

# Reduced histologic neo in-stent restenosis after use of a paclitaxel-coated cutting balloon in porcine coronary arteries

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**Summary.** The incidence of in-stent restenosis (ISR) has declined dramatically, but once it develops, no current treatment option, such as drug-eluting stents, drug-coated balloons, or cutting balloons (CBs), prevents re-narrowing of the stented atherosclerotic artery. In this preclinical study, we aimed to improve the efficacy of ISR treatment by coating CBs with paclitaxel (paclitaxel-eluting cutting balloon; PECB) and to characterize the histological features of neo-ISRs that arise after ISR treatment. ISR was induced by bare metal stent (BMS) implantation in coronary arteries in pigs. After one month of follow-up, BMS-induced ISR was treated with either CB or PECB. After another month, we performed quantitative coronary angiography, explanted the treated arteries and assessed histopathological and histomorphometric parameters. In addition, we compared histological features of neo-ISRs with pre-treatment ISRs. Injury, inflammation, fibrin deposition, and endothelialization scores were similar between the CB and PECB groups at one month after ISR treatment. Neointimal area ( $0.87 \pm 0.61$  vs.  $1.95 \pm 1.14$  mm<sup>2</sup>,  $p=0.02$ ), mean neointimal thickness ( $0.40 \pm 0.39$  vs.  $0.99 \pm 0.56$  mm,  $p=0.01$ ), and percent area stenosis ( $27.3 \pm 20.4$  vs.  $48.3 \pm 22.9\%$ ,  $p=0.04$ ) were decreased in PECB-treated coronary arteries compared to CB-treated arteries, respectively. Density of cells (predominantly smooth muscle cells; SMCs) was increased in neo-ISRs ( $3.51 \pm 3.05 \times 10^3$  vs.  $6.35 \pm 2.57 \times 10^3$  cells/mm<sup>2</sup>,  $p<0.01$ ),

but significantly more CD68<sup>+</sup> and CD20<sup>+</sup> cells were found in pre-treatment ISRs. In conclusion, PECB treatment of ISRs led to better results in terms of smaller neointimal area and %area stenosis of neo-ISR. SMC density was increased in neo-ISRs in contrast with higher percentage of CD68<sup>+</sup> and CD20<sup>+</sup> cells in pre-treatment ISRs.

**Key words:** Drug-eluting ballon, Cutting ballllon, Neointima, In-stent restenosis, Paclitaxel

## Introduction

The widespread use of drug-eluting stents (DES) instead of bare metal stents (BMS) has markedly reduced the incidence and severity of in-stent restenosis (ISR) (Mohan and Dhall, 2010; Valgimigli et al., 2015). A variety of coatings have been tested, including

**Abbreviations.** %AS, % area stenosis; %DS, % diameter stenosis; BMS, bare metal stent; CB, cutting balloon; CD, cluster of differentiation; DAB, diaminobenzidine; DCB, drug-coated balloon; DES, drug-eluting stent; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; H&E, hematoxylin & eosin; IHC, immunohistochemistry; ISR, in-stent restenosis; LA, lumen area; LAD, left anterior descending artery; LCX, circumflex branch of left coronary artery; LLL, late lumen loss; MLD, minimal lumen diameter; OMA, obtuse marginal artery of the LCX; PEB, paclitaxel-eluting balloon; PECB, paclitaxel-coated cutting balloon; PES, paclitaxel-eluting stent; PTCA, percutaneous transluminal coronary angioplasty; QCA, quantitative coronary angiography; RCA, right coronary artery; SB, scoring balloon; SMC, smooth muscle cell; VC, vessel circumference;  $\alpha$ -SMA, smooth muscle actin.

sirolimus, paclitaxel, zotarolimus, everolimus, or tacrolimus (Grube et al., 2003; Moses et al., 2003; Siller-Matula et al., 2010; Raber et al., 2012; Valgimigli et al., 2015), yet ISR still occurs in up to 12.2% of all coronary stent implantations. ISR incidence depends on lesion type, implanted device, and procedural and patient-related characteristics (Cassese et al., 2014). It presents clinically as recurrent angina or myocardial ischemia and angiographically as a >50% diameter stenosis within the stent (Dangas et al., 2010). Histological hallmarks of ISR are initial transient thrombus formation, neo-intimal growth, proliferation of smooth muscle cells (SMCs), and formation of abundant extracellular matrix (ECM) (Carter et al., 1994; Virmani and Farb, 1999). Neointima formation can be divided into early (days to weeks) and late (weeks to months) phase. The early phase is characterized by thrombus formation and leukocyte (especially macrophage) migration into the vessel wall. In contrast, features of the late phase are migration and proliferation of SMCs into the intima and synthesis of ECM by SMCs. Finally, over time, the ECM largely consists of neointimal tissue (Mitra and Agrawal, 2006). Many methods have targeted ISR treatment, but most have yielded suboptimal results (Adamian et al., 2001). After successful dilation or stenting of the ISR, neoatherosclerosis or in-stent atherosclerosis can develop, leading to re-narrowing of the artery. Neoatherosclerosis is characterized by lipid-laden foamy macrophages, a necrotic core, calcifications within the neointima, thinning of the fibrous cap, and formation of a thin-cap fibroatheroma (Yahagi et al., 2016).

Coronary ISR and small vessel diseases are primary indications for using drug-coated balloons (DCBs), but fibro-calcific disease or diffuse, long lesions surpass the capacity of DCB (Cremers et al., 2014). Almost three decades ago, the first cutting balloon (CB) device with longitudinally oriented blades was introduced (Barath et al., 1991). The first clinical experience with CBs yielded less dissection and a lower restenosis rate compared to conventional percutaneous transluminal coronary angioplasty (PTCA) balloons, as well as improved angiographic parameters compared to rotational atherectomy, additional stenting, or PTCA (Unterberg et al., 1993). CB also has been used in balloon-resistant de novo lesions and a variety of non-coronary applications (Ansel et al., 2004; Rabbi et al., 2004).

Paclitaxel is a naturally occurring compound from the Pacific yew tree (Rowinsky and Donehower, 1995). Because of its lipophilicity, it penetrates readily into the arterial wall, where it is retained and exerts a long-lasting effect even after only a short exposure time (Axel et al., 1997; Posa et al., 2008). Paclitaxel induces formation of decentralized, unorganized, and extraordinarily stable microtubules, which inhibits cell replication in the G2/M phase (Rowinsky and Donehower, 1995). Coating of a PTCA balloon with paclitaxel (paclitaxel-eluting balloon; PEB) results in inhibition of proliferation and migration of SMCs in vessels, reducing neointimal tissue formation (Axel et

al., 1997).

