Pioglitazone Increased Circulating MicroRNA-24 with Decreases in Coronary Neointimal Hyperplasia in Type 2 Diabetes: OCT Analysis

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Background

- Endothelial dysfunction is the first step in the progression in atherosclerosis.
- Endothelial dysfunction has been more frequently documented in patients with type 2 diabetes.

Hong SJ et al. CCI 2010;76:924-33.
Manfrini O et al. IJC 2013
Wong WT et al. J Cardiovasc Pharma 2013

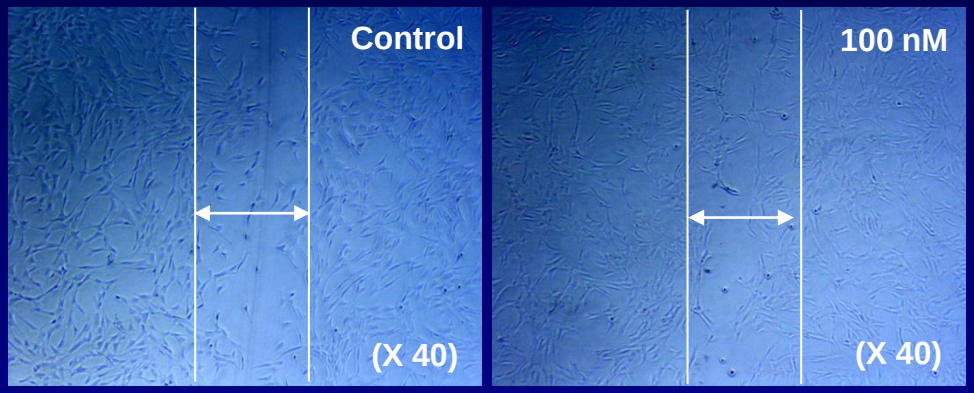
Diabetes & Pioglitazone

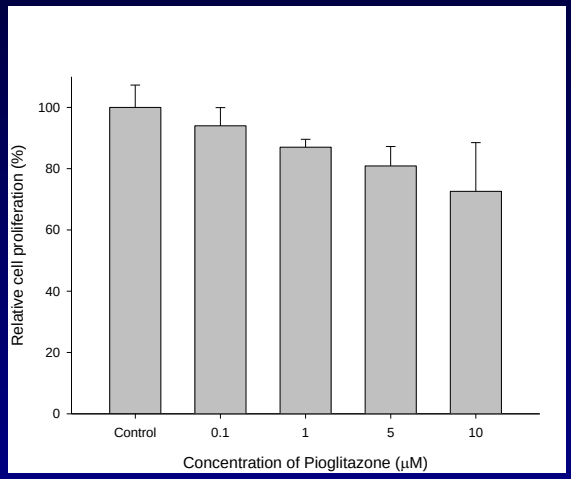
- Early decreases in the number of NK cells, circulating TNF-α , IL-6, and MCP-1 concentration, and the expression of CCR2 on circulating CD14+ cells after pioglitazone treatment may have abated inflammation, thereby reducing atherosclerosis progression.
- The early decreases in SMC migration and proliferation in the pioglitazone group have been documented in type 2 diabetic patients.

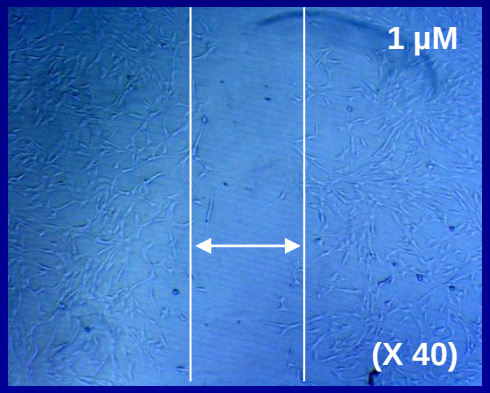
Hong SJ et al. Heart 2006;92:1119-24.
Hong SJ et al. AJC 2007
Hong SJ et al. ATVB. 2010;30:2655-65.

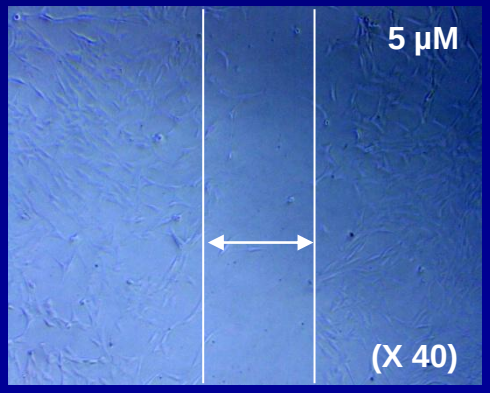
Effects of Pioglitazone on SMC Proliferation in Dose-Dependent Manner.

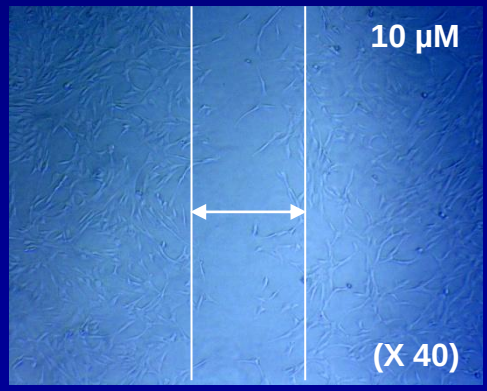
(MTT proliferation assay)







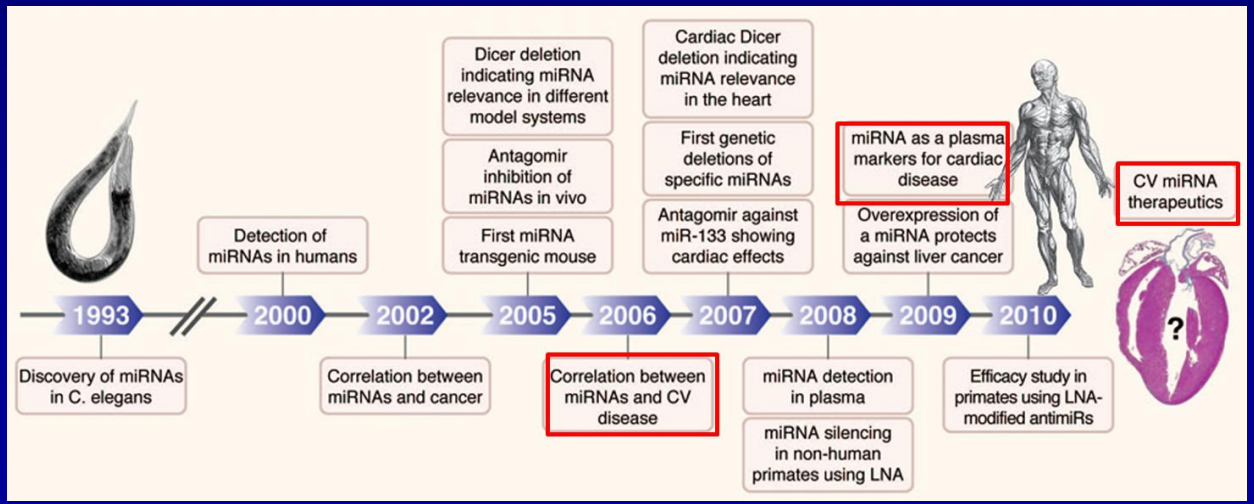




Hong SJ et al. ATVB. 2010;30:2655-65.

