

# Atherosclerotic Plaque Inflammation Varies Between Vascular Sites and Correlates With Response to Inhibition of Lipoprotein-Associated Phospholipase A<sub>2</sub>

Robert S. Fenning, MD; Mark E. Burgert, MS; Damir Hamamdžić, DVM, PhD; Eliot G. Peyster, MD; Emile R. Mohler, III, MD; Shreya Kangovi, MD; Beat M. Jucker, PhD; Stephen C. Lenhard, MS; Colin H. Macphee, PhD; Robert L. Wilensky, MD

**Background**—Despite systemic exposure to risk factors, the circulatory system develops varying patterns of atherosclerosis for unclear reasons. In a porcine model, we investigated the relationship between site-specific lesion development and inflammatory pathways involved in the coronary arteries (CORs) and distal abdominal aortas (AAs).

**Methods and Results**—Diabetes mellitus (DM) and hypercholesterolemia (HC) were induced in 37 pigs with 3 healthy controls. Site-specific plaque development was studied by comparing plaque severity, macrophage infiltration, and inflammatory gene expression between CORs and AAs of 17 DM/HC pigs. To assess the role of lipoprotein-associated phospholipase A<sub>2</sub> (Lp-PLA<sub>2</sub>) in plaque development, 20 DM/HC pigs were treated with the Lp-PLA<sub>2</sub> inhibitor darapladib and compared with the 17 DM/HC untreated pigs. DM/HC caused site-specific differences in plaque severity. In the AAs, normalized plaque area was 4.4-fold higher ( $P<0.001$ ) and there were more fibroatheromas (9 of the 17 animals had a fibroatheroma in the AA and not the COR,  $P=0.004$ ), while normalized macrophage staining area was 1.5-fold higher ( $P=0.011$ ) compared with CORs. DM/HC caused differential expression of 8 of 87 atherosclerotic genes studied, including 3 important in inflammation with higher expression in the CORs. Darapladib-induced attenuation of normalized plaque area was site-specific, as CORs responded 2.9-fold more than AAs ( $P=0.045$ ).

**Conclusions**—While plaque severity was worse in the AAs, inflammatory genes and inflammatory pathways that use Lp-PLA<sub>2</sub> were more important in the CORs. Our results suggest fundamental differences in inflammation between vascular sites, an important finding for the development of novel anti-inflammatory therapeutics. (*J Am Heart Assoc.* 2015;4:e001477 doi: 10.1161/JAHA.114.001477)

**Key Words:** arteriosclerosis • coronary disease • darapladib • inflammation • lipoprotein-associated phospholipase A<sub>2</sub> • peripheral vascular disease

Although atherosclerosis is a systemic disease and the circulatory system is uniformly exposed to risk factors such as hyperglycemia and hypercholesterolemia, plaque development varies between vascular sites. For decades, clinicians have noted such heterogeneity of presentation,<sup>1</sup> and

researchers have shown distinct risk factor profiles for arterial beds.<sup>2</sup> We have previously demonstrated variable disease development of atherosclerosis in diabetic/hypercholesterolemic (DM/HC) pigs with severe, high-risk lesion development in the coronary arteries (CORs), less severe disease in the thoracic aorta, and minimal disease in the carotid arteries.<sup>3</sup> Extensive atherosclerosis in the distal abdominal aortas (AAs) extending into the proximal iliac vessels has also been reported in these animals.<sup>4</sup> The cause of such variable site-specific disease development, observed in both humans and DM/HC pigs, is unclear but clinically important.

Lipoprotein-associated phospholipase A<sub>2</sub> (Lp-PLA<sub>2</sub>) is a potential target for atherosclerosis treatment.<sup>5</sup> An enzyme secreted by inflammatory cells, it generates the proinflammatory mediators lysophosphatidylcholine and oxidized nonesterified fatty acids from oxidized low-density lipoprotein within the arterial wall.<sup>6</sup> We previously showed that selective inhibition of Lp-PLA<sub>2</sub> with darapladib reduced the development of high-risk COR atherosclerotic plaques in a DM/HC pig model,<sup>7</sup> and

From the Hospital of the University of Pennsylvania, Philadelphia, PA (R.S.F., D.H., E.G.P., E.R.M., S.K., R.L.W.); GlaxoSmithKline, King of Prussia, PA (M.E.B., B.M.J., S.C.L., C.H.M.).

