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# Histological features of restenosis associated with paclitaxel drug-coated balloon: implications for therapy☆

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ABSTRACT

*Purpose:* To investigate the cellular and extracellular changes induced by drug-coated balloons (DCB) in the treatment of superficial femoral artery (SFA) restenosis, and to compare histopathological features with those observed after plain old balloon angioplasty (POBA) from the same patients.
*Methods and Results:* Plaque samples for five patients with SFA restenosis (first-time) after POBA were collected using atherectomy and DCB. These samples constitute the POBA restenosis group. The same five patients developed recurrent restenosis (RR) after DCB, at the same intervention site. These SFA-RR lesions were again treated using atherectomy and POBA. These samples constitute the DCB restenosis group. DCB restenosis group plaques showed significant reduction in neointima, smooth muscle cells, fibroblast densities, and Ki67 index; and increase in caspase 3, features of apoptosis and type III collagen deposition in comparison to the POBA restenosis group.
*Conclusion:* Plaque tissue from the DCB restenosis group show reductions in neointimal thickness, cellularity, and cellular proliferation, along with increased apoptosis, and Type III collagen content. These results suggest a different mechanistic pathway for DCB restenosis, in which neointimal proliferation is reduced but reparative fibrosis is increased. The treatment for SFA-RR after DCB may therefore benefit from different forms of therapy including scaffolding, rather than recurrent anti-proliferative therapy.


## 1. Introduction

Peripheral artery disease (PAD) affects 8 million people in the United States [1,2]. The clinical manifestations of lower extremity PAD span the spectrum from intermittent claudication to critical limb ischemia [3,4]. Interventional procedures, though effective in alleviation of the symptoms of limb ischemia may induce recurrent symptoms mediated by restenosis. [5]. Restenosis is a complex coordinated sequence of events initiated by mechanical vessel wall injury and facilitated by increased cellular response in the neointima [6]. The advent of drug-coated balloons (DCB), especially those coated with paclitaxel have been shown to be a promising avenue in reducing the neointimal cellular proliferation, leading to reduction of restenosis [7]. Paclitaxel induces reversible polymerization of the α and β subunits of tubulin in smooth muscle cell (SMC), preferentially binding with the β subunit of tubulin and stabilizing subcellular microtubule organization [8]. This prevents further progression in the cell cycle [8]. By preventing microtubule disassembly, paclitaxel blocks the cellular proliferation beyond G2 to M phase of the cell cycle [9]. Clinical studies of DCB in the femoropopliteal segment have shown promising results with primary patency rates of 92.1% at 1 year. [10]. Furthermore, the LEVANT I trial showed that DCBs reduced restenosis and target lesion revascularization (TLR) compared to plain old balloon angioplasty (POBA) [11]. Nevertheless, a "catch up" phenomenon in

*Abbreviations:* PAD, peripheral artery disease; DCB, drug-coated balloon; SMC, smooth muscle cell; TLR, target lesion revascularization; POBA, plain old balloon angioplasty; RR, recurrent restenosis; SFA, superficial femoral artery; H&E, hematoxylin and eosin; FSP-1, fibroblast specific protein 1; HPF, high power field; ISR, in-stent restenosis.

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late lumen loss is observed during the second year, resulting in restenosis rates of 20–30% [10–12]. While clinical superiority of DCB over POBA in PAD is established, the histomorphological differences in the restenotic process have not been completely elucidated. This study investigates the sequential cellular and extracellular histopathological features of recurrent restenosis in the same patient at the same lesion site, initially treated with atherectomy+POBA and later treated with atherectomy+DCB following subsequent restenosis.

## 2. Methods

### 2.1. Demographics of patients

Five patients with symptomatic lower extremity PAD were treated for de novo stenosis of the superficial femoral artery (SFA) with POBA as the index intervention. These patients were followed in a standard surveillance program and were found to have symptomatic restenosis of the POBA intervention site. At this time, the same patients/same lesion site confirmed by angiography underwent directional atherectomy first, followed by DCB angioplasty for therapy of the POBA restenosis (Central Illustration). All patients were treated with Lutonix (BARD) MOXY balloons. These samples represent the group of POBA restenosis. During subsequent follow up, the same five patients developed recurrent restenosis at the same lesion site, and underwent directional atherectomy first, followed by POBA for symptomatic relief (Fig. 2). These samples constitute the group of DCB restenosis.

All plaque samples were collected from the SFA, and within 20 min, washed in saline and fixed in 10% buffered formalin and immediately submitted for processing into paraffin blocks. All specimens were collected using the Silver hawk atherectomy device (Medtronic, Santa Clara, CA) at a single center. All patients in the study had Rutherford category III claudication symptoms, were TASC A/B SFA lesions without occlusions and without aorto-iliac disease with at least a single run-off vessel to the foot at both baseline and re-intervention. Relevant demographic profiles for the cases were reviewed (Table 1).

All procedures were performed after informed consent for experimentation in human subjects. The study was approved by the Institutional Review Board, conforming to the Declaration of Helsinki.

### 2.2. Morphological characteristics

#### 2.2.1. Neointimal thickness

Using Hematoxylin and Eosin (H&E) stained slides, the neointimal thickness in the restenotic plaques was outlined and measured using Zedec QuantIm computerized planimetry system attached to the Olympus BX50 microscope and documented in mm.

