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The effect of CD34-capturing coronary stents with abluminal sirolimus coating on endothelial coverage

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KEYWORDS

- drug-eluting stent
- in-stent restenosis
- stent thrombosis

Abstract

Aims: Drug-eluting stents (DES) reduce neointimal hyperplasia by inhibition of vascular smooth muscle cell proliferation, concomitantly inhibiting stent endothelialisation and increasing the risk for stent thrombosis. The present study compares a contemporary DES to an endothelial progenitor cell-capturing DES (COMBO stent), with regard to intimal hyperplasia and endothelial coverage.

Methods and results: Twelve New Zealand white rabbits were subjected to bilateral iliac artery stent placement. Each animal received both an everolimus-eluting stent (EES) and a COMBO stent. Four weeks after implantation, optical coherence tomography (OCT) was performed in six animals and tissue was harvested from the other six animals. Endothelial stent coverage assessed by scanning electron microscopy was significantly higher in COMBO stents than in EES (96.6±3.5% vs. 78.5±16.8%; $p<0.05$). Neointimal hyperplasia by OCT differed significantly (EES: 0.227±0.025 mm^2 vs. COMBO: 0.188±0.044 mm^2; $p<0.05$), but not by histology (EES: 0.823±0.200 mm^2 vs. COMBO: 0.891±0.312 mm^2; p=NS). No differences were observed in late loss between EES and COMBO stents (0.29±0.19 mm^2 vs. 0.29±0.16 mm^2; p=NS).

Conclusions: Endothelialisation is significantly improved in the COMBO stent with equal inhibition of intimal hyperplasia, which may reduce thrombotic events after DES implantation and allow earlier discontinuation of dual antiplatelet therapy.

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Abbreviations

BAR	balloon-to-artery ratio
BMS	bare metal stent
DES	drug-eluting stent
EC	endothelial cell
EES	everolimus-eluting stent
EPC	endothelial progenitor cell
H&E	haematoxylin and eosin
IV	intravenous
IM	intramuscular
LL	late loss
NIH	neointimal hyperplasia
NS	not significant
OCT	optical coherence tomography
PES	paclitaxel-eluting stent
sEM	scanning electron microscopy
SES	sirolimus-eluting stent
VSMC	vascular smooth muscle cell

Introduction

Cardiovascular disease remains the leading cause of death in the world with rising numbers especially in non-Western countries[1]. The most frequent treatment for coronary artery disease (CAD) is to restore coronary blood flow by percutaneous coronary intervention (PCI).

Though superior to solo balloon angioplasty[2], coronary stent implantation has two complications: in-stent restenosis and stent thrombosis[3]. In-stent restenosis is driven by the inflammatory response that occurs upon inflation of the balloon catheter to restore the lumen and the accompanying endothelial damage. This triggers vascular smooth muscle cell (VSMC) proliferation, leading to neointimal hyperplasia (NIH) and subsequent luminal narrowing. The advent of drug-eluting stents (DES) that reduce VSMC proliferation has largely solved this problem[4,5]. However, by non-selectively inhibiting endothelial cell proliferation as well, the risk for in-stent thrombosis is increased.

In particular, early stent endothelialisation reduces thrombotic complications and decreases neointima formation[6,7]. Endothelial progenitor cell (EPC) capturing stents use anti-CD34 antibody coatings to facilitate colonisation of circulating EPCs onto the stent struts. In comparison to bare metal stents (BMS) or DES, they have been shown to improve stent endothelialisation and decrease stent thrombosis in both *in vitro* and *in vivo* studies[8-11]. However, compared to DES, neointima formation and the need for target vessel revascularisation were significantly higher due to the lack of antiproliferative coatings.

It is for these reasons that the COMBO™ Dual Therapy Stent (OrbusNeich, Hong Kong) combines a luminal anti-CD34 antibody coating to improve luminal stent endothelialisation with abluminal antiproliferative drug elution from a bioresorbable polymer matrix to inhibit VSMC proliferation and intimal hyperplasia. The REMEDEE trial has shown the non-inferiority of the COMBO stent compared to paclitaxel-eluting stents (PES) with regard to angiographic in-stent late lumen loss[12] .

