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Comparison of acute thrombogenicity and albumin adsorption in three different durable polymer coronary drug-eluting stents

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KEYWORDS

- ACS/NSTE-ACS
- stable angina
- stent thrombosis

Abstract

Background: The relative thrombogenicity and albumin adsorption and retention of different durable polymers used in coronary stents has not been tested.

Aims: This study sought to compare the thromboresistance and albumin binding capacity of different durable polymer drug-eluting stents (DES) using dedicated preclinical and *in vitro* models.

Methods: In an *ex vivo* swine arteriovenous shunt model, a fluoropolymer everolimus-eluting stent (FP-EES) (n=14) was compared with two durable polymer DES, the BioLinx polymer-coated zotarolimus-eluting stent (BL-ZES) (n=9) and a CarboSil elastomer polymer-coated ridaforolimus-eluting stent (EP-RES) (n=6), and bare metal stents (BMS) (n=10). Stents underwent immunostaining using a cocktail of antiplatelet antibodies and a marker for inflammation and were then evaluated by confocal microscopy (CM). Albumin retention was assessed using a flow loop model with labelled human serum albumin (FP-EES [n=8], BL-ZES [n=4], EP-RES [n=4], and BMS [n=7]), and scanned by CM.

Results: The area of platelet adherence (normalised to total stent surface area) was lower in the order FP-EES (9.8%), BL-ZES (32.7%), EP-RES (87.6%) and BMS (202.0%), and inflammatory cell density was least for FP-EES <BL-ZES <EP-RES <BMS. Although nearly full coverage by albumin binding was shown for all durable polymer DES, FP-EES showed significantly greater intensity of albumin as compared to BL-ZES, EP-RES and BMS (FP-EES 79.0%; BL-ZES 13.2%; EP-RES 6.1%; BMS 1.5%).

Conclusions: These results suggest that thromboresistance and albumin retention vary by polymer type and that these differences might result in different suitability for short-term dual antiplatelet therapy.

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Abbreviations

BMS bare metal stent(s)
BL-ZES BioLinx polymer-coated zotarolimus-eluting stent
CM confocal microscopy
EP-RES CarboSil elastomer polymer-coated ridaforolimus-eluting stent
FP-EES fluoropolymer everolimus-eluting stent
DAPT dual antiplatelet therapy
DES drug-eluting stent(s)
HAS human albumin serum
PBMA poly n-butyl methacrylate
ST stent thrombosis

Introduction

Stent thrombosis (ST) continues to be one of the most feared complications after percutaneous coronary interventions[1]. Thus, thromboresistance is an ideal property for coronary stents. Previous data have suggested that polymeric coatings influence blood-materials interactions depending upon their adsorbing capacity with regard to specific plasma proteins which drive the stent's interaction with platelets and leukocytes, potentially leading to cell adhesion, activation and thrombosis[2-4]. For biomaterials, it has been proposed that surfaces with high levels of albumin adsorption are desirable as albumin could passivate the polymer surface by preventing more reactive components, such as platelets and fibrinogen, from binding[5]. It is well known that fluorinated polymers such as poly(vinylidene fluoride-co-hexafluoropropylene) (PVDF-HFP) used in both the CoCr everolimus-eluting stents (Abbott Vascular, Santa Clara, CA, USA) and the platinum-chromium everolimus-eluting stent (Boston Scientific, Marlborough, MA, USA) possess this reaction[4]. While it is generally believed that polymeric coatings, regardless of type, have some antithrombotic effect compared to bare metal surfaces, the relative effects of different durable polymers used in coronary DES have never been examined with respect to thrombogenicity and albumin adsorption and retention.

We compared the relative thromboresistance of three currently available durable polymer DES with distinct coatings using an *ex vivo* porcine shunt model and their albumin binding capacity to understand the association between albumin adsorption and thromboresistance for each polymeric DES (i.e., fluoropolymer everolimus-eluting stent [FP-EES; Abbott Vascular, BioLinx™ polymer-coated zotarolimus-eluting stent [BL-ZES; Medtronic, Minneapolis, MN, USA] and CarboSil® elastomer polymer-coated ridaforolimus-eluting stent [EP-RES; Cordis, Milpitas, CA, USA]).

Methods

Detailed methods are described in **Supplementary Appendix 1**.

TEST DEVICES

The control arm for three different studies was the XIENCE Alpine™ (Abbott Vascular) (FP-EES). Comparators included two contemporary durable polymer DES: 1) BL-ZES which is coated with a mixture of hydrophilic C10 polymer, hydrophilic C19 polymer, and polyvinylpyrrolidone (PVP) (BioLinx polymer), and 2) EP-RES which is coated with a blend of CarboSil and PBMA. The MULTI-LINK 8™ BMS (Abbott Vascular) served as the third comparator. **Table 1** lists experimental models and devices, and **Supplementary Table 1** shows the description of all devices.

Table 1. Summary of animal model and stents tested.

Type of study		Thrombogenicity study	Human albumin study
Model		Ex vivo AV shunt model	In vitro flow model
Animal		Porcine	NA
Period		1 hour	30 min
Number of animals		7	NA
Type of stent	1	FP-EES (n=14)	FP-EES (n=8)
	2	BL-ZES (n=9)	BL-ZES (n=4)
	3	EP-RES (n=6)	EP-RES (n=4)
	4	BMS (n=10)	BMS (n=7)

AV: arteriovenous; BMS: bare metal stent; BP-ZES: BioLinx polymer zotarolimus-eluting stent; EP-RES: elastomer polymer ridaforolimus-eluting stent; FP-EES: fluoropolymer everolimus-eluting stent; NA: not applicable

SWINE EX VIVO ARTERIOVENOUS SHUNT MODEL

For assessment of acute thrombogenicity and inflammation, platelets (CD61/CD42b), monocytes (CD14) and neutrophils (PM-1) were evaluated by confocal microscopy (CM)[3]. For quantification of platelet aggregation, the areas of positive staining within each stented segment were measured by ZEN software (Zeiss ZEN 2012; Carl Zeiss Microscopy, Jena, Germany). The number of inflammatory cells was counted manually and expressed as cell density (cells/mm^2) relative to strut surface area.

ALBUMIN

PREPARATION OF STENTS

Stents were cut longitudinally to make uniform lengths (6-7 mm) and inserted into a bottomless channel slide used for perfusion application (sticky-Slide I Luer 0.8 mm channel height, Cat. No. 80198; ibidi GmbH, Gräfelfing, Germany) **(Supplementary Figure 1A, Supplementary Figure 1B)**.

ALBUMIN IN VITRO FLOW LOOP

Labelled human albumin was circulated through silicone tubing in line with the ibidi slide using a perfusion pump. The first 30 minutes under flow was for albumin binding **(Supplementary Figure 1C)**, and the second 30 minutes under flow was for washing with FluoroBrite™ DMEM (Thermo Fisher Scientific, Waltham, MA, USA) **(Supplementary Figure 1D)**. Albumin binding was imaged only during latter phases because of background noise generated by dissolved fluorescent proteins during the first 30 minutes.

Randomly selected regions of interest from one different area of each stent were scanned. In addition, the entire area of each stent

was scanned by CM and quantified by ZEN software. High and low signals of fluorescence were defined in each experiment to assess all stents in the same condition. Each image was recorded at one-minute intervals.

ASSESSMENT OF ALBUMIN ADHERENCE TO VARYING STENT SURFACES

The positive area of albumin labelling (green channel) was quantitated within defined regions of interest. Intensity scale was defined as the average of total light intensity of the green signal from every pixel by the strut area. The analysis was performed every one minute, and the covered strut area was quantified and expressed as percent albumin coverage (%) and intensity scale of albumin.

A REAL-TIME MANNER USING HUMAN PLATELET IN VITRO FLOW LOOP

In addition, human platelet adherence to stent surfaces was evaluated in a real-time manner using an *in vitro* flow loop live-cell assay to determine the temporal distribution of platelet adherence.

STATISTICAL ANALYSIS

In an *ex vivo* shunt model and an albumin flow loop model, nested generalised linear mixed models with Dunnett's correction for multiple testing were employed in order to investigate group differences in consideration of multiple measurements per individual. Within these models, stent type was considered as fixed effect, while the experimental factor variables animal or experiment, shunt number and linear position were considered as nested random effects. Values are expressed as estimated mean with 95% confidence interval. The analyses were performed with SPSS Advanced Statistics, Version 25 (IBM Corp., Armonk, NY, USA). The statistical tests were two-tailed and a value of $p<0.05$ was considered to indicate statistical significance.

Results

ACUTE THROMBOGENICITY IN A PORCINE ARTERIOVENOUS SHUNT MODEL

There was no evidence of blood coagulation or platelet function abnormalities in any of the animals studied. Additionally, the measures of coagulation were similar in all shunt runs **(Supplementary Table 2)**.

Table 2 summarises the results of platelet adhesion by CM. **Figure 1** shows representative CM images with immunofluorescent staining against dual platelet markers (CD61/42b) in FP-EES, BL-ZES, EP-RES and BMS. When total platelet fluorescence area was normalised to stent surface area, platelet adhesion was lower in the order FP-EES, BL-ZES, EP-RES and BMS. **Table 2** lists the results of immunofluorescent staining against a neutrophil marker (PM-1) and a monocyte marker (CD14) by CM; **Figure 2** shows representative images from each group. The cell density of PM-1 was least for FP-EES <BL-ZES <BMS <EP-RES. Similarly, the cell density of CD14 was lower in the order FP-EES, BL-ZES, BMS and EP-RES.

Table 2. Ex vivo arteriovenous shunt model by confocal microscopy.

