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**Evaluation of platelet adhesion and activation on polymers:
Round-robin study to assess inter-center variability**

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Evaluation of platelet adhesion and activation on polymers: round-robin study to assess inter-center variability

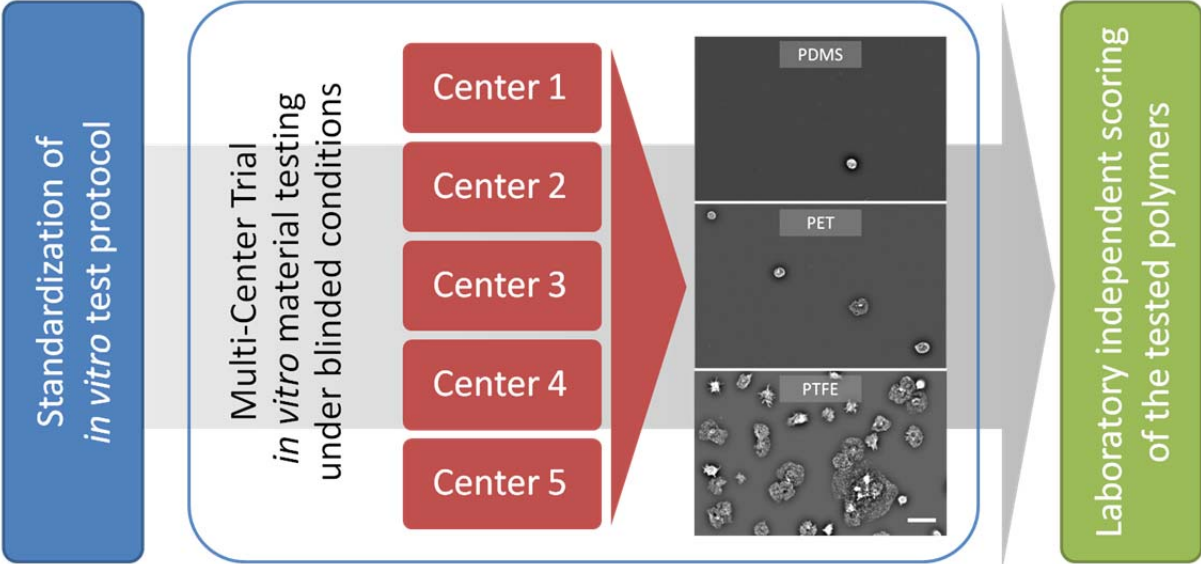
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Graphical abstract

Inter-center variability in the *in vitro* evaluation of platelet adhesion and activation on polymers



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Abstract

The regulatory agencies provide recommendations rather than protocols or standard operation procedures for the hemocompatibility evaluation of novel materials e.g. for cardiovascular applications. Thus, there is a lack of specifications with regard to test setups and procedures. As a consequence, laboratories worldwide perform *in vitro* assays under substantially different test conditions, so that inter-laboratory and inter-study comparisons are impossible. Here, we report about a prospective, randomized and double-blind multicenter trial which demonstrates that standardization of *in vitro* test protocols allows a reproducible assessment of platelet adhesion and activation from fresh human platelet rich plasma as possible indicators of the thrombogenicity of cardiovascular implants. Standardization of the reported static *in vitro* setup resulted in a laboratory independent scoring of the following materials: poly(dimethyl siloxane) (PDMS), poly(ethylene terephthalate) (PET) and poly(tetrafluoro ethylene) (PTFE). The results of this *in vitro* study provide evidence that inter-laboratory and inter-study comparisons can be achieved for the evaluation of the adhesion and activation of platelets on blood-contacting biomaterials by stringent standardization of test protocols.

Introduction

Clinical use of cardiovascular devices, such as synthetic vascular grafts and stents, is continuously increasing along with concerns on the hemocompatibility of these implants [1–5]. The hemocompatibility evaluation of novel materials for cardiovascular applications has to fulfill the ISO standard 10993 Part 4 [6–8]. Since the regulatory agencies provide recommendations rather than protocols or standard operation procedures, definitive specifications how the test should be performed are lacking [9,10]. This has led to a situation that laboratories worldwide perform *in vitro* assays under substantially different test conditions, so that inter-laboratory and inter-study comparisons are impossible [11].

