

Evaluating the Interactions between Endothelial and Smooth Muscle Cells in the Presence of Drug-Eluting Stents and Platelets: Is Interleukin6 a New Possible Therapeutic Target for In-Stent Restenosis?

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Research Article

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Abstract

The success of percutaneous coronary intervention (PCI) procedures is limited by in-stent restenosis (ISR). The use of drug-eluting stents (DES) decreased the incidence of ISR; however, ISR continues to affect a percentage of patients with ischemic cardiac events. Therefore, there is room for improvement considering not only the effectiveness of DES but also the adjunct pharmacological interventions for patients after PCI. Interleukin 6 (IL-6) can stimulate the growth of vascular smooth muscle cells (SMCs) in a platelet-derived growth factor (PDGF)-dependent manner and that antibodies against the IL-6 receptor reduced intimal hyperplasia in mice. In this study, our aim is to develop an *in vitro* study, based on co-cultures of endothelial cells (EC) and SMCs in the presence of platelets and stents, to analyze the cytokine responses (IL-6 and other inflammation markers) associated with ISR, in the quest for new therapeutic targets. Overall, in the presence of platelet-rich plasma (PRP), there was an increase in the production of reactive oxygen species in the groups that received the stents, probably because platelets potentiate stent-associated oxidative stress. Moreover, the analysis of the expression of inflammatory markers showed a difference in the expression of IL-6 only in the groups that received stents, which further increased in the presence of platelets. Altogether, our findings suggest that the down regulation of IL-6 expression using pharmacologic inhibitors is a possible new therapeutic strategy in the context of ISR.

Keywords: Percutaneous coronary intervention; In-stent restenosis; Interleukin 6

Abbreviations

ATCC: American Type Culture Collection; BMS: Bare-Metal Stents; CAD: Coronary Artery Disease; DES: Drug-Eluting Stents; EC: Endothelial Cells; FACS: Fluorescence-Activated Cell Sorting; FBS: Fetal Bovine Serum; HUVEC: Human Umbilical Vein Endothelial Cells; ICAM-1: Intercellular Adhesion Molecule 1; IL-1: Interleukin 1; IL-6: Interleukin 6; IL-6R, IL-6 Receptor; ISR: in-stent restenosis; MCP-1: Mitochondrial Pyruvate Carrier 1; MDA: Malondialdehyde; p27: cyclin-dependent kinase inhibitor; PCI: Percutaneous Coronary Intervention; PCNA: Cell Nuclear Antigen; PGDF: platelet-derived growth factor; PRP: Platelet-Rich Plasma; ROS: Reactive Oxygen Species; SMC: Vascular Smooth Muscle Cell; TBA: Thiobarbituric Acid; TNF-DR4: Tumor Necrosis Factor Death Receptor 4; VEGFR-1: Vascular Endothelial Growth Factor Receptor 1; VSMC: Coronary Artery Smooth Muscle Cell

Introduction

The use of percutaneous coronary interventions (PCI) for the treatment of coronary artery disease (CAD) has increased in the recent decades, becoming the predominant revascularization method for patients with CAD [1]. However, despite the benefits; PCI is associated with mechanical injury to the endothelium. Vascular damage can result in neointimal hyperplasia and the consequent in-stent restenosis (ISR), the great Achilles heel of PCI [2,3].

Although the rate of ISR has decreased with the use of drug-eluting stents (DES) [4-7], presenting more commonly as stable angina, in 10% of cases, there is the development of acute coronary syndrome, associated with high morbidity and mortality, as well as with increased costs for the health-care systems [8,9]. Therefore, PCI can further be improved, particularly considering the adjunct pharmacological therapy, either at the local or systemic levels.

In the CANTOS Trial [10], canakinumab, an inhibitor of interleukin (IL)-1 β demonstrated clinical benefit in the context of CAD, but with an increase in the rate of severe systemic infection and death. This trial paved the way for the investigation of other agents targeting inflammation and immunity with more robust benefits than those of canakinumab, and without serious adverse effects.

IL-6 stimulates the growth of SMCs in a platelet-derived growth factor (PDGF)-dependent manner [11]. Niida et al., demonstrated that the use of anti-IL-6 receptor antibodies in mice significantly reduced neointimal hyperplasia [12]. This finding was confirmed more recently by Kitano et al, [13]. Additionally, the effect of the IL-6 receptor inhibitor tocilizumab in the context of giant cell arteritis and Takayasu's arteritis has been studied by Stone et al. [14] and Nakaoka et al. [15] respectively with good results and without new safety concerns.

For the above findings, we hypothesize that pharmacologic inhibitors (also termed blockers) of IL-6 such as the commercially available monoclonal antibodies tocilizumab, sarilumab, and satralizumab, which block the IL-6 receptor (IL-6R), or siltuximab, targeting IL-6 could show promising results in the context of the prevention/management of ISR. Based on this premise, we developed an *in vitro* study based on cellular co-cultures of ECs and SMCs in the presence of platelets and stents, to analyze the cytokine responses (particularly of IL-6) associated with ISR.

Materials and Methods

Cell lines and study design

The present study was performed using human ECs and SMCs: human umbilical vein endothelial cells (HUVECs) and human primary coronary artery smooth muscle cells (VSMCs), respectively. The HUVECs (ATCC[®] CRL-1730[™]) and VSMCs (ATCC[®] PCS-100-021[™]) were obtained from the American Type Culture Collection (ATCC, Gaithersburg, MD, USA) and were maintained in RPMI 1640 medium (Cultilab, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (FBS), 1% human insulin S (Humulin 100 IU/mL; Eli Lilly and Company, Indianapolis, IN, USA), and 200 nM sodium bicarbonate (pH 7.4). These cells were grown as either monocultures or co-culture systems, alone, or together with bare-metal stents (BMS) or DES, in the presence or absence of 10% platelet-rich plasma (PRP) for 24 and 48 h (**Figure 1A-F; Table 1**).