The aim of our study was to investigate the potential benefits of paclitaxel-eluting cutting balloons (PECBs) in a preclinical porcine ISR model to explore the efficacy and safety of PECBs. In addition, we explored the histological differences between pre-treatment and neo-ISRs and investigated the effect of PECBs on neo-ISR formation and composition, as well as on local inflammation and cell proliferation.

## Materials and methods

### *Paclitaxel-coated cutting balloons*

PECBs (3.0/10 mm, 3  $\mu\text{g}$  paclitaxel per  $\text{mm}^2$  balloon surface area; effective delivery dose 2  $\mu\text{g}/\text{mm}^2$ ) were constructed and provided by Eurocor (GE) specifically for this experiment. Detailed data on type of scoring elements, excipient, acute drug transfer, and pharmacokinetic curves are available from Eurocor.

### *Animal model*

We used a porcine preclinical model to induce ISR (Fig. 1). Overdilation of coronary arteries with a stent balloon, causing tunica intimal and media injury, reliably results in neointimal proliferation that mimics restenosis after stenting (Schwartz et al., 2008). Eight domestic pigs (30-35 kg, male) were fasted overnight and premedicated with 250 mg acetylsalicylic acid and 300 mg clopidogrel. On the next day, pigs received an intramuscular injection of 12 mg/kg ketamine hydrochloride, 1 mg/kg xylazine, and 0.04 mg/kg atropine. Anesthesia was deepened with isoflurane and  $\text{O}_2$  via mask and maintained with 1.5-2.5 vol% isoflurane, 1.6-1.8 vol%  $\text{O}_2$ , and 0.5 vol%  $\text{N}_2\text{O}$  via an intratracheal tube. After induction of general anesthesia, access to the femoral artery was obtained through surgical preparation of the artery under sterile conditions, and a 6-F introducer sheath (Medtronic, USA) was inserted. A total of 200 IU/kg of heparin sodium was administered before coronary artery angiographies were performed by insertion of a 6-F guiding catheter (Medtronic, USA) with regular contrast media (Ultravist, Bayer, GE). Heart rate, arterial blood pressure, electrocardiography,  $\text{O}_2$  saturation, and temperature were monitored throughout the procedure.

To induce moderate to high-grade ISR, BMS (length: 15 mm; diameter: 3 mm) were placed in the left anterior descending artery (LAD), left circumflex (LCX), or right coronary artery or diagonal branch of the LAD or the obtuse marginal artery of the LCX (Fig. 1). Except for one animal with three stents, each animal received four stents, so that the total number of implanted stents was 31. Stent balloon inflation pressure (8-16 mmHg) was chosen to achieve a 1.2:1 stent/artery ratio and inflated for 30 s. After control angiography, the guiding catheter and introducer sheath were removed, the arteriotomy ligated, and the skin closed in two

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layers.

After one month, we performed coronary angiography to evaluate the degree of ISR. Of the 31 stented arteries, 20 showed visually significant (>50%) ISR. These 20 in-stent lesions were 1:1 randomized to ISR treatment with either PECB (n=10) or plain CB (balloon diameter: 2.5-3.0 mm, length: 15 mm; n=10). Coronary angiography confirmed full contact of the balloon with the stented vessel wall during each balloon inflation with 2-5 atm.

During the 2-month follow-up, daily doses of 100 mg acetylsalicylic acid and 75 mg clopidogrel were administered orally. Two months after BMS implantation (equivalent of 1 month post-PECB or -CB treatment), control coronary angiography was performed to evaluate neo-ISR. Euthanasia was performed under general anesthesia with 10 ml intravenous saturated potassium chloride. Coronary arteries were harvested for histomorphometric, histopathological, and immunohistochemistry assessments.

Experiments were conducted at the Institute of Diagnostics and Oncoradiology, University of Kaposvar, Hungary. All animal facilities met the standards of the American Association for Accreditation of Laboratory Animal Care. Animal investigations were performed in accordance with the "Position of the American Heart Association on Research Animal Use" as adopted by the AHA on November 11, 1984, and animal care complied

with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. Washington: National Academy Press, 1996. The study was approved by the Ethics Committee on Animal Experimentation at the University of Kaposvar, Hungary (EC number: KA-1787).

### Quantitative coronary angiography (QCA)

Using QCA, minimal lumen diameter (MLD), % diameter stenosis (%DS), and late lumen loss (LLL) were evaluated at the 1- and 2-month follow-up time points using a computer-assisted, quantitative, angiographic edge-detection algorithm (QCA, ACOMPC, Siemens, GE). An independent observer with no knowledge of the group status assessed QCA.

### Histopathology and histomorphometry in stented arteries

At the final follow-up, coronary arteries were flushed with 100 ml saline and pressure fixed in situ with 4% buffered formaldehyde for 30 min. Subsequently, arteries were dissected from the epicardial surface and fixed in 2% formaldehyde, and the CB or PECB-treated arteries were embedded in Technovit 9100 (Heraeus Kulzer, GE). Samples were cut in 6- $\mu$ m sections with a microtome and melted on slides at 65°C

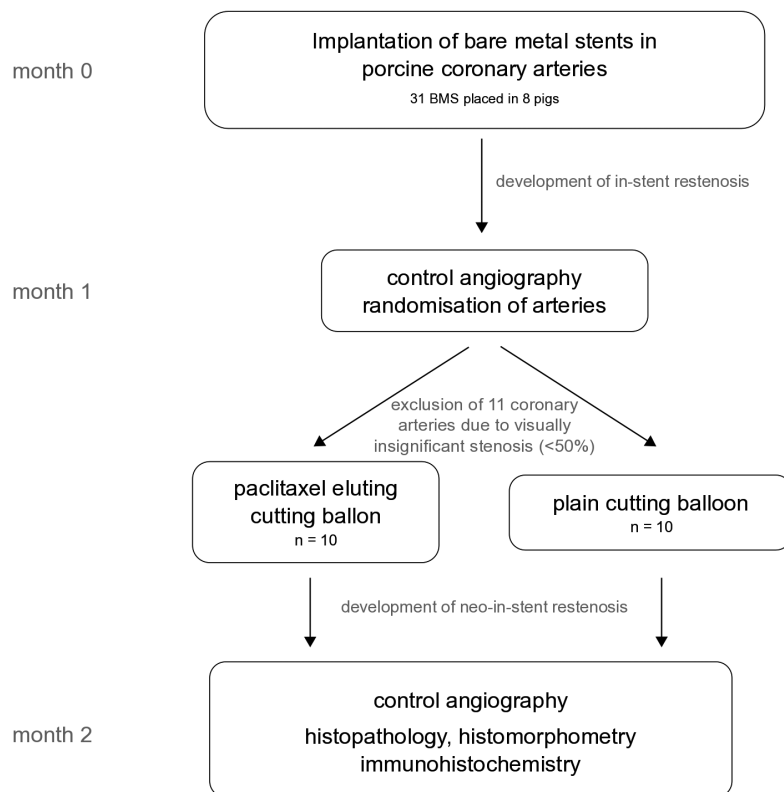


Fig. 1. Flow chart of the study.

for  $\geq 24$  h.