Percent CD42b/CD61 positive area of total stent strut (%)				
Stent type	Estimated mean (95% CI)	*p*-value		
		vs BL-ZES	vs EP-RES	vs BMS
FP-EES	9.8 (8.5-17.3)	<0.01	<0.01	<0.01
BL-ZES	32.7 (17.6-60.8)	NA	0.03	<0.01
EP-RES	87.6 (45.4-168.9)	0.03	NA	0.01
BMS	202.0 (111.5-365.8)	<0.01	0.01	NA
PM-1 positive cells density (number/mm²)				
Stent type	Estimated mean (95% CI)	*p*-value		
		vs BL-ZES	vs EP-RES	vs BMS
FP-EES	38.1 (29.6-49.0)	<0.01	<0.01	<0.01
BL-ZES	465.8 (340.0-638.1)	NA	0.23	0.64
EP-RES	637.7 (433.7-937.6)	0.23	NA	0.4
BMS	515.5 (382.4-694.9)	0.64	0.4	NA
CD14 positive cells density (number/mm²)				
Stent type	Estimated mean (95% CI)	*p*-value		
		vs BL-ZES	vs EP-RES	vs BMS
FP-EES	34.9 (25.1-48.7)	<0.01	<0.01	<0.01
BL-ZES	286.9 (189.6-433.7)	NA	0.25	0.83
EP-RES	428.0 (257.9-70.3)	0.25	NA	0.32
BMS	305.2 (206.1-451.9)	0.83	0.32	NA

BMS: bare metal stent; CI: confidence interval; EP-RES: elastomer polymer ridaforolimus-eluting stent; FP-EES: fluoropolymer everolimus-eluting stent; NA: not applicable

ALBUMIN ADSORPTION AND RETENTION IN A FLOW LOOP MODEL USING HSA

The amount of fluorescent albumin on the BMS surface increased over time; there were significant differences between times 0 and 30 minutes and between 30 minutes and 48 hours **(Supplementary Figure 2)**. Also, BMS with 48-hour exposure to albumin showed a significantly lesser amount of platelet attachment as compared to BMS with no exposure to albumin **(Supplementary Figure 3, Supplementary Table 3)**. **Table 3** summarises the results of albumin coverage by CM, and **Figure 3** shows representative CM images with immunofluorescent albumin in FP-EES, BL-ZES, EP-RES and BMS. **Figure 4** shows representative quantitated images in all stents as analysed by ZEN software. When total albumin fluorescence area was normalised to stent surface area, an area of total albumin coverage was significantly greater in FP-EES, BL-ZES and EP-RES than in BMS, whereas there were no significant differences in area between FP-EES, BL-ZES and EP-RES. However, the area of albumin coverage as determined by high signal intensity was greater in FP-EES versus all other stents. Although FP-EES, BL-ZES and EP-RES showed nearly complete albumin coverage on the stent struts, the majority of sampled areas showed high albumin signal intensity in FP-EES, whereas it was a low signal intensity in BL-ZES and EP-RES **(Figure 4)**.

A real-time analysis over the 30-minute washing phase also showed that coverage of albumin was significantly greater in FP-EES, BL-ZES and EP-RES relative to BMS **(Supplementary Table 4, Figure 5, Moving image 1)**. The intensity of albumin, indicative of a higher amount of adsorption, was greater in the

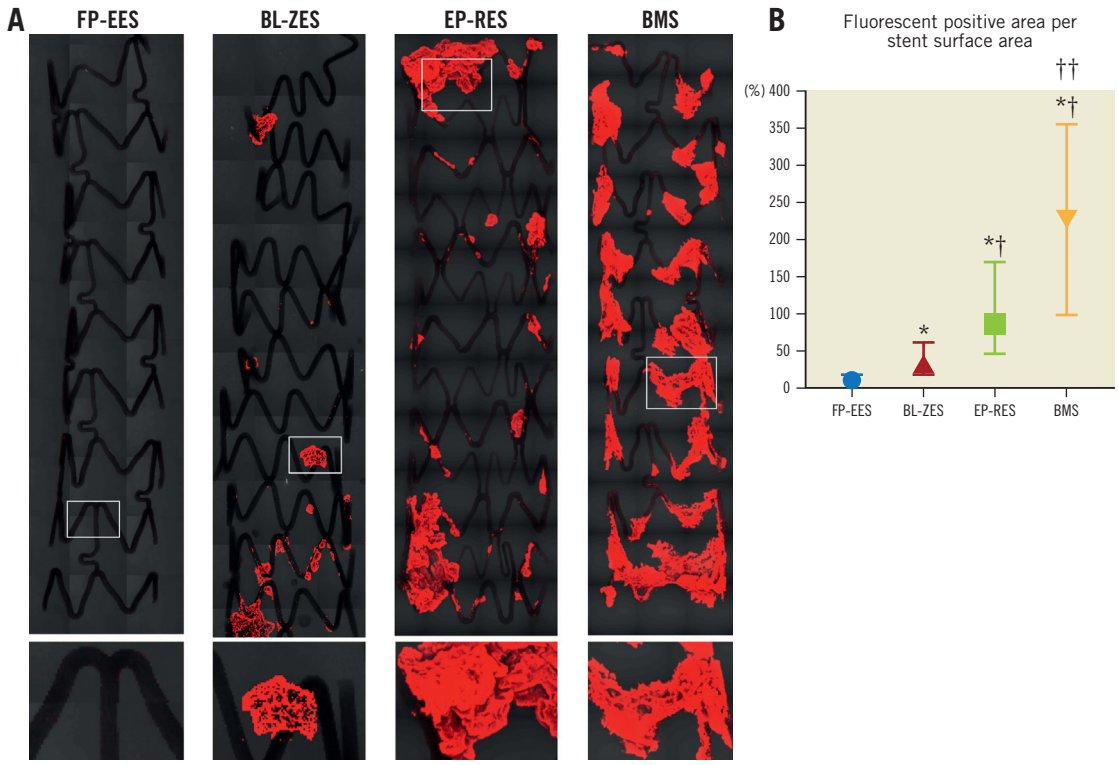

Figure 1. *Representative confocal microscopic images using immunofluorescent staining against dual platelet markers (CD61/CD42b) in an ex vivo shunt model. A) Low (upper) and high (lower) power confocal microscopic images showed minimal platelet aggregation in FP-EES, whereas obvious platelet aggregation was observed in BL-ZES, EP-RES and BMS. B) Graph shows fluorescent positive area per stent surface. Data are presented as mean±standard deviation for each. * p<0.05 vs FP-EES; † p<0.05 vs BL-ZES; †† p<0.05 vs EP-RES.*

Table 3. Human albumin adsorption in in vitro flow model.

Percent low signal area of total stent strut (%)				
Stent type	Estimated mean (95% CI)	*p*-value		
		vs BL-ZES	vs EP-RES	vs BMS
FP-EES	17.8 (11.3-27.8)	0.02	0.02	0.02
BL-ZES	76.6 (40.6-144.6)	NA	0.94	<0.01
EP-RES	79.0 (41.9-149.3)	0.94	NA	<0.01
BMS	7.2 (4.5-11.7)	<0.01	<0.01	NA
Percent high signal area of total stent strut (%)				
Stent type	Estimated mean (95% CI)	*p*-value		
		vs BL-ZES	vs EP-RES	vs BMS
FP-EES	79.0 (45.7-136.6)	<0.01	<0.01	<0.01
BL-ZES	13.2 (6.1-28.5)	NA	0.20	0.03
EP-RES	6.1 (2.8-13.2)	0.20	NA	0.06
BMS	1.5 (0.8-2.6)	0.03	0.06	NA
Percent total signal area of total stent strut (%)				
Stent type	Estimated mean (95% CI)	*p*-value		
		vs BL-ZES	vs EP-RES	vs BMS
FP-EES	96.9 (66.5-141.3)	0.79	0.67	<0.01
BL-ZES	89.5 (52.5-152.3)	NA	0.89	<0.01
EP-RES	85.0 (49.9-144.7)	0.89	NA	<0.01
BMS	8.3 (5.6-12.4)	<0.01	<0.01	NA

BMS: bare metal stent; BP-ZES: BioLinx polymer zotarolimus-eluting stent; CI: confidence interval; EP-RES: elastomer polymer ridaforolimus-eluting stent; FP-EES: fluoropolymer everolimus-eluting stent; NA: not applicable

order FP-EES, BL-ZES, EP-RES and BMS. All stents did not show a significant decrease in albumin retention over the 30-minute washing phase **(Supplementary Table 5)**. Albumin intensity correlated negatively with platelet attachment in the shunt model **(Supplementary Figure 4)**.

PLATELET ADHESION IN A REAL-TIME FLOW LOOP MODEL USING HUMAN PLATELETS

A novel *in vitro* flow loop model using freshly isolated fluorescently labelled human platelets from healthy volunteers was constructed. Stents were exposed to circulating platelets, imaged and analysed by CM over the course of 60 minutes. Consistent with the shunt study, the amount of platelet aggregation was numerically least in FP-EES, followed by BL-ZES and EP-RES and then BMS (n=3 per group), although there were non-significant differences between each **(Supplementary Table 6, Supplementary Figure 5, Moving image 2)**. Platelet adhesion in the flow model correlated nicely with that in the shunt model **(Supplementary Figure 6)**.