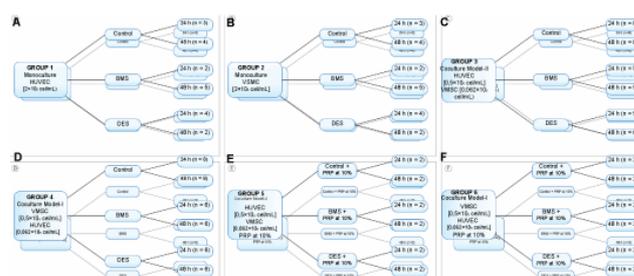


Figure 1: Study design: different treatment groups. (A) Group 1: HUVECs monocultures (2×10^5 cells/mL). **(B) Group 2:** VSMCs monocultures (2×10^5 cells/mL). **(C) Group 3:** Co-culture of HUVECs (0.5×10^5 cells/mL) and VSMCs (0.062×10^5 cells/mL) - Model-I. **(D) Group 4:** Co-culture of VSMCs (0.5×10^5 cells/mL) and HUVECs (0.062×10^5 cells/mL) - Model-I. **(E) Group 5:** Co-culture of HUVECs (0.5×10^5 cells/mL) and VSMCs (0.062×10^5 cells/mL) - Model-II - with 10% PRP. **(F) Group 6:** Co-culture of VSMCs (0.5×10^5 cells/mL) and HUVECs (0.062×10^5 cells/mL) - Model-I - with 10% PRP. All experiments were conducted for either 24 or 48 h. Cells were incubated either in the absence or presence of stents (BMS or DES). HUVECs, human umbilical vein endothelial cells; VSMCs, vascular smooth muscle cells; BMS, bare-metal stent (Pro-Kinetic[®], Biotronik, Switzerland); DES, drug-eluting stent (Orsiro[®], Biotronik, Switzerland); n, sample number; PRP, platelet-rich plasma.

In vivo, ISR is a slow and gradual process that begins within hours and is completed after a couple of months. In our study, we focused on the 24 and 48 h time points, which are short, resulting from reduced vascular cell viability in culture, either because of senescence or cell death. Of note, after 48 h, a high proportion of the cells in the culture underwent apoptosis. This said, the time frames explored in this study are consistent with those used in previous studies [16-19].

Table 1: Experimental design: Different groups used in this study

Study groups	Culture type	Cell type	PRP	Stent	Cell density*	Sirolimus concentration**
Group 1-control	Monoculture	HUVECs	no	None	2 x 10 ⁵	70
Group 1-BMS	Monoculture	HUVECs	no	BMS	2 x 10 ⁵	70
Group 1-DES	Monoculture	HUVECs	no	DES	2 x 10 ⁵	70
Group 2-control	Monoculture	VSMCs	no	None	2 x 10 ⁵	220
Group 2-BMS	Monoculture	VSMCs	no	BMS	2 x 10 ⁵	220
Group 2-DES	Monoculture	VSMCs	no	DES	2 x 10 ⁵	220
Group 3-control	Co-culture Model-II	HUVECs and VSMCs	no	None	0.5 x 10 ⁵ HUVECs and 0.062 x 10 ⁵ VSMCs	55
Group 3-BMS	Co-culture Model-II	HUVECs and VSMCs	no	BMS	0.5 x 10 ⁵ HUVECs and 0.062 x 10 ⁵ VSMCs	55
Group 3-DES	Co-culture Model-II	HUVECs and VSMCs	no	DES	0.5 x 10 ⁵ HUVECs and 0.062 x 10 ⁵ VSMCs	55
Group 4-control	Co-culture Model-I	HUVECs and VSMCs	no	None	0.5 x 10 ⁵ VSMCs and 0.062 x 10 ⁵ HUVECs	55
Group 4-BMS	Co-culture Model-I	HUVECs and VSMCs	No	BMS	0.5 x 10 ⁵ VSMCs and 0.062 x 10 ⁵ HUVECs	55
Group 4-DES	Co-culture Model-I	HUVECs and VSMCs	No	DES	0.5 x 10 ⁵ VSMCs and 0.062 x 10 ⁵ HUVECs	55
Group 5-control	Co-culture Model-II	HUVECs and VSMCs	Yes	None	0.5 x 10 ⁵ HUVECs and 0.062 x 10 ⁵ VSMCs	27.5
Group 5-BMS	Co-culture Model-II	HUVECs and VSMCs	Yes	BMS	0.5 x 10 ⁵ HUVECs and 0.062 x 10 ⁵ VSMCs	27.5
Group 5-DES	Co-culture Model-II	HUVECs and VSMCs	Yes	DES	0.5 x 10 ⁵ HUVECs and 0.062 x 10 ⁵ VSMCs	27.5
Group 6-control	Co-culture Model-I	HUVECs and VSMCs	Yes	None	0.5 x 10 ⁵ VSMCs and 0.062 x 10 ⁵ HUVECs	27.5
Group 6-BMS	Co-culture Model-I	HUVECs and VSMCs	Yes	BMS	0.5 x 10 ⁵ VSMCs and 0.062 x 10 ⁵ HUVECs	27.5
Group 6-DES	Co-culture Model-I	HUVECs and VSMCs	Yes	DES	0.5 x 10 ⁵ VSMCs and 0.062 x 10 ⁵ HUVECs	27.5

* The cellular density is given in cells/mL.

** The sirolimus concentration is given in µg/well.

(A) Group 1: HUVECs monoculture (2×10⁵ cells/mL). **(B) Group 2:** VSMC monoculture (2×10⁵ cells/mL). **(C) Group 3:** Co-culture Model-II comprising HUVECs (0.5×10⁵ cells/mL) and VSMCs (0.062×10⁵ cells/mL). **(D) Group 4:** Co-culture Model-I comprising VSMCs (0.5×10⁵ cells/mL) and HUVEC (0.062×10⁵ cells/mL). **(E) Group 5:** Co-culture Model-II comprising HUVECs (0.5×10⁵ cells/mL) and VSMCs (0.062×10⁵ cells/mL) with 10% PRP. **(F) Group 6:** Co-culture Model-I comprising VSMCs (0.5×10⁵ cells/mL) and HUVEC (0.062×10⁵ cells/mL) with 10% PRP. All experiments were conducted for either 24 or 48 h. This was done in the absence or presence of a stent (BMS or DES). HUVECs, human umbilical vein endothelial cells; VSMCs, vascular smooth muscle cells; BMS, bare-metal stent (Pro-Kinetic®, Biotronik, Switzerland); DES, drug-eluting stent (Orsiro®, Biotronik, Switzerland); PRP, platelet-rich plasma at 10%.

PRP preparation and use

The preparation of PRP was a simple procedure. Samples from more than one donor were used to ensure that the platelets were fully activated during handling. Briefly, an anticoagulant-containing blood sample, collected from a human volunteer was centrifuged under controlled conditions, using a density gradient. This centrifugation was performed to obtain plasma with the highest concentration of platelets compared to the basal peripheral blood counts. 10% PRP was added to at least 90% confluent cultures. Briefly, cells were first cultured with 10% FBS for 24 h to reach 90% confluence. Then, the

medium was removed, and cells were cultured for 24 h with a serum-free medium. Only after, the cells were cultured with medium containing 10% PRP for 24 h, followed by different treatments for the indicated durations [20].