**Table 1**
Clinical demography features of POBA and DCB groups

| Clinical and angiographic parameters at restenotic intervention ($n$=5) | |
|---|---|
| Age (years ± CI) | 67.0±2.8 |
| Gender (male) | 2 |
| Body mass index (kg/m$^2$ ± CI) | 28.2±1.5 |
| Diabetes mellitus | 4 |
| Hypertension | 5 |
| Hyperlipidemia | 5 |
| Coronary artery disease | 5 |
| Smoker | 0 |
| Onset of restenosis after intervention of POBA group (months) | 8.0±3.8 |
| Onset of restenosis after intervention of DCB group (months) | 10.8±3.9 |
| Post-atherectomy stenosis | 0% |

#### 2.2.2. Smooth muscle cells and fibroblast density

To identify SMC and fibroblast density, immunochemistry was performed using specific primary and secondary antibodies; for SMCs, α-actin (mouse monoclonal α-actin; Enzo Lab Inc., Farmingdale, NY, 1:40 dilutions) was used for labeling and for fibroblast, fibroblast specific protein 1 (FSP-1) (rabbit polyclonal; Abcam, 1:100 dilutions) was used. Appropriate positive control (colon for SMC, skin for FSP-1) and negative (mouse and rabbit IgG) controls were included to distinguish nonspecific binding. Using the α-actin and FSP-1 immunostained slides, total number of positively stained α-actin and FSP-1 cells in 10 random high power fields (HPF) (Olympus BX50 microscope in 40X) for each plaque were enumerated. The total plaque area occupied in HPF was measured in mm$^2$ using a computerized planimetry system. The density of α-actin and FSP-1 positive stained cells were calculated by dividing the total number of α-actin/FSP-1 positive stained cells by the total plaque area occupied per HPF (numerator is # SMC/fibroblast: denominator is total area) .

#### 2.2.3. Smooth muscle cell loss in tunica media

Plaque sections stained with α-actin were examined under the HPF for the presence or absence of medial loss. This histological feature was evaluated by the discontinuity or loss of SMC content in the tunica media.

#### 2.2.4. Cellular proliferation index by Ki67

Ki67 antibody (rabbit monoclonal Ki67; Abcam, Cambridge, MA, USA, 1:100 dilutions) was used to quantify proliferating cells in the neointima. Appropriate positive (human tonsil tissue) and negative (mouse IgG) controls were included to distinguish nonspecific binding. The total number of Ki67-positive nuclei in the plaque intima that exhibited nuclear Ki67 protein expression was enumerated using light microscopy in 10 random HPF per specimen and estimated in relation to total number of nuclei studied in each field and quantified as percentage of Ki67-positive nuclei among the intimal cells and reported. Ki67 index is a percentage variable where the denominator is the total number of cells in the field, and the numerator is the Ki67 positive nuclei.

#### 2.2.5. Apoptosis index by active caspase-3

Immunohistochemistry for active caspase-3 expression was performed using rabbit monoclonal IgG antibody (ab44976-Abcam Inc., Cambridge, MA, 1:200 dilution) with 3,3-diaminobenzidine chromogen and Elite Vectastain kit (Vector Laboratories) using an appropriate secondary biotinylated antibody. The active caspase-3 grade was manually quantified in 20 random HPF per specimen using percentage of positive cells stained per HPF [13], and a semi quantitative score was adopted to grade as follows: grade 0: absent; grade 1: 1–25%; grade 2: 26–50%; grade 3: >51%.

#### 2.2.6. Nuclear/cytoplasmic morphological features of apoptosis

Morphological features of nuclear proliferation and apoptosis were measured under oil immersion field focus (100X) using H&E sections as previously published [14]. (1) nuclear division which represents nuclear chromatin division; (2) nuclear spindling that illustrates mitotic spindle formation and appears as split nuclear strands that pulls apart each other during mitosis process; (3) nuclear fragmentation: represents discontinuity/fragmentation of nuclear chromatin; (4) chromatin condensation: nuclear chromatin with dense basophilic (intense blue) staining; (5) chromatin margination: nuclear chromatin (basophilic) staining with peripheral margination or rimming of the chromatin in the nucleus; (6) cytoplasmic bleb: abnormal vacuolation of the cytoplasm exhibiting protrusions or blebs (bulging); (7) cytoplasmic eosinophilia: dense eosinophilic (acidophilia; pinkish color) staining of cytoplasmic content, indicating an abnormal increase in protein

content and (8) cytoplasmic apoptotic body that represent multiple necrotic vesicles in the cytoplasm, were carefully screened under oil immersion field at 100×.

#### 2.2.7. Extra cellular matrix collagen content

Plaque tissue sections were stained with specific immunochemistry for collagens using primary mouse monoclonal antibody to identify type I collagen (ab90395, Abcam, MA, USA), at 1:100 dilution and rabbit polyclonal antibody to type III collagen (ab7778, Abcam, MA, USA), at 1:200 dilutions. Appropriate positive and negative control for type I and III collagen was included using human uterine tissue. Collagen grade was semi quantitatively scored as published previously [15,16].