Discussion

The current study evaluated acute thrombogenicity and albumin retention in currently available durable polymer DES. The main findings of this preclinical study were as follows: 1) antithrombotic and anti-inflammatory reactions were greater in the order FP-EES, BL-ZES, EP-RES and BMS in an *ex vivo* porcine shunt

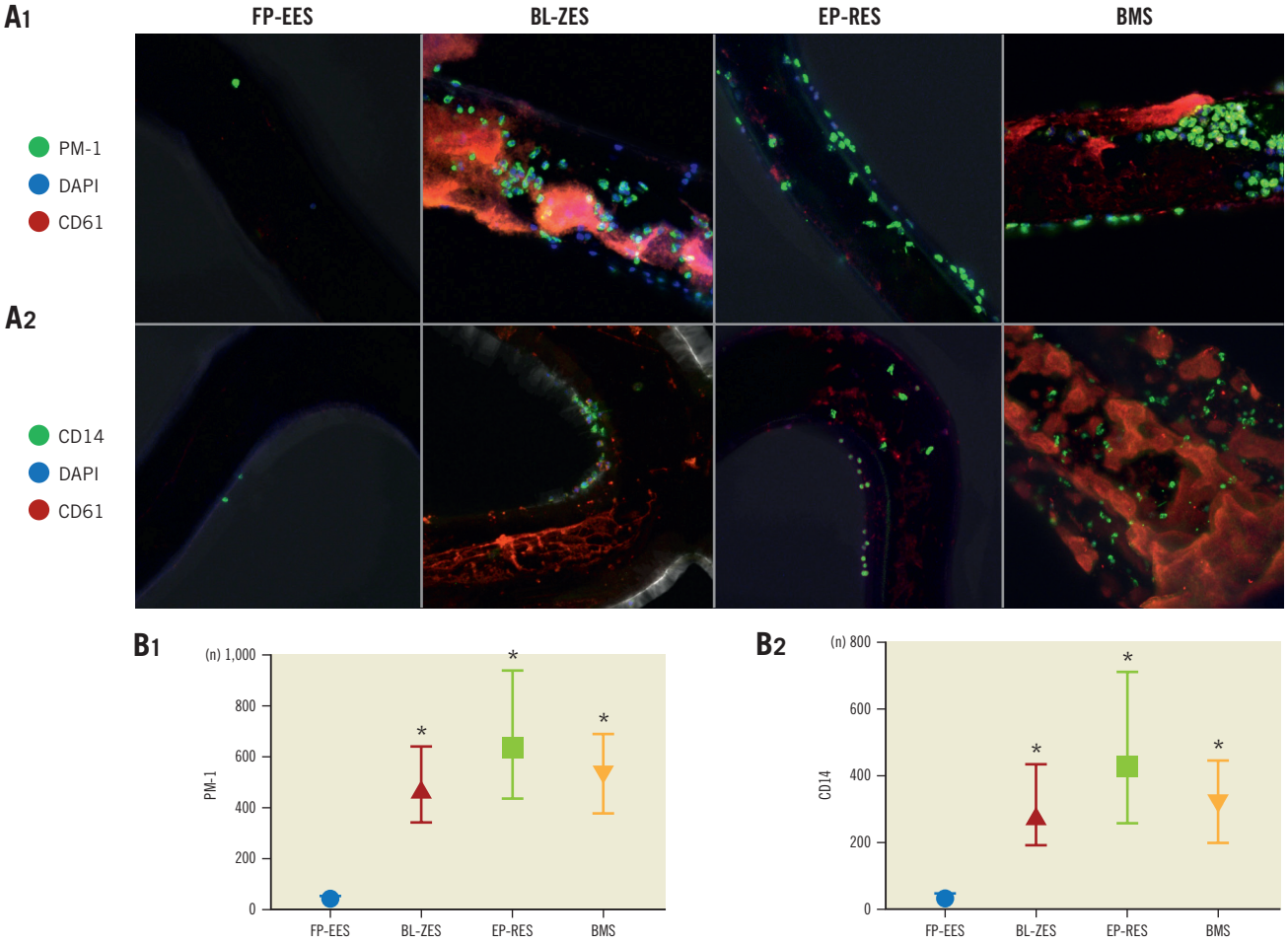

Figure 2. *Representative confocal microscopic images using immunofluorescent staining against a neutrophil marker (PM-1) and a monocyte marker (CD14) in an ex vivo shunt model. A) The upper panels (A1 and A2) show confocal microscopic images with PM-1 staining and the lower panels show confocal microscopic images with CD-14 staining. FP-EES showed minimal neutrophil and monocyte attachment. However, BL-ZES, EP-RES and BMS showed many neutrophils and monocytes on the stent surfaces. B) Graphs show the number of PM-1 (B1) and CD-14 (B2) positive cells on the stent struts. The number of PM-1 and CD-14 was significantly the least in FP-EES relative to BL-ZES, EP-RES and BMS. Data are presented as mean±standard deviation for each group. * p<0.05 vs FP-EES.*

model; 2) albumin coverage was greater in all durable polymer DES relative to BMS, and 3) albumin intensity indicating concentration of albumin was significantly the greatest in FP-EES, followed by BL-ZES and EP-RES, with the least being in BMS. Taken together, these data suggest that durable polymer coatings provide some level of thromboresistance compared to BMS, which may be the mechanism by which polymeric coatings to some extent have thromboresistant properties. From this standpoint, durable polymers used on DES are not equivalent.

DIFFERENT DURABLE POLYMER COATING DES IN AN EX VIVO SHUNT MODEL

This shunt study suggests that polymeric coating provides a protective barrier against acute thrombus formation. Clinical data also suggest a protective effect of polymer-coated stents in patients with coronary artery diseases[6]. Among durable polymer DES, fluoropolymer EES showed the strongest thromboresistance. BL-ZES showed some degree of thromboresistance when compared with BMS as the control. This suggests that the BioLinx polymer confers some ability to avoid platelet adhesion. The outer surface of the polymer consists of hydrophilic polyvinyl-pyrrolidinone which inhibited monocyte adhesion in one study[7] and, according to our study, allows some degree of albumin adsorption. On the other hand, PBMA/CarboSil coating showed comparable thrombogenicity to BMS. Previous *in vitro* testing showed initially good adsorption of albumin to PBMA polymer with very little retention after detergent washing and a relatively low albumin/fibrinogen binding ratio compared to the PVDF-HPF fluoropolymer[4]. These data support the observation that, even if durable polymers are applied, these polymers show differing individual potential with respect to thrombogenicity even in the current era.

Platelet aggregation on the surface of stent struts exists along with circulating leukocytes consisting mainly of neutrophils and monocytes[8]. Moreover, leukocyte tissue factors are transferred to platelets,

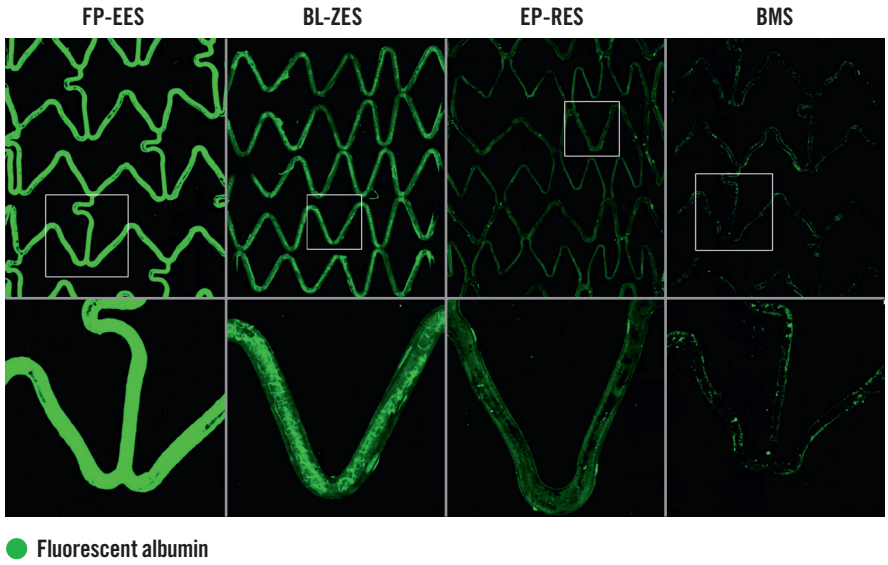

Figure 3. *Representative confocal microscopic images using fluorescent human serum albumin in the flow loop model. The low (upper) and high (lower) power images show the stent surface fully covered by an obvious strong signal of fluorescent albumin in FP-EES. On the other hand, although BL-ZES and EP-RES also showed near complete coverage by albumin, the albumin signals (green) on the surface in BL-ZES and EP-RES were less intense relative to FP-EES. Minimal albumin signal was observed in BMS.*