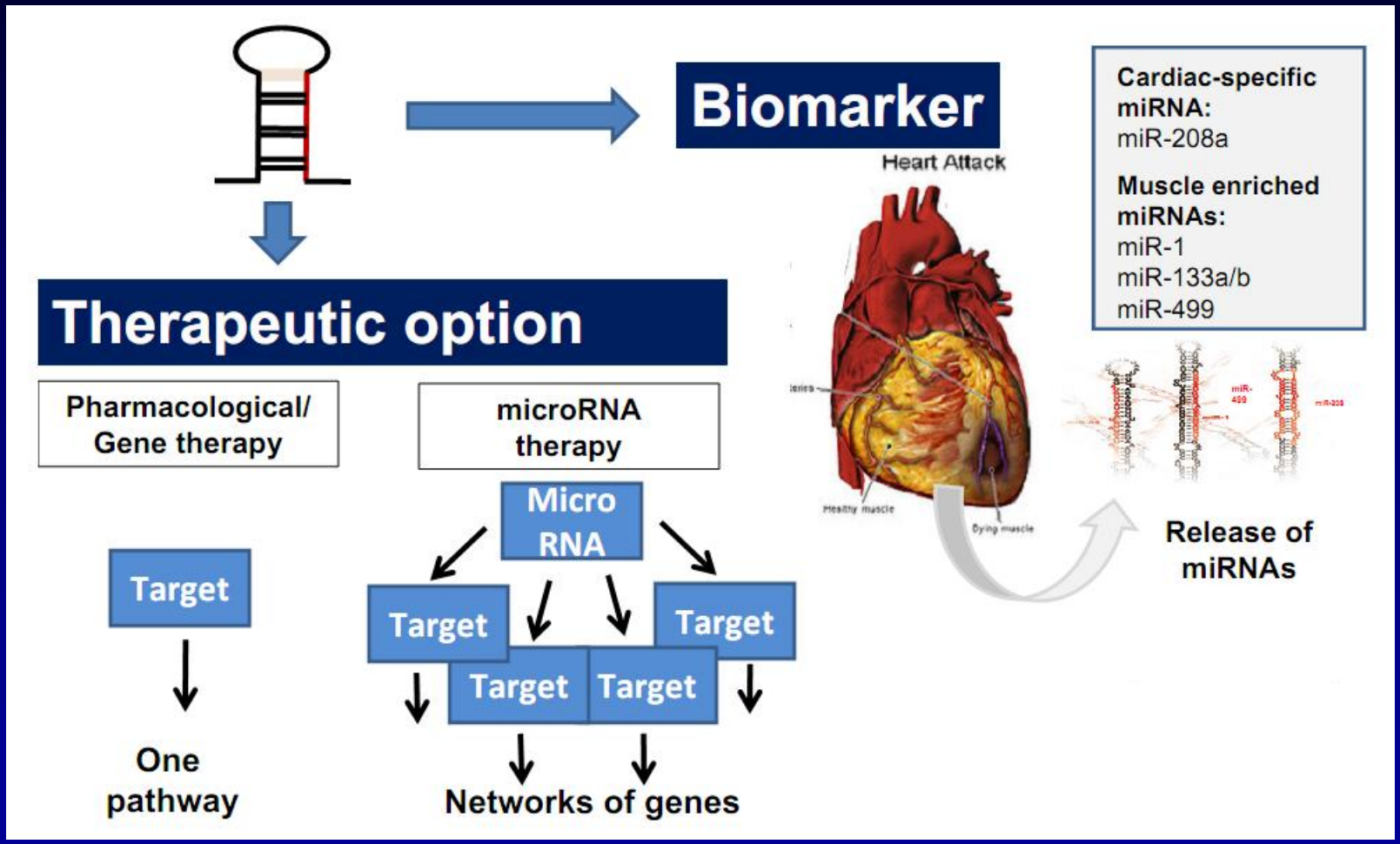
What is MicroRNAs (miRNAs)?

- Has very few nucleotides (an average of 22).
- Post-transcriptional regulators that bind to complementary sequences on target mRNAs.
- The human genome may encode > 1,000 miRNAs.
- Target about 60% of mammalian genes.
- Aberrant expression of miRNAs implicated in numerous disease states.



Bartel DP et al. Cell 2009;136:215-233.
Circ Res 2011;108:219-234.

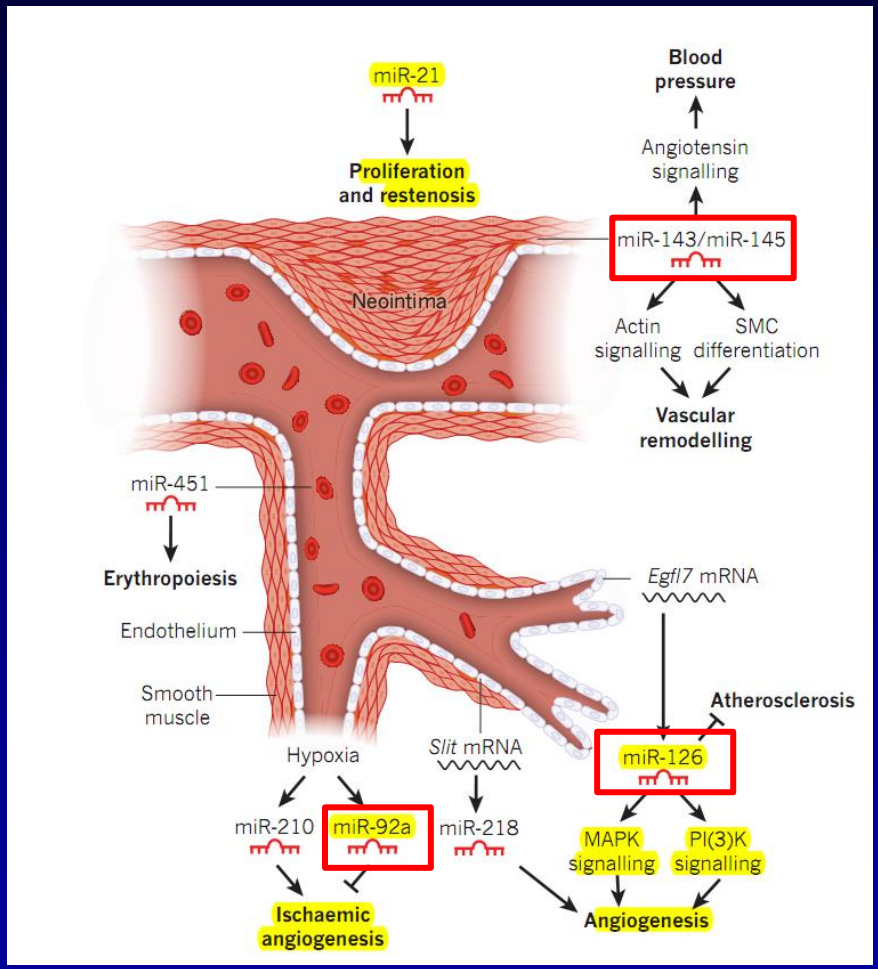
miRNA Function



Dimmeler EHJ 2010.

Background

- miRNA-126, -24 expresssed in endothelial cells and essential for vascular development.
- miRNA-17~92 cluster modulate angiogenesis.
- miRNA-143/145 expressed in SMC.
- miRNA-1 and -133 expressed in cardiomyocytes and control myogenesis.
- miRNA-208a, -208b expressed by introns of myosin heavy chains.



Nature 2011;469:336-342.
Circ Res 2010;107:677-684.

Objectives

- We prospectively compared

1. The effects of pioglitazone on coronary neointimal hyperplasia and changes in microRNAs with their correlation to neointimal hyperplasia in type 2 diabetic patients during the 9-month f/u.
2. The effects of pioglitazone in improving endothelial function
3. The effects of pioglitazone in systemic inflammation

Methods:

Identification of Endothelial Function-Related miRNAS

- Pilot Study: Detection of miRNAs expressed in peripheral blood of patients with >10% FMD (n=5), <10% FMD (n=3)

