BioLegend	304906
VWR	612-1874
OMNILAB-LABORZENTRUM GmbH & Co. KG	5186480
Th. Geyer GmbH & Co. KG	9409410
Th. Geyer GmbH & Co. KG	0030 000.870
Th. Geyer GmbH & Co. KG	0030 000.919
Fisher Scientific	BMS269
CARL ROTH	K082.1
ebo kunze industriedesign, Neuffen, Germany	CLS 011-20
BioLegend	420301
VWR	613-1184
ebo kunze industriedesign, Neuffen, Germany	CLS 010-20
OMNILAB-LABORZENTRUM GmbH & Co. KG	5409320
OMNILAB-LABORZENTRUM GmbH & Co. KG	5409331
TECOmedicalGroup	A029
Fisher Scientific	NC9999403
Philips	n.a.
Carl Roth GmbH + Co. KG	X715.1
Carl Roth GmbH + Co. KG	X714.1
Sarstedt	67.746
Corning BV	4488
Corning BV	4489
Corning BV	4487
VWR	BURK8803-0812
Biozym	552034
BioLegend	77316
Fuhrmann GmbH	32055
Becton Dickinson	300296
ebo kunze industriedesign, Neuffen, Germany	CLS 020-20
Fisher Scientific	10000730
BioLegend	421101
Medtronic	161100107100103
AppliChem	A4974,0250
Perkin Elmer	33539
Biozym	55BV101-B-E
Sysmex Europe	n.a.
GE Sensing & Inspection, Wunstorf, Germany	n.a.

Comments/Description

Referred to as hepPVC tube

Referred to as PVC tube

Referred to as polyPVC tube

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The whole manuscript was thoroughly proofread and spelling or grammer issues were corrected.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.

The paragraph indentation has been changed to 0 for left and right, and line spacings are single. Further, a single line space between each step and substep and note in the protocol section has been added. The font is Calibri 12 points.

3. Please define all abbreviations during the first-time use.

All abbreviations have been defined during the first use.

4. Please make the title concise to reflect the technique being presented and remove any punctuation marks i.e., colons etc.

The title was changed to “An in vitro hemodynamic loop model to investigate the hemocytocompatibility and host cell activation of vascular medical devices“

5. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "Chandler Loop" within your text. The term can be introduced once or twice but use generic term throughout.

The authors agree with the editor and apologize for using the term Chandler Loop all over the manuscript. As the name of the model goes back to the researcher A.B. Chandler, it was not the author´s intention to present a technique as an advertisement for a specific item. We therefore removed the word Chandler Loop and replaced it with generic terms throughout the manuscript except for one sentence each in the introduction and discussion part.

6. Please remove the Chandler Loop from the keywords.

The keyword “Chandler Loop” was removed.

7. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Presented here is a protocol …"

The short summary has been rephrased and is now within the word limit.

8. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

The long abstract was revised to clearly state the goal of the protocol and is within the word limit.

9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

The protocol section has been revised and is now written throughout in the imperative tense. All phrases as "could be", "should be" etc. were removed and sentences were rephrased. Any text that could not be written in the imperative tense was added as a note.

10. The Protocol should contain only action items that direct the reader to do something.

The protocol section has been revised and sections without action items have been removed.

11. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

The protocol section has been revised and steps containing more than 3 action steps have been divided .

12. Please ensure you answer the "how" question, i.e., how is the step performed?

The authors have revised the whole protocol section and are now confident that the "how" question is addressed in each step.

13. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

The protocol was adjusted to fit inside the 10-page limit including all headings and spacing. Futhermore, 2.75 pages have been highlighted with yellow colour for video production. Headings and spacings included, the yellow highlighted text is now within the 2.75-page limit (121 lines max.).

14. Please ensure the results are described in the context of the presented technique. Please ensure all figures are discussed in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.

All figures are discussed in the representative results, and figure 1 parts showing the experimental setup were referenced in the protocol section.

15. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

The authors would like to state that all figures submitted along with this manuscript are under the copyright of the corresponding author Max Wacker. No figures have been modified from other publications.

16. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

The discussion has been revised and all issues raised by the editors (a-e) have been covered by the discussion.

17. Please do not abbreviate the journal titles in the references section.

The journal titles have been written in full length in the reference section.

18. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg).

The images were submitted as .eps files.

Reviewers' comments:

Reviewer #1:

1. The authors indicate the flow velocity of 25 cm/s, however, shear rate and shear force are further dependent on the diameter of the tube, which is not indicated. The authors correctly mention, that calculation of shear forces and shear rate in Chandler loop is still not possible, however, they should provide references to the relevant literature.1-3 Even a vague estimation of the shear rate in this manuscript might be more informative than the indicated flow velocity.

1. Gardner R.A., An examination of the fluid mechanics and thrombus formation time parameters in a Chandler rotating loop system. J. Lab. Clin. Med. 84, 494-508 (1974).
2. Touma H., Sahin I., Gaamangwe T., Gorbet M.B., Peterson S.D., Numerical investigation of fluid flow in a chandler loop. J. Biomech. Eng. 136, (2014).
3. Gaamangwe T., Peterson S., Gorbet M., Investigating the Effect of Blood Sample Volume in the Chandler Loop Model: Theoretical and Experimental Analysis. Cardiovascular Engineering and Technology 5, 133-144 (2014).

The authors agree that indicating the wall shear stress would be interesting for the readers. We therefore estimated the wall shear stress, based on a model for laminar boundary layer (see discussion). This estimation would result in values between 0.01 and 22.00 pascal, depending on the distance tot he wall (1.00-0-0.01 mm). The references suggested by the reviewer were also implemented in the discussion part and the diameter of tubings were given (ID 5 mm).

2. The authors state (as their ref [7]) that the tube diameter affects the activation processes in blood. It is more likely, that shear forces are the driving parameters. The authors should express it that way.

The authors agree with the reviewer and rephrased the sentence accordingly.

3. In the introduction, line 101, the authors state that there is "no contact between blood and any other material except the material to be tested". This statement does not consider the interface with air (at the cross-sections of the loop and blood film at the loop surface), which has very high interface energy, leading to protein denaturation.4,5 The interface with air appears correctly in the discussion, but the sentence in the introduction should be adjusted.

4. Leiske D.L., Shieh I.C., Tse M.L., A Method To Measure Protein Unfolding at an Air-Liquid Interface. Langmuir 32, 9930-9937 (2016).
5. Maa Y.F., Hsu C.C., Protein denaturation by combined effect of shear and air-liquid interface. Biotechnol. Bioeng. 54, 503-512 (1997).

The authors agree with the reviewer. The sentence in the introduction has been changed accordingly.

4. The authors mention roller pump systems as air-free systems. In this context also the ball-valve ('hemobile') system should be mentioned expressively.6

> **van Oeveren W., Tielliu I.F., de Hart J., Comparison of modified chandler, roller pump, and ball valve circulation models for in vitro testing in high blood flow conditions: application in thrombogenicity testing of different materials for vascular applications. Int J Biomater 2012, 673163 (2012).**

The authors agree and the ball-valve system has been mentioned in the discussion part.

5. The authors compare different tube materials. These materials have different gas permeability, as also mentioned by the authors in the discussion. Different CO2 permeability may also lead to different blood pH during incubation. As coagulation is highly sensitive to pH, a pH-shift may cause misleading results.7-9 The reviewer generally suggests incubation in a CO2 incubator and blood gas/pH validation aufter incubation.

> 7. Foley M.E., McNicol G.P., An in-vitro study of acidosis, platelet function, and perinatal cerebral intraventricular haemorrhage. Lancet 1, 1230-1232 (1977).
> 8. Engström M., Schött U., Romner B., Reinstrup P., Acidosis impairs the coagulation: A thromboelastographic study. J. Trauma 61, 624-628 (2006).
> 9. Dirkmann D., Hanke A.A., Gorlinger K., Peters J., Hypothermia and acidosis synergistically impair coagulation in human whole blood. Anesth. Analg. 106, 1627-1632 (2008).