MOVAT, hematoxylin and eosin and toluidine blue staining were performed according to the manufacturer's protocol to assess proteoglycans and cell density in both neointimal areas. Histopathology and histomorphometry of the stented coronary arteries were performed without operator awareness of the CB type used. Three sections of stented coronary arteries were analyzed — the proximal, middle, and the distal stent parts — and the mean histopathological and histomorphometric values of these three segments were calculated and entered into the analyses.

Histopathological analysis included scores for fibrin deposition, inflammation, endothelialization, and injury. Fibrin deposition score around stent struts was graded from 0-4, as follows: 0 for no fibrin deposition, 1 for fibrin deposition involving  $<25\%$  of the vessel circumference (VC), 2 for fibrin deposition involving 25%-50% of the VC, 3 for fibrin deposition involving 50%-75% of the VC, and 4 for fibrin deposition involving 75%-100% of the VC. The inflammation score was graded from 0-4 as follows: 0 for no inflammation, 1 for scattered inflammation in  $<25\%$  of the VC, 2 for inflammation covering 25%-50% of the VC, 3 for inflammatory infiltration in 50%-75% of the VC, and 4 for inflammatory infiltration in 75%-100% of the VC. Endothelialization score was graded from 0-4, with 0 for no endothelial cells present on the strut, 1 for  $<25\%$  of the strut covered by endothelial cells, 2 for 25%-75% of the strut covered by endothelial cells, 3 for 75%-100% of the strut covered by endothelial cells, and 4 for strut covered by neointimal tissue. We used the injury score according to Schwartz et al. (1992).

Histomorphometric analysis included quantification of the neointimal area, maximal neointimal thickness, % area stenosis (%AS), and lumen area.

#### *Immunohistochemical and immunofluorescence staining of stented coronary arteries*

Immunohistochemical and immunofluorescence staining were performed on formalin-fixed, Technovit 9100-embedded tissue specimens according to the manufacturer's protocol. Briefly, sections 6  $\mu\text{m}$  thick were cut with a microtome and melted on slides at 65°C for  $\geq 24$  h. Sections were deplastinized for 3 $\times$ 20 min in 2-methoxyethyl-acetate, followed by xylene, alcohol in decreasing concentrations, and distilled water for re-hydration. For demasking of the antigens, slides were put into plastic jars with citrate buffer (pH 6) and heated in a microwave oven. The slides were allowed to cool and then were washed in phosphate-buffered saline. Endogenous peroxidase activity was quenched with 0.3%  $\text{H}_2\text{O}_2$ , and sections were washed again in phosphate-buffered saline. Slides were incubated with ready-to-use normal serum. After excess serum was blotted from the slides, they were incubated with a primary antibody in respective dilutions. Later, sections were washed in PBS and incubated with ImmPRESS

reagent anti-mouse/rabbit (Vector Laboratories, Burlingame, California). Following another wash in phosphate-buffered saline, DAB substrate (Sigma Aldrich, USA) was added, and the reaction was visualized under a microscope, followed by washing in PBS and tap water and counterstaining with Mayer's Haemalum Solution (Merck, GE). Then sections were washed with tap water, put in alcohol in increasing concentrations, and mounted with a non-aqueous mounting medium (Eukitt®).

For immunofluorescence staining, a FITC-labeled anti-rabbit antibody (Sigma Aldrich, USA) was used as secondary antibody. Counterstain was performed using Hoechst stain, and quenching endogenous peroxidase activity was omitted. Anti-CD68 (1:50, orb1856, Biorbyt, UK, IHC), anti-CD20 (1:100, ab8237, Abcam, UK, IHC), anti-ki-67 (1:50, ab833, Abcam, UK, IHC), and anti- $\alpha$ -SMA (1:50, ab5694, Abcam, UK, IF) were used as primary antibodies. For negative control, primary antibodies were omitted.

Samples were analyzed for presence of CD68<sup>+</sup> (macrophage marker), CD20<sup>+</sup> (B-lymphocyte marker), and ki67<sup>+</sup> (cell proliferation marker) cells and  $\alpha$ -SMA<sup>+</sup> (SMC marker). The percentage of positive cells was calculated in high power fields.

#### *Statistics*

Continuous parameters are expressed as mean $\pm$ SD. Continuous variables were compared by unpaired Student's t tests. P values  $\leq 0.05$  were considered as statistically significant. Data obtained were evaluated statistically using IBM SPSS Statistics version 23 (SPSS Inc., USA) and GraphPad Prism 6 software (GraphPad Software Inc., USA). All tests were performed in a two-sided manner.

## **Results**

### *Stenting procedural data*

We observed no procedural or post-procedural complications (e.g., bleeding, myocardial ischemia) after the use of PECB or CB.

### *QCA*

QCA at the one-month follow-up showed varying degrees (from mild to severe) of ISR. Based on the visual evaluation, 20 stents with  $>50\%$  ISR were randomized to treatment with either PECB or CB. According to randomization, the groups did not differ before treatment with PECB or CBs (MLD:  $p=0.87$ ; %DS:  $p=0.86$ ) (Fig. 2A,B). One month after CB dilation of the ISR, angiographic parameters were similar between PECB and CB groups (Fig. 2C,D): MLD (2.01 $\pm$ 0.74 vs. 2.13 $\pm$ 0.98 mm,  $p=0.76$ ; Fig. 2E), %DS (34.6 $\pm$ 25.4 vs. 38.4 $\pm$ 25.1%,  $p=0.74$ ; Fig. 2F), and LLL (0.60 $\pm$ 0.81 vs. 0.67 $\pm$ 0.91,  $p=0.86$ ; Fig. 2G), respectively.