The lack of defined and certified positive or negative standards and experimental protocols does not allow a conclusion if a material is superior to another. Hence, the literature is full with claims for “new” or “better” biomaterials based on the wide variety of tests, performed under static, venous or arterial shearing conditions, which have not undergone *in vitro* side by side comparisons to a standard [12–16]. If such *in vitro* studies would be performed with accepted reference materials and according to standardized protocols, respective reports could contribute in a meaningful way to a database, which ultimately would provide the basis for a comparison of the candidate implant materials [17].

To demonstrate that platelet adhesion and activation on material surfaces can be assessed in different laboratories consistently, a round-robin study was performed. The same standardized static *in vitro* test with fresh human citrated platelet-rich plasma was performed in all centers and was coordinated in detail before the start of the study to ascertain whether differences between materials can be detected over the “noise of inter-center variability”.

Material and Methods

Study design

This *in vitro* study was performed in 2015 at 5 centers in Germany. The *in vitro* study protocol was reviewed by the ethics committees or investigational review boards at each participating site. The study was designed as a randomized and double blinded multicenter *in vitro* study and was performed according to the guidelines of the International Society on Thrombosis and Haemostasis and British committee for standards in haematology [18–20]. Randomization and blinding was performed utilizing the Research Randomizer software

(Version 4.0) in another participating center by researchers, who were not involved in the experiments [21]. *In vitro* tests, microscopy and image-based data analyses were carried out under blinded conditions. Material samples - prepared by each participating test center - were shipped to a reading center for microscopic and image-data analysis. Microscopy was carried out within 4 weeks after receipt of the sample. Subsequently, to the collection of all data sets, results were unblinded for statistical analysis. For each donor, samples were tested in duplicate.

Polymer substrates

Table 1 summarizes physical-chemical characteristics of the three tested polymers, which include medical grade poly(dimethyl siloxane) (PDMS, Bess Medizintechnik GmbH, Berlin, Germany), poly(ethylene terephthalate) (PET, Nalge Nunc International, New York, USA) and poly(tetrafluoro ethylene) (PTFE, Bess Medizintechnik GmbH, Berlin, Germany).

Table 1. Root mean square surface roughness and advancing water-air contact angle measurements of the tested polymers.

Polymer	Root mean square surface roughness (R_q in nm) n=3	Advancing water-air contact angle (Θ_{adv} in $^\circ$) n=10
Poly(dimethyl siloxane)	14 ± 2	109 ± 2
Poly(ethylene terephthalate)	24 ± 2	79 ± 1
Poly(tetrafluoro ethylene)	62 ± 6	119 ± 2
One-way ANOVA	P<0.01	P<0.01

Measurements of the dynamic contact angles were carried out subsequent to a preconditioning phase (24 hours) in ultra-pure deionized water (conductivity of $0.055 \mu\text{S}\cdot\text{cm}^{-1}$) and at ambient temperatures. Advancing contact angles (Θ_{adv} in $^\circ$) were determined under the same conditions by using the captive bubble method (DSA 100, Krüss GmbH, Hamburg, Germany) [22]. Values were recorded on at least six positions with ten repeating measurements in total (values given here represent the respective arithmetic mean and standard deviation).

Surface roughness of the three polymers was characterized by atomic force microscopy (MFP-3D, Asylum Research, Santa Barbara, USA) [23,24]. The polymer samples were immersed in water and scans (area: $60 \times 60 \mu\text{m}^2$) were performed at ambient temperatures in dynamic tapping-mode (scan rate: 0.8 or 1.0 Hz) and on three positions (values are given as arithmetic mean and standard deviation) utilizing an OMCL-AC200TS-R3 silicon cantilevers

(Olympus, Tokyo, Japan). Root-mean-square roughness values (R_q) were calculated for each image (Igor Pro 6.22A software, WaveMetrics, Inc., Portland, OR, USA).

Table 2. Demographical and laboratory data of the participating donors.