#### 2.2.8. Extra cellular matrix proteoglycan content

To measure proteoglycans (PG), plaque tissue sections were stained with 1% Alcian blue at pH 2.5 per manufacturer's instruction (Polyscientific, NY, USA) and the proteoglycan density was evaluated using a semi quantitative score to grade as follows: grade 0: absent PG stain; grade 1: 1–25% PG stain; grade 2: 26–50% PG stain; grade 3: >51% PG stain with Olympus BX50 microscope.

### 2.3. Statistical analysis

Patients were divided into two groups: POBA restenosis group and DCB restenosis group (see Central Illustration). Clinical and demographic parameters were compared among groups using Fischer's Exact test or Student's t test for categorical or continuous variables, respectively. Null hypothesis is that there are no difference between POBA restenosis group and DCB restenosis group. A *P* value less than .05 was considered significant. IBM SPSS/PASW 22.0 software (SPSS Inc., Chicago, IL).

## 3. Results

### 3.1. Demographic and clinical profiles

There were three female and two male subjects in the study. Other relevant demographic profiles are shown in Tables 1 and 3.

### 3.2. Morphological features (Table 2)

#### 3.2.1. Neointimal thickness

Mean neointima thickness was significantly reduced in DCB when compared to POBA restenosis (0.102±0.01 vs. 0.482±0.05; *P*=.001) (Fig. 1A–C).

#### 3.2.2. Smooth muscle cells and fibroblasts density

Mean SMC density was significantly reduced in DCB when compared to POBA restenosis (1184.05±59.46 vs.2587.01±68.21; *P*=.0001), (Fig. 3A, B, C). Furthermore, mean fibroblast density was also significantly reduced in DCB when compared to POBA restenosis. (911.06±44.04 vs.1731.52±131.48; *P*=.002) (Fig. 3D–F).

#### 3.2.3. Smooth muscle cell loss in tunica media

Partial loss of SMCs was observed in the plaques from DCB restenosis, whereas no loss in the media was observed in plaques from POBA restenosis.

#### 3.2.4. Cellular proliferation index by Ki67

Mean percentage of nuclei expressing Ki67 was significantly reduced in DCB when compared to POBA restenosis. (2.77%±0.95 vs. 16.95% ± 1.78; *P*=.0001) (Fig. 3G–I).

#### 3.2.5. Apoptosis index by activated caspase-3

Mean score of activated caspase 3 was significantly increased in DCB when compared to POBA restenosis (2.74±0.04 vs. 1.96±0.11; P=.0001), (Fig. 3J–L).

#### 3.2.6. Nuclear/cytoplasmic morphological features of apoptosis (Fig. 4)

Morphological features of apoptosis including nuclear division, nuclear spindling, chromatin fragmentation/lysis, chromatin condensation, chromatin margination, cytoplasmic bleb, cytoplasmic eosinophilia and apoptotic body were increased in DCB restenotic plaques when compared to POBA restenotic plaques. (Fig. 4A–C).

### 3.3. Extra cellular matrix content evaluations (Fig. 5)

#### 3.3.1. Collagen content

In DCB restenosis plaques, mean grade of type III collagen was significantly increased when compared to POBA restenosis (2.28±0.22 vs. 0.96±0.23; *P*=.003), whereas type I collagen grade was decreased in DCB restenosis plaques compared to POBA restenotic plaques (0.44±0.04 vs. 0.72±0.04; *P*=.001).

#### 3.3.2. Proteoglycan content

Proteoglycan grade showed no difference between DCB restenosis plaque versus POBA restenosis plaque. (2.38±0.17. vs. 2.40±0.10; p = NS).

No evidence of significant acute or chronic inflammatory cells were observed by histology. As well, no foreign materials or foreign body giant cells were noticeable in the study material.

**Table 2**
Pathological features of POBA and DCB

| Histopathological parameters at restenotic intervention | Plain old balloon angioplasty (n=5) | Paclitaxel drug-coated balloon angioplasty (n=5) | *P* |
|---|---|---|---|
| Neointima thickness (mm) | 0..482±0.045 | 0.102±0.007 | .001 |
| Smooth muscle cell density | 2587.01±68.22 | 1184.05±59.46 | .0001 |
| Fibroblast cell density | 1731.52±131.48 | 911.06±44.03 | .002 |
| Ki 67 (Percentage) | 16.95±0.16 | 2.77±0.95 | .0001 |
| Caspase-3 grade | 1.96±3.8 | 2.74±0.04 | .0001 |
| Collagen I density | 0.780±0.139 | 0.660±0.129 | NS |
| Collagen III density | 0.960±0.23 | 2.28±0.22 | .003 |
| Proteoglycan grade | 2.4±0.105 | 2.38±0.165 | NS |

## 4. Discussion

Development of DCB therapy for management of femoral popliteal disease has offered a significant advancement given its durability in the intermediate term [10–12]. Beyond being a treatment for native vessel femoro-popliteal stenosis, DCB is also a therapeutic option for in-stent restenosis (ISR) [17]. As the DCB use increases and long-term data is accrued, there seems to be a late catch up phenomenon of ~30% restenosis at 3 years [18]. The optimal therapy to address DCB restenosis remains unclear, largely due to the lack of understanding of the pathophysiology of this process.