Accompanying Tables S1 through S3 are available at <http://jaha.ahajournals.org/content/4/2/e001477/suppl/DC1>

**Correspondence to:** Robert L. Wilensky, MD, Hospital of the University of Pennsylvania, 3400 Spruce St, 9 Gates, Philadelphia, PA 19104. E-mail: robert.wilensky@uphs.upenn.edu

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The Stabilization of Atherosclerotic Plaque by Initiation of Darapladib Therapy Trial (STABILITY) has shown that darapladib significantly reduced the risk of the secondary end points of major coronary events and total coronary events in patients with stable coronary heart disease, although it failed to demonstrate significant reductions in the risk of the primary end point of cardiovascular death, myocardial infarction, and stroke.<sup>8</sup> In the current study, we addressed the question of whether the mechanism of atherosclerosis in 2 areas of extensive lesion development—the CORs and the distal AAs—differed with regard to inflammatory pathways, specifically those using Lp-PLA<sub>2</sub>. We assessed differences in plaque development by comparing plaque severity, macrophage infiltration, and inflammatory gene expression profiles caused by DM/HC induction between the CORs and AAs. In addition, we assessed for differences in the role of Lp-PLA<sub>2</sub> in plaque development and gene expression by analyzing differences in response to darapladib treatment between the CORs and AAs.

## Methods

### Animals and Experimental Protocol

The DM/HC porcine model has been previously described.<sup>3,7</sup> Briefly, 37 male Yorkshire domestic pigs weighing 20 to 25 kg (Archer Farms) were made DM/HC with 3 healthy pigs serving as controls (non-DM/HC control). DM was induced by 125 mg/kg of intravenous streptozotocin (Sicor Pharmaceuticals), and exogenous insulin was administered via a sliding scale for blood glucose levels >350 mg/dL to avoid ketoacidosis. Hypercholesterolemia was induced with a hyperlipidemic diet containing 0.5% cholesterol, 10% lard, and 1.5% sodium cholate (Animal Specialties) to achieve a target cholesterol level of 400 to 800 mg/dL. Four weeks after DM/HC induction, pigs were randomly assigned into a control group (DM/HC control, n=17) or a treatment group (DM/HC darapladib, n=20) receiving 10 mg/kg/d orally of the selective Lp-PLA<sub>2</sub> inhibitor darapladib (SB480848; GlaxoSmithKline). Twenty-eight weeks after DM/HC induction (24 weeks from treatment), pigs were killed (Eutasol; Virbac AH) and tissue was harvested for analysis. A distal section of AA including the proximal iliac and the right CORs were processed for gene expression. Following fixation with formaldehyde, the remaining distal AA underwent magnetic resonance imaging (MRI). Then, the tissue was processed for histologic and immunohistochemical analyses. The left anterior descending coronary artery was used for the analyses. Glucose and cholesterol levels for each animal were measured monthly for the duration of the study and were plotted over time. The cumulative total plasma glucose or cholesterol level was determined by calculating the area under the curve for the respective levels for each animal. All studies were

approved by the University of Pennsylvania Animal Care and Use Committee. These experiments are a further analysis derived from the 40 pigs from which only the coronary data were published.<sup>7</sup> In the current experiment, the magnitude of effect of DM/HC induction and darapladib treatment on the AAs is compared with the magnitude of effect of DM/HC induction and darapladib treatment on the CORs. Further details of the methods have been previously published.<sup>7</sup>