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### Histomorphometry of neo-ISR

Histomorphometric analyses of neo-ISR of coronary arteries treated with PECB showed decreased neointimal area ( $0.87 \pm 0.61$  vs.  $1.95 \pm 1.14$  mm,  $p=0.02$ ; Fig. 3C), mean neointimal thickness ( $0.40 \pm 0.39$  vs.  $0.99 \pm 0.56$  mm,  $p=0.01$ ; Fig. 3D), and %AS ( $27.3 \pm 20.4$  vs.  $48.3 \pm 22.9\%$ ,  $p=0.04$ ; Fig. 3E). Lumen area was marginally higher in the PECB group ( $2.57 \pm 1.04$  vs.  $1.94 \pm 0.76$  mm<sup>2</sup>,  $p=0.13$ ; Fig. 3F).

### Histopathology

Histopathology of neo-ISR showed similar scores between groups. Injury score ( $1.14 \pm 1.01$  vs.  $1.04 \pm 0.62$ ,  $p=0.78$ ; Fig. 4A), inflammation ( $1.69 \pm 0.90$  vs.  $1.27 \pm 0.94$ ,  $p=0.23$ ; Fig. 4B), and fibrin deposition score ( $0.89 \pm 0.78$  vs.  $0.80 \pm 0.90$ ,  $p=0.79$ ; Fig. 4C) did not differ between PECB- and CB-treated pigs. Furthermore, endothelialization was complete in all lesions.

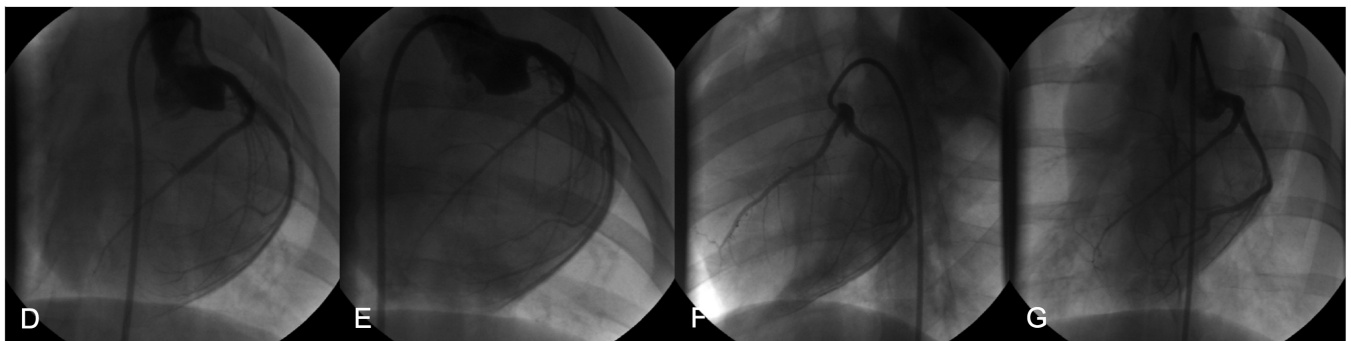
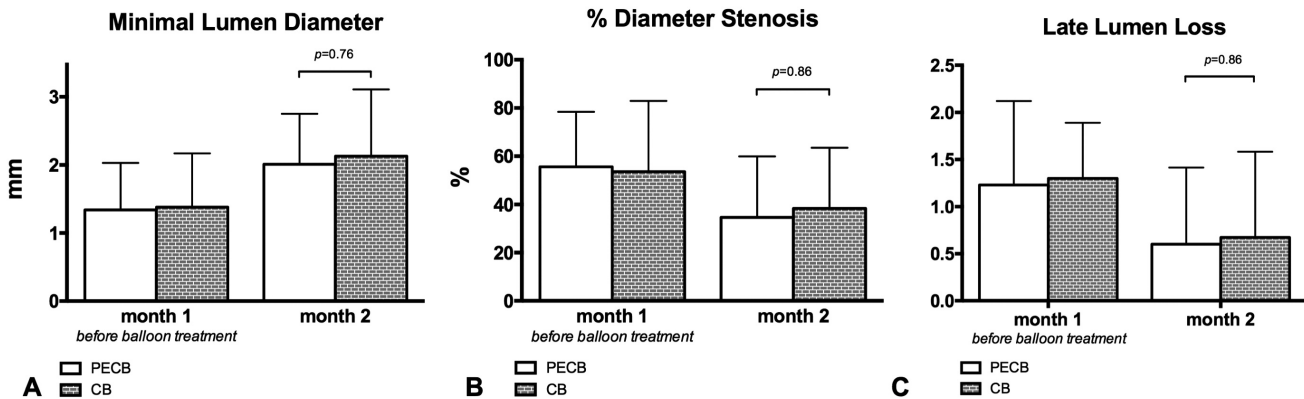
MOVAT staining showed ECM stained blue, consistent with the presence of proteoglycans. The neointimal layer of the neo-ISR featured a higher cell

density (pre-treatment ISR vs. neo-ISR:  $3.51 \pm 3.05 \times 10^3$  vs.  $6.35 \pm 2.57 \times 10^3$  cells/mm<sup>2</sup>,  $p < 0.01$ ; Fig. 4E,F). PECB-treated arteries presented increased cell density in the neointimal layer of the neo-ISR compared to pre-treatment ISR ( $6.08 \pm 2.86 \times 10^3$  vs.  $3.29 \pm 2.26 \times 10^3$  cells/mm<sup>2</sup>,  $p=0.005$ , respectively; Fig. 4D). However, the groups did not differ (Fig. 4D). Previously published characteristic features of human neoatherosclerosis (necrotic cores, lipid pools, thin cap fibroatheroma) were not observed.