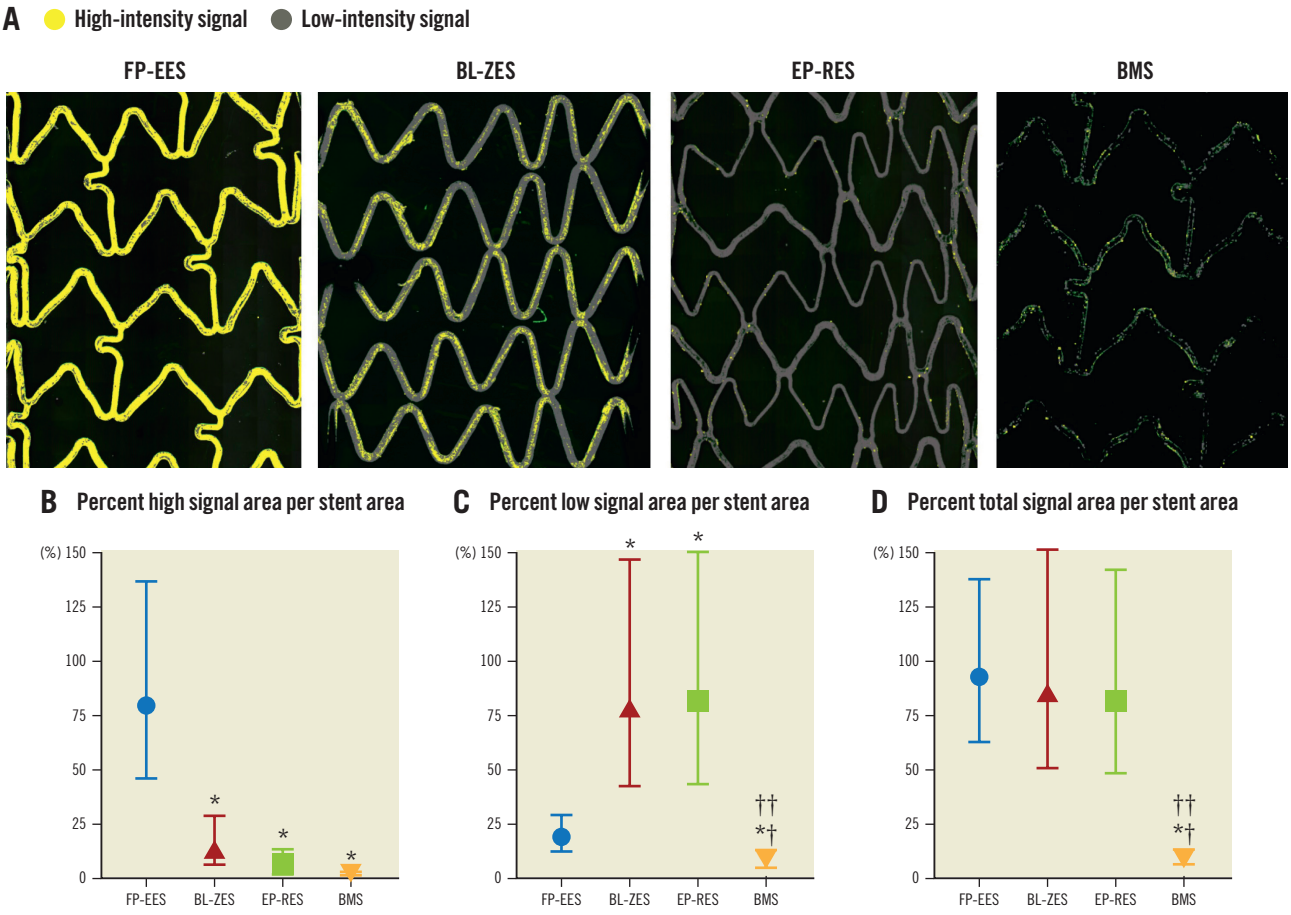

Figure 4. *Quantitated images of confocal microscopy with fluorescent human albumin. A) High signal of fluorescent albumin was coloured yellow, whereas low signal of albumin was coloured grey. FP-EES was fully covered by a yellow colour, whereas BL-ZES and EP-RES were covered mostly by grey. BMS showed minimal grey colour. B), C) & D) Graphs show high signal of albumin, low signal and total signal in each stent. Data are presented as mean±standard deviation for each group. * p<0.05 vs FP-EES; † p<0.05 vs BL-ZES; †† p<0.05 vs EP-RES.*

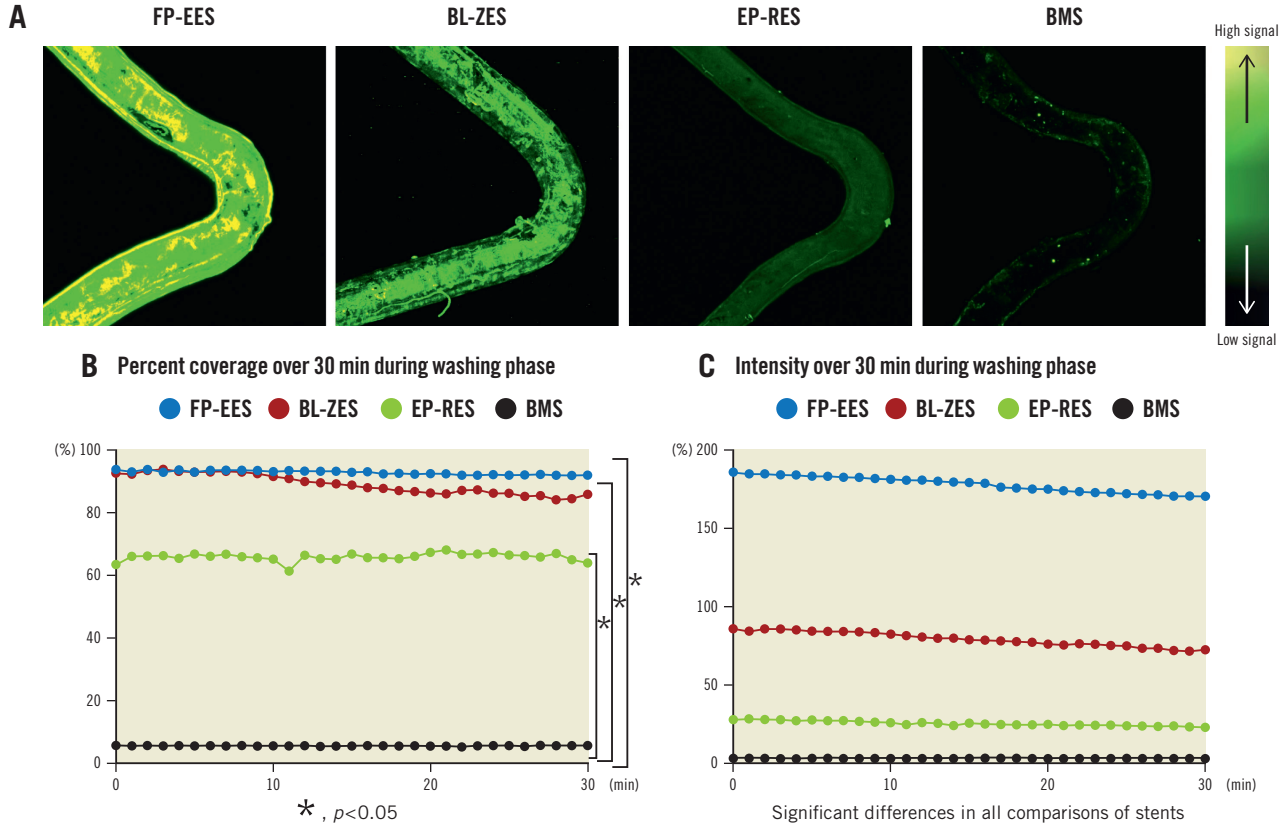

Figure 5. *Percent coverage and intensity of albumin in each group over 30 minutes during washing phases. A) These images show quantitated coverage and intensity of albumin at the end of washing phases. All durable polymer DES showed nearly full albumin coverage. However, albumin intensity was higher in the order FP-EES, BL-ZES and EP-RES. Coverage and intensity of albumin were minimal in BMS. B) & C) Graphs show estimated mean percent coverage and intensity over 30 minutes during washing phases. There were significant differences in FP-EES, BL-ZES, and EP-RES versus BMS, although significant differences were not observed between FP-EES, BL-ZES and EP-RES. FP-EES had the highest intensity, followed by BL-ZES and EP-RES, with the least being in BMS. Significant differences were seen in all comparisons. In percent coverage and intensity, minimal changes were observed over 30 minutes.*

promoting propagation of thrombus[9,10]. In the current study, the density of inflammatory cells on the surface of struts was consistent with the amount of platelet adherence to the stent surfaces.

ALBUMIN BINDING AND RETENTION IN DIFFERENT POLYMER COATINGS IN FLOW LOOP MODELS

Albumin has traditionally been used as a passivating protein. High albumin retention on the surface of the strut might be a beneficial mechanism to prevent a thrombogenic profile. The albumin-rich layer tempers host-material interactions and competitively inhibits the adhesion of fibrinogen and von Willebrand factor[4,11]. However, in an *in vivo* setting, surface adsorption is a reversible process; the composition of adsorbed proteins may change over time, a phenomenon known as the Vroman effect[12]. Albumin can be replaced with other proteins leading to thrombosis such as von Willebrand factor and fibrinogen, etc. However, absorptivity between albumin and stent surfaces may vary to a great extent based on materials. In fact, previous preclinical studies showed various capability of keeping albumin on stent surfaces by different materials after washing by sodium dodecyl sulphate[4]. Previously, Szott et al reported that fluoropolymer can retain non-significantly more albumin on the stent surface relative to PBMA and polystyrene-b-polyisobutylene-b-polystyrene (Kaneka, Japan; SIBS-1 and SIBS-2) even after using sodium dodecyl sulphate, which is supposed to detach albumin from the surface of struts. In addition to differences in albumin retention, the superior thromboresistance of FP-EES was shown. Each stent offers a different amount of albumin adsorption; the level of affinity of albumin on the stent surface appears to affect thrombogenicity.

APPLICATION TO SHORT DUAL ANTIPLATELET THERAPY (DAPT)

Differences in intrinsic thrombogenicity between stents is important when considering shortening DAPT therapy as stent struts may not be fully covered by endothelium at the time DAPT is discontinued. Bare struts, especially those which do not possess intrinsic thromboresistance, may become a nidus for thrombus formation. Superior thromboresistant features of polymers may contribute to a short duration of DAPT (i.e., <3 months).

In multicentre prospective trials using a standard strategy for DAPT duration, the rate of ST was very low (0.1-0.6%) and comparable in different types of current DES[6,13-15]. However, information about the rate of ST after a short DAPT strategy (i.e., 1 to 3 months) is limited. To date, a few studies are available where a one-month duration of DAPT was evaluated[16]. The LEADERS FREE trial was a landmark trial of one-month DAPT in which polymer-free DES were compared with BMS with the same one-month DAPT protocol. However, this study showed a high rate of ST in both groups due to greater thickness and bare surface although it also suggested that it is feasible to perform a short duration of DAPT after DES implantation. More recently, the STOP DAPT II trial also showed the feasibility of one-month DAPT after implantation of FP-EES when compared to 12-month DAPT[17]. The current preclinical study further supports these results. The potential differences in thromboresistance seen within this study for different DES suggest that a one-month DAPT duration for different types of DES may not be standardised (regardless of DES type) and that each needs to be tested in dedicated clinical trials.

Limitations

There are some limitations in this study. First, this study was performed in a swine *ex vivo* shunt model without antiplatelet agents and with low-dose heparin to evaluate the potential of the material itself. Therefore, this result cannot simply be applied to the clinical setting where patients are treated with mono or dual antiplatelet therapy. Second, the drug can affect inflammatory cell adhesion and platelet attachment of the adjacent stent in this model[18]. However, the effect of the drug on platelet attachment is minimal **(Supplementary Figure 7)**. Third, an *in vitro* flow loop model was used in this study to assess human serum albumin adsorption and retention to different DES. Although this cannot completely mimic *in vivo* conditions, we believe the results of these studies simulate *in vivo* conditions since the concentration of albumin used was similar to that in human blood. Fourth, this study focused on albumin since albumin is considered to be a protein protecting against platelet attachment. Other types of protein may also play an important role in protecting against thrombogenicity. Previous data regarding the fact that fluoropolymer binds greater amounts of albumin as well as fibrinogen sounds counterintuitive given that fibrinogen[4] is a known promoter of clot formation through its conversion to fibrin by thrombin. Some emerging data suggest that albumin has an inhibitory property against fibrin polymerisation and that this may be one of the mechanisms by which fluoropolymers promote thromboresistance[19]. Further basic research is needed for a greater understanding of this relationship. Finally, studying platelet adherence using the *in vitro* flow loop model is different from that seen in the setting of vascular injury.