### Histologic and Immunohistochemical Evaluation

Arteries were cut into 5-mm sections and embedded in paraffin. Histologic sections were stained with Movat's pentachrome and analyzed with the use of Image Pro 6.2 software (MediaCybernetics). Morphometric analysis of all arterial sections was performed to determine lesion area, area of calcification, necrotic core area, presence of intraplaque hemorrhage, medial destruction, and lesion classification as previously described.<sup>7</sup> The normalized plaque area, defined as the ratio of the lesion area to medial area, was used to adjust to compare arteries of different sizes. The normalized calcification area was defined as the ratio of the calcification area to lesion area of the most severe lesion, and the normalized necrotic core area was defined as the ratio of the necrotic core area to lesion area of the most severe lesion. Each section was classified by using the modified American Heart Association (AHA)/Virmani score (0=no disease, 1=intimal thickening, 2=intimal xanthoma, 3=pathologic intimal thickening, 4=fibrous cap atheroma, and 5=thin fibrous cap atheroma)<sup>9</sup> with the maximum AHA score of the artery used for analysis. The maximum medial destruction score was determined using the following scale from 0 to 4: 0=normal, 1=internal elastic lamina disrupted, 2=destruction of <50% of the medial thickness, 3=destruction of >50% of the medial thickness, and 4=destruction of >50% of the medial thickness along with disruption of the external elastic lamina.<sup>7</sup> Intraplaque hemorrhage was identified by the presence of extravasated red blood cells outside of the vasa vasorum.<sup>9</sup> Adjacent sections of the AAs and CORs were stained with a goat polyclonal cathepsin S antibody (Santa Cruz Biotechnology Inc) for inflammatory cells with augmented protease activity, mostly macrophages, as previously described.<sup>7</sup> The normalized macrophage area was defined by the ratio of cathepsin S staining area divided by the lesion area of the most severe lesion.

### Gene Expression Using Quantitative Real-Time Polymerase Chain Reaction

The details for the gene expression analysis have been previously published.<sup>7</sup> Briefly, a Taqman plate was constructed using 87 genes shown to be expressed in human atherosclerotic plaque<sup>10</sup> that have pig orthologs (see Table S1

for sequences). The effect of DM/HC induction on gene expression was analyzed by comparing the non-DM/HC control group (n=3) with the DM/HC control group (n=17). The effect of darapladib treatment on gene expression was analyzed by comparing the DM/HC control group (n=17) with the DM/HC darapladib group (n=20). Whole minced arteries were homogenized on ice in Trizol reagent (Sigma). Total RNA was extracted, purified, and, after on-column DNase treatment, eluted with RNase-free water. Genomic DNA contamination was removed with DNase I (Ambion). Quantification of the RNA was performed and converted to cDNA via reverse transcription. TaqMan gene expression data were analyzed on the basis of normalized expression values, by using scaled geometric mean of selected reference genes for the normalization factor calculations.

### Ex Vivo MRI

The distal AAs were placed in 0.2% gadopentetate dimeglumine-doped water solution, and imaging was performed with a 9.4-T  $\mu$ -imaging system (Bruker). A gradient echo coronal scout image to properly orient the axial high-resolution T2-weighted images had the following parameters: TE/TR=4.2/137 ms, 256 $\times$ 256 matrix, excitations=1, field of view=4 cm, slice width=50 kHz, flip angle=30°, 10 slices, 1-mm slice thickness. Once the scout image was acquired, a T2-weighted spin echo image was acquired in the axial plane with the following parameters: TE/TR=40/2000 ms, slice width=50 kHz, 256 $\times$ 256 matrix, excitations=16, field of view=1.6 cm, 20 slices, 1-mm slice thickness.

### Statistical Analysis

To assess for site-specific effects of DM/HC induction on plaque severity and macrophage infiltration, the effect size of DM/HC induction on the CORs was compared with the effect size on the AAs among the 17 DM/HC pigs. As appropriate for the data type and sample distributions, paired-response or mixed-model analysis methods were used to allow each animal to serve as its own control. The paired analysis approach was used for all the categorical responses (fibroatheroma, intraplaque hemorrhage, AHA score, and medial destruction score) with a signed rank comparison for the paired difference of the artery responses. The paired analysis approach was also used for the continuous response values that did not have normal distributions (normalized calcification area and normalized necrotic core area) with a nonparametric signed rank comparison for the paired difference of the artery values. The mixed model analysis was used for continuous response values with approximately normal distributions after log transformations (normalized macrophage area and normalized plaque area) with a 2-sided

*t* test of the contrast estimates of the log-transformed values. The *P* values test for a significant interaction between the effect size of DM/HC induction and vascular site.