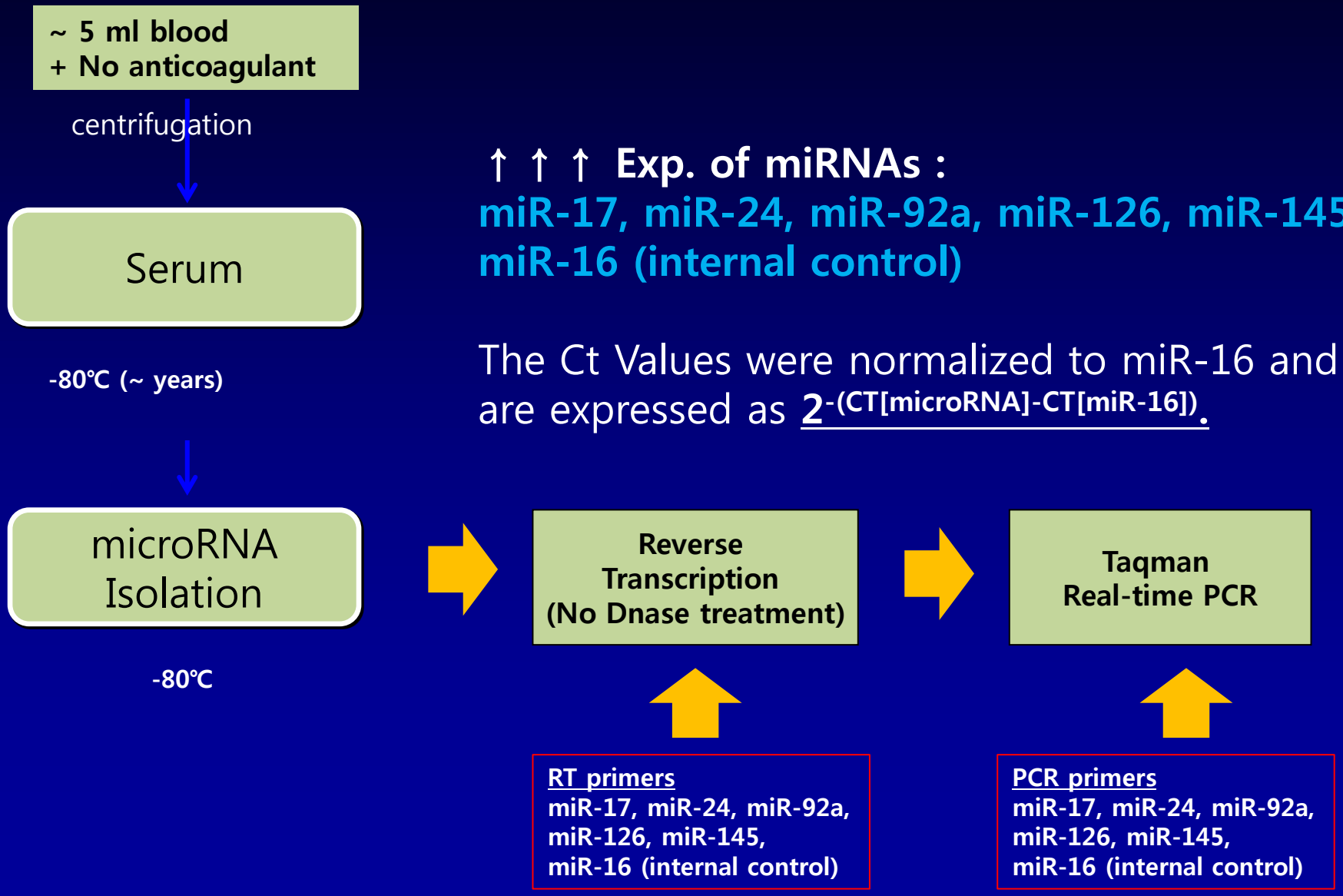
↑ ↑ ↑ Exp. of miRNAs : miR-17, miR-24, miR-92a, miR-126, miR-145
miR-16 (internal control)

↑ Exp. of miRNAs : miR-21, miR-26, miR-143, miR-155, miR-423-5p

No Exp. of miRNAs : miR-1, miR-10a, miR-100, miR-204, miR-208a

Methods:

Identification of Endothelial Function-Related miRNAs



↑ ↑ ↑ **Exp. of miRNAs :**
miR-17, miR-24, miR-92a, miR-126, miR-145
miR-16 (internal control)

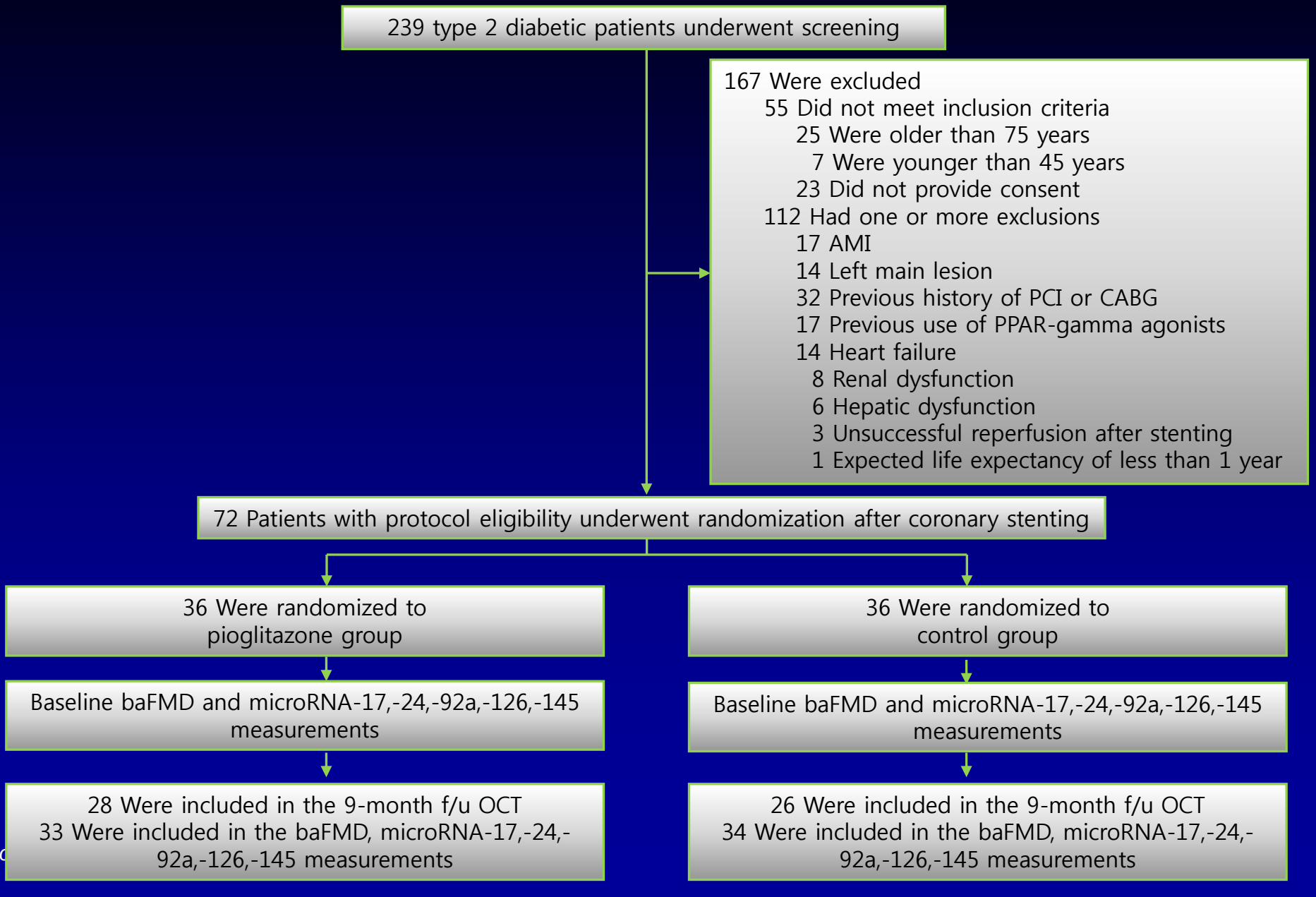
The Ct Values were normalized to miR-16 and are expressed as $2^{-(CT[microRNA]-CT[miR-16])}$.

Methods

- **The Inclusion Criteria:**
  1. Type 2 diabetic patients
  2. Aged 45 to 75 years

- **The Exclusion Criteria:**
  1. Use of pioglitazone within 3 months
  2. AMI
  3. Abnormal LFT (AST or ALT > 3 times upper normal limit)
  4. Renal dysfunction (Cr > 2.0 mg/dL)
  5. LVEF < 40%
  6. LM lesion
  7. Previous history of PCI or CABG
  8. Unsuccessful reperfusion after stenting
  9. Expected life expectancy of less than 1 year