Conclusions

Overall, antithrombogenicity was better in the order FP-EES, BL-ZES, EP-RES and BMS. Although three durable polymer DES showed greater albumin coverage as compared to BMS, albumin intensity indicating concentration of albumin was not equivalent in three durable polymer DES. The results of albumin adsorption might be the cause of differences in platelet adhesion seen in the three different types of durable polymer DES and BMS, although other factors such as the adhesion of leukocytes also probably play a role. Because we also showed that the antiproliferative agents loaded onto DES have some thromboresistant properties, it is important to acknowledge the potential contribution of the antiproliferative agents as contributing to the thromboresistant effects seen in the different DES studied. These experiments may provide insight into the suitability of different durable polymer DES for shortening DAPT duration.

Impact on daily practice

FP-EES showed greater thromboresistance and concentration of superficial albumin on the stents as compared to other types of durable polymer DES. These results suggest that there are variations among polymer types in terms of thromboresistance, and albumin binding and retention. These differences may result in different suitability for short-term DAPT.

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Conflict of interest statement

R. Virmani has received honoraria from 480 Biomedical, Abbott Vascular, Boston Scientific, Cook Medical, Lutonix, Medtronic, Terumo Corporation and W.L. Gore, and is a consultant for 480 Biomedical, Abbott Vascular, Medtronic, and W.L. Gore. A.V. Finn has received honoraria from Boston Scientific, Abbott Vascular, and CeloNova. L. Perkins is a full-time employee of Abbott Vascular. S. Hossainy is a full-time employee of Abbott Vascular. S. Pacetti is a full-time employee of Abbott Vascular. The other authors have no conflicts of interest to declare.

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Supplementary data

Supplementary Appendix 1. Methods.

Supplementary Figure 1. The system of a flow loop model.

Supplementary Figure 2. Relationship between exposure time and amount of albumin on BMS.

Supplementary Figure 3. Protection of albumin against platelet attachment on the surface of BMS.

Supplementary Figure 4. Relationship between platelet adhesion, albumin retention and intensity.

Supplementary Figure 5. Percent of platelet coverage in the flow model.

Supplementary Figure 6. Percent of platelet positive area in the shunt and flow loop models.

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Supplementary Table 1. All device descriptions.

Supplementary Table 2. Summary of the mean values from all animals' blood coagulation (PT, PTT), platelet quantification (platelet counts, platelet EST), platelet function (LTA), and activated clotting time (ACT) in an *ex vivo* swine acute shunt model.

Supplementary Table 3. Percent of platelet coverage on bare metal stent surface at 60 minutes in an *in vitro* flow model.

Supplementary Table 4. Human albumin retention over 30 minutes during washing phase in an *in vitro* flow model.

Supplementary Table 5. Percent difference in albumin coverage over 30 minutes in an *in vitro* flow model.

Supplementary Table 6. Percent of platelet coverage at 60 minutes in an *in vitro* flow model.

Moving image 1. Real-time albumin retention by percent coverage and intensity over 30 minutes. The top videos show percent coverage and intensity of albumin analysed by Nikon NIS-Elements AR 5.02 64-bit within defined regions of interest in each group. The bottom videos show graphs of percent coverage and intensity corresponding to each group one-to-one.

Moving image 2. Real-time platelet adhesion over 60 minutes. The top videos show positive areas of platelet analysed by Nikon NIS-Elements AR 5.02 64-bit within defined regions of interest in each group. The bottom videos show graphs of strut coverage by platelets corresponding to each group one-to-one.

The supplementary data are published online at: https://eurointervention.pcronline.com/doi/10.4244/EIJ-D-19-00938

Supplementary data

Supplementary Appendix 1. Methods

The study protocol was approved by the Institutional Animal Care and Use Committee of the MedStar Health Research Institute.

Test devices

Using non-commercial stent backbones is ideal to test specific blood material interactions. However, stent designs including polymer, drug, strut thickness and strut platform comprehensively affect thromboresistance. Because using commercial DES can provide practical information to help in real-world practice, commercial DES were used in this study. Four stents were compared for assessment of acute thrombogenicity and inflammation in an *ex vivo* arteriovenous shunt model, and albumin retention and platelet adhesion in *in vitro* flow loop models using fluorescent human albumin serum (HSA) and human platelets, respectively. In an *ex vivo* shunt study, 3.0 x 12 mm stents for each shunt run were used. In the flow loop models, stents with a diameter of 3.0 mm were sectioned to put into slides.

Swine ex vivo arteriovenous shunt model

A total of 42 stents were deployed in 14 shunts from seven swine for the assessment of acute thrombogenicity and inflammation. A porcine *ex vivo* arteriovenous shunt model involving a test circuit of three different in-line test stents was performed for 60 minutes to evaluate platelet adherence, thrombus formation, and acute inflammation on the devices. Each shunt model had three different stents and each animal had two shunt experiments for a total of n=6 stents per animal.

Low-dose intravenous heparin (100 IU/kg) was used to minimise the effect of platelet aggregation and thrombus formation on the surface of the stents in order to examine the inherent platelet-mediated thrombus formation induced by each stent

type. Target blood activated clotting times were measured every 20 minutes and blood activated clotting time was kept between 150 and 190 s using bolus and maintenance dosing. At the end of each shunt run, stents were gravity perfused with Ringer's lactate until cleared of blood, fixed in 10% neutral buffered formalin, and longitudinally bisected for dual immunofluorescent staining.

Assessment of platelet aggregation and inflammation

In the shunt model, one half of the stent was stained with dual immunofluorescence of the antiplatelet markers (anti-CD61/CD42b) (anti-CD61; Immunotech, Commerce, CA, USA, and anti-CD42b; sc-7070, Santa Cruz, Dallas, TX, USA) and monocyte marker (CD14; Novus Biological, Littleton, CO, USA). The other stent half was immunostained using the platelet markers (anti-CD61/CD42b) and neutrophil marker (PM-1; BMA Biomedicals, Switzerland; dilution 1:800). For quantification of inflammatory cells, images with regions relatively free of platelet thrombus were obtained.

A real-time manner using human platelets in an in vitro flow loop

Preparation of stents

XIENCE Alpine (FP-EES) (3.0 x 12 mm; Abbott Vascular, Santa Clara, CA, USA) was compared to Resolute Onyx (BL-ZES) (3.0 x 12 mm; Medtronic, Minneapolis, MN, USA), EluNir (EP-RES) (3.0 x 12 mm; Cordis, Milpitas, CA, USA) and Vision (3.0 x 12 mm; Abbott Vascular). One stent from each group was used. Stents were expanded at nominal pressure on the bench under sterile conditions, cut longitudinally and transversely to make them all of uniform length. Stents were carefully cut in order to fit into sticky slides. If stent tips did not fit into the lumen of a slide, those tips were discarded and then new stent tips were cut. Stent tips were inserted into a bottomless channel slide used for perfusion applications (sticky-Slide I Luer 0.8 mm channel height, Cat No. 80198; ibidi GmbH). Each stent was primed with human serum at 37°C before initiating the flow.

Preparation of human platelets

Whole blood (60 ml) was collected (final concentration: 0.32% sodium citrate [M/V]) by venipuncture from healthy volunteers, none of whom was on any antiplatelet therapy or other regular prescription. The blood was centrifuged for 10 min at 200×g and RT without brake. The supernatant was collected with a plastic Pasteur pipette. After collection of the supernatant, the supernatant was centrifuged again for 17 min at 700×g without brake. The lower one third was platelet-rich plasma (PRP) and the upper two thirds were platelet-poor plasma (PPP). Platelet pellets were formed from the PRP and suspended in the PRP. CMFDA (5-chloromethylfluorescein diacetate) (Molecular Probes, Eugene, OR, USA) at final concentrations of 15 μM was used to label PRP through incubation in the dark for 45 minutes at 37°C. After labelling the PRP, it was centrifuged again for 10 min at 1,300×g without brake. At the bottom of the tube, platelet pellets formed. Plasma with exception of platelet pellets was removed and the platelet pellets were suspended in fresh plasma from PPP by gently shaking the tube to a volume of 25-30 ml.

In vitro flow loop and confocal microscopy

The temporal distribution of fluorescent platelets to various stent designs was studied by serial acquisition of confocal images after placement of the ibidi chamber slide/stent positioned on a microscope stage (Zeiss LSM700) equipped with a live-cell incubation chamber and Plan-Apochromat 10×/0.45 objective. Labelled platelets were circulated through silicone tubing (Cole-Parmer, Tygon Silicone Tubing, 1/8"IDx3/16"OD) at a rate of 8 mL/min (estimated shear stress: 2.0 dyn/cm^2) in line with the ibidi slide using a perfusion pump (ISMATEC, |IPC Series, Model No. SM931A; Cole-Parmer GmbH, Wertheim, Germany). Randomly selected regions of interest (ROIs) from each stent were scanned and exported in tagged image file format (TIFF) micrographs from Zen original image files using software (LSM 700, Zen 2011; Zeiss, Oberkochen, Germany). Each image was recorded at 1-1.5 min intervals, which are dependent on Z-stacks, for 60 minutes to create a movie file.

Assessment of platelet adherence to varying stent surfaces

The positive area of platelet labelling was analysed by Nikon NIS-Elements AR 5.02 64-bit within defined ROIs for the first 60 minutes. The analysis was run on each frame of the time lapse photography (MK). The covered strut area was quantified and expressed as percent of platelet coverage (%). See the Methods section in the manuscript.

A real-time manner using human albumin in an in vitro flow loop

Albumin is considered to be passivating to platelets. The thromboresistance of fluoropolymer surfaces is believed to be related to their strong affinity for albumin, so-called “fluoropassivation”. Therefore, this study focused on relative albumin adsorption and thromboresistance of FP-EES to other durable polymer DES.