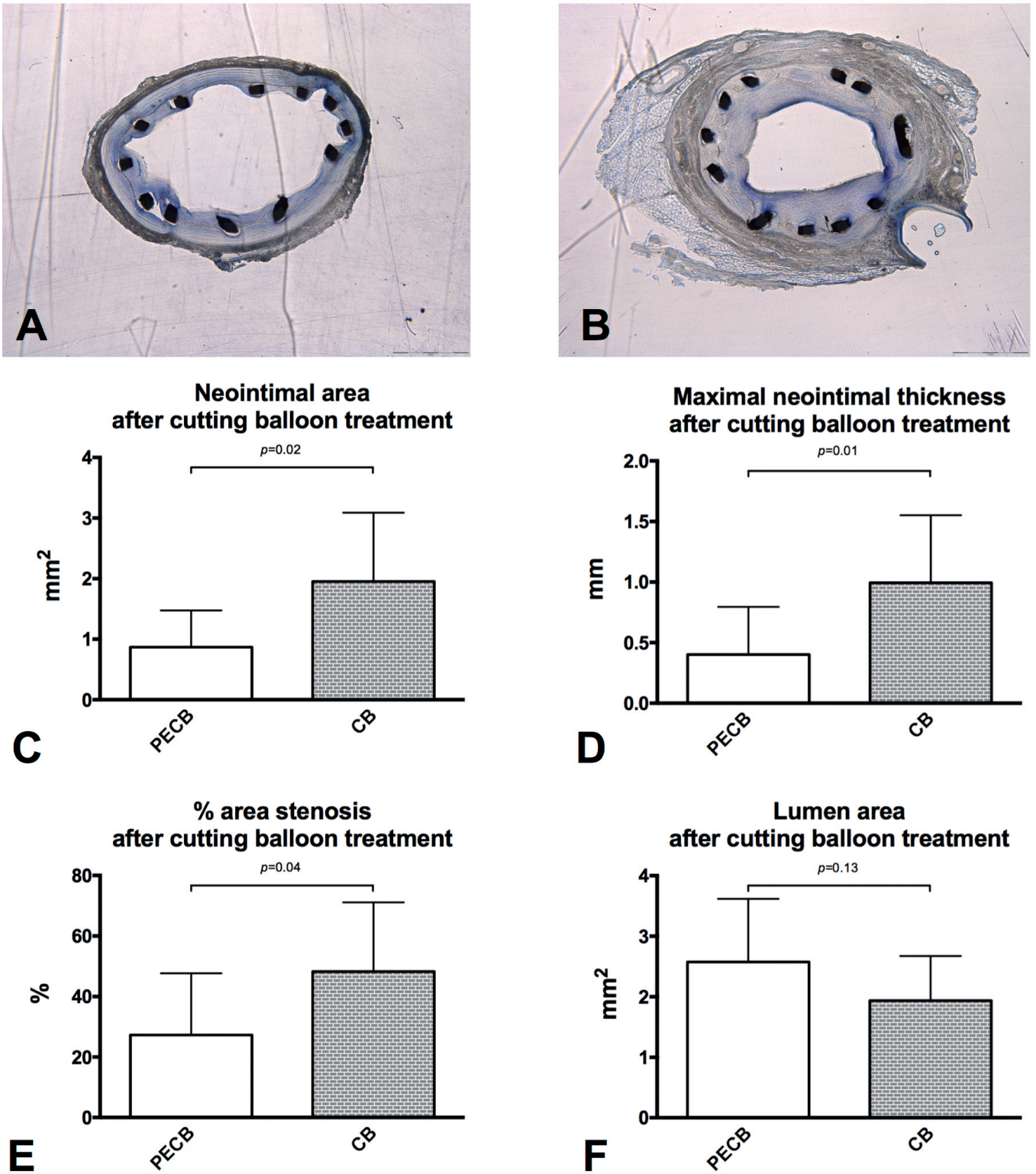
### Immunohistochemistry

Both macrophages and B cells were present especially in the pre-treatment ISR layer, particularly adjacent to the stent struts. The pre-treatment ISR layer contained more CD68<sup>+</sup> and CD20<sup>+</sup> cells (CD68<sup>+</sup>:  $22.0 \pm 30.5$  vs.  $11.8 \pm 23.5\%$  of total cells,  $p=0.005$ ; Fig. 5A; CD20<sup>+</sup>:  $22.0 \pm 30.0$  vs.  $15.7 \pm 25.2\%$  of total cells,  $p=0.04$ ; Fig. 5B). A difference between both CB was not observed (Fig. 5C,D).