Monoculture experiments

Post neutralization, the cells in suspension were counted using a Neubauer's chamber, and their concentration was adjusted to 2 × 10⁵ cells/mL, while cell viability was determined using the Blue Trypan exclusion test. For experimental purposes, the percentage of viable cells was considered ideal at > 94%. After achieving confluence, the cells were sub-cultured for amplification and used in

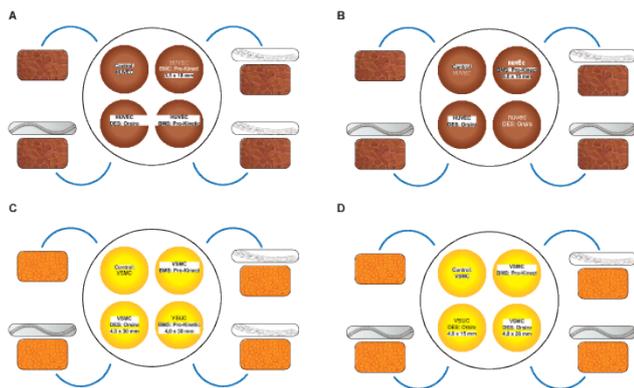


Figure 2: Monoculture experiments

Group 1: HUVEC monoculture (2×10^5 cells/mL) after 24-h (A) and 48-h (B) periods. **Group 2:** VSMC monoculture (2×10^5 cells/mL) after 24-h (C) and 48-h (D) periods. Passages were performed in the absence or presence of a stent (BMS or DES). HUVEC, human umbilical vein endothelial cells; VSMC, vascular smooth muscle cells; BMS, bare metal stent (Pro-Kinetic®, Biotronik, Switzerland); DES, drug-eluting stent (Orsiro®, Biotronik, Switzerland); VSMC, vascular smooth muscle cells.

subsequent experiments. Cell stocks were frozen in culture medium containing 10% dimethyl sulfoxide (DMSO) (C6164, Sigma Aldrich, St. Louis, MO, USA) and stored in liquid nitrogen (Figure 2A–D). The number of cells per well used was 2×10^5 for both cell types. The HUVECs used in these monoculture experiments originated from the fourth passage, while the VSMCs from the seventh. BMS or DES were added to the culture wells; cells cultured without stents were used as the control group.

Co-culture experiments

The growth of multiple cell types together allows for synergistic or antagonistic interactions between them, which are fundamental for cell differentiation, induction of apoptosis, and cell signaling [21,22]. To establish the ideal cell concentrations of the different cell types used in the two co-cultures models, we seeded HUVECs and VSMCs at different concentrations; the optimal cell concentrations were then defined based on the growth curves of the two cells. Our results revealed that the highest degree of significance was achieved at 1:8 ratios for both cell types. Of note, a 1:8 ratio represents the co-culture of 0.5×10^5 cells/mL and 0.062×10^5 cells/mL. Therefore, for the co-culture systems, two different cell concentrations of each cell line were used. In the co-culture Model-I (used in Groups 4 and 6), we seeded VSMCs at a concentration of 0.5×10^5 cells/mL, and then HUVECs at a concentration of 0.062×10^5 cells/mL, while in the co-culture Model-II (used in Groups 3 and 5), we seeded HUVECs first at a concentration of 0.5×10^5 cells/mL, and then VSMCs at a concentration of $0.062 \times$

10^5 cells/mL. Additionally, to groups 5 and 6, we added 60 μ L of PRP per well. The cell culture passages used in these experiments were the seventh passage for VSMCs and the third passage for HUVECs (Figures 3A and B and Figure 4).

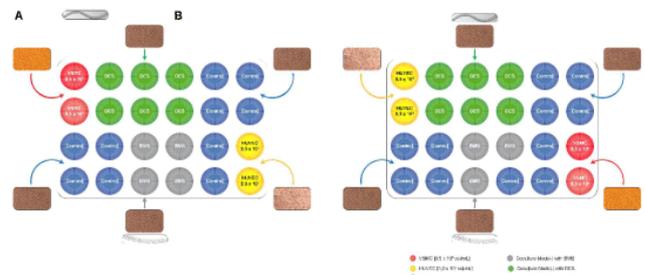


Figure 3: Co-culture experiments without PRP

The co-culture experiment was repeated for 24 h for Groups 3 (A) and 4 (B). **Group 3:** Co-culture Model-II comprising HUVEC (0.5×10^5 cells/mL) and VSMC (0.062×10^5 cells/mL). **Group 4:** Co-culture Model-I comprising VSMC (0.5×10^5 cells/mL) and HUVEC (0.062×10^5 cells/mL). All experiments were also conducted for 48 h (data not shown). Co-culture was performed in the absence or presence of a stent (BMS or DES). HUVEC, human umbilical vein endothelial cells; VSMC, vascular smooth muscle cells; BMS, bare-metal stent (Pro-Kinetic®, Biotronik, Switzerland); DES, drug-eluting stent (Orsiro®, Biotronik, Switzerland).

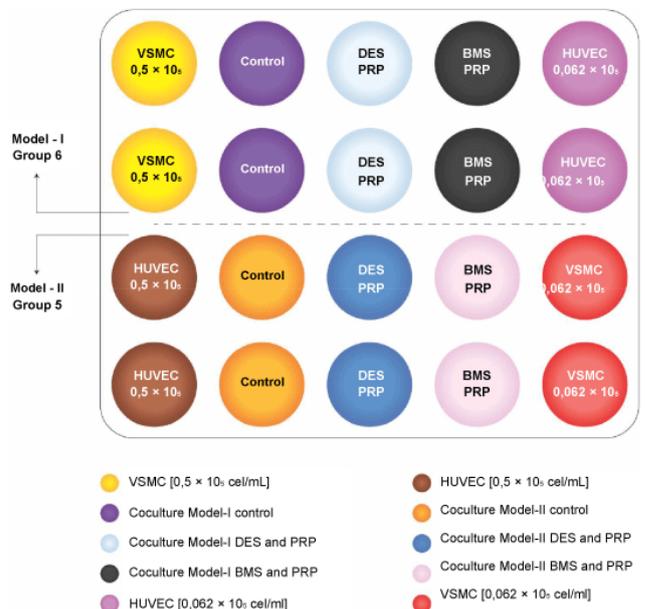


Figure 4: Co-culture experiments with PRP

The co-culture experiment was repeated for Groups 5 (A) and 6 (B) supplemented with PRP. **Group 5:** Co-culture Model-II comprising HUVEC (0.5×10^5 cells/mL) and VSMC (0.062×10^5 cells/mL) with 10% PRP at 24 h. **Group 6:** Co-culture Model-I comprising VSMC (0.5×10^5 cells/mL) and HUVEC (0.062×10^5 cells/mL) with 10% PRP at 24 h. All experiments were also conducted for 48 h (data not shown). This was done in the absence or presence of a stent (BMS or DES). HUVEC, human umbilical vein endothelial cells; VSMC, vascular smooth muscle cells; BMS, bare-metal stent (Pro-Kinetic®, Biotronik, Switzerland); DES, drug-eluting stent (Orsiro®, Biotronik, Switzerland); PRP, platelet-rich plasma.