Labelling methods for fluorescent albumin

The FITC variety averaged 1.6 fluoresceins per molecule. Fluorescent molecules react mostly through primary amines, so most likely they were attached to the lysines. Fluorescein was added using isothiocyanate chemistry.

Preparation of albumin

HSA was used in eight experiments (Product code: HSF; Protein Mods, Madison, WI, USA). FluoroBrite™ DMEM (Gibco, Waltham, MA, USA) was added to HSA of 750 mg to make it 20 ml final volume consistent with the concentration of albumin in human blood (3.5-5.0 g/dL).

Albumin in an in vitro flow loop

Labelled albumin was circulated through silicone tubing (Tygon Silicone Tubing, 1/8"IDx3/16"OD; Cole-Parmer) at a rate of 8 mL/min in line with the ibidi slide using a perfusion pump (ISMATEC, |IPC Series, Model No. SM931A; Cole-Parmer, Wertheim, Germany).

The temporal distribution of fluorescent albumin binding to various stent designs was studied by serial acquisition of confocal images after placement of the ibidi chamber slide/stent positioned on a microscope stage (Zeiss LSM800) equipped with Plan-Apochromat 10x/0.45 objective.

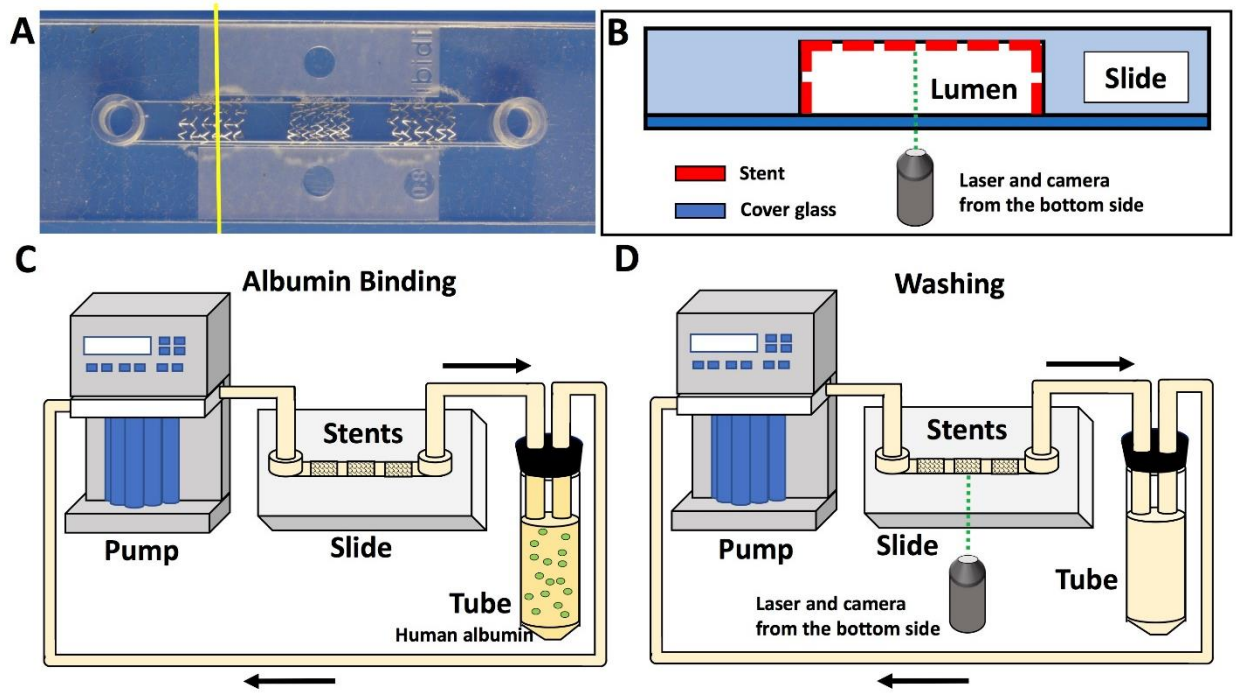
Assessment

Scanned images were exported in tagged image file format micrographs from ZEN original image files using software (LSM 700, Zen 2011; Zeiss, Oberkochen, Germany). Analysis was performed by Nikon NIS-Elements AR 5.02 64-bit. For assessment of the washing phases of albumin, one area was randomly selected from each type of stent. Therefore, selection bias cannot be excluded. However, the selected high-power areas are considered as the representative areas from each type of stent since the assessment using the entire stents showed the same tendency as the assessment using the selected high-power areas.

Statistical analysis

A power calculation

Based upon a previous shunt study [20], we estimated that a minimal sample size of six stents per group with an expected difference of roughly 30% in terms of endpoint (percentage of CD42b/CD61 immunofluorescence relative to total scanned stent surface area) with a standard deviation of 20% would provide 80% power to detect differences between groups using a two-sided alpha of 0.05. Also, based upon preliminary data for albumin study, we estimated that a minimal sample size of four stents per group with an expected difference of roughly 25% in terms of endpoint (percent low and higher signal area per stent area) with a standard deviation of 10% would provide 80% power to detect differences between groups using a two-sided alpha of 0.05.



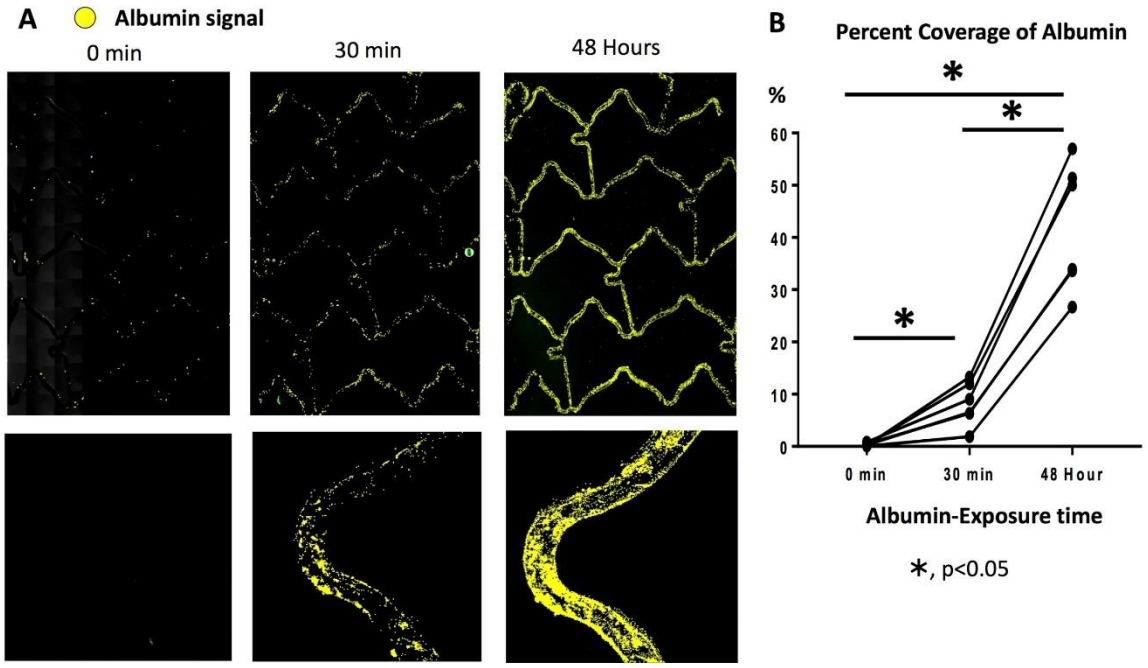
Supplementary Figure 1. The system of a flow loop model.

A) FP-EES (left), EP-RES (middle) and BMS (right) are inserted into a sticky Slide I Luer (ibidi, Germany).

B) Cross-sectional images at the yellow line in panel A.

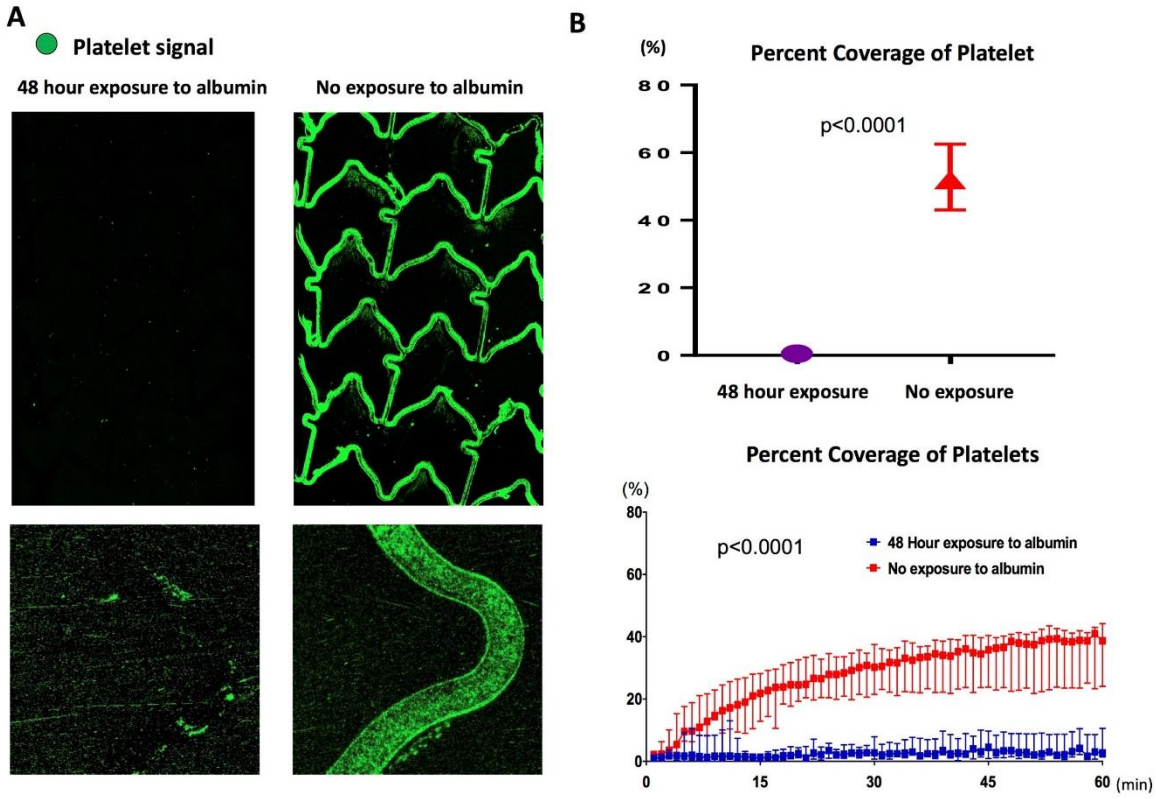
C) Model set for imaging by confocal microscopy during an albumin binding phase.