Data from coronary balloon angioplasty restenosis showed that restenotic tissue is primarily composed of cellular elements interspersed among the extracellular matrix [19]. In PAD, histopathological studies of restenosis have suggested a similar composition [13,20]. Lack of understanding of the clinical and angiographic predictors of DCB failure has hindered development of effective therapies to address DCB restenosis. In addition, the absence of histomorphological studies of DCB restenosis creates a void in the understanding of the DCB restenosis treatment.

**Table 3**
Size of atherectomy specimens

| Patient | Reference vessel diameter first intervention (mm) | Size of first atherectomy specimens | Reference vessel diameter second intervention (mm) | Size of second atherectomy specimens |
|---|---|---|---|---|
| 1 | 5 | 5 Pieces 0.3 cm to 2.0 cm in length | 5 | Multiple Pieces 0.3 cm to 0.8 cm |
| 2 | 4 | Multiple irregular fragments 1.0 cm×0.2 cm×0.1 cm | 4 | Multiple cylindrical pieces 0.6 cm to 3.0 cm |
| 3 | 4 | 2 Fragments 1.0 cm×0.1 cm×0.1 cm | 4 | Multiple pieces 0.5 cm to 1.4 cm |
| 4 | 4 | Several irregular fragments 0.5cm×0.4 cm×0.2 cm | 4 | Multiple pieces 1.3 cm×1.0 cm×0.2 cm |
| 5 | 4 | 4 Fragments 0.6 cm to 1.1 cm in length | 4 | 1 Piece 0.3 cm |

The unique feature of this study is that we compared histomorphological features of DCB restenosis with POBA restenosis, within the same segment of the SFA, at the same lesion site. This provides an opportunity to evaluate and understand the differences in the vessel wall response to two different therapies (POBA vs. DCB), at the same lesion site.

Directional atherectomy provides a source of ex vivo pathological tissue which aids in better understanding of the processes in the vessel wall. Atherectomy specimens have been used previously to study the processes within the vessel wall in both coronary and peripheral vasculature and the results have been accepted and validated. Earlier work has demonstrated that restenotic plaque is associated with an increased cellular proliferation of myofibroblast [13]. This is consistent with data from prior coronary [21] and PAD restenosis studies.

In comparison to POBA restenosis, DCB restenosis results in a decrease in proliferation of cellular elements, (smooth muscle cells and fibroblasts) decrease in collagen type I, and increase in collagen type III. (Figs. 1 and 5). The bulk of POBA restenotic plaque is composed of cellular elements and mature collagen (type I).

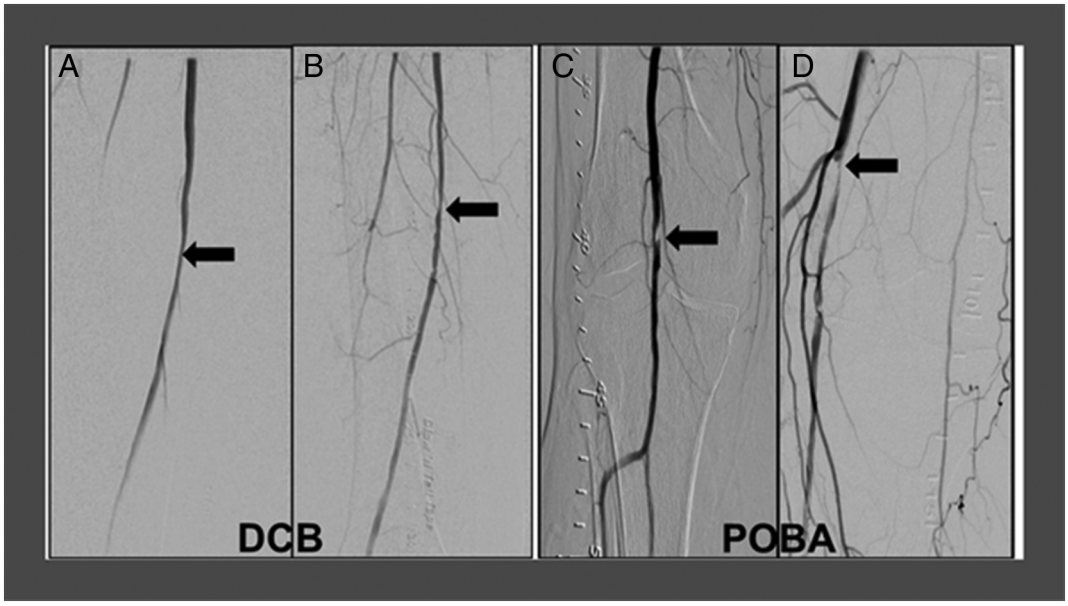



**Fig. 1.** Neointimal thickness in restenosis: (A) Hematoxylin and eosin (H&E) stain of the DCB restenotic plaque from superficial femoral artery (SFA) showing reduction in the thickness of neointima (small arrow) compared to (B) thickness of neointima (large arrow) of POBA restenosis (20×). (C) Significant difference in neointimal thickness between DCB restenosis and POBA restenosis.