Stents and sirolimus

In the present study, two types of stents were used: BMS (PRO-Kinetic Energy®) and DES (Orsiro®) were kindly donated by Biotronik AG, Bülach, Switzerland. The DES used elute the antiproliferative drug, sirolimus, [23] at a concentration of 1.4 µg/mm². Because of the different sizes of stents used, several groups received different concentrations of the drug. Of note, we calculated the sirolimus concentration present in each well using a table provided by the manufacturer, considering the diameter and length of the stents. To assess different concentrations of sirolimus, for monocultures (Groups 1 and 2), the sirolimus concentration varied between 70 and 200 µg. In Groups 3 and 5, the concentration was 55 µg per well, while in Groups 4 and 6, the concentration was 27.5 µg per well (Table 2).

Table 2: Total drug load in the Orsiro*

Stent Design	Total Drug Load		
		Small (µg)	Medium (µg)
Stent Length	9	55	70
	13	80	95
	15	93	113
	18	109	131
	22	134	162
	26	159	193
	30	184	224
	35	213	261
	40	247	298

Drug loading by measure. The diameters considered small are from 2.25 to 3.0 mm and the average diameters of 3.5 and 4.0 mm.

*Biotronik data in the file.

Microscopy

The effects of BMS and DES on the morphology of HUVECs and VSMCs grown in monoculture or co-culture conditions were observed via phase contrast using an inverted microscope (Nikon Eclipse TS 100, Nikon, Tokyo, Japan) and the NIS-Elements F software (Nikon). In all images were obtained at 30X magnification and are enlarged 20X.

Determination of lipid peroxidation products

The rate of lipid peroxidation in cells was measured using the technique reported by Ohkawa et al., [24] based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) [25]. Aliquots (100 µL) of the supernatants from the primary cultures of each group (as described above) were collected at the 24 h and 48 h time-points. Two sets of duly marked tubes containing the aliquots were separately prepared, one with 200 µL of 20% trichloroacetic acid (TCA; T4885; Sigma-Aldrich, St.

Louis, MO, USA), the other with 200 µL of thiobarbituric acid (TBA; S388033; Sigma-Aldrich) containing TCA (86% TBA/14% TCA). The samples were held for 20 min in a water bath at 100 °C. Subsequently, the corresponding TCA and TBA/TCA tubes were immediately immersed in an ice bath for 20 min and centrifuged (327 x g) at 8.000 rpm for 4 min at -20 °C. Next, 100 µL of the supernatant from each sample was collected and added to a 96-well plate, which was used to measure the formation rate of substances reactive to thiobarbituric acid (TBARS). The absorbance readings were collected at a wavelength of 535 nm in a spectrophotometer for microplates, ELISA reader, Model TP-READER (Thermo Fisher Scientific).

The lipoperoxidation rate was calculated by subtracting the *white* values from each series per group. The mean absorbance of the TCA series was given using the following mathematical expression:

$$LPO = \frac{(\text{mean absorbance TCA} - \text{white}) - (\text{mean absorbance TCA} / \text{TBA} - \text{white}) \times 5}{0.0312 \text{ molar coefficient (MDA)}}$$

where TCA = 20% trichloroacetic acid; TBA = 20% thiobarbituric acid; MDA = malondialdehyde.

Values were expressed as nmol of MDA/mL formed in the supernatants from cell cultures.

Cell cycle analysis via flow cytometry

After serum-starvation for 24 h, the cells were treated with bare-metal or drug-eluting stents in the presence or absence of 10% platelet-rich plasma for 24 and 48 h. After treatment, the cells were re-suspended in 300 µL of 0.2% trypsin solution (T3924, Sigma-Aldrich) at 37°C and blocked with the addition of complete culture medium with 20% FBS. The cells were then centrifuged for 5 min at 327 g, and the resulting cell pellets were re-suspended in 1 mL of cold growth medium (6.1 mM glucose, 137 mM NaCl, 4.4 mM KCl, 1.5 mM 14 Na₂HPO₄, 0.9 mM KH₂PO₄, and 0.5 mM EDTA). Thereafter, absolute ethanol (4°C) was slowly added while stirring; cells were fixed for 30 min. After this period, cells were centrifuged, re-suspended in 200 µL fluorescence-activate cell sorting (FACS) buffer, 10 µL Triton X-100, 10 µL propidium iodide (PI; 50 µg/mL, Sigma®), and 1 µL RNase-A (0.3 mg Sigma®), and incubated at room temperature for 1 h in the dark [26]. The reading was performed using a FACS Calibur™ flow cytometer (BD Biosciences, San Jose, CA, USA), particularly,

the FL2-H fluorescence channel (Alexa Fluor 488). The cell cycle phases (fragmented DNA or sub-G1, G0/G1, S phase, and G2/M) were analyzed using the Cell-Quest program (BD Biosciences). The results obtained were analyzed using the WinMDI 2.8 software. Values are expressed as the mean percentage of cells distributed in different phases of the cell cycle.

Analysis of the expression of cellular markers using flow cytometry

Flow cytometry was used to determine the expression of specific markers associated with cell apoptosis and the control of cell proliferation such as caspase-3, proliferating cell nuclear antigen (PCNA), cyclin-dependent kinase inhibitor (p27), and cyclin D1 [27-29]. The determination of markers involved in the inflammation, such as intercellular adhesion molecule (ICAM)-1, IL-1, tumor necrosis factor (TNF)-death receptor (DR)-4, IL-6, mitochondrial pyruvate carrier (MPC)-1, and vascular endothelial growth factor receptor (VEGFR)-1 (also involved in angiogenesis) was also performed [30-34].