D) The model set during a washing phase.



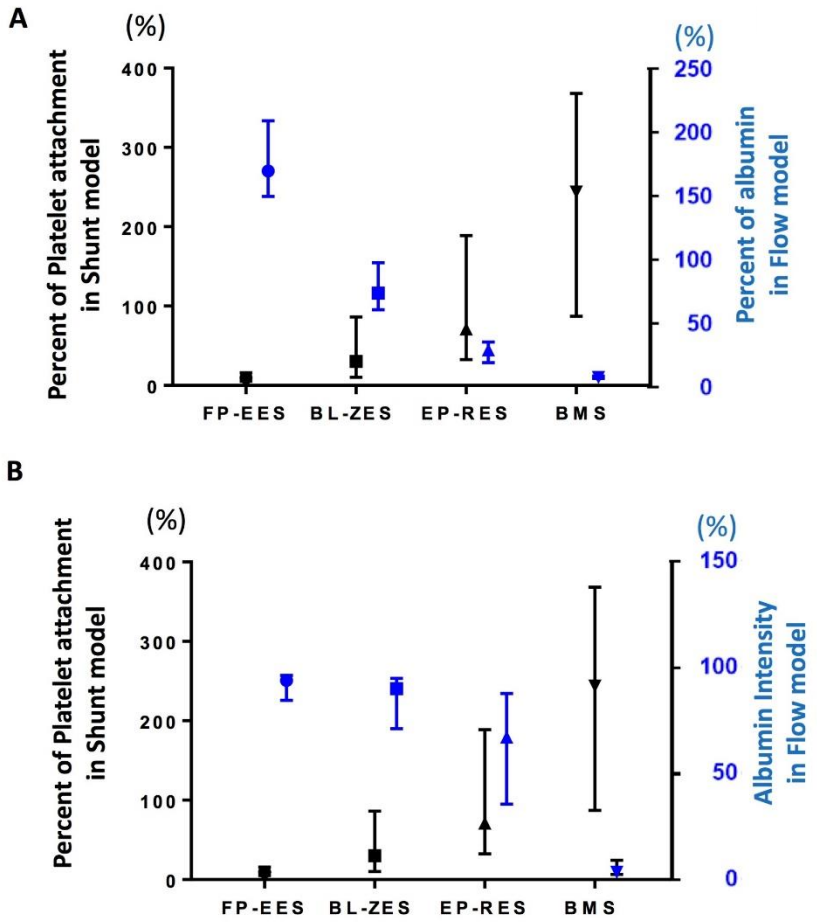
Supplementary Figure 2. Relationship between exposure time and amount of albumin on BMS. A) BMS was exposed to fluorescent albumin to confirm whether exposure time could play a role in increasing the amount of albumin surface of the stents. Three different time points were evaluated (0, 30 minutes and 48 hours) and each time point group had three BMS. Two predefined areas from each stent were analysed (n=6 for each time point group). The upper panels show low-power images of the BMS with albumin signal, while the lower panels show high-power images.

B) The amount of fluorescent albumin increased significantly over time (between 0 minutes and 30 minutes; p=0.013, between 30 minutes and 48 hours; p=0.009, between 0 minutes and 48 hours; p=0.0004). Data were analysed by Tukey's multiple comparisons test taking into account each pair.



Supplementary Figure 3. Protection of albumin against platelet attachment on surface of BMS. A) Labelled human platelets were measured by confocal microscopy in BMS with 48-hour exposure to fluorescent albumin versus BMS not exposed to albumin. Each group had three BMS and two predefined areas of high-power images were analysed (n=6 high-power regions per group). Upper panels show low-power images of BMS with platelet signals, while lower panels are high-power images on a representative individual strut.

B) BMS with 48-hour exposure to albumin showed significantly lower platelet attachment than those with no exposure ($p<0.0001$). Upper graph shows percent coverage of platelet from low-power images; data were expressed as estimated mean and 95% confidence interval. Lower graph shows percent coverage of platelet from high-power images; data were expressed as mean and interquartile.

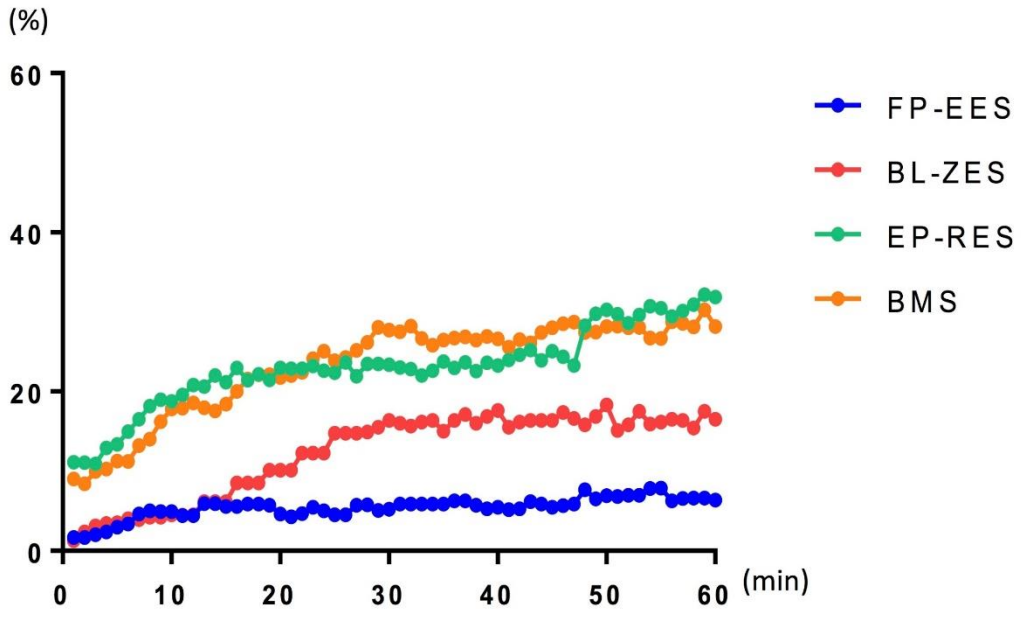


Supplementary Figure 4. Relationship between platelet adhesion, albumin retention and intensity.

Albumin intensity correlated negatively with platelet adhesion in the shunt model.

Data were expressed as median and interquartile range.

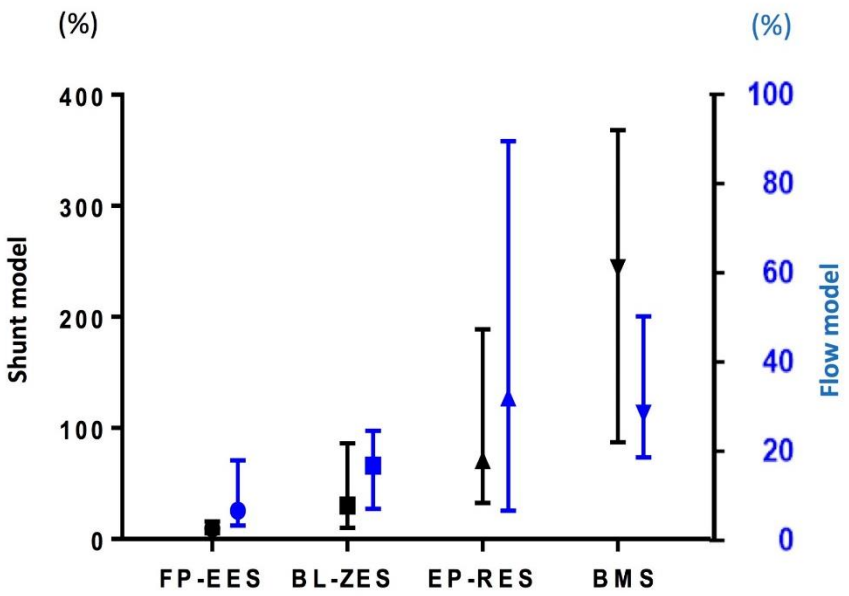
BMS: bare metal stent; BP-ZES: BioLinx polymer zotarolimus-eluting stent; EP-RES: elastomer polymer ridaforolimus-eluting stent; FP-EES: fluoropolymer everolimus-eluting stent



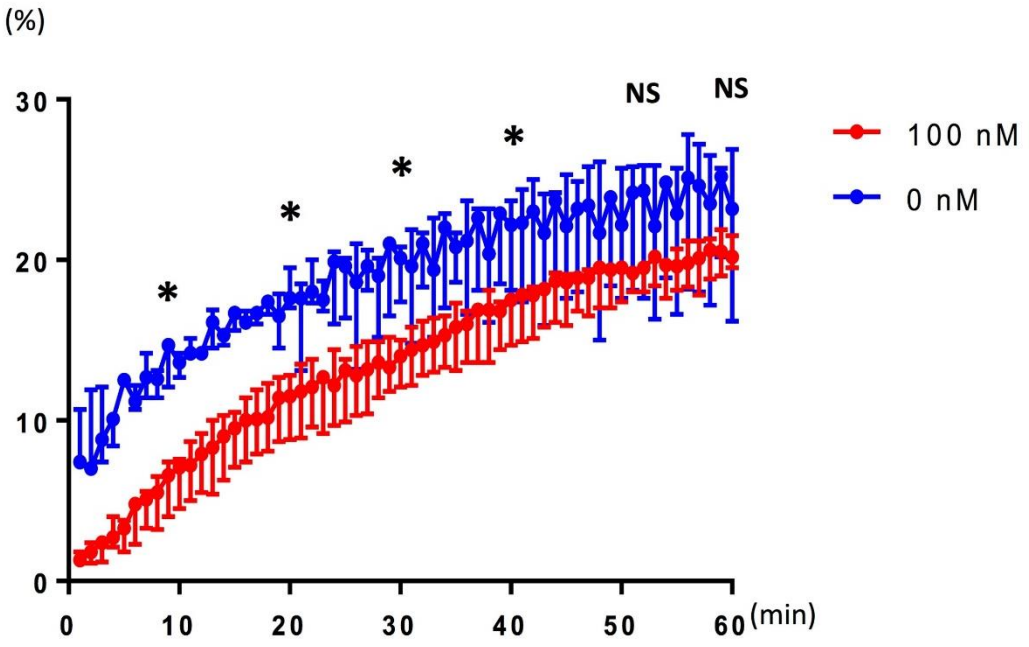
Supplementary Figure 5. Percent of platelet coverage in the flow model.