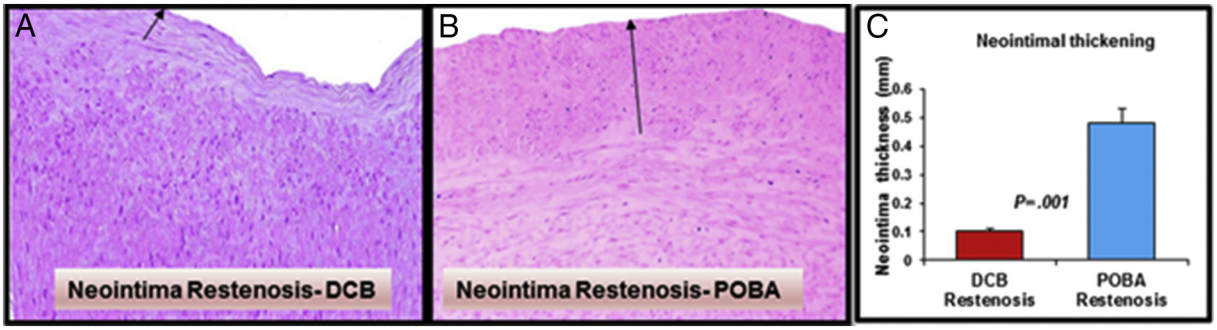



**Fig. 2.** Representative digital subtraction angiograms depicting baseline stenosis and restenosis in the superficial femoral artery. In case one, (a) shows baseline stenosis and (b) depicts restenosis following drug-coated balloon angioplasty. In case two, (c) shows baseline stenosis and (d) depicts stenosis following balloon angioplasty. in (a), (b) and (c)mark the area of stenosis. In(d) marks the expected course of occluded artery.