Briefly, cells were fixed in FACS buffer with 4% paraformaldehyde for 1 h. Next, cells were centrifuged at 1,500 rpm for 5 min, the cell pellets were re-suspended in the FACS buffer, and the membranes were permeabilized with 0.1% Triton X-100 for 30 min at 4°C. Then, 1 µg of each indicated primary antibody was incubated for 24 h at 26 °C (Table 3). Human IgG Labelling Kit with Alexa-Fluor®

Table 3: List of primary antibodies used in this study

Primary Antibodies	Producer (catalog number)
VEGF-R1	R&D Systems (AF-293-NA)
p-27	Santa Cruz (sc-1641)
MCP-1	Santa Cruz (sc-377082)
TNF-DR4	Abcam (ab13890)
ICAM-1	R&D Systems (BBA3)
IL-1	Santa Cruz (sc-32294)
IL-6	Santa Cruz (sc-57315)
cyclin D1	Santa Cruz (sc-8396)
caspase-3	Abcam (ab13847)
PCNA	Abcam (ab29)

VEGF-R1: Receptor 1 for vascular endothelial growth factor; p-27: p-27 protein; MCP-1: monocyte-1 chemotactic protein; TNF-DR4: TNF-receptor 4 (tumor necrosis factor); ICAM-1: intercellular adhesion molecule-1; IL-1: interleukin 1; IL-6: interleukin-6; PCNA: proliferating cell nuclear antigen. Antibodies were from either R&D Systems (Minneapolis, MN, USA), Santa Cruz (Dallas, TX, USA), or Abcam (Cambridge, MA, USA).

488 (Z25402; Life Technologies; Carlsbad, CA, USA) and 0.2% bovine serum albumin (BSA) were incubated for an additional 24h at 4 °C in the dark.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism™ 5 (GraphPad Software, La Jolla, CA,

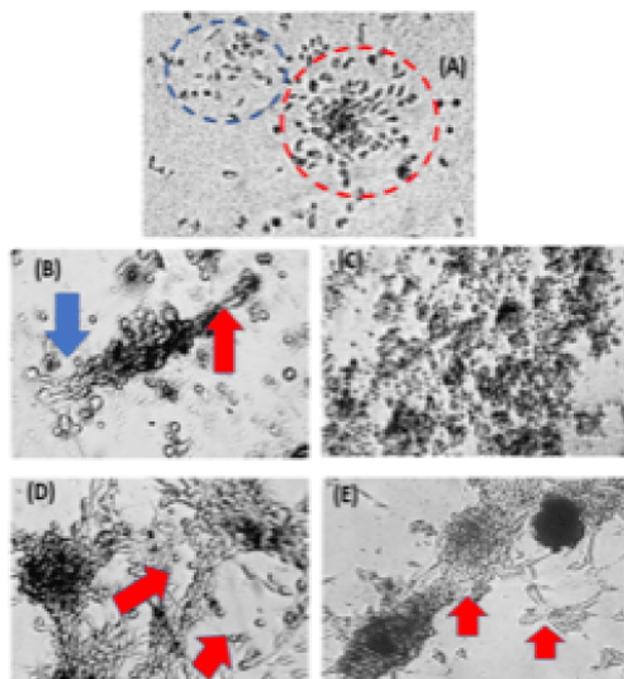


Figure 5: Representative photomicrographs of the cell culture models.

Cell organization of the 48-h culture of HUVECs and VSMCs in a medium supplemented with 10% PRP and exposed to BMS and DES. (A) Arrangement of human endothelial cell disposition – HUVECs (blue circle) and VSMC (red circle), cultured with the addition of PRP (Groups 5-control and 6-control); (B) co-culture of HUVEC and VSMC cells with 10% PRP for 48h in the presence of the BMS (Groups 5-BMS and 6-BMS). Note the formation of a primitive vascular network with the central disposition of endothelial cells and the superposition of VSMC; (C) co-culture of HUVEC and VSMC without 10% PRP for 48h in the presence of DESBMS (Groups 5-BMS and 6-DES). A significant increase was observed in the cell population in the culture supernatant, with dead, pyknotic cells and a large amount of cell debris; (D) co-culture of HUVEC and VSMC cells supplemented with 10% PRP for 48h in the presence of the BMS (Groups 5-BMS and 6-BMS). Note the formation of a vascular network, with tubular disposition of endothelial cells (red arrow) and increased density of smooth muscle cells; (E) co-culture of HUVEC and VSMC supplemented with 10% PRP for 48-h in the presence of DES (Groups 5-BMS and 6-BMS). The blue arrow highlights the arrangement of smooth muscle cells in the human artery and the presence of lower density endothelial cells, with characteristics of a cell death process (red arrow). All experiments were conducted for either 24 or 48 h; cells were cultured in the absence or presence of stents (BMS or DES); not all conditions are represented in the Figure. HUVECs, human umbilical vein endothelial cells; VSMCs, vascular smooth muscle cells; BMS, bare-metal stent (ProKinetic, Biotronik, Switzerland); DES, drug-eluting stent (Orsiro, Biotronik, Switzerland); n, sample number; PRP, platelet-rich plasma. All images were taken at 30X magnification and are shown with 20X enlargement.

USA). Statistical significance was determined using anon-parametric multivariant two-way ANOVA analysis, followed by a post-hoc analysis using the Tukey's multiple comparisons test (0.5%). $p < 0.05$ was considered significant: ns, not significant; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Results

Morphological analysis of HUVECs and VSMCs treated with BMS or DES

The effects of BMS and DES on the morphology of HUVEC and VSMC grown in monoculture or co-culture conditions were observed via phase contrast using an inverted microscope (Nikon Eclipse TS 100, Nikon, Tokyo, Japan) and the NIS-Elements F software (Nikon). Images obtained under an inverted light microscope at 30X magnification and are enlarged 20x. Representative images demonstrating changes in morphology are shown in (Figure 5A-E).

Lipid peroxidation profiles

The rate of formation of TBARS (specifically MDA-related) generated by HUVECs in monocultures (Group 1) was measured after 24 and 48 h of treatment. At the 24 h time-point a significant decrease ($p < 0.01$) was observed in the DES group (Group 1-DES) compared with both the control group (group without stent) and the BMS group (Group 1-BMS); however, after 48 h of treatment, there was no significant difference among the three groups (Figure 6A, B). Additionally, in the context of VSMCs monocultures (Group 2), there was also a significant decrease ($p < 0.01$) in the DES group (Group 2-DES) after 24 h of treatment compared with both the control and BMS groups. However, after 48 h, there was also no significant difference among the groups (Figure 6C, D).