To assess for site-specific effects of darapladib treatment on plaque severity and macrophage infiltration, the effect size of treatment on the CORs was compared with the effect size on the AAs by comparing the DM/HC control pigs with the DM/HC darapladib pigs. As in the comparisons of the induction differences, the comparisons between the treatments by artery location used paired-response or mixed-model analysis methods to allow each animal to serve as its own control. For categorical responses, the Exact Cochran–Mantel–Haenszel test was used. For continuous response values that did not have normal distributions, the Wilcoxon–Mann–Whitney test was used. For continuous response values with normal distributions after log transformation, 2-sided *t* tests of the contrast estimates of log-transformed values were used.

To assess for site-specific effects of DM/HC induction and darapladib treatment on gene expression, mixed-model analysis of log-transformed expression levels was used. For each gene at each vascular site, the change in expression was compared with the baseline normal expression levels from the 3 normal pigs. The assessment of the effects of darapladib treatment and of the treatment differences between the vascular sites also used the same mixed-model analysis methods for comparing the 17 DM/HC control pigs with the 20 DM/HC treatment pigs. Log-transformed values were tested using 2-sided *t* tests for the contrasts, and the unadjusted *P* values were reported for all mixed-model comparisons. The log-transformed comparisons were retransformed into fold-change values. Positive fold-change values indicate increased expression compared with control, while negative fold-change values indicate decreased expression.

The correlation estimation used the Spearman rank method to assess for associations between cumulative total plasma cholesterol levels or glucose levels and normalized plaque area in the 2 vascular sites with induction of DM/HC, as well as to assess for the association of normalized plaque area between the CORs and the AAs.

All statistical tests were assessed for significance at the 0.05 level, regardless of the number of tests performed. Specifically, the *P* values reported for the gene expression comparisons were not adjusted for the 87 separate genes with comparisons.