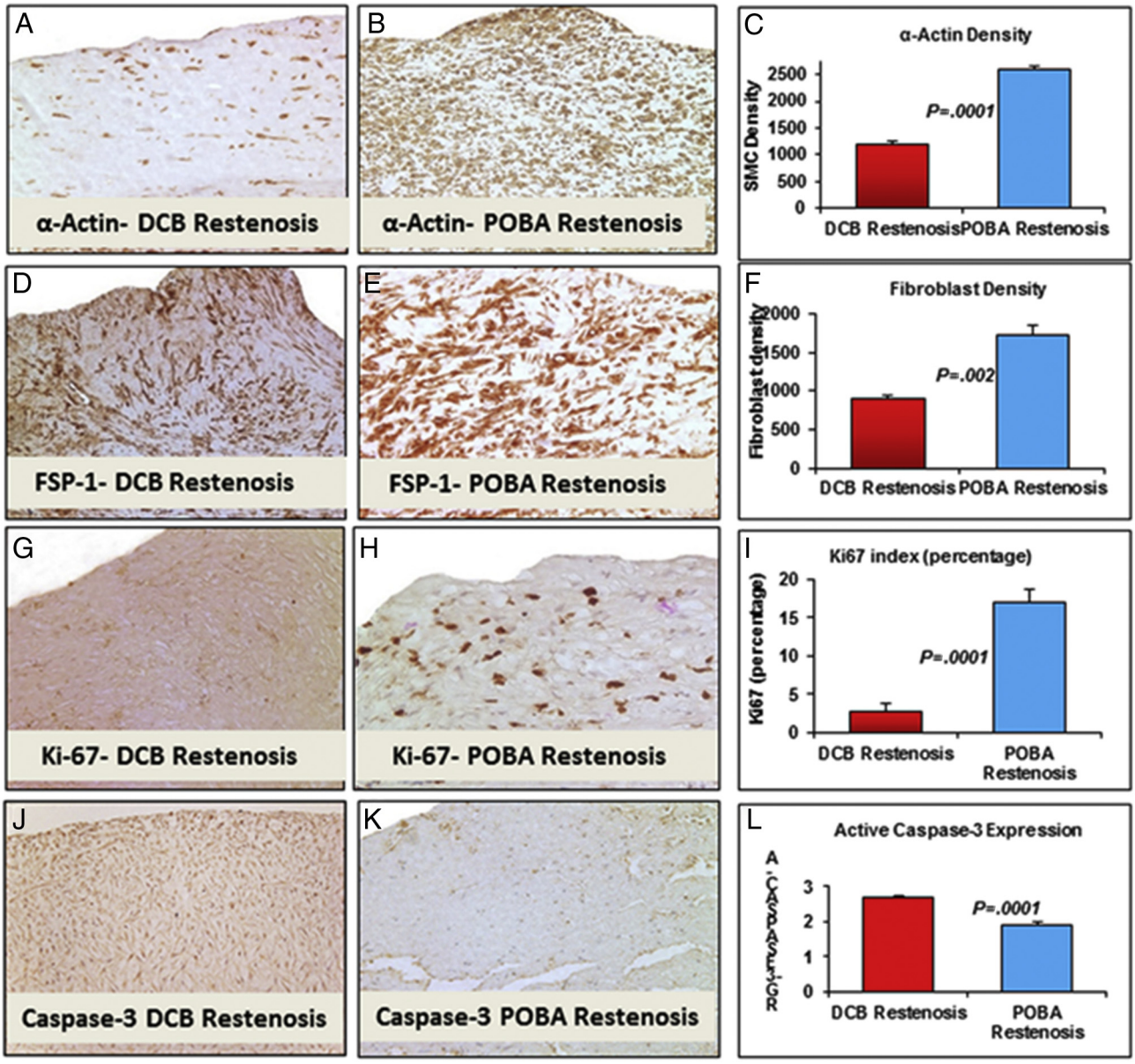



**Fig. 3.** Immunostained section for SMCs with α-Actin shows reduced expression in DCB restenotic plaque (A) compared to POBA restenotic plaques (B) (20×). (C) Significant decrease in SMC density in DCB restenotic plaques. (D) Immunostained section of fibroblasts with FSP-1 in DCB restenotic plaque showing reduced FSP-1 expression compared to (E) POBA restenotic plaque. (I) (20×). Significant decrease in fibroblast density in DCB restenotic plaque. (G) Immunostained section of Ki67 in DCB restenotic plaque compared to (H) POBA restenosis plaque showing reduced proliferative cells by Ki67 expression in DCB restenotic plaques. (20X). (I) Significant decrease in percentage of cells by Ki67 in DCB restenotic plaque. (J) Immunostained section of caspase-3 expression in DCB restenotic plaque showing increased caspase-3 expression compared to (K) POBA restenotic plaque (20×). (I) Significant increase in active caspase 3 expression in DCB restenotic plaque.

However, DCB restenotic plaque is poor in cellularity, and composed predominantly of immature collagen (type III). This difference may be important in risk-stratifying and selecting the most effective therapy for patients with SFA restenosis. For patients with POBA restenosis, an anti-proliferative approach is needed. Drug based therapy would likely be most effective in the treatment of these lesions. For patients with DCB restenosis, the lack of cellularity and the increase in type III collagen may benefit from mechanical scaffolding rather than an anti-proliferative approach. This co-adjuvant therapy directed towards maintaining the structural integrity of the vessel wall may reduce the risk of recurrent restenosis after DCB. However, this sequential, therapeutic approach needs further clinical investigation.

Differential expression of apoptotic markers lends further support to this hypothesis. In this study, we demonstrated increased apoptosis in DCB vs. POBA plaques. Paclitaxel, a drug which is known to increase apoptosis of cellular elements would have minimal additional benefit in DCB restenosis because of decreased cellularity, whereas in POBA and in-stent restenosis [22], the pro-apoptotic, anti-proliferative effect of paclitaxel may render a greater benefit given the increased cellularity.

Presence of immature collagen phenotype in DCB restenosis provides a potential therapeutic target. Data exists from coronary restenosis studies, where a temporal trend has been established in ratio of types I and III collagen, where type III dominates early in this process, and as the plaque 'matures' and stabilizes, type I collagen tends to predominate [23]. Future therapies may be developed to help 'mature' the plaque phenotype, improving healing, while at the same time preventing excess extracellular matrix deposition [24].

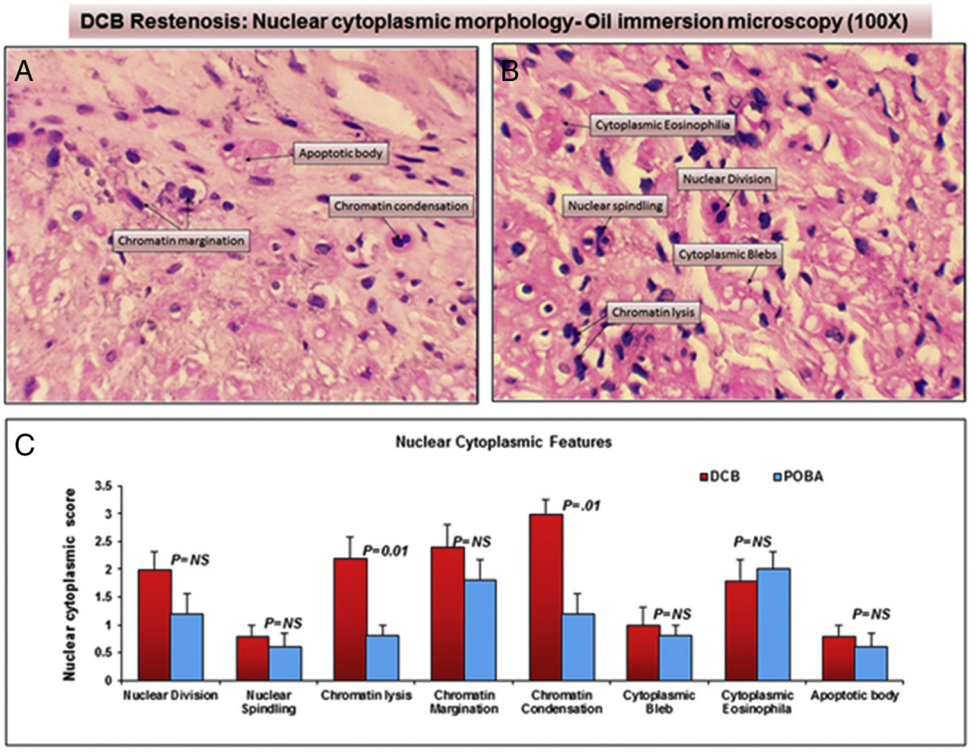



**Fig. 4.** H&E-stained sections (A, B) showing morphological features of apoptosis including nuclear division, spindling, chromatin lysis, margination, condensation of chromatin and cytoplasmic blebbing and eosinophilia (Eosin) and apoptotic body (indicated by arrows) under oil immersion fields (×100) in the DCB and POBA restenotic plaque (upper panel). (C) Quantification of these morphological features is displayed as bar graphs (lower panel).