However, PES belong to the first-generation DES, which nowadays have been largely replaced by safer and more effective second-generation DES[13,14]. Yet, histological data regarding stent endothelialisation in combination with clinical standard optical coherence tomography (OCT) have not been reported so far. The aim of the current study was to compare these stent types in rabbits, using both histology and OCT to assess endothelial cell coverage and intimal hyperplasia.

Methods

EXPERIMENTAL DESIGN

Twelve female New Zealand white rabbits (Charles River, Chatillon-sur-Chalaronne, France; 3.5-4.0 kg) were subjected to iliac artery stenting. Two different types of stent were implanted in the left and right iliac arteries in an alternating fashion (switching sides). The COMBO stent combines a sirolimus-eluting bioresorbable coating on the abluminal side with an anti-CD34 antibody coating on the luminal side. The XIENCE PRIME® stent (Abbott Vascular, Santa Clara, CA, USA) has a conformal, everolimus-eluting permanent polymer coating with omnidirectional release of the drug.

Data acquisition and measurements were performed by a blinded observer. All animal experiments were approved by the Ethical Committee on Animal Experimentation of the University Medical Center Utrecht (Utrecht, The Netherlands) and conform to the "Guide for the care and use of laboratory animals".

ANAESTHESIA

The rabbits were fasted overnight prior to surgery. From the day before implantation until termination at 28 days, rabbits received 10 mg/kg aspirin (Aspro; Bayer, Mijdrecht, The Netherlands) daily, dissolved in 400 mL freshly prepared drinking water after closely monitoring the average water intake per day. Subcutaneous meloxicam (1 mg/kg) was given before surgery as analgesia.

Acepromazine and methadone (both 1.5 mg/kg) were injected intramuscularly for premedication. Etomidate (1.5-2 mg/kg) was injected via the ear vein, after which rabbits were intubated and ventilated with a mixture of oxygen/air (1:2) and 1.5% isofluorane. Sufentanil (1 µg/kg/hr) was continuously administered intravenously.

STENT IMPLANTATION

Heparin (150 IU/kg IV) was injected prior to cannulation of the left carotid artery. A 4 Fr sheath was inserted through which a 3 Fr Fogarty balloon (Edwards Lifesciences, Irvine, CA, USA) was inserted. After inflation, the balloon was retracted through both iliac arteries twice for approximately 4 cm to induce endothelial denudation. Afterwards, the stents (3.0×15.0 mm) were implanted in the iliac artery. Nominal pressure was applied to inflate the balloon to a diameter of 3.0 mm, followed by a second angiogram.

QUANTITATIVE ANGIOGRAPHY

Angiograms of the iliac arteries were obtained before and after stent implantation and at termination. Luminal diameters were measured using ImageJ. Calibration was performed on the guiding catheter in the same image. The balloon-to-artery ratio (BAR) was

defined as the luminal diameter after stenting/luminal diameter before stenting. Late loss was defined as the difference between the angiographic diameter directly after stenting and the angiographic diameter at 28 days of follow-up.

OPTICAL COHERENCE TOMOGRAPHY (OCT)

To avoid detection of iatrogenic endothelial damage in the scanning electron microscopy (sEM), OCT was performed in six of the 12 rabbits. Four weeks after implantation, the rabbits were heparinised with 150 IU/kg prior to cannulation of the right carotid artery. A 6.5 Fr SheathLess Eaucath multipurpose guiding catheter (ASAHI Intecc, Aichi, Japan) was inserted and selectively placed in the iliac artery. A C7 Dragonfly™ Duo imaging catheter (St. Jude Medical, St. Paul, MN, USA) was positioned with the proximal and distal markers on both sides of the stent. Pure contrast agent was injected through the guiding catheter for temporary removal of signal-distorting blood flow from the iliac artery. A manually triggered pullback was performed using the OCT ILUMIEN™ OPTIS™ OCT system (St. Jude Medical). Image analysis was performed using dedicated software (Curad B.V., Amsterdam, The Netherlands)[15], including automated contour detection algorithms. For each cross-sectional frame (n=10/mm; n=150/stent), the lumen contour and the stent contour were automatically delineated and manually corrected where needed. Neointima formation was defined as the difference between stent area and luminal area, expressed both as mm^2 and as a percentage of the total stent area (i.e., two separate outcome measurements). All 150 cross-sectional frames were used to calculate the average neointimal area and neointimal area as a percentage of total stent area. These were then used to calculate the mean and standard deviation for each group (EES or COMBO). Stent struts were classified into three categories: embedded, if buried in the vessel wall; protruding, if protruding in the lumen but still in contact with the vessel wall; and malapposed, if protruding and not in contact with the vessel wall. For the latter, the distance between stent strut and luminal contour was automatically measured and classified malapposed if greater than its strut thickness (EES: 88 µm; COMBO: 104 µm).