The authors agree with the reviewer, that significant changes in blood pH can be the result of different gas permeabilities of the tested tubes, and incubation in a CO_2 incubator and pH validation might help to further standardize this method. However, with this manuscript, it was the author's intention to test the blood compatibility based on the natural properties of the materials that were tested under real life conditions, mimicking a clinical scenario of extracorporeal perfusion of blood under room air. We thank the reviewer for this comment and implemented this issue further in the discussion part of the manuscript, including the references given by the reviewer and giving suggestions for future improvements of the presented model by incubation in a CO_2 chamber.

6. Method Section 2: Authors indicate the length of the tube, but not its diameter. Without this information, blood volume and rotation speed are useless data. (The value 5mm appears only in the materials list)

The information of the inner diameter has been added.

7. Method 1.6: "collect blood in a beaker glass" - Glass is highly activating and used by some groups as the positive control.10

10. Streller U., Sperling C., Hübner J., Hanke R., Werner C., Design and evaluation of novel blood incubation systems for in vitro hemocompatibility assessment of planar solid surfaces. J. Biomed. Mater. Res. B Appl. Biomater. 66B, 379-390 (2003).

The authors agree with the reviewer, that glass is potentially highly activating the coagulatory system, as demonstrated by Streller et al. However, in this manuscript, the results clearly show that incubation in a beaker glass did not lead to activation of the plasma or cellular components of the coagulatory system. Therefore, the authors did not change the protocol, but mentioned this issue in the discussion part of the manuscript.

8. Method Section 1: Heparin: Is there any preference for unfractionated or low molecular weight heparin?

We normally work with unfractionated heparin, because the monitoring of heparinization is possible if needed in other setups by measuring the activated clotting time. The information that the heparin used in this study was unfractionaed heparin has been added to the manuscript in the respective part of the method section.

9. Method section 6: The authors obviously do not describe an Enzyme Linked Immunosorbent Assay (ELISA), as stated in 6.2. - The fHb kit is not mentioned in the equipment list.

The authors apologize for this mistake. The method part has been changed accordingly. Here, only the fHb reagent is necessary, and indeed, no kit is needed. The fHb reagent has been added to the materials list.

10. Abstract: The statement "is a reproducible method" appears better as a conclusive than as an introductory statement.

The authors agree and the abstract has been changed accordingly.

11. The stent is not further specified, whether bare-metal or drug-eluting stent. The terms "polyPVC", "hepPVC", PVC do not appear in this form in the materials table.

The authors added the information on the stent material in the introduction and further specified the acronyms polyPVC, hepPVC and PVC in the materials table.

12. Line 436f: The label 'FMO-CD41' appears twice.

The second appearing FMO-CD41 was a typo error instead of FMO-CD162, which was used for the leukocyte integrin panel. We thank the reviewer for pinpointing this error.

Reviewer #2:

1. The procedure for blood collection is carefully described (1.5) and it is mentioned that the blood donors should not be on medication. However, it should be emphasized that this also

includes NSAID and that medication should have been withdrawn for at least 10 days (=the normal life span of platelets in the circulation).

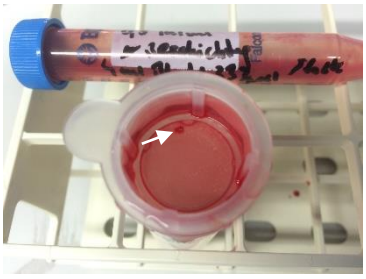
The respective information has been added in the method section.

2. I do not agree that it is preferred to use the same donor when testing different devices. Obviously different compounds (devices, tubings etc) should be tested together simultaneously as is done in this paper, but testing should then be repeated using blood from different donors. The reason for this is that the plasma cascade systems differ also between healthy individuals, so information obtained from more than one donor will give more realistic information about the response during the intended use (i.e, during medical treatment).