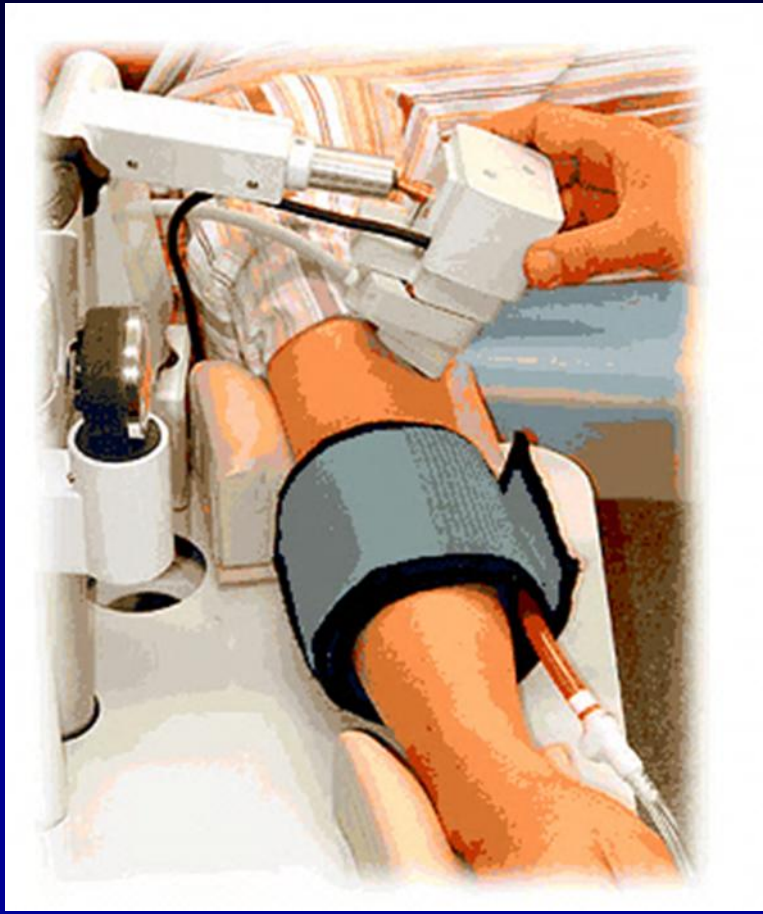
Study Protocol



Endpoints

- **Primary Endpoints:**
  1. To compare changes in neointimal volume with OCT and in the circulating levels of microRNA-17, -24, -92a, -126 and -145 which have been known as indicators of endothelial cell migration and atherosclerosis progression during the 9-month f/u.
- **Secondary Endpoints:**
  1. To compare changes in baFMD between the 2 groups during the 9-month follow-up.
  2. To compare changes in inflammatory markers such as hsCRP, IL-6, TNF-α, adiponectin, sICAM-1, and sVCAM-1
  3. To compare changes in the insulin resistance index such as the HOMA index during the 9-month f/u.

Measurements of Flow-Mediated Dilation



- → Increase forearm cuff pressure up to 200 mmHg
- → Obstruct forearm blood flow for 5 minutes and then release the cuff quickly
- → Measure changes in brachial artery dilatation
- → Normal ≥ 10% increase in baFMD
- → With brachial artery endothelial dysfunction, <10% increase

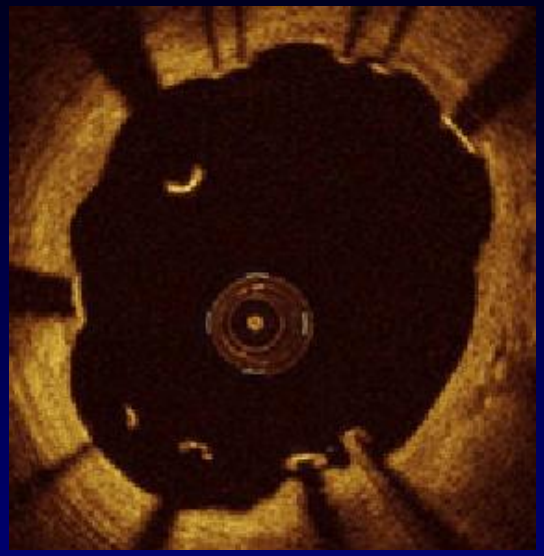
Optical Coherence Tomography Image Analysis

- OCT data were analyzed at the Korea University OCT Core Laboratory.
- OCT was performed after 200μg intracoronary nitroglycerin injection.
- OCT images have been acquired using a nonocclusive technique with the C7XR system (LightLab Imaging, Inc., Westford, MA),
- Mean area and volumes of lumen, stent, and neointimal hyperplasia were calculated along the entire stented segment.
- The center of the luminal surface of the strut was determined for each strut, and its distance to the lumen contour was calculated to determine strut-level neointimal thickness.
- The number of struts without coverage was counted for each frame in order to count the total number of uncovered struts per lesion.

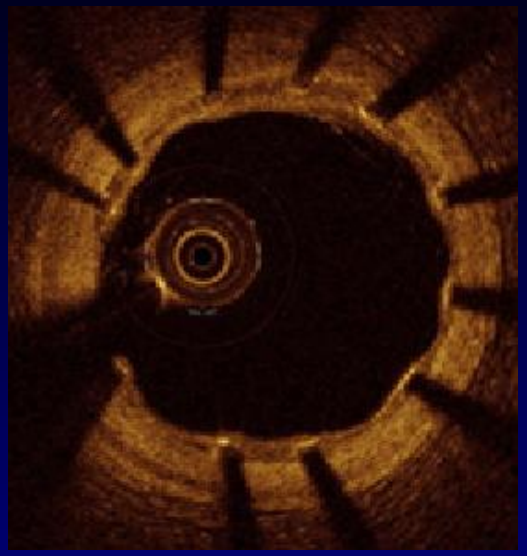
Optical Coherence Tomography Image Analysis

- Struts were categorized as
  1. **Uncovered** when a tissue layer on the endoluminal surface was not visible,
  2. **Covered embedded** struts when covered by tissue and not interrupting the smooth lumen contour
  3. **Covered rhombus** struts when covered by tissue but extending into the lumen
  4. **Malapposed** if the distance from the endoluminal surface of the strut to the adjacent lumen contour was greater than the sum of the metal and polymer thickness
- Neointima was the tissue between the luminal border and the inner border of the struts.