TISSUE PREPARATION, HISTOLOGICAL ANALYSIS AND SCANNING ELECTRON MICROSCOPY

The remaining six rabbits were also sacrificed after 28 days. After heparinisation (1,000 IU/kg IV), catheters were placed in the aorta and caval vein under general anaesthesia as described above. An angiogram was performed to visualise the stents and surrounding arteries. After sacrifice, the aorta was perfused with Ringer's lactate to remove blood cells from the stents, followed by pressure fixation with 4% formalin. Subsequently, the stents and adjacent arteries were dissected. The stent was cut axially so that one part comprised one third of the stent and the other part comprised two thirds of the stent.

The larger part was used for morphometric and inflammation analyses. After an additional formalin fixation for at least 72 hours, the stents were embedded in methyl methacrylate for histological analysis. Sections were cut with a diamond-coated saw at three levels. A haematoxylin and eosin (H&E) staining was performed for morphometric analysis. Luminal contours and internal elastic laminae (IEL) were traced manually using pictures made at a 20x magnification using ImageJ. The amount of neointima was calculated by subtracting the luminal area from the IEL area. Inflammation was evaluated as previously described[16]. At 40x magnification, each stent strut in a tissue section was scored for inflammation as follows: 0= no inflammatory cells surrounding the strut; 1= very light, non-circumferential cellular infiltrate surrounding the strut; 2= localised moderate to dense cellular aggregate surrounding the strut non-circumferentially with or without slight expansion into the neointima not in direct contact with the strut; 3= circumferential dense cellular infiltrate of the strut with extensive expansion into the neointima not in direct contact with the stent strut. The scores of the individual stent struts were averaged per tissue section and tissue sections were averaged per stent. The averages of all the stents in one group (EES or COMBO) were used to calculate the means and standard deviations.

The smaller part was cut longitudinally and used for sEM. Stents were fixed in a 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). A secondary fixation using 1% osmium tetroxide in 0.1 M cacodylate buffer was performed, followed by dehydration. Liquid was removed from the samples using critical point drying. The samples were sprayed with platinum and analysed using sEM (Phenom Desktop SEM; Phenom-World BV, Eindhoven, The Netherlands). Of each single stent, eight to 12 images at 360x magnification were made. Stent strut contours could easily be visualised as slightly elevated areas in the image. In each image, the covered area of the stent strut contour was measured as well as the total area of the stent strut contour, using ImageJ. The covered area was then expressed as a percentage of the total stent strut area. In each animal, the individual scores (i.e., percentages) of these eight to 12 images were averaged per rabbit. These averages were then used to calculate the mean and standard deviation for each group (EES or COMBO).

Statistical analysis

Values are presented as mean±standard deviation (SD). Data distribution was evaluated for normality using the Shapiro-Wilk test. All data were normally distributed and a paired-samples t-test was performed to test for significant differences ($p<0.05$). Statistical analyses were performed using SPSS software, Version 21 (IBM Corp., Armonk, NY, USA).

Results

QUANTITATIVE ANGIOGRAPHY

Angiograms were taken before, directly after stent implantation and at 28-day follow-up **(Figure 1)**. No differences in vessel diameters, BAR or late loss at four-week follow-up were observed **(Table 1)**.