## Results

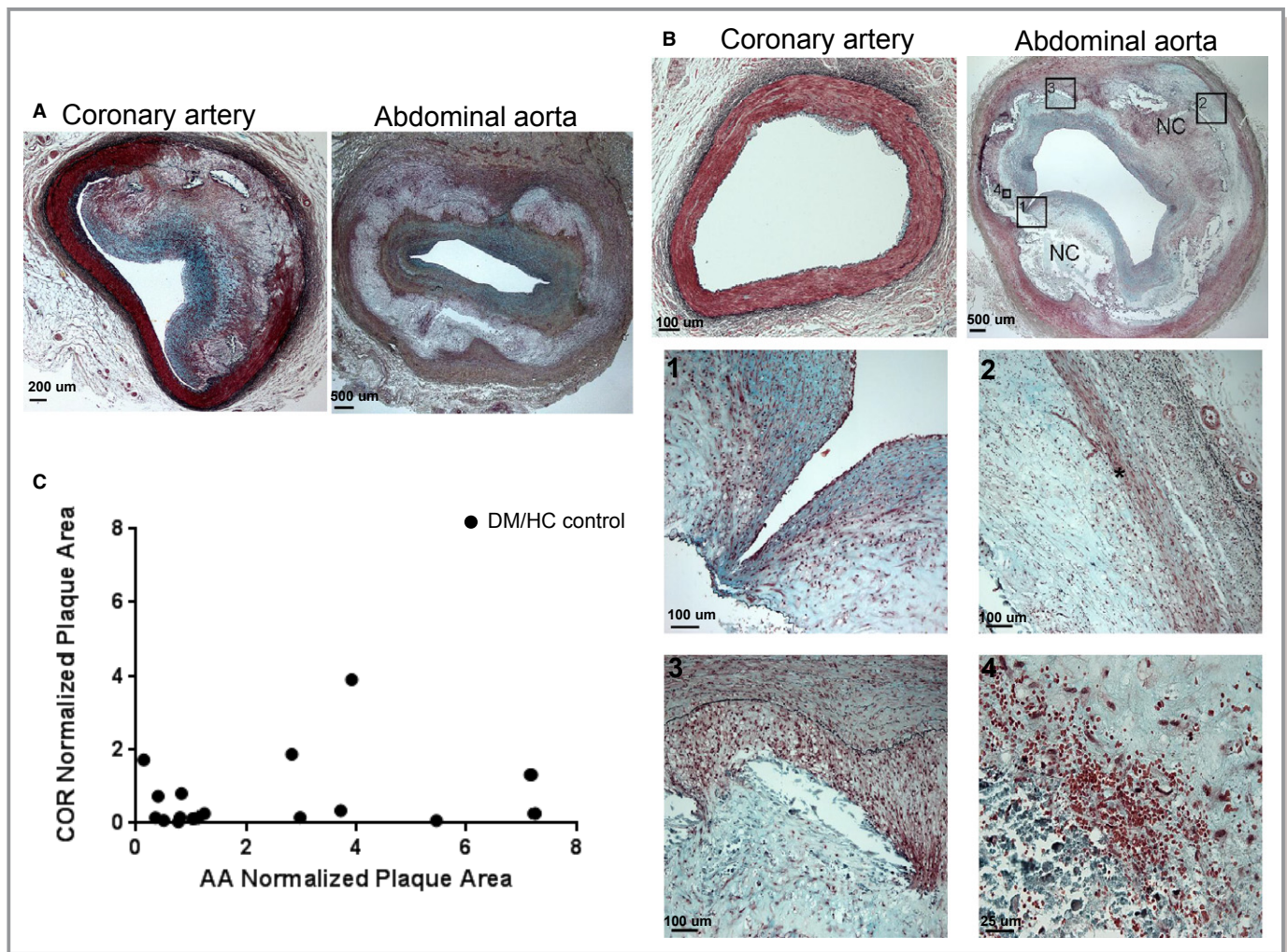
### Induction of DM/HC Causes More Severe Lesion Development in AAs Compared With CORs

The induction of DM/HC led to a sustained elevation of glucose and cholesterol levels, respectively, as previously



pigs had fibroatheromas in the AAs (9/17 animals were discordant with all the discordant pairs positive for fibroatheroma in the AAs,  $P=0.004$ , Figure 1C). For intraplaque hemorrhage, more pigs had this high-risk finding in the AAs (6/17 animals were discordant with all the discordant pairs positive for intraplaque hemorrhage in the AAs,  $P=0.031$ ). The median normalized calcification area was 0.17 higher in the AAs (0.08 to 0.26 for lower and upper quartiles,  $P=0.0042$ , Figure 1D), and the normalized macrophage area was 1.5-fold higher in the AAs (95% CI 0.7 to 3.1,

$P=0.011$ , Figure 1E). Normalized necrotic core size and medial destruction scores were not significantly different between the vascular sites. Figure 1F shows for each DM/HC control animal the normalized plaque area of the AA and COR, illustrating more severe atherosclerosis of the AA. Examples of concordant severe disease involving vascular sites as well as discordant minimal disease in the CORs but severe, complex disease in the AAs are shown in Figure 2A and 2B, respectively. Figure 2B (1 to 4) highlights advanced atherosclerotic features of the AAs, including thin cap fibroathero-



**Figure 2.** Illustrative example of more severe atherosclerosis involving the AA. A, Example of a DM/HC control pig with the development of advanced, complex atherosclerosis in both the COR and the AA. B, In contrast, an example of a DM/HC control pig with very minimal intimal thickening of the COR but advanced plaque in the AA. All images represent the most severe lesion of the arterial bed for the animal. B, Magnify different findings of advanced disease of the AA plaque: (1) Thin cap fibroatheroma as evident by a thin layer of smooth muscle and collagen overlying a necrotic core. (2) Atherosclerotic destruction of the medial layer. This represents a medial destruction score of 4 since >50% of the medial layer is destroyed with loss of a clear external elastic lamina border. (3) An area of calcification as seen by an absence of Movat’s staining. (4) Intraplaque hemorrhage as seen by extravasated red blood cells into a plaque. All histology slides are stained with Movat’s pentachrome. C, There is no significant correlation of plaque size between the 2 sites in an analysis of the DM/HC control pigs ( $n=17$ ), as shown by a plot of COR normalized plaque area to AA normalized plaque area with a Spearman correlation coefficient of 0.16,  $P>0.5$ . AA indicates abdominal aorta; COR, coronary artery; DM/HC, diabetes mellitus and hypercholesterolemia.