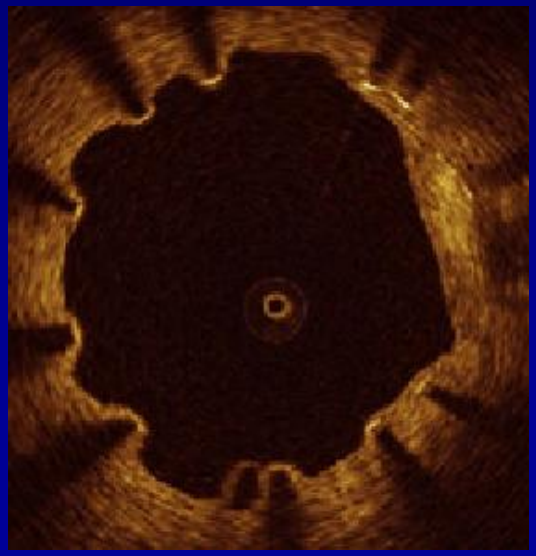
Stent Strut Apposition & Coverage



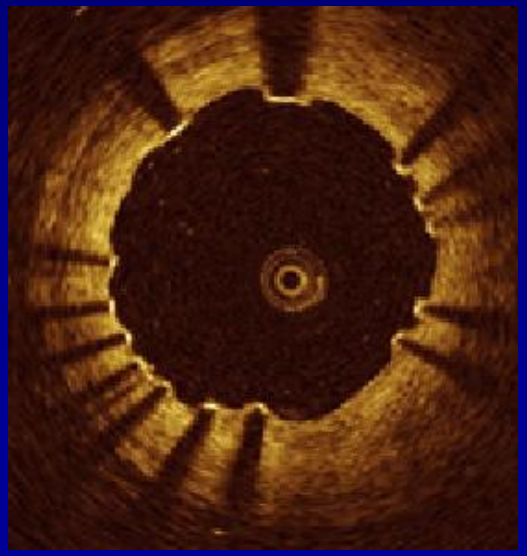
Malapposed Not covered



Apposed Covered

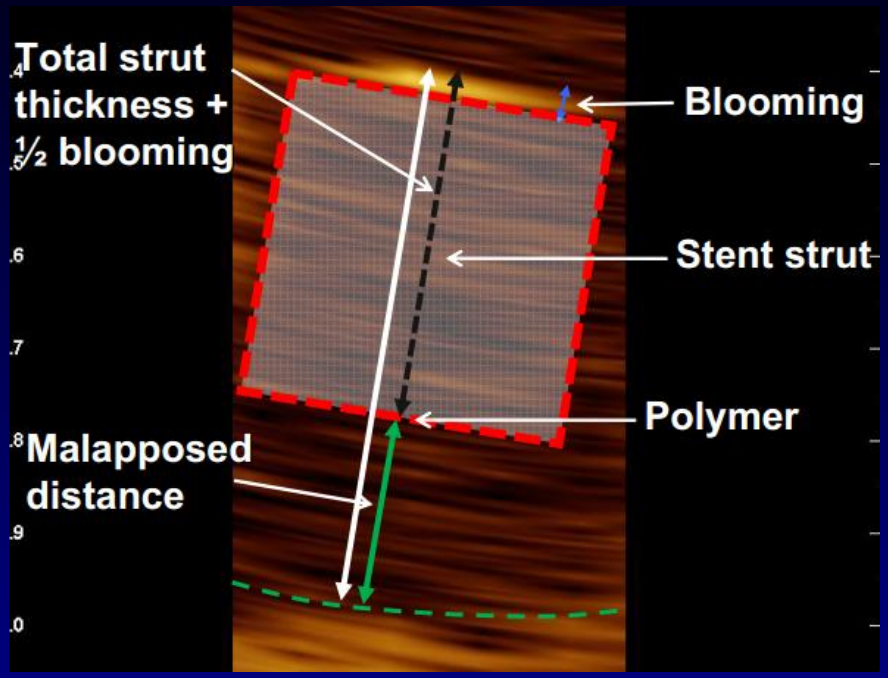


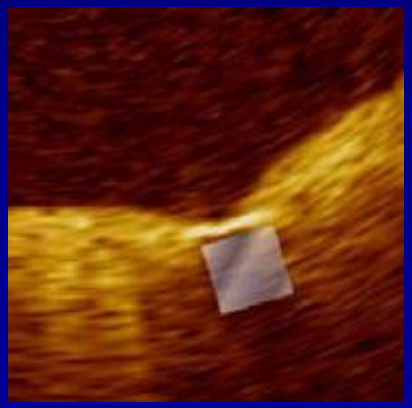
Malapposed Covered



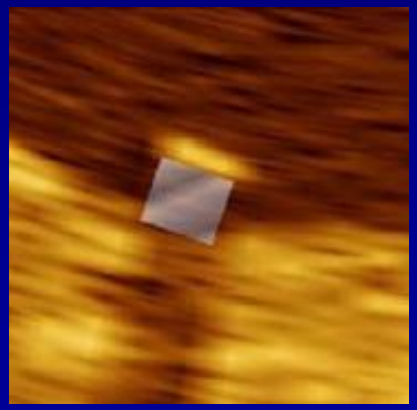
Apposed Not covered

Classification of Stent Strut Apposition

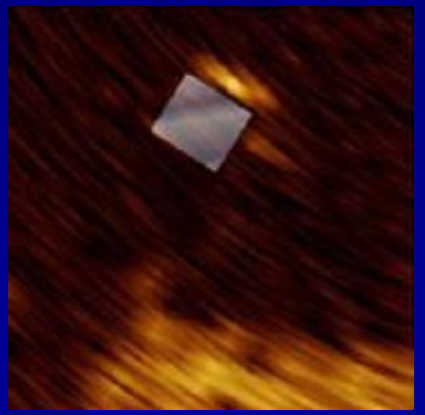




Embedded



Protruding



Malapposed

Results: Baseline Characteristics

Variable	Pioglitazone Group (n=36)	Control Group (n=36)	P value
Age (years)	58.3 ± 11.8	60.0 ± 12.7	0.282
Male sex	19 (52.8 %)	21 (58.3 %)	0.635
Body mass index (kg/m^2)	24.6 ± 3.9	24.4 ± 3.5	0.801
Risk factors			
Hypertension	14 (38.9 %)	13 (36.1 %)	0.808
Hyperlipidemia	12 (33.3 %)	13 (36.1 %)	0.804
Current smoking	9 (25.0 %)	7 (19.4 %)	0.571
Family history of CAD	5 (13.9 %)	8 (22.2 %)	0.358
Past history of TIA or stroke	1 (2.8 %)	1 (2.8 %)	1.000
LVEF (%)	57.1 ± 9.5	56.9 ± 10.1	0.894
Stable angina	20 (55.6 %)	18 (50.0 %)	0.637
Unstable angina	16 (44.4 %)	18 (50.0 %)	0.637
Duration of diabetes (months)	28 ± 24	26 ± 24	0.676

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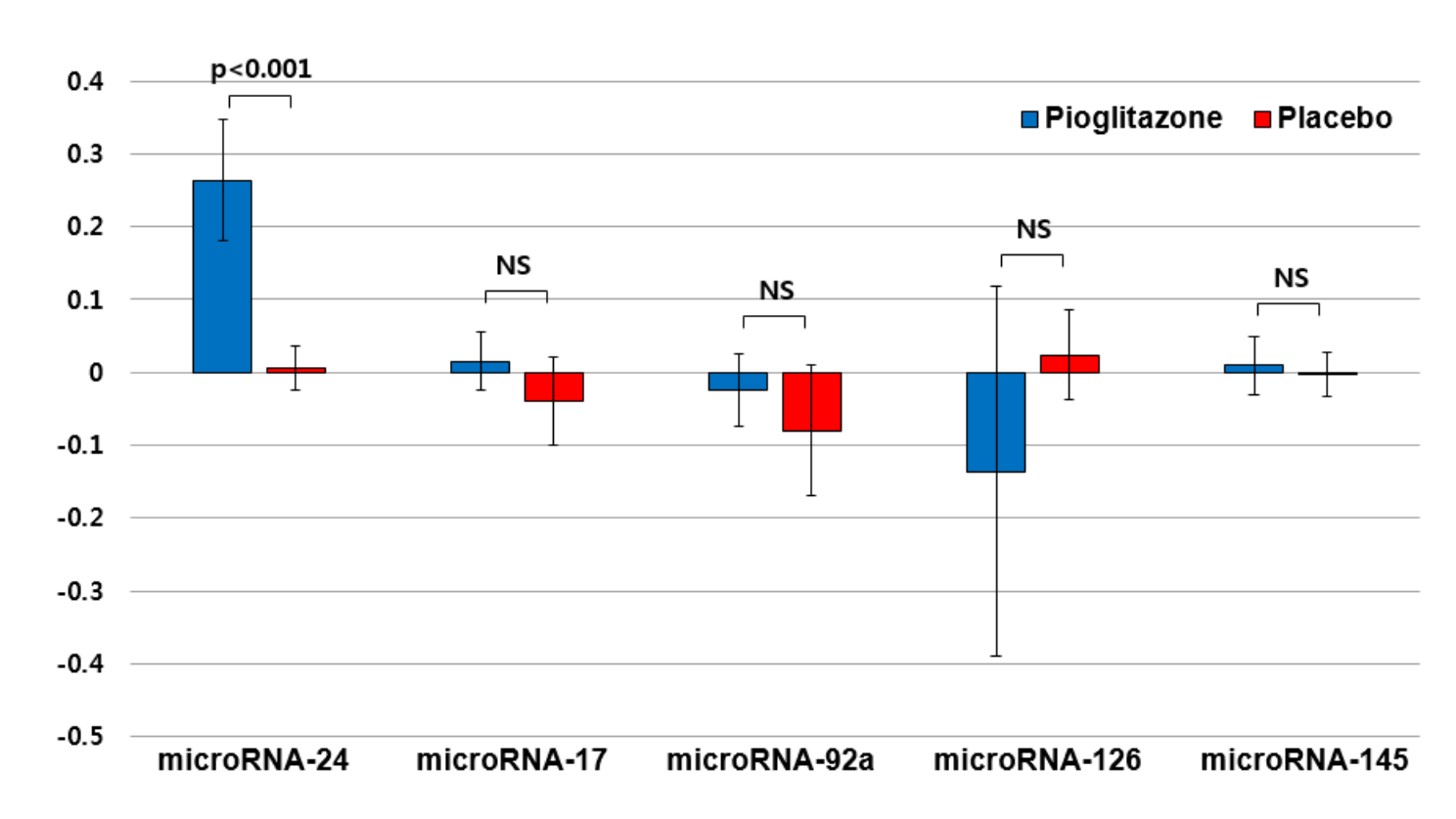
OCT Parameters at 9-Month F/U