STENT ENDOTHELIALISATION

Figure 2A-Figure 2D show overview images of the EES and COMBO stent by sEM. Four weeks after stent implantation,

Table 1. Angiography measurements at baseline and 28-day follow-up.

Stent	Before stent placement, diameter (mm)	After stent placement, diameter (mm)	B:A ratio	Follow-up diameter (mm)	Late loss
EES (n=12)	2.14±0.23	2.99±0.18	1.42±0.19	2.70±0.20	0.29±0.19
COMBO (n=12)	2.15±0.21	2.91±0.18	1.37±0.16	2.62±0.16	0.29±0.16

Values are represented as mean±standard deviation (SD). No significant differences were observed between the two groups. B:A ratio: balloon-to-artery ratio

the COMBO stent showed visually improved strut coverage at intermediate (**Figure 2A-Figure 2D**, upper right panels) and higher magnification (**Figure 2A-Figure 2D**, lower right panels). Quantification of strut coverage confirmed a lower endothelial coverage in the EES and a significantly improved endothelial coverage in the COMBO stent (78.5±16.8% vs. 96.6±3.5%; p=0.038, **Figure 2E**).

NEOINTIMAL HYPERPLASIA AND INFLAMMATION

Four weeks after stent implantation, intravascular OCT was performed in six of the 12 animals. All images per stent were semi-automatically analysed and luminal and stent areas were quantified **(Figure 3A, Figure 3B)**. Absolute neointimal area by OCT analysis was significantly higher in EES compared to COMBO stents (0.227±0.025 mm^2 vs. 0.188±0.044 mm^2; p=0.013; **Figure 3C**), but did not differ when expressed as a percentage of the total stent area (EES: 3.78±0.45% vs. COMBO: 3.49±0.95%; p=NS; **Figure 3D**). The percentage of protruding stent struts as a measure of the vascular healing response did not differ significantly between the two stent types (EES: 35.1±14.7% vs. COMBO: 29.7±17.1%; p=NS; **Figure 3E**).

Neointimal hyperplasia was also assessed in H&E stained tissue sections **(Figure 4A, Figure 4B)**. In contrast to OCT, no significant differences were observed with respect to neointima formation between EES and COMBO stents (0.823±0.200 mm^2 vs. 0.891±0.312 mm^2; p=NS; **Figure 4C**). This may be due to the higher accuracy of histology or the lower number of analysed sections compared to OCT.

Finally, H&E stained tissue sections were evaluated for inflammation **(Figure 4D, Figure 4E)**. In the majority of stent struts, cellular infiltrate was absent or only minimally present. Hence, average inflammatory scores did not differ significantly between EES and COMBO stents (0.530±0.380 vs. 0.435±0.295; p=NS; **Figure 4F**).

Discussion

The significant reduction of in-stent restenosis in DES that we have witnessed so far has come at the expense of reduced endothelialisation and the corresponding higher risk for stent thrombosis[8-11]. To overcome these drawbacks, different approaches have been shown to be promising. Amongst these are using a sole abluminal antiproliferative drug coating[17], seeding stents with human trophoblastic endovascular progenitor cells[18] and using anti-CD34 for endothelial progenitor cell (EPC) capturing[7]. It is this latter approach that is investigated in the current study, comparing an abluminal sirolimus-eluting stent with luminal anti-CD34 coating (COMBO stent) to a second-generation everolimus-eluting stent (EES) with respect to endothelial cell coverage and neointimal hyperplasia. At 28 days, the COMBO stent showed significantly improved endothelial coverage by sEM compared to the EES.