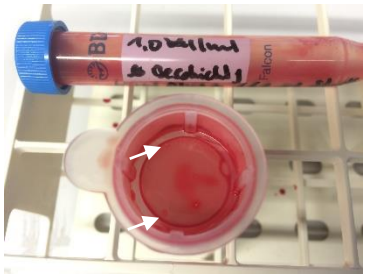
We completely agree with the reviewer concerning the investigation on different donors, due to their differences in the plasma cascade system. However, in this study, we are evaluating these medical devices for the first time and therefore as a first step procedure we expected different outcomes while compare the different types of medical devices. To ensure the fact that the differences in the result outcome are mainly due to the devices and not due to the donor sample, we have utilized the blood samples from a same donor and thereby kept one parameter as constant (i.e., blood samples from the same donor, in this case). Henceforth, we believed that the differences in the outcome are mainly mediated by the devices. Thereafter, as a next step procedure and in the subsequent future studies, we will definitely consider to evaluate different donors on the investigative medical devices that exhibited a best outcome from this study. Therefore, we changed the “Note” paragraph under 1.5 respectively.

For the experiments I wonder why the rather high concentration of heparin (1.5 IU/ml) and the long incubation time (3h) were chosen. It is possible to get a more sensitive system by lowering both parameters.

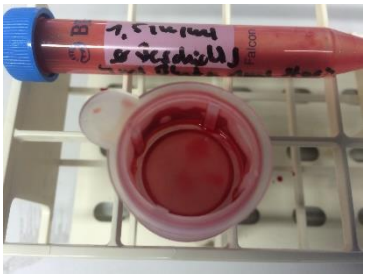
Although we might achieve more sensitive system by reducing both the parameters, we have selected the parameters with respect to heparin concentration of 1.5 IU/ml and time point that was based on our own very recently accepted article, where the hemocompatibilities of different donor samples with different types of in-house vascular prosthesis have been evaluated. Here, a constant heparin concentration of 1.5 IU/ml was used to curtail the inherent variability in coagulation capacity from different donors. Here, we tested different heparin concentrations from 0.5-1,0 IU/ml and found that sometimes clotting occurred even in the 1.0 IU/ml group, but 1.5 IU/ml seemed to be the lowest concentration to provide a proper heparinization (see image, white arrows indicating thrombi). We therefore adapted the same heparin concentration for the current study.



0.5 IU/ml



1.0 IU/ml



1.5 IU/ml

However, we took away the word “moderate” in the context with heparin concentration of 1.5IU/ml in the main text. Furthermore, the time (in hours) was also selected based on our very recently accepted article, where we used the time points 2 and 4 hrs to evaluate the hemocompatibilities, where we observed no obvious changes in 2 hrs but could observe concrete changes at 4hrs. Therefore, our recently accepted study remained as a base to select a time-point of 3 hrs as we hoped to visualize any obvious changes with the blood to the materials that are being currently tested in this study. This study will soon be published in PLOS ONE (title: In vitro hemo- and cytocompatibility of bacterial nanocelluose small diameter vascular grafts: Impact of fabrication and surface characteristics.).

4. Please state the recommended brand of the heparin since preparations differ in function.

The brand of the heparin has been added in the method section and is also available in the materials list.

5. It is stated that the blood is pooled in a glass beaker prior to the experiment (1.6, 2.2.11) and after termination (3.3). Glass is a very potent activator of coagulation and platelets so I suggest to use vials of inert polymers such as polypropylene. This protocol should also give a recommended time frame between drawing the blood and starting the experiment. In our lab we aim at 5 min in order to minimize preactivation of the blood.

The authors agree with the reviewer, that glass is a potent activator of coagulation and platelets, as this issue was raised by the second reviewer. However, as presented in this manuscript, the authors did not see excessive activation of the coagulatory system for both plasma and cellular components, as well as complement activation compared to baseline levels, indicating that the collection in a beaker glass did not lead to activation. But, as this issue might be important for others reading the manuscript, the authors implemented a paragraph on this issue in the discussion.