Parameter	Unit	Center 1	Center 2	Center 3	Center 4
Female/Male		7/3	5/5	3/7	5/5
Age	Years	35.0±7.0	32.8±8.2	24.8±3.2	28.2±4.3
Height	m	1.72±0.08	1.74±0.08	1.75±0.07	1.78±0.11
Weight	kg	67±9	71±10	96±10	70±14
Body mass index		22.4±2.5	23.5±2.8	22.2±1.7	22.0±2.3
Systolic blood pressure	mmHg	116±12	126±13	119±13	123±13
Diastolic blood pressure	mmHg	71±10	78±8	79±10	71±9
Heart rate	bpm	65±8	68±7	n.a.	66±10
White blood cell count	Gpt·L ⁻¹	5.9±1.9	5.1±0.9	5.6±1.0	5.8±1.1
Red blood cell count	Tpt·L ⁻¹	4.6±0.3	4.23±0.4	4.8±0.3	5.1±0.7
Hemoglobin	mmol·L ⁻¹	8.4±0.7	7.9±0.8	8.7±0.7	8.8±1.1
Hematocrit	%	39.0±2.1	38.7±3.6	41.4±3.6	42.7±4.6
Mean red blood cell volume	fL	85.1±3.9	91.5±3.4	86.7±3.0	84.0±5.2
Platelet count	Gpt·L ⁻¹	218.8±36.9	199.5±61.2	226.4±42.7	256.0±35.8
Mean platelet volume	fL	11.1±0.9	8.45±0.7	6.78±0.4	10.1±1.0
CD41 ⁺ /42 ⁺ & CD62P ⁺ events	% in WB	5.5±5.3	3.4±2.1	14.1±8.4	11.5±5.4
CD62P mab concentration	µg·mL ⁻¹	0.2	0.2	0.4	0.4
CD62P mab supplier		BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences
CD62P mab clone		AC1.2	AK-4	AC1.2	AC1.2
Flow cytometer supplier		Miltenyi	BD Biosciences	Be. Dickinson	BD Biosciences,
Flow cytometer model		MACSQuant	FACSCalibur	FACScan	FACSCanto II

Study population

In each test center, blood was taken from ten apparently healthy subjects (according to criteria of the Nordkem-Workshop). Only donors, which were free from platelet function affecting medication for at least 10 days (such as: inhibitors of the prostaglandin metabolism e.g. Aspirin; platelet surface receptor blocker e.g. Clopidogrel; calcium availability reducing substances e.g. intensely consumed herbal medicines (e.g. garlic, ginseng or ginkgo biloba) and β -blockers) were included in the study [25]. Donors suffering from diabetes mellitus, lipid metabolism disorder or hypertension were excluded. Blood was withdrawn from the cubital vein following an atraumatic protocol. Further steps of the blood withdrawal were not defined and carried out according to the standardized procedure of each test laboratory. Blood samples were collected into e.g. S-Monovettes[®] (Sarstedt, Germany) or into medical syringes

with a 19-21 gauge needle (Braun, Melsungen, Germany) prefilled with sodium citrate ($0.106 \text{ mol}\cdot\text{L}^{-1}$, 1+9 anticoagulant to blood ratio). In addition, EDTA was used as anticoagulant for the complete blood cell count (hemogram). Homogenization of blood and anticoagulant was achieved by immediate agitation of the collection device during the blood donation. Samples were discarded if clotting was observed during visual inspection of the whole blood containing syringes and during hemogram analysis (e.g. drop of platelet count). Demographic data of the donors were collected (sex, age, height, weight, body mass index) as well as blood pressure and heart rate. Hemogram data (red- and (differential) white-blood cell count, hemoglobin, hematocrit) were obtained from EDTA anticoagulated whole blood. Platelet function was tested utilizing flow cytometry (anti-human CD41 or CD42 (CD41/CD42) and CD62P antibody positive events) [26]. C-reactive protein levels were assessed semiquantitatively. Subjects were excluded when values were not within the reference ranges for healthy humans, abnormalities in platelet count/function and early inflammatory processes were noticed. Table 2 summarizes demographical and laboratory data of the participating donors.

Blood preparation and in vitro platelet adhesion assay

Protocols for blood preparation and *in vitro* platelet adhesion testing were previously described elsewhere [26]. Briefly, Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP) were prepared by centrifugation of resting (15 min) citrated whole blood (PRP: $140 \times g$, 20 min; PPP: $1500 \times g$, 20 min). PRP was diluted with PPP to adjust the platelet concentration to $5 \times 10^4 \text{ platelets}\cdot\mu\text{L}^{-1}$ (PRPadjust). Prior to any further testing, PRPadjust rested for 15 minutes at room temperature (RT) under gentle agitation. Polymer discs (13 mm diameter) were placed in 24 well suspension cell culture plates (CELLSTAR[®], Greiner Bio-One, Frickenhausen, Germany) and fixed with a Teflon ring. After phosphate buffered saline (PBS) equilibration, samples were covered with 1 mL PRPadjust for one hour (37°C humidified atmosphere, 5% CO_2). To ensure a homogeneous distribution of platelets, PRPadjust was gently agitated and transferred into the wells according to basic cell culture techniques [27]. During the exposure of the polymer samples to PRPadjust, the test system (the well plate) was not agitated and platelets were allowed to sediment onto the material surfaces. After incubation, non adherent platelets were gently washed off with PBS. Material surface adherent platelets were fixed and fluorescently labelled by glutardialdehyde (2 wt%, 30 min, RT). Samples were embedded in mounting medium (e.g. Mowiol 4-88, Carl Roth GmbH, Karlsruhe, Germany) and covered with glass coverslips (e.g. Menzel GmbH, Braunschweig,