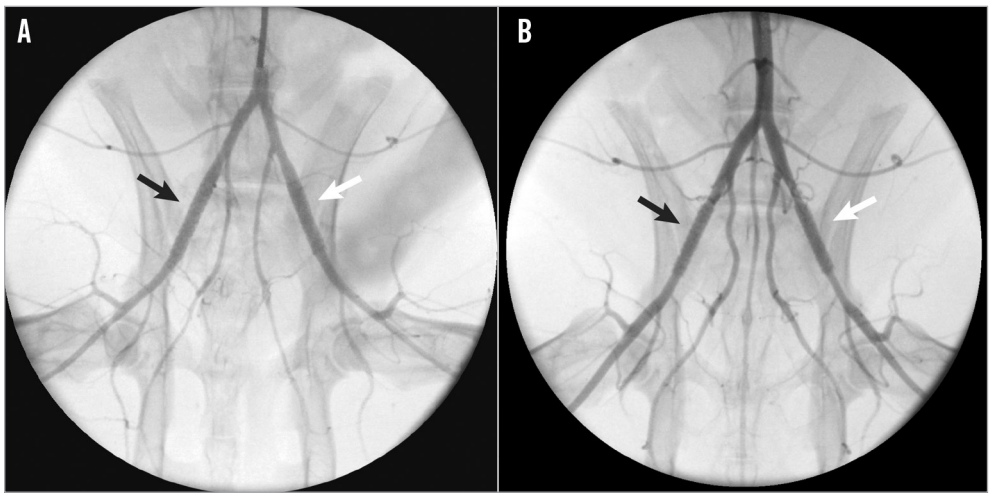

Figure 1. *Angiographic images. Angiograms obtained directly after implantation (A) and after 28 days of follow-up (B). Stent location is indicated by arrows, black for the everolimus-eluting stent and white for the COMBO stent.*