Additionally, with respect to Groups 3 and 4, which were co-cultures of HUVECs and VSMCs without PRP, there was no difference in the rate of the production of TBARS (MDA-related) in either treatment period (Figure 6E-H). Meanwhile, considering Model-II cell co-cultures with PRP (Group 5), there was a significant increase ($p < 0.01$) in the production rate of MDA in the DES subgroup (Group 5 DES) versus the same condition without PRP (Group 5-DES) in both treatment periods (Figure 6I, J). Similarly, in Group 6, consisting of Model-I cell co-cultures with PRP, there was a significant increase ($p < 0.01$) in the MDA production rate in the DES+PRP subgroup (Group 6 DES+PRP) compared with the respective DES subgroup without PRP (Group 6-DES) in

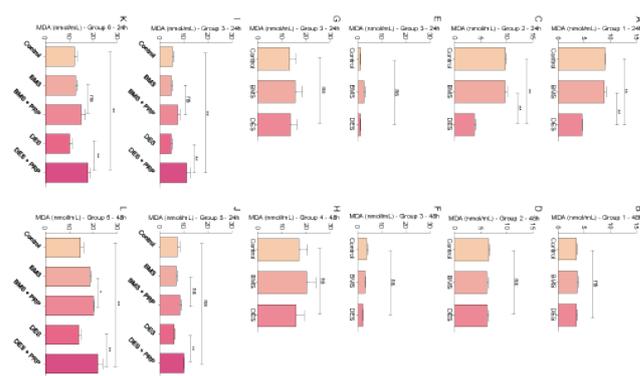


Figure 6: Characterization of the formation of lipid peroxides under different experimental conditions. (A, B) Group 1: HUVECs monocultures (2×10^5 cells/mL). **(C, D) Group 2:** VSMCs monocultures (2×10^5 cells/mL). **(E, F) Group 3:** Co-culture of HUVECs (0.5×10^5 cells/mL) and VSMCs (0.062×10^5 cells/mL) - Model-II. **(G, H) Group 4:** Co-culture of VSMCs (0.5×10^5 cells/mL) and HUVECs (0.062×10^5 cells/mL) - Model-I. **(I, J) Group 5:** Co-culture of HUVECs (0.5×10^5 cells/mL) and VSMCs (0.062×10^5 cells/mL) - Model-II - with 10% PRP. **(K, L) Group 6:** Co-culture of VSMCs (0.5×10^5 cells/mL) and HUVECs (0.062×10^5 cells/mL) - Model-I - with 10% PRP. All experiments were conducted for either 24 or 48 h. Cells were incubated either in the absence or presence of stents (BMS or DES). MDA, malondialdehyde; HUVECs, human umbilical vein endothelial cells; VSMCs, vascular smooth muscle cells; BMS, bare-metal stent (Pro-Kinetic®, Biotronik, Switzerland); DES, drug-eluting stent (Orsiro®, Biotronik, Switzerland); n, sample number; PRP at 10%, platelet-rich plasma at 10%. Data are shown as the mean \pm SD. Statistical differences were assessed using the Kruskal Wallis test with Tukey's post hoc analysis (0.5%): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, non-significant.

both treatment periods. During the 48-h treatment period, there was also an increase in the MDA production rate in the BMS+PRP subgroup (Group 6-BMS+PRP) compared to the rate observed in the BMS without PRP subgroup (Group 6-BMS) (Figure 6K, L).

Evaluation of the cell cycle by flow cytometry

The distribution of cell populations in the different phases of the cell cycle in the context of the different culture conditions was evaluated. With respect to monocultures of HUVECs in the presence of DES (Group 1-BMS) for 24 h there was a significant decrease ($p < 0.001$) in the percentages of cells in the G0/G1, and a significant increase ($p < 0.001$) in the frequency of cells in the G2/M phase compared with the control group (Figure 7A). Importantly, after 48-h of treatment, the trend seen in the context of the G2/M phase was similar to that after 24 h of treatment ($p < 0.01$). Curiously, the cell cycle profile of Group 1-BMS, completely changed from the 24 h to the 48-h time-points, with a decrease in the proportion of cells in the G0/G1 phase ($p < 0.01$), and a consequent increase in the proportion of cells in the S and G2/M phases ($p < 0.001$) (Figure 7B).