Variable	Pioglitazone (n=36)	Control (n=36)	P value
Number of patients with 9-month follow-up	28 (77.8 %)	26 (72.2 %)	0.586
Number of target lesions	38	38	
Mean stent length (mm)	26.3 ± 6.8	27.7 ± 5.8	0.155
Neovascularization	2 (7.1 %)	3 (11.5 %)	0.663
Frequency of intracoronary thrombus	2 (7.1 %)	1 (3.8 %)	1.000
Cross-section level analysis			
Number of struts analyzed per cross section	6.7 ± 1.9	6.5 ± 1.8	0.872
Mean lumen area, mm^2	5.85 ± 2.07	5.08 ± 1.88	<0.001
Mean stent area, mm^2	6.78 ± 2.34	6.98 ± 2.19	0.768
Mean neointimal area, mm^2	0.93 ± 0.78	1.90 ± 1.43	<0.001
Lumen volume, mm^3	157.23 ± 79.44	143.61 ± 67.04	0.021
Stent volume, mm^3	181.43 ± 104.91	196.32 ± 110.19	0.115
Neointimal volume, mm^3	25.02 ± 17.78	55.10 ± 30.01	<0.001
Percentage net volume obstruction, %	13.9 ± 10.1	28.5 ± 13.4	<0.001

OCT Parameters at 9-Month F/U

Variable	Pioglitazone (n=36)	Control (n=36)	P value
Strut-level analysis			
Total number of analyzed struts (total)	15,820	16,654	
Number of covered struts (total)	15,283	16,203	
Frequency of covered struts per lesion, %	96.7 ± 5.3	97.0 ± 6.2	0.780
Covered embedded struts	94.9 ± 8.1	95.1 ± 8.7	0.879
Covered rhombus struts	1.7 ± 2.8	2.0 ± 3.2	0.803
Number of uncovered struts (total)	537	451	
Frequency of uncovered struts per lesion, %	3.3 ± 5.2	3.0 ± 5.8	0.803
Uncovered well apposed struts	3.2 ± 4.0	2.9 ± 4.8	0.837
Uncovered malapposed struts	0.1 ± 1.3	0.1 ± 1.1	0.914
Mean neointimal thickness of covered struts, mm	0.16 ± 0.15	0.28 ± 0.34	<0.001
Neointimal unevenness score	1.68 ± 0.31	1.73 ± 0.35	0.571
Peri-strut low-intensity area, %	2.92 ± 1.75	3.12 ± 1.65	0.666

Changes in MicroRNA-17, -92a, -126, -145 During the F/U



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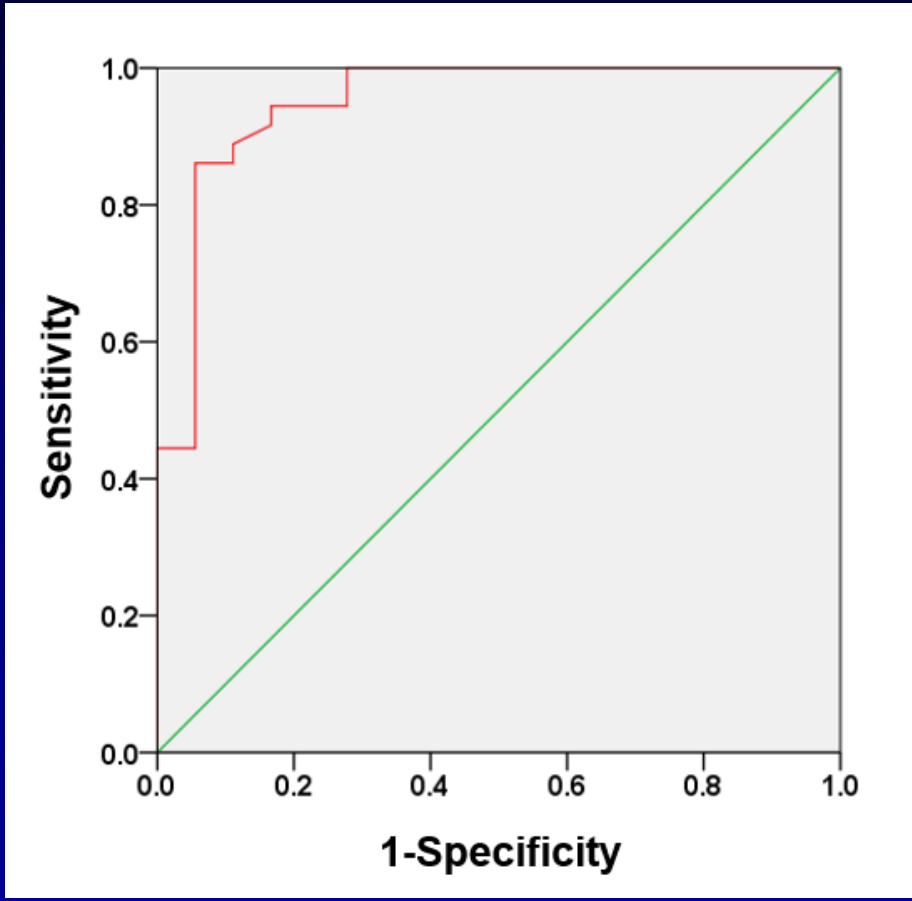
Receiver-Operating-Characteristic Curve and the Corresponding Area Under the Curve for the Changes in MicroRNA-24



In detecting neointimal volume greater than 25 mm3. Cut-off value for the changes in microRNA-24 was 0.1715 with sensitivity of 0.861 and specificity of 0.944.

Changes in Brachial Artery FMD During the 9-Month F/U

Variables	Pioglitazone Group (n=36)		Control Group (n=36)	
	Baseline	At 9-month	Baseline	At 9-month
Brachial artery diameter at rest (mm)	3.96±0.42	4.00±0.40	3.99±0.39	4.01±0.41
Flow-mediated dilation (mm)	4.18±0.41	4.46±0.39*†	4.25±0.38	4.30±0.40
Changes from at rest (mm)	0.22±0.11	0.47±0.14*†	0.25±0.17	0.28±0.18
Nitroglycerin-mediated dilation (mm)	4.48±0.48	4.58±0.45	4.52±0.47	4.60±0.46
Changes from at rest (mm)	0.53±0.20	0.58±0.23	0.53±0.22	0.60±0.24

Changes in Inflammatory Markers

	Pioglitazone Group (n=36)		Control Group (n=36)	
Variables	**Baseline**	**After 9 months**	**Baseline**	**After 9 months**
IL-6 (pg/ml)‡	4.37 ± 4.01	1.81 ± 1.31 †*	4.77 ± 3.94	2.90 ± 2.28*
Changes from baseline (pg/ml)	-2.57 ± 2.19 †		-1.87 ± 1.71	
TNF-α (pg/ml)‡	6.83 ± 4.76	2.82 ± 3.05 †*	6.16 ± 5.27	4.61 ± 3.60*
Changes from baseline (pg/ml)	-4.02 ± 1.77 †		-1.52 ± 1.37	
hsCRP (mg/L)‡	4.18 ± 3.01	1.24 ± 1.22*	4.56 ± 4.10	1.52 ± 1.60*
Changes from baseline (mg/L)	-2.93 ± 2.62		-3.03 ± 3.09	
Adiponectin (μg/ml)‡	3.98 ± 3.99 †	7.98 ± 5.65 †*	5.41 ± 4.66	5.65 ± 5.32
Changes from baseline (μg/ml)	4.01 ± 2.93 †		0.23 ± 1.15	
sICAM-1 (ng/mL)‡	742 ± 501	657 ± 508	575 ± 432	502 ± 337
Changes from baseline (ng/mL)	-85 ± 80		-75 ± 94	
sVCAM-1 (ng/mL)‡	976 ± 588	769 ± 393 †*	1065 ± 692	1069 ± 811
Changes from baseline (ng/mL)	-207 ± 213 †		2 ± 460	

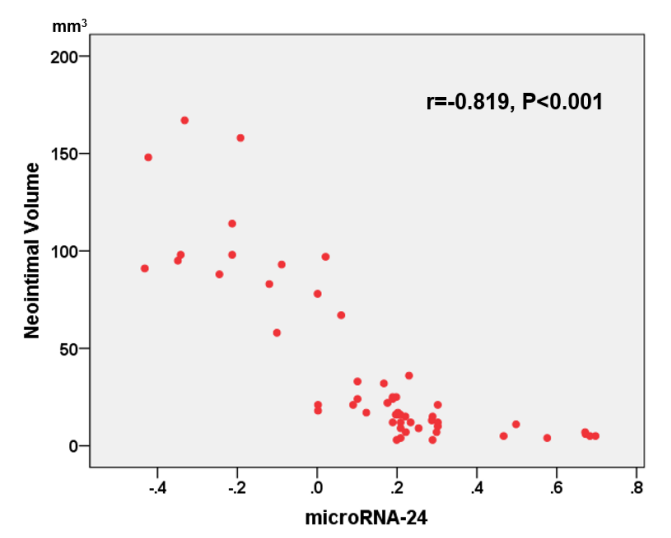
* $p < 0.05$ compared with baseline. † $p < 0.05$ compared with the Control Group.