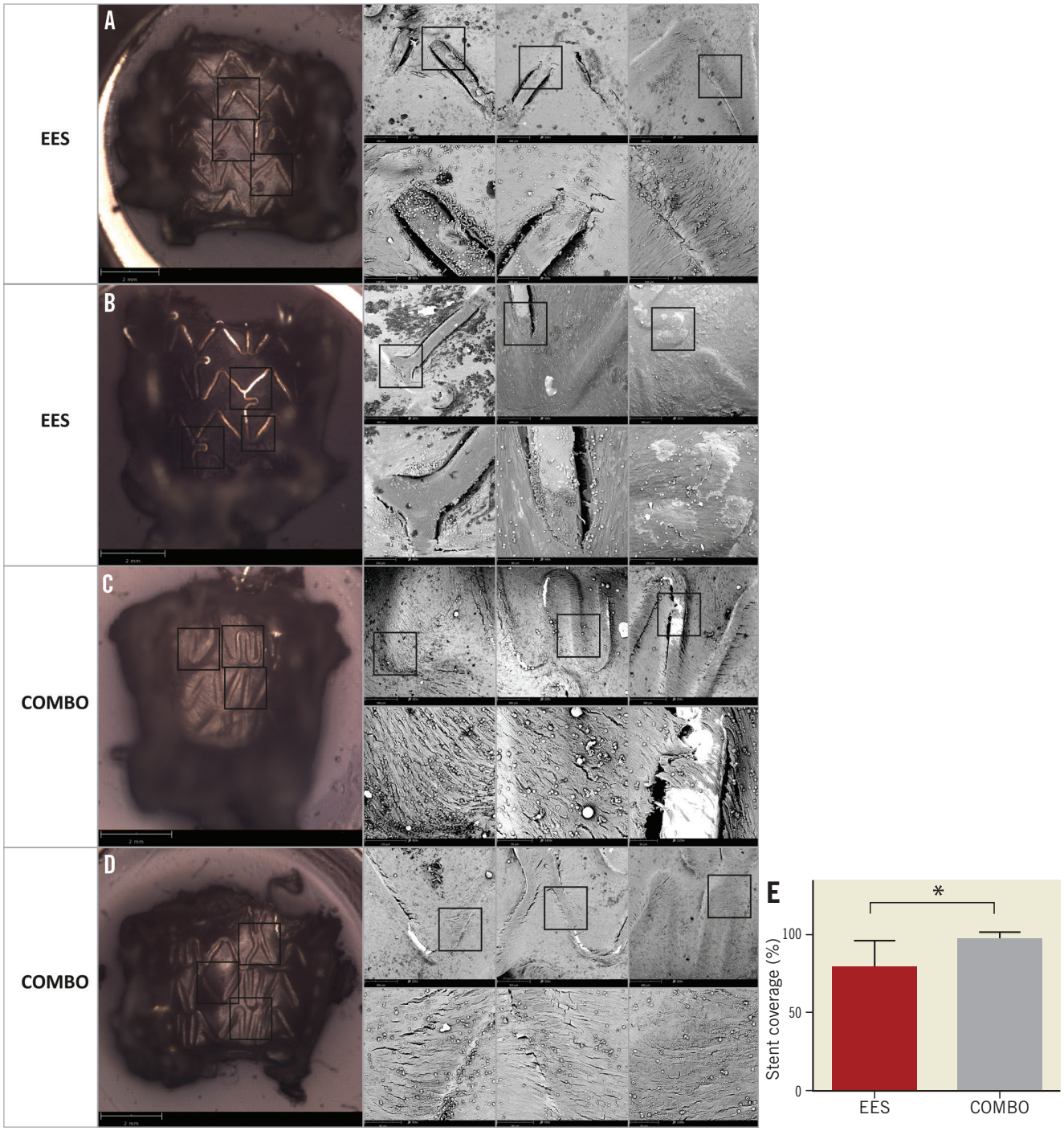

Figure 2. *Assessment of stent endothelialisation using scanning electron microscopy (sEM). Scanning electron microscopy imaging of the luminal surface of the everolimus-eluting stent (EES) (A, B) and the COMBO stent (C, D); intermediate (upper right panels) and high magnification (lower right panels). At high magnification, the COMBO stent showed confluent stent coverage, whereas the EES struts were not completely endothelialised. Quantification of stent strut coverage showed a significantly improved endothelialisation of the COMBO stent compared to the EES (E). *: p<0.05*

DES are known to interfere with endothelial cell proliferation and function, leading to delayed strut endothelialisation. Strut coverage in our study was decreased in the EES to a comparable degree to that previously reported[19]. In contrast, the COMBO stent showed almost complete endothelial coverage. This finding indicates that the anti-CD34 coating accelerates stent coverage, even in the presence of an antiproliferative component, similar to stents without antiproliferative coatings[20]. As stent endothelialisation is a major determinant of stent thrombosis[5], the COMBO stent might therefore reduce stent-related thrombotic events.