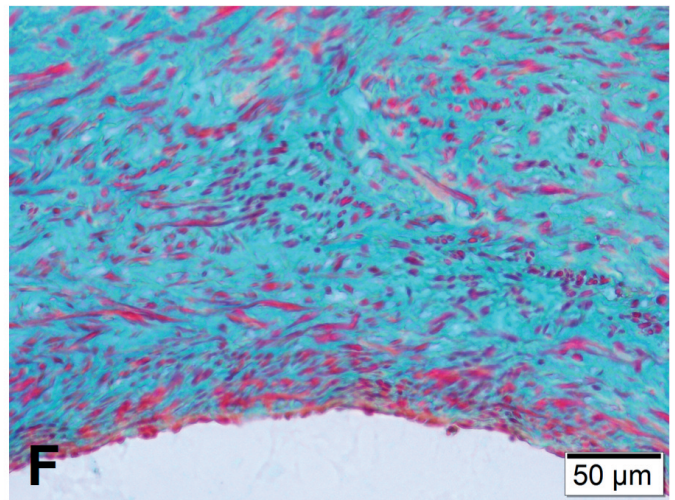
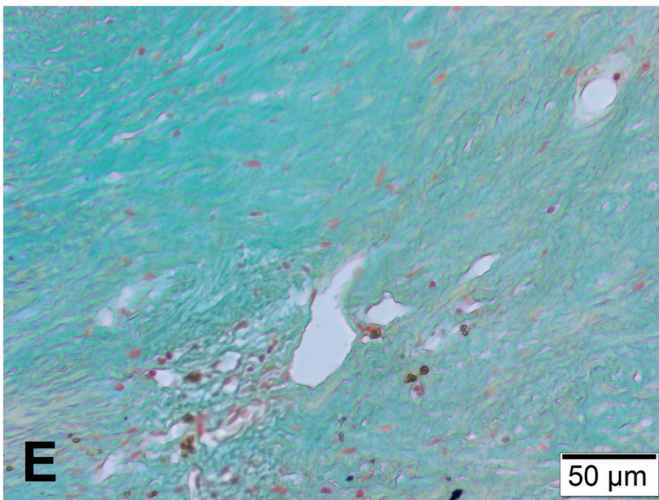
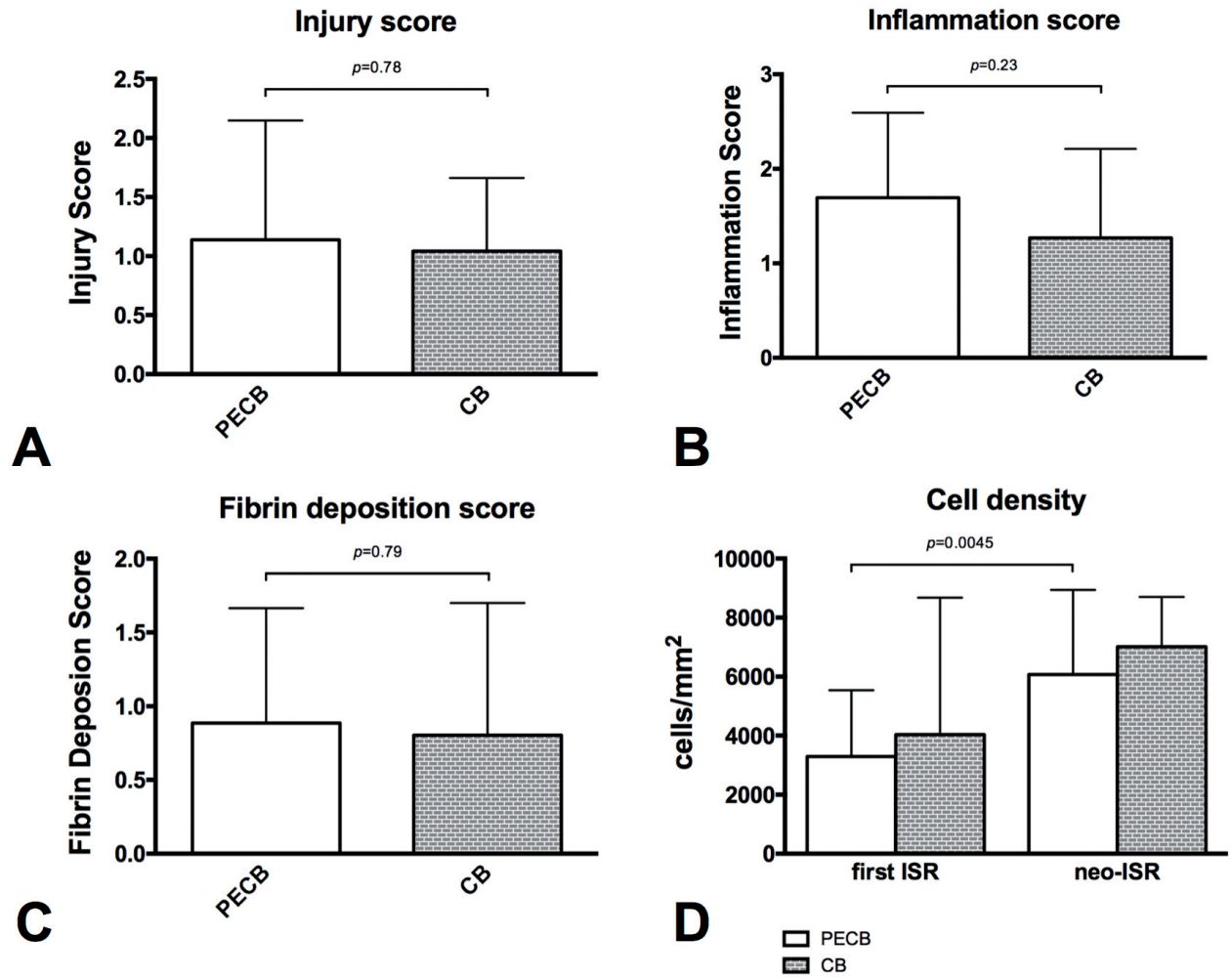
The predominant cell type was SMCs.  $\alpha$ -SMA<sup>+</sup> cells were found in the pre-treatment and neo-ISR. However,



**Fig. 2.** Coronary angiography and quantitative analysis at month 1 (before cutting balloon treatment) and month 2 (final follow-up). **A-C.** Final quantitative angiographic results: minimal lumen diameter (MLD), percent diameter stenosis (%DS), and late lumen loss (LLL). **D.** Coronary angiography directly after bare metal stent placement. **E.** Coronary angiography showing moderate in-stent restenosis 1 month after implantation of bare metal stent, with lesions randomized to treatment with either cutting balloon (CB) or paclitaxel-eluting cutting balloon (PECB). **F.** Angiographic results of in-stent restenotic lesions 1 month after treatment with CB. **G.** Angiographic results of in-stent restenotic lesions 1 month after treatment with PECB.