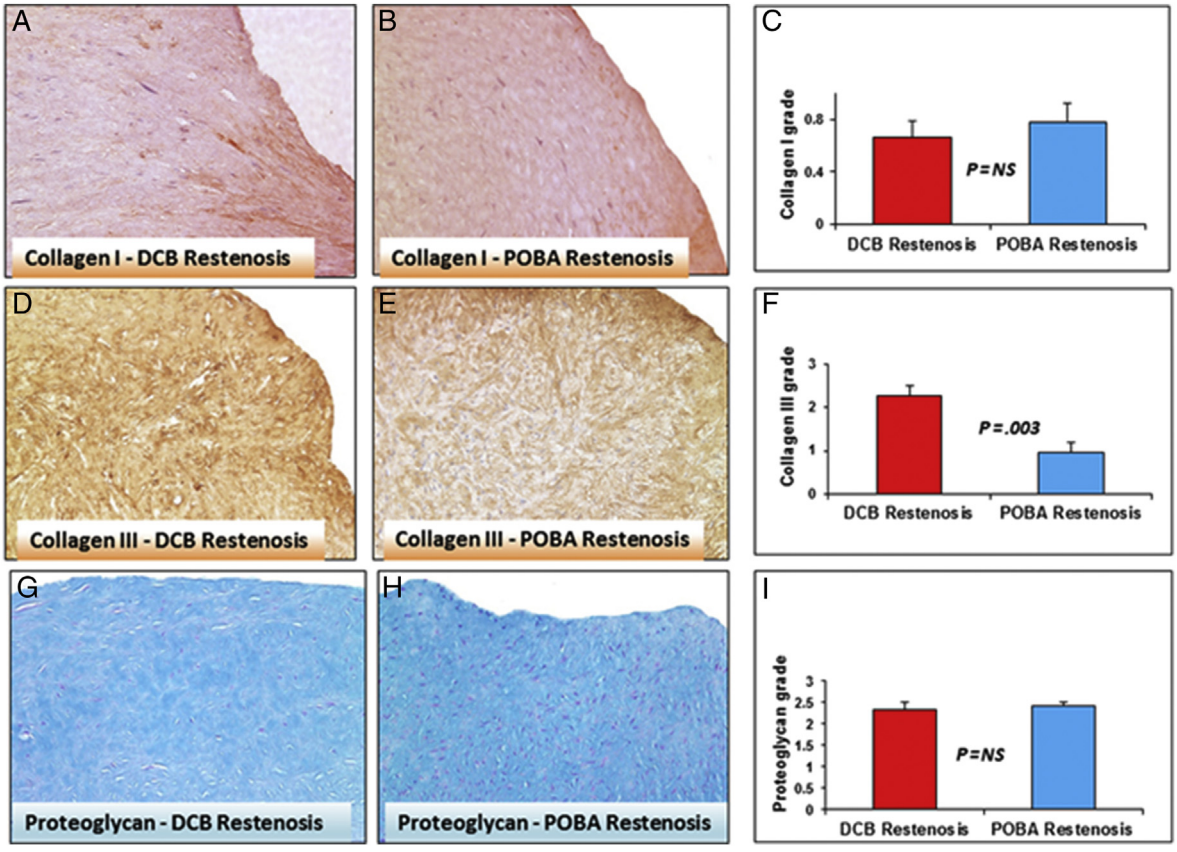
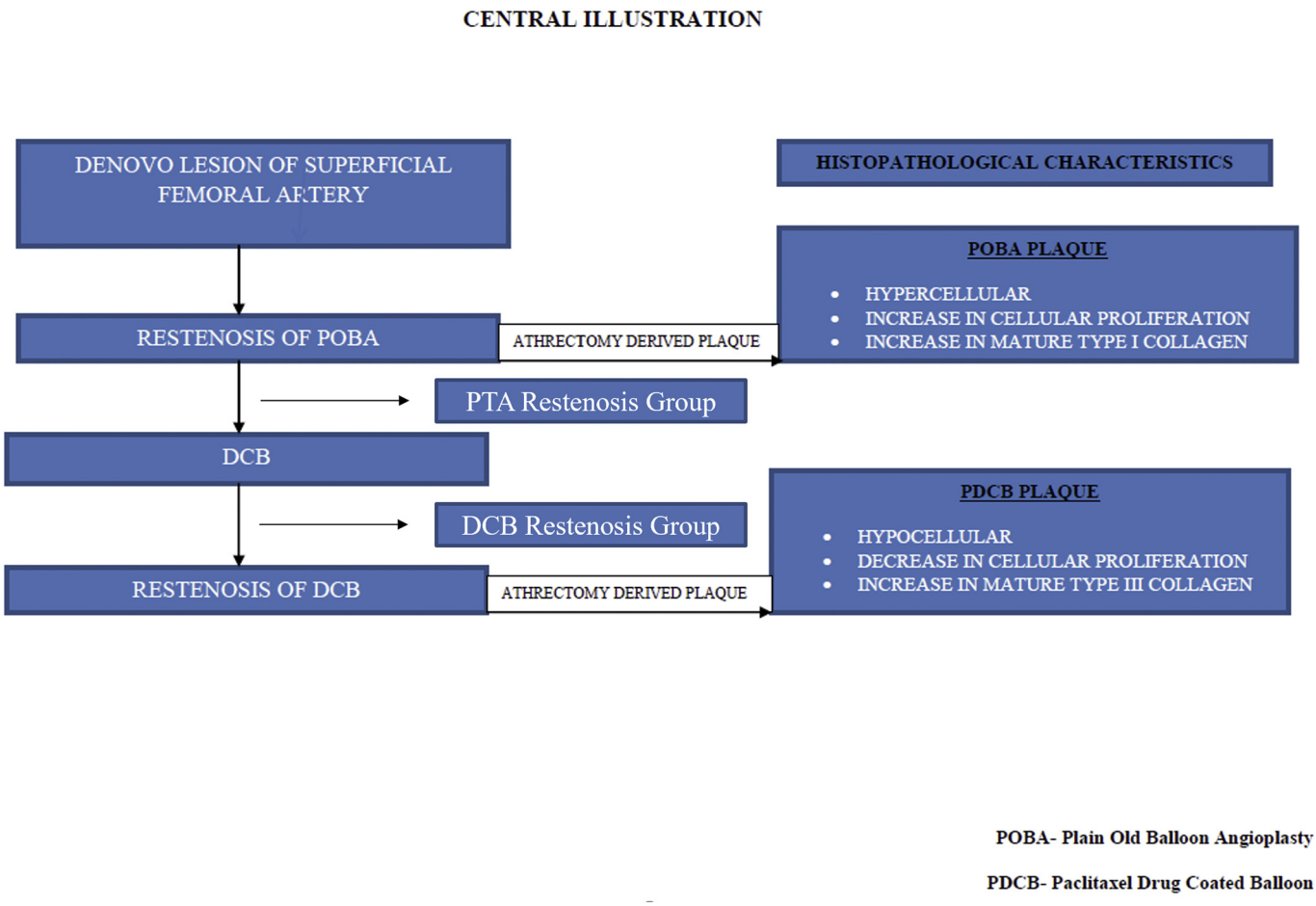