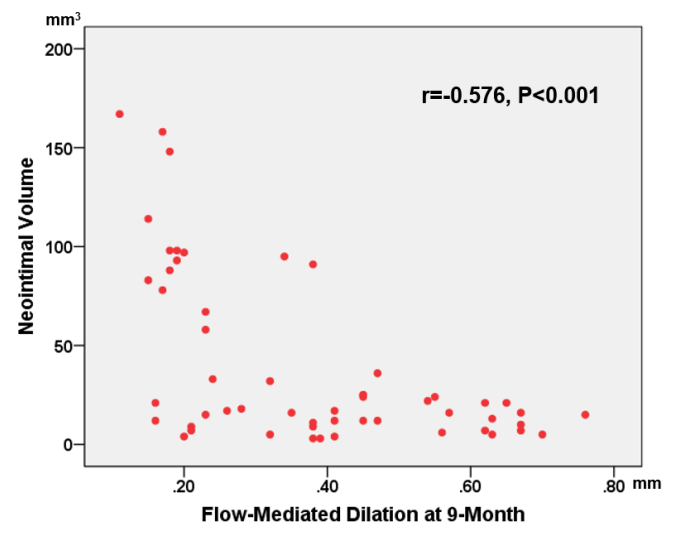
Changes in Lipid Profiles

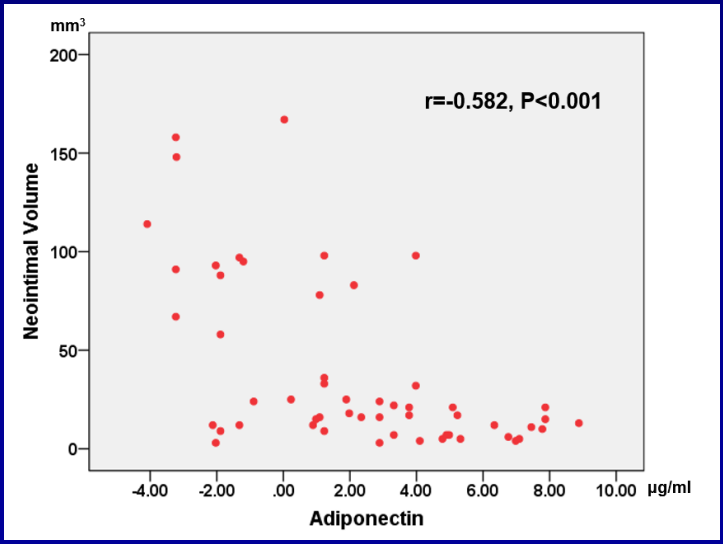
	Pioglitazone Group (n=36)		Control Group (n=36)	
Variables	**Baseline**	**After 9 months**	**Baseline**	**After 9 months**
Total cholesterol (mg/dl)	214 ± 60	161 ± 47*	219 ± 48	156 ± 42*
Changes from baseline (mg/dl)	-53 ± 60		-63 ± 56	
LDL-cholesterol (mg/dl)	149 ± 66	90 ± 46*	159 ± 77	89 ± 45*
Changes from baseline (mg/dl)	-60 ± 45		-68 ± 55	
HDL-cholesterol (mg/dl)	39 ± 29	43 ± 22	37 ± 28	40 ± 19
Changes from baseline (mg/dl)	3 ± 10		3 ± 8	
Triglyceride (mg/dl)‡	135 ± 99	119 ± 79	129 ± 83	123 ± 60
Changes from baseline (mg/dl)	-16 ± 57		-7 ± 60	

$p < 0.05$ compared with baseline. † $p < 0.05$ compared with the Control Group.

Correlation Between Neointimal Volume and Various Parameters







Comparison of Adverse Clinical Events Between the 2 Groups During the 9-Month F/U

Variable	Pioglitazone Group (n=36)	Control Group (n=36)	P value
Death (%)	0 (0.0 %)	0 (0.0 %)	NA
Myocardial infarction (%)	0 (0.0 %)	1 (2.8 %)	1.000
New onset CHF (%)	1 (2.8 %)	0 (0.0 %)	1.000
Fracture (%)	0 (0.0 %)	0 (0.0 %)	NA
Stroke	0 (0.0 %)	0 (0.0 %)	NA
Bladder cancer (%)	0 (0.0 %)	0 (0.0 %)	NA

Summary

- Type 2 diabetic patients treated with pioglitazone not only benefit from its known hypoglycemic and LDL-cholesterol lowering effects but also from its anti-inflammatory and increasing circulating microRNA-24 levels

  → improving endothelial dysfunction and eventually decreasing neointimal proliferation in type 2 diabetic patients.

Conclusions

1. We have found that circulating level of microRNA-24 was aberrantly decreased in type 2 diabetic patients with excessive neointimal hyperplasia.
2. Therefore, modulation of microRNA-24 expression by pharmacological approach such as administering pioglitazone has strong down-regulating effects on neointimal proliferation in type 2 diabetic patients.
3. Circulating microRNA-24 could be used as a potential novel biomarker for predicting excessive neointimal hyperplasia in type 2 diabetic patients after coronary stent implantation.

Thank You For Your Attention!

Background

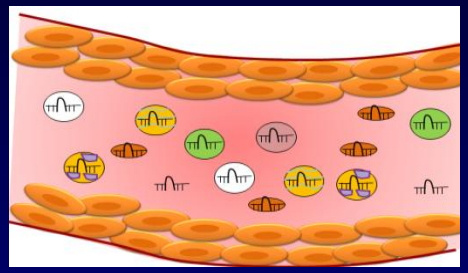
- Several studies highlight the beneficial effect of pioglitazone in reducing coronary atherosclerosis in type 2 diabetic patients.
- However, the U.S. FDA has informed the public that use of the pioglitazone for more than 1 year may be associated with an increased risk of bladder cancer especially for men.
- A meta-analysis suggests that the pioglitazone confers excess risk for fractures especially for women.

Hong et al. AJC 2007
Loke YK et al. CMAJ 2009;180:32.
Strom A et al. *Circ Res.* 2007;101(8):e83-89.
Finn AV et al. *Circulation.* 2005;112(2):270-278.

Methods:

Quantitative Assay of Circulating microRNAs

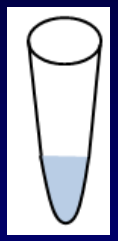
250 µl serum



miRNA isolation

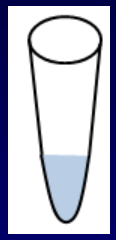
TRI Reagent BD Solution

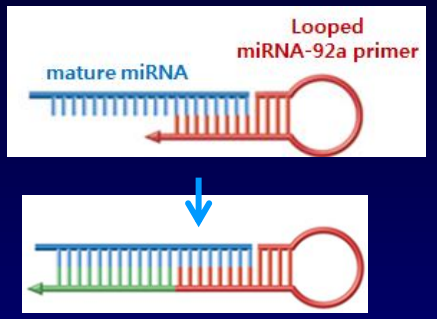
100 ~ 200 ng miRNAs



~10 ng microRNA (25 ~ 50 µl serum)