Germany). In the reading center, platelets were visualized by confocal laser scanning microscopy (see Figure 1, LSM 510 Meta, Carl Zeiss, Jena, Germany, 100 fold primary magnification, excitation: 488 nm Argon laser, emission: bandpass filter: 560 nm – 615 nm) [28–30].

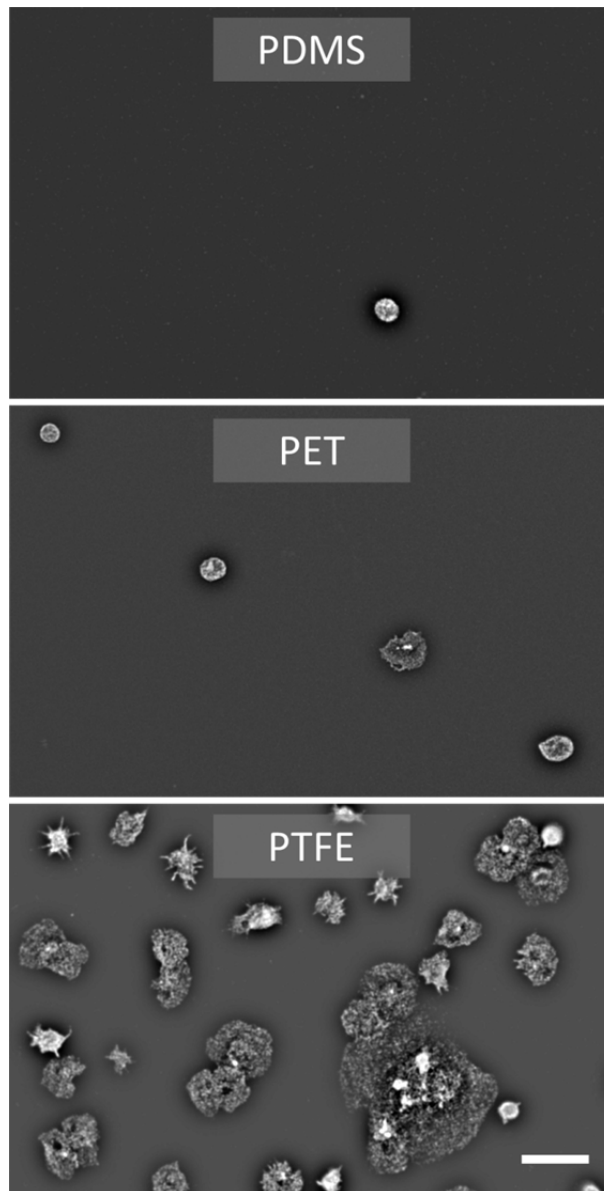


Figure 1. Representative images of adherent platelets from healthy subjects on poly(dimethyl siloxane) (PDMS), poly(ethylene terephthalate) (PET) and poly(tetrafluoro ethylene) (PTFE) (Scale bar=10 μ m, confocal laser scanning microscopy, anti-CD42a antibody staining, 100 fold primary magnification).

The material samples were tested in duplicate per donor. In order to ensure the evaluation of a representative area of each polymer sample, microscopic images of the adherent platelets were taken at five pre-defined positions (four cardinal directions and center). The area of each image was $127.28 \times 127.28 \mu\text{m}^2$ (total analyzed area = $162 \mu\text{m}^2$ per material and donor). Microscopic images were analyzed with a custom made ImageJ software plugin (ImageJ software, National Institutes of Health, USA). The plugin comprised a two-stage filter-set (rolling ball background subtraction- and a watershed segmentation-algorithm) that allowed counting and measuring of the platelets adherent on the material surface. The parameters: platelet density (number of adherent platelets per surface area) [$1 \cdot \text{mm}^{-2}$], total covered surface area [%] and the covered surface area per platelet [μm^2] were obtained.

Statistics

For all samples, arithmetic mean \pm standard deviation is given. For testing the physical-chemical data of the three polymers, a one-way ANOVA was performed. Differences between the three materials and the laboratories were evaluated using a two-way ANOVA. P-values less than 0.05 were considered significant. Pairwise comparisons were performed using the Tukey test. Standard mean differences (effect size) between the tested polymers were calculated for all centers. All statistics, including generation of the Levy-Jenning charts and calculation of the coefficients of variation, were compiled in GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA).