Platelet aggregation in the flow model was tested in FP-EES, BL-ZES, EP-RES and BMS (n=3 each). Using generalised linear mixed models, FP-EES had numerically less platelet aggregation among the four stents tested, followed by BL-ZES and BMS, with the greatest platelet aggregation being seen in EP-RES, although there was no significant difference between each group.

BMS: bare metal stent; BP-ZES: BioLinx polymer zotarolimus-eluting stent; EP-RES: elastomer polymer ridaforolimus-eluting stent; FP-EES: fluoropolymer everolimus-eluting stent



Supplementary Figure 6. Percent of platelet positive area in the shunt and flow loop models. Two studies using the shunt and flow loop models (60 minutes) consistently showed the least platelet aggregation in FP-EES followed by BL-ZES. EP-RES and BMS had the most attachment of platelets in both types of experiment. Data were expressed as median and interquartile range.

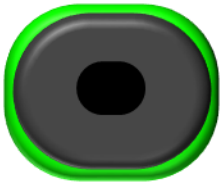


Supplementary Figure 7. Comparison of platelet attachment between BMS without drug and with drug (100 nM) in the flow model.

We compared everolimus 0 nM with 100 nM (n=3 for each) in order to investigate the effect of drug on platelet attachment. The drug dose of everolimus was determined based on our previous study [21]. There were significant differences between the groups at 10, 20, 30, and 40 minutes; however, percent of platelet coverage did not differ between them at 50 and 60 minutes.

Data were expressed as median and interquartile range.

BMS: bare metal stent; NS: not significant

Device	Xience Xpedition	Resolute Onyx	EluNIR	Vison
Shape				
Platform	MULTI-LINK 8	Onyx	-	MULTI-LINK 8
Material	CoCr	CoNi / Ptlr Core	CoCr	CoCr
Strut thickness	81 µm	81 µm	87 µm	81 µm
Drug type	Everolimus	Zotarolimus	Ridaforolimus	-
Drug dose	100 µg/cm^2	1.6 µg/cm^2	1.1 µg/mm2	-
Materials of the polymer	PBMA/PVDF-HFP	BioLink polymer	PBMA/CarboSI® 20 55D	-
Coating type	Circumferential	Circumferential	Circumferential	-
Coating thickness	7-8 µm / side	6 µm / slide	7 µm	-

Supplementary Table 1. All device descriptions.

Supplementary Table 2. Summary of the mean values from all animals' blood coagulation (PT, PTT), platelet quantification (platelet counts, platelet EST), platelet function (LTA), and activated clotting time (ACT) in an *ex vivo* swine acute shunt model.

Test	Mean (min-max)			Fold change (%)		
	Baseline	After 1st shunt	After 2nd shunt	1st shunt vs baseline	2nd shunt vs. baseline	2nd shunt vs. 1st shunt
Prothrombin time (seconds)	9.2 (8.0–10.7)	10.1 (9.9–13.8)	11.9 (10.71–15.40)	1.22	1.30	1.07
PTT (seconds)	11.4 (9.1–13.2)	69.6 (33.6–100)	84.6 (50–100)	6.11	7.42	1.22
Platelet count (x1,000/μL)	231.8 (103–472)	253.2 (87–297)	233.9 (83–401)	1.09	1.01	0.92
Platelet EST	Adequate	Adequate	Adequate	N/A	N/A	N/A
LTA (ADP=20 uM) (% platelet aggregation)	58.7 (33–99)	33.0 (12–51)	23.2 (4–44)	0.56	0.39	0.70
LTA (ADP=5 μM) % platelet aggregation	32.7 (11–41)	12.8 (3–31)	10.6 (4–39)	0.39	0.32	0.83
ACT (seconds)	102.14 (97–114)	165.20 (143–189)	172.42 (131–204)	1.62	1.69	1.04

Platelet EST was considered to be adequate when the platelet count was estimated to be within the reference interval.
ADP: adenosine diphosphate; LTA: light transmission aggregometry; PTT: partial thromboplastin time

Supplementary Table 3. Percent of platelet coverage on bare metal stent surface at 60 minutes in an *in vitro* flow model.

Group	48-hour exposure to albumin	No exposure to albumin	*p*-value
Low power (%)	0.45 (0.27-0.77)	51.89 (43.08-62.50)	<0.0001
High power (%)	4.07 (1.93-8.58)	37.73 (34.74-40.98)	<0.0001

Analysed by GLMM (generalised linear mixed models).
Data were expressed as estimated mean and 95% confidence interval.

Supplementary Table 4. Human albumin retention over 30 minutes during washing phase in an *in vitro* flow model.

Percent coverage of albumin at 0 min (%)				
		p-value		
Stent type	Estimated mean (95% CI)	vs BL-ZES	vs EP-RES	vs BMS
FP-EES	93.6 (70.8-123.7)	0.96	0.09	<0.01
BL-ZES	92.6 (62.4-137.3)	NA	0.18	<0.01
EP-RES	63.3 (42.7-93.9)	0.18	NA	<0.01
BMS	5.5 (4.1-7.5)	<0.01	<0.01	NA
Percent coverage of albumin at 30 min (%)				
		p-value		
Stent type	Estimated mean (95% CI)	vs BL-ZES	vs EP-RES	vs BMS
FP-EES	91.9 (68.3-123.6)	0.78	0.14	<0.01
BL-ZES	85.8 (56.4-130.5)	NA	0.32	<0.01
EP-RES	63.9 (42.0-97.2)	0.32	NA	<0.01
BMS	5.5 (4.0-7.6)	<0.01	<0.01	NA
Intensity scale of albumin at 0 min				
		p-value		
Stent type	Estimated mean (95% CI)	vs BL-ZES	vs EP-RES	vs BMS
FP-EES	185.5 (137.5-250.4)	<0.01	<0.01	<0.01
BL-ZES	85.7 (56.2-130.7)	NA	<0.01	<0.01
EP-RES	27.7 (18.2-42.2)	<0.01	NA	<0.01
BMS	3.1 (2.2-4.2)	<0.01	<0.01	NA
Intensity scale of albumin at 30 min				
		p-value		
Stent type	Estimated mean (95% CI)	vs BL-ZES	vs EP-RES	vs BMS
FP-EES	170.2 (122.3-236.9)	<0.01	<0.01	<0.01
BL-ZES	72.5 (45.4-115.7)	NA	<0.01	<0.01
EP-RES	22.9 (14.3-36.5)	<0.01	NA	<0.01
BMS	3.0 (2.1-4.3)	<0.01	<0.01	NA

BMS: bare metal stent; BP-ZES: BioLinx polymer zotarolimus-eluting stent; CI: confidence interval; EP-RES: elastomer polymer ridaforolimus-eluting stent; FP-EES: fluoropolymer everolimus-eluting stent; NA: not applicable

Supplementary Table 5. Percent difference in albumin coverage over 30 min in an *in vitro* flow model.

Percent difference of coverage between 0 to 30 min (%)				
Stent type	Estimated mean (95% CI)	*p*-value		
		vs BL-ZES	vs EP-RES	vs BMS
FP-EES	3.2 (1.35-7.66)	0.24	0.35	0.10
BL-ZES	14.1 (3.58-55.4)	NA	0.57	0.94
EP-RES	8.3 (2.1-32.5)	0.57	NA	0.43
BMS	15.0 (5.7-39.4)	0.94	0.43	NA
Percent difference of intensity scale between 0 to 30 min (%)				
Stent type	Estimated mean (95% CI)	*p*-value		
		vs BL-ZES	vs EP-RES	vs BMS
FP-EES	10.4 (6.3-17.2)	0.27	0.24	0.79
BL-ZES	17.2 (8.9-33.5)	NA	0.79	0.21
EP-RES	19.7 (9.1-42.5)	0.79	NA	0.19
BMS	9.4 (5.5-16.3)	0.21	0.19	NA

BMS: bare metal stent; BL-ZES: BioLinx polymer zotarolimus-eluting stent; CI: confidence interval; EP-RES: elastomer polymer ridaforolimus-eluting stent; FP-EES: fluoropolymer everolimus-eluting stent; NA: not applicable

Supplementary Table 6. Percent of platelet coverage at 60 minutes in an *in vitro* flow model.

Percent of platelet coverage at 60 min (%)				
Stent types	Estimated mean (95% CI)	*p*-value		
		vs BL-ZES	vs EP-RES	vs BMS
FP-EES	6.76 (2.49-18.37)	0.12	0.11	0.11
BL-ZES	25.63 (8.99-73.10)	NA	0.95	0.97
EP-RES	26.42 (9.630-72.48)	0.95	NA	0.91
BMS	25.24 (9.20-69.25)	0.97	0.91	NA

Analysed by GLMM (generalised linear mixed models).

BMS: bare metal stent; BL-ZES: BioLinx-polymer zotarolimus-eluting stent; CI: confidence interval; EP-RES: elastomer polymer ridaforolimus-eluting stent; FP-EES: fluoropolymer everolimus-eluting stent; NA: not applicable