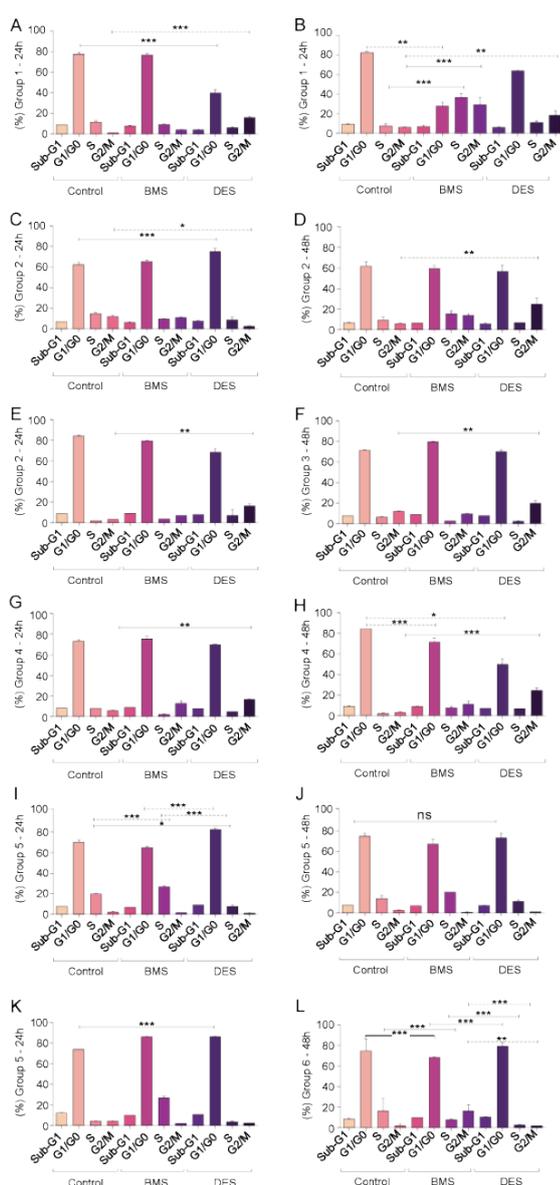


Figure 7: Evaluation of cell cycle progression under different experimental conditions. Cells at the four different phases of the cell cycle were resolved using flow cytometry and defined as follows: sub-G1, G0/G1, S, and G2/M. **(A, B) Group 1:** HUVECs monocultures (2×10^5 cells/mL). **(C, D) Group 2:** VSMCs monocultures (2×10^5 cells/mL). **(E, F) Group 3:** Co-culture of HUVECs (0.5×10^5 cells/mL) and VSMCs (0.062×10^5 cells/mL) - Model-II. **(G, H) Group 4:** Co-culture of VSMCs (0.5×10^5 cells/mL) and HUVECs (0.062×10^5 cells/mL) - Model-I. **(I, J) Group 5:** Co-culture of HUVECs (0.5×10^5 cells/mL) and VSMCs (0.062×10^5 cells/mL) - Model-II - with 10% PRP. **(K, L) Group 6:** Co-culture of VSMCs (0.5×10^5 cells/mL) and HUVECs (0.062×10^5 cells/mL) - Model-I - with 10% PRP. All experiments were conducted for either 24 or 48 h. Cells were incubated in the absence or presence of stents (BMS or DES). HUVECs, human umbilical vein endothelial cells; VSMCs, vascular smooth muscle cells; BMS, bare-metal stent (Pro-Kinetic®; Biotronik, Switzerland); DES, drug-eluting stent (Orsiro®; Biotronik, Switzerland); PRP, platelet-rich plasma at 10%. Data are shown as the mean \pm SD. Statistical differences were assessed using the ANOVA and the with Tukey's post hoc analysis (0.5%): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, non-significant.

group, after both 24 h ($p < 0.01$; **Figure 7E**) and 48 h ($p < 0.01$; **Figure 7F**) of culture. Interestingly, the same results were observed in the context of Model-I co-cultures (**Figure 7G**). Moreover, after 48 h of culture, there was a significant decrease in the frequency of cells in the G0/G1 phase in Group 4-BMS ($p < 0.001$) and Group 4-DES ($p < 0.05$) compared with the control group (**Figure 7H**).

Furthermore, in Group 5 (Model-II co-cultures with PRP) after 24 h of culture, there was a significant decrease in the ratio of cells in the S phase in Group 5-DES versus both the control ($p < 0.05$) and BMS ($p < 0.001$) groups, whereas there was a significant increase in the frequency of cells in the G0/G1 phase ($p < 0.001$) (**Figure 7I**). However, at the 48-h time-point, there were no differences among the different groups (**Figure 7J**). Last but not least, in Group 6 (Model-I co-cultures with PRP) there was an increase in the frequency of cells in the G0/G1 phase in DES groups, compared with that in control cells ($p < 0.001$) (**Figure 7K**). Notably, at the 48-h time-point, the profile changed; the frequency of cells in the G0/G1 phase was lower in the BMS group than in both the control and DES groups ($p < 0.001$), and the frequency of cells in the G2/M phase was significantly higher ($p < 0.01$) in the BMS group compared with both the control and DES groups. Curiously, both the addition of BMS and DES decreased the frequency of cells in the S phase versus that in control cells ($p < 0.001$; **Figure 7L**).

Analysis of the expression of cellular markers

In this study, we analyzed the expression of various cellular markers involved in apoptosis and cell proliferation, such as caspase-3, PCNA, p27, and cyclin D1 in the different groups after 24 and 48 h of culture. Of note, there was no difference in the expression of these markers, indicating that sirolimus did not induce apoptosis in HUVECs and VSMCs, and consequently that the drug concentrations used were not cytotoxic.

Additionally, also the analysis of the expression of cellular markers involved in inflammation (ICAM-1, IL-1 β , TNF-DR4, IL-6, and MCP-1) and angiogenesis (VEGF-R1) overall showed no differences among groups, with one exception, IL-6. Curiously, after 24 h of culture, we observed that the expression of IL-6 was higher in all DES-treated groups versus the BMS control counterparts, except for group 2. Moreover, after 48 h, we observed that there was a significant increase in the expression of IL-6 in groups 3 and 5 (PRP-treated) in the context of DES (**Figure 8A-B**).

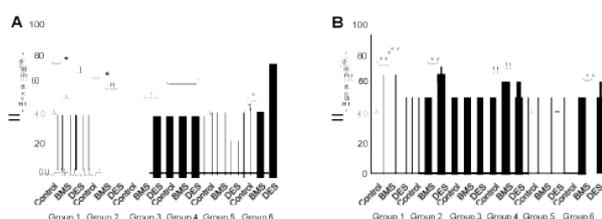


Figure 8: Expression of IL-6.

IL-6 expression quantified in the different groups at 24 or 48 h using flow cytometry. IL-6, interleukin 6; HUVECs, human umbilical vein endothelial cells; VSMCs, vascular smooth muscle cells; BMS, bare-metal stent (Pro-Kinetic, Biotronik, Switzerland); DES, drug-eluting stent (Orsiro, Biotronik, Switzerland); n, sample number.

Data are shown as the mean \pm SD. Statistical differences were assessed using the ANOVA with Tukey's post hoc analysis (0.5%): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, non-significant.

Altogether, these findings suggest that endothelial cell-mediated inflammatory responses in the presence of DES occurred earlier than that detected in the presence of BMS, and in a higher extent in the presence of PRP.

Discussion

Drug-eluting stents suppress neointimal hyperplasia *in vivo*, reducing the rate of ISR detected in the follow-up examinations of patients subjected to PCI [5,6,8]. Although sirolimus and its analogs are effective antiproliferative agents when applied lumenally through stents, their effects on human ECs and SMCs *in vitro* have not been well studied. Therefore, here, we constructed different *in vitro* models to fill this gap in knowledge; we cultivated, for the first time, HUVECs and VSMCs in the context of monocultures or co-cultures in the presence of platelets and added the stents directly into the culture wells.

Habib et al., reported that the tissue concentration of sirolimus reaches 500 nmol/L after implantation of sirolimus-eluting stents *in vivo*, which is equivalent to 0.457 $\mu\text{g}/\text{mL}$ [35]. Other research groups have reported that the average tissue concentration of sirolimus is 0.014 $\mu\text{g}/\text{mL}$ *in vivo* [36]. Therefore, in our *in vitro* study, we used a higher sirolimus concentration than the tissue concentration eluted by DES *in vivo*. Of note, since there are no reports of *in vitro* studies like ours in the literature, we could not assess if the dose used in this study was too high.

Interestingly in the context of monocultures treated with DES, we observed a decrease in the production of TBARS after 24 h. Additionally, in the co-cultures treated with PRP, there was an increase in the production of reactive oxygen species in the groups that received stents

(either BMS or DES), but in a more significant fashion in the context of DES. These findings indicate that platelets potentiate the oxidative stress generated by the presence of a stent, which may result from the amplification of the inflammatory responses.