Results

Intra-individual analysis

Figure 2 summarizes the intra-individual variability (10 blood donations on different days from the same donor) tested in study center 5, which is considered to describe the respective reproducibility of the test method. All measurements were within the 2s decision limits for the parameters platelet density and total covered area with 60 % - 70 % of all measurements within the 1s limits. Only one single 1_{2s} event was observed within measurements for the parameter area per platelet, so that 96.7 % were within 2s limits and 70 % of the measurements within the 1s limits.

The coefficient of variation (CV) was lowest for PTFE (CV=3.6 % - 6.2 %) for all three analyzed parameters. For the parameters platelet density and total covered area, the CV of

PET appeared to be highest (CV=35.2 % - 38.2 %) and intermediate for PDMS (CV=25.8 % - 31.6 %). Values of the parameter area covered per single platelet differed only marginally between these materials (PDMS: CV=12.4 %, PET: CV=11.7 %).

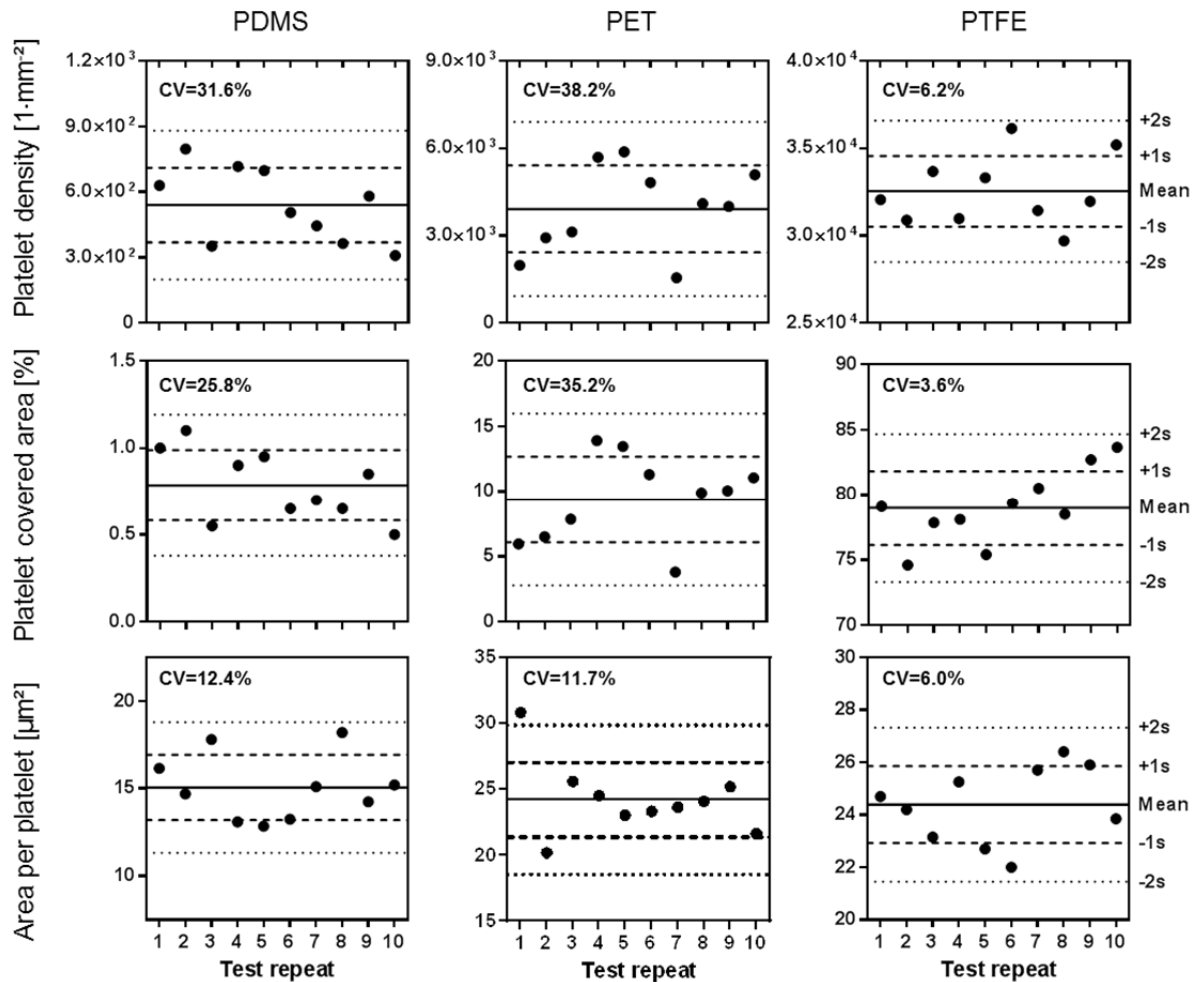


Figure 2. Levey-Jennings charts demonstrating the intra-individual variability in series (reproducibility of the test method) and coefficients of variation of all tested materials and parameters. Data are shown for n=10 independent tests with blood from one donor, performed in study center 5. Decision limits are defined as: $\pm Ns$ [mean \pm (standard deviation)(N)]. Arithmetic mean value=mean, coefficient of variation=CV, standard deviation=s (poly(dimethyl siloxane) (PDMS), poly(ethylene terephthalate) (PET), poly(tetrafluoro ethylene) (PTFE)).

Inter-individual analysis

Despite absolute values for the three polymers differed slightly among the centers, identical trends were observed for all measurements. In all centers, PTFE was found with the highest platelet density, covered surface area and area per single platelet, PET values were scored intermediate and PDMS values were lowest (Figure 3). All parameters differed significantly for the three polymers (ANOVA for repeated measures: $p < 0.0001$) and pairwise comparisons revealed that all materials differed markedly from each other (non-adjusted: $p < 0.0001$ each).