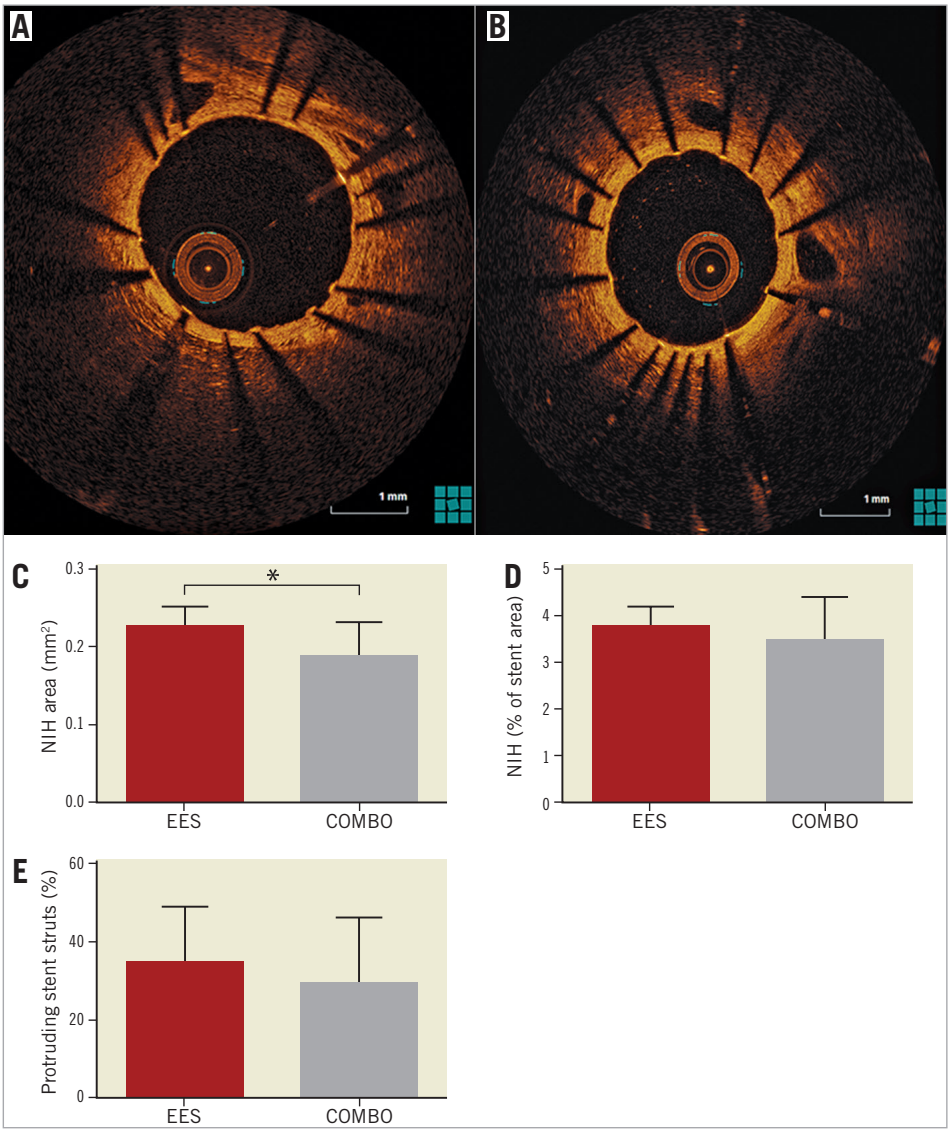

Figure 3. *Assessment of neointima formation by optical coherence tomography (OCT). Representative optical coherence tomography images of the everolimus-eluting stent (EES) (A) and the COMBO stent (B). The difference between stent and lumen contour represents neointima formation, which was significantly decreased in the COMBO stent (C). Neointima formation expressed as a percentage of the total stent area did not differ between both stent types (D). Classification of stent struts as being buried in the vessel wall (embedded) or protruding into the lumen (protruding) was not different between both groups (E). *: p<0.05*

In our previous findings with the anti-CD34 capturing stents (without a drug-eluting component) in comparison with BMS we found superior endothelial coverage with the anti-CD34 stent at seven days (82.21% vs. 77.92%)[21]. Our current results with the COMBO stent (96.6% endothelial coverage at 28 days) are largely in line with the earlier findings, suggesting that the abluminal elution of the antiproliferative drug has no negative effect on stent endothelialisation.

Neointima formation was significantly higher in EES compared to COMBO stents when measured by OCT, whereas histologic measurements did not show significant differences. In histological sections, the internal elastic membrane (IEM) can be easily detected and very accurately traced. In OCT as the clinical standard, the stent strut contour is semi-automatically detected using the endoluminal stent strut reflections. This method excludes the abluminal part of the stent struts and corresponding neointimal area. In situations with very low amounts of neointima as present in the current study, OCT is therefore less accurate than histology. However, the differences between both techniques are very small and therefore clinically not significant.

Previous studies have shown similar underestimation of neointima formation in OCT compared to histology[22]. Moreover, our current results are in line with previous experiments, comparing