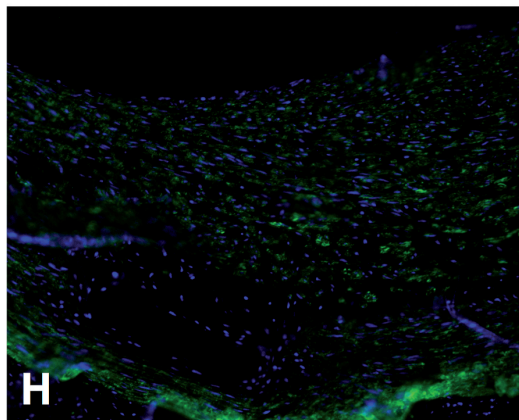
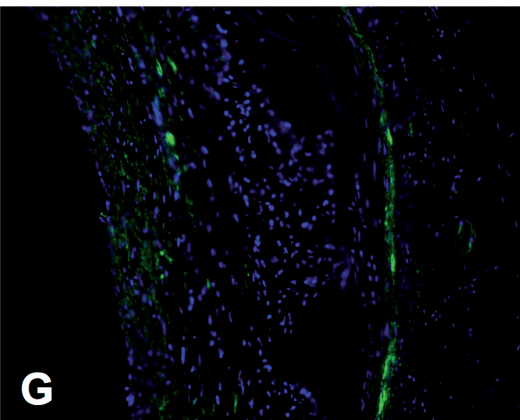
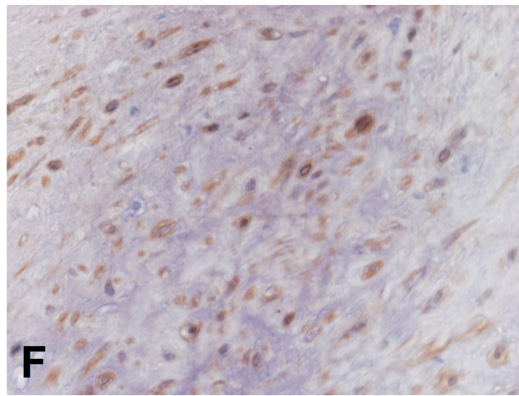
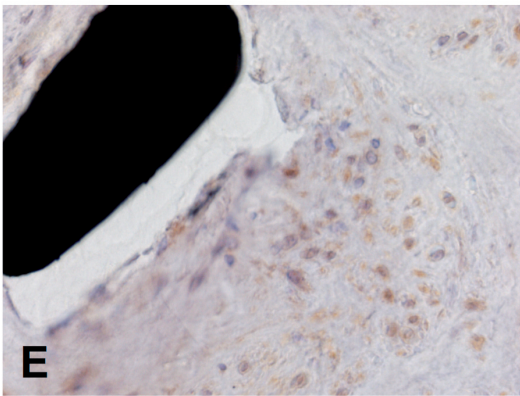
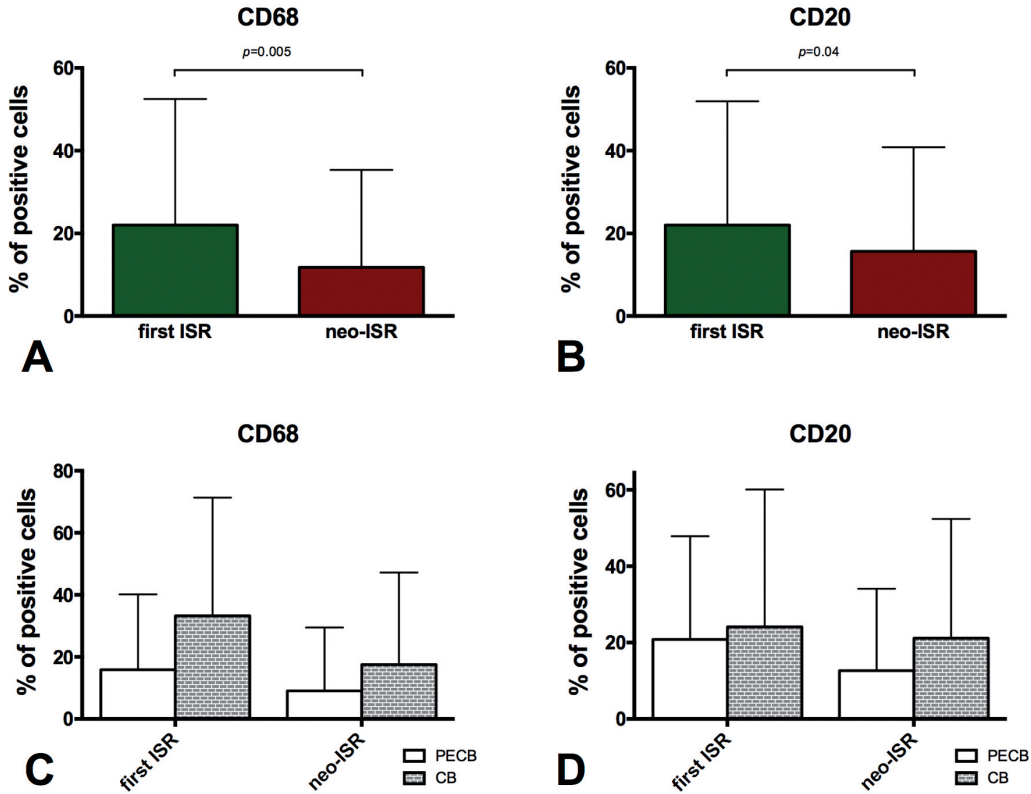


**Fig. 3.** Representative histological images and histomorphometric analyses of coronary arteries with in-stent restenosis, treated with either a paclitaxel-eluting cutting balloon (PECB) or cutting balloon (CB). **A, B.** Histological images of PECB- or CB-treated in-stent restenosis. **C.** Quantitative neointimal area. **D.** Quantitative maximal neointimal thickness. **E.** Quantitative percent area stenosis. **F.** Quantitative lumen area.  $\times 2$ .



**Fig. 4.** Histopathological analysis of coronary arteries with in-stent restenosis, treated with either a paclitaxel-eluting cutting balloon (PECB) or cutting balloon (CB). **A.** Injury score. **B.** Inflammation score. **C.** Fibrin deposition score. **D.** Cell density. **E.** MOVAT staining of the pre-treatment in-stent restenosis layer **F.** Neo in-stent stenosis layer.  $\times 20$ .

PECB reduces histologic neo-ISR



**Fig. 5.** Presence of inflammatory cells in the pre-treatment in-stent restenosis and neo in-stent restenosis layers. **A, B.** Comparisons of CD68<sup>+</sup> and CD20<sup>+</sup> cell presence in the pre-treatment and neo in-stent restenosis layers; pooling of all arteries, irrespective of treatment mode. **C, D.** Comparison of CD68 and CD20 cell positivity in the pre-treatment and neo in-stent restenosis layers in the arteries with in-stent restenosis treated either with paclitaxel-eluting cutting balloon (PECB) or cutting balloon (CB). **E, F.** Representative histological sections of images with CD68<sup>+</sup> (E) and CD20<sup>+</sup> (F) staining B cells, respectively. **G, H.** Representative images of  $\alpha$ -SMA staining in a coronary artery treated with a PECB and CB, respectively.  $\times 20$ .



cells within the immediate vicinity of the stent struts did not express  $\alpha$ -SMA (Fig. 5G,H). Immunolabeling for ki67 showed no ki67<sup>+</sup> proliferating cells in restenotic areas in either group.

## Discussion

Our study demonstrated the feasibility, safety, and histological efficacy of PECB use in a porcine model of ISR. We applied the term “neo-ISR” to distinguish repeat ISR from the pre-treatment ISR. Neo-ISR in originally healthy porcine coronary arteries showed histological features that were partially similar to the neoatherosclerosis of diseased human coronary arteries (e.g., presence of macrophages and immune cells), but lacked necrotic cores, lipid pools, and thin cap fibroatheromas. Histomorphometric analysis revealed significantly decreased neointimal area, mean neointimal thickness, and %AS of the neo-ISRs in the PECB group compared to the CB group, suggesting histologic efficacy in the treatment of the initial ISRs.