For the three parameters, the coefficient of variation was lowest for PTFE (platelet density: CV=34.9 %, covered surface area: CV=39.2 %, area per platelet: CV=13.9 %) and highest for PET (platelet density: CV=82.2 %, covered surface area: CV=92.1 %, area per platelet: CV=24.2 %). Intermediate values were observed for PDMS (platelet density: CV=46.2 %, covered surface area: CV=52.1 %, area per platelet: CV=18.0 %).

Inter-individual differences ($\Delta\%$) between the polymers exceeded the intraindividual variations between the different donors markedly in any case. For all analyzed parameters, differences between PDMS and PTFE were highest (platelet density: $\Delta\% = 190.8 \% \pm 6.9 \%$, covered surface area: $\Delta\% = 195.2 \% \pm 3.9 \%$, area per platelet: $\Delta\% = 65.0 \% \pm 16.6 \%$), intermediate between PDMS and PET (platelet density: $\Delta\% = 148.7 \% \pm 37.0 \%$, covered surface area: $\Delta\% = 166.2 \% \pm 28.5 \%$, area per platelet: $\Delta\% = 46.4 \% \pm 24.7 \%$) and lowest between PET and PTFE (platelet density: $\Delta\% = 132.3 \% \pm 36.2 \%$, covered surface area: $\Delta\% = 141.5 \% \pm 34.1 \%$, area per platelet: $\Delta\% = 19.5 \% \pm 25.2 \%$) (Figure 3).

Standardized differences according to Cohen (d) ranged between 1.0 and 5.5. For all parameters, highest differences were observed between PDMS and PTFE (platelet density: $d = 5.5$, covered surface area: $d = 5.0$, area per platelet: $d = 4.3$). For the parameters platelet density ($d = 2.8$) and covered surface area ($d = 2.8$), differences were intermediate between PTFE and PET, and low between PET and PDMS (platelet density: $d = 2.1$, covered surface area: $d = 2.0$). For the parameter area per platelet, the difference between PET and PTFE was lowest ($d = 1.0$) and intermediate between PET and PDMS ($d = 2.2$) (Figure 3).