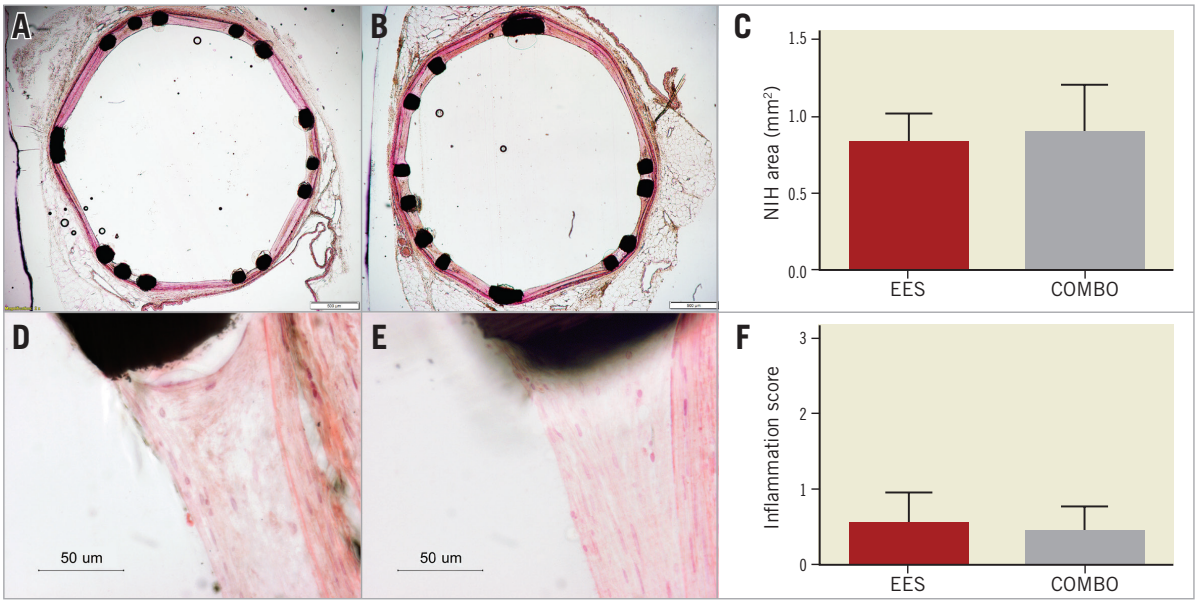

Figure 4. *Neointima formation assessment by HE-stained tissue sections. Representative images of tissue sections of the everolimus-eluting stent (A) and the COMBO stent (B). The difference between internal elastic membrane (IEL) and lumen area represents neointima formation, which did not differ between the two groups (C). Representative high magnification images of both everolimus-eluting (D) and COMBO stent (E) with minor inflammatory cell deposition near the stent strut. Quantification of inflammation on a 0–3 scale confirmed no differences between the two groups (F).*

different DES types with BMS. While BMS showed significantly more neointima formation compared to any DES, there was no difference between DES types[19,23]. The comparable neointimal areas found with EES compared to the COMBO stent suggest that the effect of the improved endothelialisation on VSMC mobilisation is relatively small in comparison with the inhibitory action of the antiproliferative drug. Inflammatory cell deposition was also not affected by accelerated endothelialisation.

Limitations

Because two thirds of each stent was used for morphometric analysis (H&E), we were unable to describe the effect of the COMBO stents on endothelialisation in the middle part of the stent. In comparison to most of the contemporary preclinical studies that assessed the entire stent for endothelialisation[10,18,23], the assessment of only one third of the stent limits its translation to stent re-endothelialisation in the middle part of the stent. In addition, though the COMBO stent can be expected to reduce neointima formation both by accelerated endothelialisation[24] and by the elution of an antiproliferative drug, our current study was not designed to discriminate between the relative effects of these mechanisms. Moreover, since sEM and histology data on one side and OCT data on the other side were not assessed in the same rabbits, the present study does not allow direct comparison of stent coverage and neointima formation. Comparisons and associations between OCT and microscopy (i.e., histology or sEM) should therefore be interpreted with caution.

Conclusion

In summary, when compared to the EES, the COMBO stent shows improved endothelialisation and equal inhibition of neointimal hyperplasia in rabbits at 28 days post PCI. Large-scale clinical trials are warranted to show how the accelerated endothelialisation in the COMBO stent translates into clinical benefits in terms of reduced stent thrombosis and neo-atherosclerosis as well as the ability to reduce the duration of antiplatelet therapies after PCI.

Impact on daily practice

This study shows that, compared to the everolimus-eluting stent, the COMBO stent shows improved endothelialisation and equal inhibition of neointimal hyperplasia in rabbits at 28 days post PCI. As stent endothelialisation is an important determinant of stent thrombosis, this finding increases the evidence for preferred use of endothelial cell-capturing DES in patients with an increased risk for stent thrombosis or with contraindications for dual antiplatelet therapy.

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Conflict of interest statement

E. Ligtenberg and S. Rowland are employees of OrbusNeich Medical. The other authors have no other relevant affiliations or financial involvement with any organisation or financial involvement with any organisation or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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