6. The original reference describing the CHC corline should be given since this surface is distinct from other heparin surfaces (Larm, et al Biomaterials Medical Devices, and Artificial Organs, 1983; 11:2-3, 161-173. Furthermore, the intert CHC-surface was utilized to optimize and characterize different parameters (incubation time, size of air bubble in the loop etc) in a Chandler loop system (Gong, et al. J Clin Immunol, 1996; 16 (4) 222), which should also be included in the reference list.

The authors agree with the reviewer that the original references regarding the CHC surface should be given in the manuscript. The references were added to the introduction part.

7. I don't understand the rationale for pooling the blood from duplicate samples instead of running the analyses in parallel (3.3). Multiple analyses will give more information about the accuracy of the loop model and the analytical techniques.

Yes, we do agree with the reviewer that running the analysis in parallel would give accuracy of the loop. But, we have pooled the samples for two main reasons: (i) we intend, mainly, to save the plasma samples by pooling them and running the samples in duplicates as we had to measure 5 different analytes using ELISA, the technique that consumes quite some volume of the samples. Moreover, we also have saved some plasma, if any repetitions were required; (ii) during the statistics, the data are normally represented as mean value (averaged) of a respective group. Henceforth, we had pooled the samples that were already averaged even before the commencement of measurements, which were again run in duplicates.

8. Why is citrate and not EDTA used to prepare the blood for FACS analysis after termination of the experiment? I would assume that it would save some time to use EDTA for both cell counting and preparation of the FACS samples. Also, it is easy (and much cheaper) to prepare a stock solution of EDTA (0.2M, titrated to pH 7.4) and add directly to the blood after finishing the experiment.

For many years, citrate was used as anticoagulant for platelet based research and is therefore preferred by most investigators undertaking platelet studies. This was the main reason for us to choose citrate as anticoagulant of choice for FACS studies, involving platelets. Moreover, EDTA is known to induce platelet activation by influencing the platelet membrane dynamics and since platelet remains to be one of the main target cell population in our current study, we used citrate.

Handling of plasma samples, especially when measuring products of cascade system activation markers such as FPA and sC5b-9 (7, 8) as is done here is of utmost importance. Rapid freezing at -80°C (3.6.5) is mandatory but the condition for thawing is equally important. Quick thawing in a 37°C water bath and immediate transfer to ice (containing some water) to lower the temperature. Information should be added.

The authors agree with the reviewer on this issue. The respective information has been added as a note.

10. Figs 2, 3, 4: please indicate the numbers of experiments since this will give indication of the accuracy of the methods. Also, state in the legends to Figs 6, 7, 8 which is the selected "representative plot" which is presented in each figure. Presumably the one with the highest bar. In these figures I assume that the bars are from single measurements but it should be pointed out.

The number of experiments has been added to the figure legends of figure 2,3 and 4 (N=1). Here, we would like to state that each loop was already run in duplicates, thus, the pooled blood is already a duplicate.

Furthermore, the information on the selected representative FACS plot has been given in each figure. Since the complete FACS gating strategies were optimized with the baseline parameter where the cell population is very distinct and clear, we have used the baseline of donor blood as representative FACS plots. The reviewer is correct, the bars are from single measurements. This has been included in the figure legend.

11. At the end of the discussion (line 625) it is appropriate to cite Nordling et al, J. Vis. Exp. (93), e52112 (2014).

The authors thank the reviewer for suggesting this interesting study. We therefore added it as a reference at the end of the discussion.

12. TNF-alfa is today only called TNF.

TNF-α was changed to only TNF all over the manuscript.

13. Please check the cross-references to the numbered sections, there are some inconsistencies, in particular on page 4 (lower part).

The authors apologize for the mistakes. All cross references have been checked.

3. Line 294: the word "of" is repeated.

The duplicate has been removed.