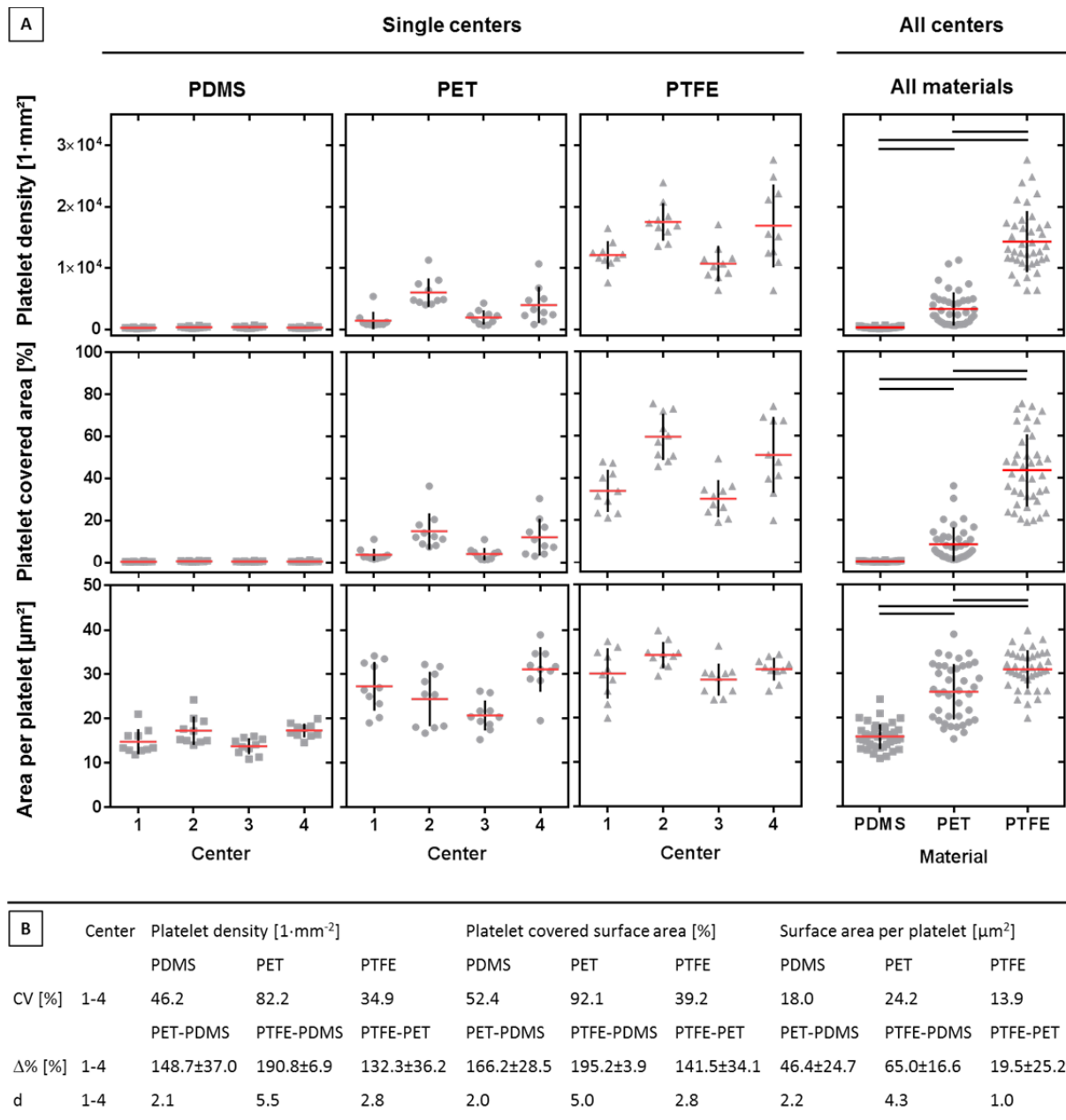


Figure 3. (A) Scatter plots of the parameters platelet density [$1 \cdot \text{mm}^{-2}$], platelet covered area [%] and covered area per platelet [μm^2]. Data sets are plotted for each material (poly(dimethyl siloxane) (PDMS), poly(ethylene terephthalate) (PET), poly(tetrafluoro ethylene) (PTFE) and center (1-4) with $n=10$ donors (left: “Single centers”). On the right (“All centers”), a summary of the data from all centers ($n=40$ donors) is shown for each material (horizontal red lines=arithmetic mean, crossing black lines = standard deviation). (B) Summary of the interindividual analysis for the summarized center data (center 1-4). Coefficient of variation [%], percentage differences [%] and standardized difference (effect size, d) are given for the parameters mentioned above (Arithmetic mean \pm standard deviation).