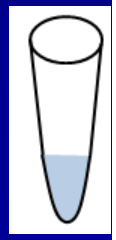
Reverse transcription

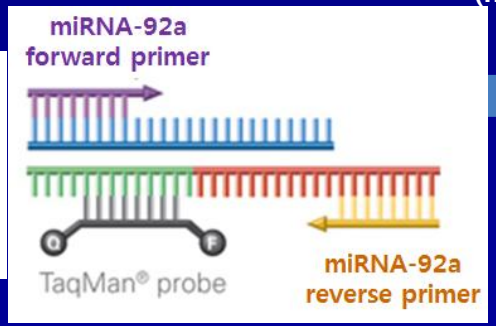




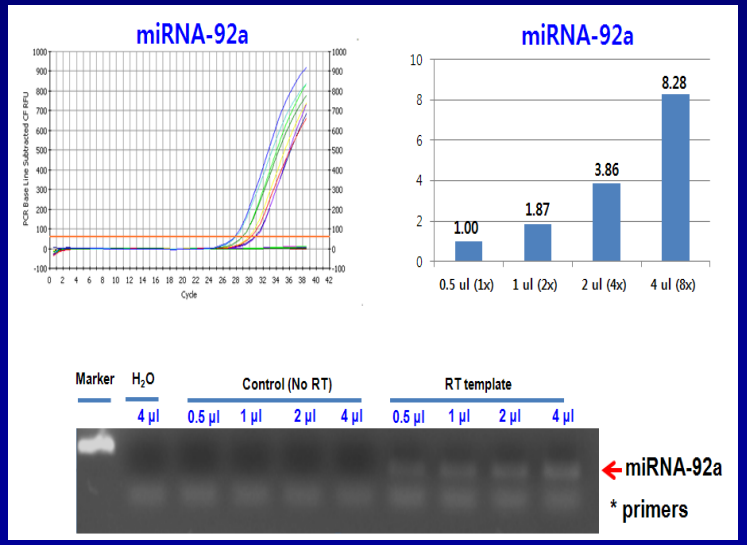
1/10 RT-Product (2.5 ~ 5 µl serum)

Real-time PCR (Taqman miRNA Assay)





Normalization (microRNA-16)



Medications at Baseline

Variable	Pioglitazone Group (n=36)	Control Group (n=36)	P value
Medication at baseline			
Oral antidiabetic therapy	29 (80.6 %)	31 (86.1 %)	0.527
Biguanides	20 (55.6 %)	24 (66.7 %)	0.334
α-Glucosidase inhibitors	8 (22.2 %)	11 (30.6 %)	0.422
Sulfonylureas	16 (44.4 %)	17 (47.2 %)	0.813
Insulin	5 (13.9 %)	4 (11.1 %)	1.000
Aspirin	26 (72.2 %)	22 (61.1 %)	0.317
ACE inhibitor	3 (8.3 %)	4 (11.1 %)	1.000
Angiotensin II receptor blocker	11 (30.6 %)	9 (25.0 %)	0.599
β-blocker	9 (25.0 %)	6 (16.7 %)	0.384
Calcium channel blocker	12 (33.3 %)	14 (38.9 %)	0.624
Diuretics	5 (13.9 %)	3 (8.3 %)	0.710
Nitrate	13 (36.1 %)	17 (47.2 %)	0.339
Nicorandil	3 (8.3 %)	3 (8.3 %)	1.000

Comparison of Angiographic Parameters During the 9-Month F/U

Variable	Pioglitazone Group (n=36)	Control Group (n=36)	P value
Number of target lesions	46	48	
Target Vessel			
Left anterior descending artery	24 (52.2 %)	22 (45.8 %)	**0.539**
Left circumflex artery	7 (15.2 %)	10 (20.8 %)	**0.479**
Right coronary artery	15 (32.6 %)	16 (33.3 %)	**0.940**
Baseline			
Reference diameter (mm)	2.81 ± 0.50	2.76 ± 0.45	**0.533**
In-stent minimum lumen diameter (mm)	0.68 ± 0.27	0.61 ± 0.29	**0.672**
In-stent percentage of stenosis	75.8 ± 8.7	77.9 ± 9.6	**0.801**
Mean lesion length (mm)	23.1 ± 9.1	24.2 ± 9.4	**0.729**
Postprocedure			
Reference diameter (mm)	2.84 ± 0.46	2.83 ± 0.45	**0.859**
In-stent minimum lumen diameter (mm)	2.61 ± 0.31	2.58 ± 0.29	**0.906**
In-stent percentage of stenosis	8.1 ± 9.4	8.8 ± 9.3	**0.722**
Acute gain (mm)	1.94 ± 0.34	1.97 ± 0.36	**0.635**
Mean stent length (mm)	26.1 ± 7.0	27.9 ± 6.0	**0.191**
Mean stent diameter (mm)	2.8 ± 0.4	2.8 ± 0.3	**0.937**
Number of patients with 9-month f/u	28 (77.8 %)	26 (72.2 %)	**0.586**
9-month follow-up			
Reference diameter (mm)	2.86 ± 0.51	2.87 ± 0.49	**0.914**
In-stent minimum lumen diameter (mm)	2.50 ± 0.20	2.39 ± 0.17	**0.023**
In-stent percentage of stenosis	12.6 ± 9.1	16.7 ± 7.5	**0.039**
Late lumen loss (mm)	0.10 ± 0.15	0.19 ± 0.24	**0.058**
Binary restenosis	2 (5.6 %)	3 (8.3 %)	**1.000**
Target lesion revascularization	**1 (2.8 %)**	**2 (5.6 %)**	**1.000**

Changes in Insulin Resistance

	Pioglitazone Group (n=36)		Control Group (n=36)	
Variables	**Baseline**	**After 9 months**	**Baseline**	**After 9 months**
Fasting insulin (μU/mL)‡	12.8±4.5	9.4±3.8*	13.2±6.0	10.0±6.4*
Changes from baseline (pmol/l)	-3.4±3.5		-3.1±3.3	
Fasting glucose (mmol/l)‡	7.9±3.1	6.3±1.9*	8.0±3.2	6.4±2.0*
Changes from baseline (mmol/l)	-1.6±2.3		-1.5±3.1	
HOMA index‡	4.5±4.5	2.6±2.3*	4.7±4.3	2.8±2.5*
Changes from baseline (%)	-1.9±2.2		-1.9±2.0	
HbA_{1c} (%)‡	7.4±1.6	6.8±0.9*	7.5±1.9	6.9±0.8*
Changes from baseline (%)	-0.6±0.9		-0.6±0.7	
RBP4 (μg/ml)‡	70.2±20.2	54.5±21.1*	67.9±22.8	49.9±19.6*
Changes from baseline (μg/ml)	-15.9±5.7		-17.8±6.0	

$p < 0.05$ compared with baseline. † $p < 0.05$ compared with the Control Group.

Methods:
Isolation of Serum Samples

- Peripheral blood samples (5 mL) were drawn into serum collection tubes

→ allowed to stand for about 30 min at RT

→ centrifuged at 1,800 g for 10 min at RT.

→ the supernatant (serum) aliquoted into eppendorf tubes and stored at -80ºC.

RNA Preparation

- Total RNAs from human serum were isolated by using TRI Reagent BD (MRC, TB126).
- In Brief,

→ 250 μl of serum per eppendorf tube was added to 0.75 ml of TRI Reagent BD

→ stored for 5 min at RT.

→ the samples were extracted with 200 uL of chloroform

→ the supernatant was isopropanol precipitated by centrifugation for at 12,000 g 15 min 4°C.

→ The pellet was washed in 1 ml of 75% ethanol by centrifugation

→ finally the pellet was re-suspended in 5 μl of RNase-free water.

→ The samples isolated from the same patients were gathered. Total RNA was quantitated by using a spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, DE).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction
(qRT-PCR, Real-time PCR)

- Total RNAs isolated from the serum were synthesized into single-stranded cDNA by using TaqMan MiRNA reverse transcription kit and miRNA-specific stem-loop primers (Applied BioSystems, Inc.).
- 10 ng of total RNA per 15 μL RT reaction was reverse transcribed using the TaqMan microRNA Reverse Transcription kit (ABI).
- MiR-17, miR-24, miR-92a, miR-126, miR-145 and miR-16 primers were used for RT reaction.
- Subsequently, 2 μL of the RT product was used for detecting miRNA expression by quantitative (q)PCR using TaqMan microRNA Assay kits (ABI) for the corresponding microRNA.
- Real-time PCR was performed using an iQ™ Cycler (Bio-Rad Laboratories, CA, USA) using the following program: 10 minutes pre-incubation at 95℃ and 40 cycles of 15 seconds of denaturation at 95℃ and 60 seconds of annealing/extending at 60℃.
- MiR-17, miR-24, miR-92a, miR-126 and miR-145 primers and miR-16 primers as an endogenous control were used. The amount of miRNA not detected after 40 cycles of a real-time PCR was regarded in the present study as a CT equivalent to 40.
- Negative controls were included with every real-time RT-PCR assay, and no amplification of the signal was observed when water was added instead of RNA or cDNA sample.
- The measurement of miRNA expression was assayed in duplicate.
- The Ct Values were normalized to miR-16 and are expressed as $2^{-(CT[microRNA]CT[miR-16])}$.