**Fig. 5.** Collagen distribution in restenosis: (A) Immunostained section of collagen I, in DCB restenotic plaque compared to (B) POBA restenotic plaque, type I collagen (20×). (C) Decrease in type I collagen in DCB restenotic plaque compared to POBA. (D) Immunostained section of collagen III, in DCB restenotic plaque compared to (E) POBA restenotic plaque, show increase in type III collagen (20×). (F) Significant increase in type III collagen in DCB restenotic plaque compared to POBA. Proteoglycan distribution in restenosis: (G) Proteoglycans stained by Alcian Blue at pH 2.5 in DCB restenotic plaque compared to (H) POBA restenotic plaque, show no differences (20×). (C) Proteoglycans were similar in DCB and POBA restenotic plaque.




**Central Illustration.** Study design flow chart.

Finally, a predominance of type III collagen, a feature of less evolved plaques, may confer biological behavior to DCB restenosis analogous to vulnerable plaques. Data from coronary literature suggests that plaques from sudden cardiac death patients are enriched in collagen type III, and stable plaques predominantly have type I collagen. This may suggest that DCB restenotic plaques may have a predisposition to clinical thrombotic events, but further studies and temporal follow up is needed to establish this paradigm. [6].

The main limitation of this study is the small number of patients, limiting our ability to perform extensive statistical adjustment. To counteract this limitation an extensive, meticulous and time-consuming morphological and histopathological analysis was performed. In addition to immunohistochemistry and planimetric, quantitative morphometry, individual nuclei and cytoplasmic changes were blindly recorded cell-by-cell. It also cannot be ignored that there may be varying "biology" of migrated cell in the neointima during different restenosis. Although we detected significant differences in several parameters, larger studies are welcome to confirm these observations. Second, the non-randomized nature of this report precludes causal inferences. Third, our findings reflect the unique patient-mix treated at a large-volume tertiary care center with experienced operators, which may not generalize to other institutions.

This study suggests DCB restenotic plaque is hypocellular with immature type III collagen. This completely different mechanism when compared to POBA restenosis suggests that therapies directed towards prevention of POBA restenosis may not apply to prevent DCB restenosis. Contemporary treatment options that are not drug based, such as a mechanical scaffold may represent a viable option for the therapy of DCB restenosis. Further investigations directed at modifying collagen deposition and maturation may provide future potential therapeutic targets.

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*Conflict of interest*

"All the authors have no conflict of interest to declare".

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