Discussion

In vitro hemocompatibility evaluation involves several parameters such as the cellular and humoral immune system as well as the blood coagulation cascade and platelets. Understanding the ability of synthetic blood contacting devices – particularly cardiovascular implant materials – to accumulate platelets is thought to be crucial, because early post-operative platelet adherence might be responsible for the formation of thrombi, which has an adverse impact on postoperative outcome [31]. Thus, platelet adhesion and activation are reported to be early indicators of the thrombogenicity of blood contacting implants [32–39]. To demonstrate that these parameters can be assessed *in vitro* reproducibly and reliably in different laboratories, polymers with varying degrees of thrombogenicity were tested in five independent test centers in Germany. The results of this study confirm that standardization of the applied static *in vitro* test resulted in a reproducible and laboratory-independent evaluation of the tested polymers. Despite single center data varied slightly in the absolute numbers for all analyzed parameters, scoring of the materials was identical for the parameters platelet density and platelet covered surface area (Figures 1 and 3). Considering the data of each center (n=10 donors each) and the summarized numbers, values were lowest for PDMS, highest for PTFE and intermediate for PET (see Figure 3). For the parameter covered surface area per platelet (spreading/activation of the single platelet), differences between PET and PTFE were contrarily scored (50% found a significant difference, 50% did not). However, both observations revealed a relatively strong activation of the adherent platelets on both polymers. An unweighted summarized scoring of all three parameters ($\Sigma = \text{parameter A} + \text{parameter B} + \text{parameter C}$) results in an equal ranking of the tested materials in all centers (PDMS<PET<PTFE). In concert with this, standardized differences (d) between all tested polymers exceeded by far 0.8, which is a decision limit for strong effects/differences [40,41].

Analysis of the reproducibility in series (intra-individual differences) revealed that the analytical process of the *in vitro* test system (all polymers and parameters) is in control, with 96.6% - 100% of the data within 2s limits and 60% - 70% of the data within 1s limits [42]. Intra-individual variations were markedly lower (up to 38.2%) than the inter-individual variations, which reached up to 92.1%. However, percentage differences ($\Delta\%$) and further the effect sizes (d) between the materials exceeded significantly these variations for any analyzed parameter (Figures 2 and 3). This demonstrates that the influence of the material substrate on the platelet adhesion and activation was considerably higher than the variability of the

measuring method so that adhesion and activation of platelets on the materials could be scored in a robust, reproducible, center-independent and precise way.

We could show that inter-laboratory and inter-study comparisons are possible in the evaluation of platelet adhesion and activation on polymers if critical steps of the *in vitro* assay are standardized. Among these, preparation and processing of the blood have been reported to influence functionality of the platelets (e.g. centrifugation protocols, duration of resting and testing times) [43,44]. The selection of sodium citrate allowed an adequate standardization of the blood anticoagulation at concentrations, which are commonly applied for the evaluation of platelet function in the clinical laboratory ($0.105\text{--}0.109\text{ mol}\cdot\text{L}^{-1}$ final concentration) [19,45–47]. Differences between centers, particularly those in the absolute numbers of the tested parameters (e.g. platelet density), might presumably be attributed to those steps in the test protocol, which were not harmonized. Also, it cannot be excluded that the different distributions of age and sex in the four patient groups as well as the donor-specific initial activation of platelets (percentages of $\text{CD41}^+/\text{42}^+$ and CD62^+ positive cells) might have attributed to these results. However, it is noteworthy to say that particularly the type of antibody, the staining protocol, the type of cytometer and the gating strategy has been shown to influence the results of the flow cytometry analysis. Nevertheless these factors were clearly weaker than the influence of the materials on the adherence and activation of fresh human platelets.

The aim of this study was to emphasize that certain standardization of *in vitro* test protocols is necessary to enable inter-laboratory and inter-study comparisons of thrombogenicity test results. An *in vitro* assay for platelet adhesion and activation was chosen, which does not take into account the full spectrum of hemocompatibility tests demanded by the regulatory agencies, but enables the demonstration of crucial steps for a reproducible testing in different laboratories for that particular purpose. Main aspects were: utilizing fresh human blood, harmonizing blood sample management as well as quantitative analysis of the same test materials. Perspectively, harmonization of further test parameters and setups - including e.g. dynamic whole blood assays - should be addressed in a similar way in order to allow a side by side comparison of novel materials with materials of known - low and high – thrombogenicity (reference materials). Of course, these materials have to be selected in view of the kind of tested material family (e.g. polymer- or metal-based biomaterials) and the test parameters (e.g. hemolysis or platelet adhesion).

Conclusion

The results of this study reveal that stringent standardization of the described *in vitro* assay allowed a reproducible evaluation of the adhesion and activation of human platelets on polymer-based biomaterials. Harmonization of the study protocol resulted in an equivalent scoring of the tested materials, regardless of the laboratory in which the test was performed. We concluded that the reported multi-center approach represents an accurate way to assess inter-laboratory variabilities and can help defining appropriate standards for the thrombogenicity testing of biomaterials. To achieve this, future studies will comprise the evaluation of further test setups, such as dynamic tests with fresh human whole blood.

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