### *Treatment of ISR with coated CB*

Current treatment options for ISR are unsatisfactory (Siontis et al., 2015). Even though stent-in-stent implantation or DCB are the gold standard, they carry considerable disadvantages, such as the addition of a new metal layer, which increases the risk for uncovered struts, thrombosis and repeated ISR. An advantage of CBs is more precise and predictable vessel wall injury (Albiero et al., 2004). Scoring balloons (SBs) are also associated with an increased precision of treatment of vessel wall injury (Cremers et al., 2014). Coating of SBs with paclitaxel resulted in a reduced LLL, major adverse cardiac event incidence, and target lesion revascularization rate (Scheller et al., 2016). Coating CBs with paclitaxel is another option and may be a viable alternative to, e.g., restenting with BMS or DES, using PTCA or DCB, or rotational atherectomy. Similar to using DCB, DB and PECB offer the advantage that no biostable or bioreabsorbable polymeric stent matrix remains in the vessel wall. Stent polymers induce inflammation, which may result in enhanced neointima proliferation and ISR (Byrne et al., 2009). Furthermore, drug concentration is not homogeneous in the stent area, which could also contribute to ISR (Posa et al., 2010).

The ideal drug for local delivery in DCB should be lipophilic for rapid uptake and long-term retention in the vessel wall (Zhu et al., 2015). Moreover, DCB leads to high local drug concentrations in the initial phase after treatment, in which platelet activation and acute inflammatory infiltration trigger the restenosis cascade (Posa et al., 2010; Perez de Prado et al., 2014). Paclitaxel fulfils these requirements and thus seems to be ideal. A variety of studies have been conducted regarding treatment of ISR with a paclitaxel-eluting balloon, showing decreased incidence of restenosis and adverse events compared to use of an uncoated balloon

(Scheller et al., 2006, 2008; Unverdorben et al., 2009). An uncoated CB for coronary angioplasty in ISR has already been tested and has shown improved clinical and angiographic outcome (Adamian et al., 2001). In a recent network meta-analysis by Sethi et al. (2015), diverse percutaneous coronary intervention methods to treat ISR have been compared. PECB was superior to all non-drug-eluting treatments and everolimus-eluting stents for target vessel revascularization. PECBs also led to less vessel thrombosis compared to PEB.

In our study, although quantitative angiographic parameters were similar at one month following ISR treatment with both CB types, histomorphometry indicated better performance of PECB for inhibiting neo-intimal proliferation. It is generally accepted that angiography, as a “contrast luminography,” is much less sensitive than histological analysis. In angiography, vessel diameter is calculated as the average of the distal and proximal non-stented segments, which renders it unsuitable for assessment of real vessel diameter (the denominator of the %diameter stenosis calculator) and vessel remodeling. Accordingly, histomorphometric parameters are accepted endpoints for qualitative and quantitative assessment of a new intravascular device under experimental conditions.

### *Histological characteristics of pre-treatment and neo-ISRs*

Histological features of ISRs and restenosis after PTCA are well known. However, histological hallmarks of ISRs treated with PECB or CB are not yet described. We demonstrated that macrophages and B cells are present in both (pre-treatment ISR and neo-ISR) neointimal tissue layers. The predominant cell type in both layers was  $\alpha$ -SMA<sup>+</sup> SMCs. We could not find any ki67<sup>+</sup> proliferating cells. Our findings are not surprising given that migration and proliferation of SMCs peaks at day 7 and returns to basal levels on day 28 after stent placement (Carter et al., 1994; Costa and Simon, 2005). Additionally, we show here that cell density was low in the mature neointimal tissue of the pre-treatment ISR and that the neointima consisted of abundant proteoglycan matrix. Glover et al. (2002) investigated neointimal tissue obtained from coronary atherectomies and found similar results, strengthening our findings. They also identified no proliferating cells, although macrophages were present in neointimal tissue. Furthermore, they demonstrated that neointimal tissue is dominated by proteoglycan matrix.

Formation of a second neointimal layer (neo-ISR) that lies luminal to the pre-treatment ISR after restenosis therapy is conceivable. However, the question of composition of this neo-ISR remains unaddressed. Here we demonstrated that by one month after ISR treatment with a CB or PECB, a neo-ISR can develop that differs from the pre-existing neointima. This neo-ISR exhibits a higher cell density, although in contrast to the pre-treatment ISR, CD20<sup>+</sup> and CD68<sup>+</sup>

cells are found in lesser proportions in this layer. An increased total cell density in the neo-ISR may be a sign of younger age and more active environment of this neointima compared to the pre-treatment ISRs, in which reorganization towards an abundant proteoglycan matrix has already occurred. Neoatherosclerosis has been characterized in human coronary arteries treated with BMS and DES (Nakazawa et al., 2011; Jinnouchi et al., 2017). The earliest feature of neoatherosclerosis is clusters of foamy macrophages within the peristrut region (Yahagi et al., 2016). Human neoatherosclerosis occurs at an earlier time point in DES than in BMS (Nakazawa et al., 2011). Transformation from neointimal thickening to neoatherosclerotic tissue may result from delayed or impaired reendothelialization or endothelial denudation, a hallmark of DES. Theoretically, macrophage cell density might differ between PECB- and CB-treated coronary arteries. However, we did not observe a difference between the groups, which might be explained by the short follow-up. A short follow-up also might explain the absence of other neoatherosclerosis-specific histological hallmarks, such as necrotic core formation, calcification, and thin cap fibroatheroma.

### Limitations

Our study has several limitations. First, we treated ISRs of originally healthy porcine coronary arteries, although the composition of the histological ISR is similar to that of human diseased coronary artery ISR. Accordingly, ours can be considered a proof-of-concept study that opens the way to further investigations that include animals with hypercholesterolemic diets and involve longer follow-up of greater than 30 days, such as 90 and 180 days. Second, we implanted 31 BMS in eight pigs by using all three coronary arteries with side branches of each pig to minimize the number of animals used. Thus, our hypothesis-generating study did not necessarily follow the standard rules of preclinical experiments involving coronary stents, which prescribe a maximum of 2-3 stents implanted in one animal. However, with our approach, we could better implement the 3R rules (reduce, refine, replace) of the European Commission governing animal experiments. We chose 20 of the 31 lesions to treat based on visual assessment of ISRs, as is usual in human coronary angiographic laboratories. Third, our results are descriptive, and a mechanistic explanation for histologically differing features of both ISR layers needs to be addressed in further studies, e.g., exploring whether neo-ISR and pre-treatment ISR consistently exhibit a different phenotype or if the variation we observed was the result of distinct maturing times.

### Conclusion

In this study, we demonstrated the feasibility, safety, and histological efficacy of using PECB in a porcine

preclinical model of ISR. Treating ISR with PECB resulted in decreased neointimal area, mean neointimal thickness, and %AS. These results suggest that PECB could be a viable alternative to established therapies in ISR. Here we introduced the term “neo-ISR” to distinguish the neo-atherosclerosis in human atherosclerotic arteries, which may serve as the basis of further preclinical studies involving ISR treatment.

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