Moreover, the cell cycle analysis revealed that the most consistent effect of sirolimus was an increase in the percentages of cells in the G2/M phase. When platelets were added to the co-culture models, there was an increase in the percentage of cells in the G0/G1 phase and a decrease in the percentage of cells in the G2/M phase. Notably, platelets are pro-inflammatory in the context of vascular injury [37]. The release of PDGF via platelets into the culture medium may have stimulated SMCs and influenced the impact of sirolimus on the cell cycle.

To further explore the antiproliferative mechanism of sirolimus in the two cell types, we examined the expression of cell markers involved in apoptosis and cell proliferation, such as caspase-3, PCNA, p27, and cyclin D1, which are involved in apoptosis. Importantly, no differences were found in the expression of the cell markers involved in apoptosis/cell proliferation under the different culture conditions tested. These results confirmed that sirolimus did not induce apoptosis or necrosis in HUVECs and VSMCs. Parry et al., demonstrated that sirolimus at low nanomolar concentrations caused cell cycle arrest in the G0/G1 phase in ECs and SMCs and did not induce cell death, [18] that is in line with our results.

In parallel, in this study, we also analyzed the expression of cell markers involved in inflammation, such as ICAM-1, IL-1 β , TNF-DR4, IL-6, and MCP-1, and in angiogenesis, e.g., VEGF-R1. Except for IL-6, no differences were detected among groups. Therefore, our findings differ from those of previous studies, particularly regarding the expression of p27 [38-40]. For instance, Kawamata et al., examined the intracellular events following sirolimus treatment in the context of exponentially growing T cells and reported that sirolimus positively regulated p27 [40]. The authors suggested the presence of a putative threshold level for p27 in the late phase of G1 cells that had already passed through the cell cycle. One possible explanation for the lack of an increase in p27 expression in our study is that it appears that the increase in p27 is related to cell cycle arrest at the G1 phase. However, in our study, most experiments demonstrated cell cycle arrest at the G2/M phase. The stents used in this study released sirolimus at a concentration

higher than that described in the literature, which may be also a reason for this difference. For instance, Habib et al., reported that sirolimus reaches a tissue concentration of 500 nmol/L (0.457 µg/mL) after implantation of sirolimus-eluting stents [35]. Additionally, Marx et al., reported that sirolimus concentrations as low as 1 ng/mL inhibited cell growth [41]. This value corresponds to 0.001 µg/mL, which is much lower than that used in our study; in the case of monocultures, we used sirolimus concentrations ranging from 70 to 220 µg, and in co-cultures without and with PRP, the concentration of sirolimus was 55 µg and 27.5 µg. However, in agreement with our findings, Sun et al., reported that when sirolimus was used at high doses in fibroblasts and SMCs, there was no significant difference in the expression of p27 [17]. Altogether, these findings suggest that high doses of sirolimus (IC₅₀ = 200 nmol/L, *in vivo*) impact cells via a p27-independent mechanism, whereas low doses (IC₅₀ = 2 nmol/L, *in vivo*) act on cells via a p27-dependent mechanism.

However, differences were detected with respect to the expression of IL-6, at both 24 and 48 h of culture. After 24 h, we observed that the expression of IL-6 was higher in all DES-treated groups versus BMS-treated and control counterparts, except in the context of group 2. Additionally, after 48 h, we showed that there was a more pronounced increase in the expression of IL-6 in groups 3 and 5 (PRP-treated) in the context of DES. These findings suggest that the inflammatory responses associated with ECs and SMCs in the presence of DES occurred earlier than in the presence of BMS, and in a higher magnitude in the presence of platelets. Ikeda et al. demonstrated that SMCs collected from the aorta of rats released IL-6 promoting their growth in an autocrine manner via the induction of the production of PDGF [11].

The CANTOS Trial¹⁰ has helped to move the inflammatory hypothesis of CAD forward. Canakinumab, a human monoclonal antibody against IL-1β, showed modest absolute clinical benefits and major side effects. However, with the publication of the CANTOS results, doctors have definitive evidence that directly targeting inflammation is beneficial prevention of CAD. In fact, different studies using anti-inflammatory drugs such as methotrexate and colchicine in the context of CAD and, further support this hypothesis [42-44].

Niida et al., demonstrated that the use of anti-IL-6 receptor antibodies in mice significantly reduced intimal hyperplasia [12]; this was confirmed by Kitano et al., [13].

Importantly, our *in vitro* data further support these *in vivo* findings, suggesting a major role for IL-6 in the context of ECs and SMCs and consequently of intimal hyperplasia and ISR, orchestrated by platelets. This supports the hypothesis that the blockade of IL-6 signaling using drugs targeting IL-6 or the IL-6 receptor, such as tocilizumab, sarilumab, satralizumab, and siltuximab, may be an effective treatment option in the context of ISR. Importantly, there is precedent; tocilizumab has already been used successfully in the treatment of giant cells [14] and Takayasu arteritis [15]. Therefore, we propose that tocilizumab should be tested in the context of human clinical trials for the treatment of ISR. Initially, due to the high cost of this therapeutic agent, this approach should be considered for patients with high residual inflammatory risk due to comorbidities such as diabetes, and metabolic syndrome, as well as for those with elevated high-sensitivity reactive C-protein levels, even if their LDL cholesterol levels are under control.

This study has some limitations. We acknowledge that our work is an *in vitro* study with limited translational impact for the treatment of ISR *in vivo*. Furthermore, HUVECs may not be representative of the ECs found in the vascular beds that receive the stents. Likewise, cultured VSMCs are already differentiated, and there is a possibility of variation between the SMCs derived from different patients. The MDA/TBARS assay also presents selectivity problems; recent technologies, such as the determination of isoprostane, may be more specific. We also know that ISR is so aggressive and mechanically multifactorial in nature that numerous therapeutic interventions have failed, despite the high theoretical potential. However, we believe that the study of IL-6 blockage in the context of ISR is worthy.

Platelets potentiate the oxidative stress generated by the presence of stents, possibly via the amplification of inflammatory responses. Moreover, the analysis of different cellular markers involved in inflammation showed that only the expression of IL-6 was affected; the expression was higher in all DES-treated groups and in the presence of platelets. These findings suggest that the blockade of IL-6 signaling using pharmacologic inhibitors is a possible new therapeutic strategy in the context of ISR.

Acknowledgements

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Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

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