ma, medial destruction, calcification, and intraplaque hemorrhage. Disease severity as assessed by plaque size in one vascular bed did not predict disease severity in the other vascular bed, as illustrated by a lack of correlation of normalized plaque area in Figure 2C (Spearman correlation coefficient of 0.16,  $P>0.5$ ).

### DM/HC Induces Unique Site-Specific Gene Expression Profiles

Real-time quantitative PCR on whole arteries was used to explore differences in gene expression profiles between the AAs and CORs caused by the induction of DM/HC. Eight of the 87 atherosclerotic genes studied had differential expression between the 2 sites. Interestingly, many of these genes play a significant role in the inflammatory cascade and showed expression levels that were increased in the CORs and either decreased or not as robustly increased in the AAs (Table 1). For example, CD97, expressed by active lymphocytes, monocytes, and macrophages,<sup>11</sup> showed a 1.5-fold increase in expression in the CORs versus a 1.5-fold decrease in expression in the AAs ( $P=0.013$ ). Similarly, the inflammatory cytokine CHI3L1 (also known as YKL-40), which has been shown to be important in atherosclerotic lesion development,<sup>12,13</sup> was significantly more upregulated in the CORs compared with the AAs. For CHI3L1, DM/HC resulted in an 18.1-fold increase in the CORs versus a 4.0-fold increase in the AAs ( $P=0.042$ ). While 46 of the 87 genes analyzed showed increased expression in both sites, including genes important to the inflammatory process (Table 2 for selected genes and Table S2 in supplemental material for all genes analyzed), the differential expression of these 8 genes suggests a potentially more robust inflammatory response in the CORs.

### Role of Lp-PLA<sub>2</sub> Varies Among Vascular Sites

Given this difference in gene expression profiles between the CORs and the AAs we tested the hypothesis that a targeted anti-inflammatory agent would preferably affect the site with more inflammatory genes upregulated. To this end, differences in effect size of darapladib treatment on plaque severity in the CORs and AAs was assessed by comparing 17 DM/HC control pigs with 20 DM/HC darapladib pigs. In our previous work, darapladib treatment resulted in significant attenuation of COR atherosclerosis with a notable decrease in coronary plaque and necrotic core area, medial destruction score, as well as fewer lesions with unstable features.<sup>7</sup> In the current analysis, darapladib modified disease progression to a lesser extent in the AAs. There was a statistically significant site-specific effect of treatment on normalized plaque area while the effect of treatment on the development of fibroatheromas approached statistical significance. The response to treatment with darapladib on attenuation of normalized plaque area was 2.9-fold greater in the CORs compared with the AAs (95% CI 1.0 to 8.2,  $P=0.045$  for an interaction between response to darapladib therapy and vascular site, Figure 3A). For the development of fibroatheromas, there was a trend toward a site-specific differential effect from darapladib treatment. In the CORs, darapladib treatment reduced the number of fibroatheromas (7/17 versus 2/20), while in the AAs, darapladib treatment did not affect the development of fibroatheromas (16 of 17 versus 19 of 20),  $P=0.069$  for an interaction between response to darapladib therapy and vascular site, Figure 3B). Figure 4 shows representative sections of AAs from DM/HC control and darapladib pigs demonstrating similar disease severity and similar macrophage infiltration. This is in contrast to representative sections of CORs from DM/HC control and darapladib pigs showing a significant effect of treatment on

**Table 1.** Influence of DM/HC Induction on Gene Expression for the Genes Differentially Affected by DM/HC in the CORs and AAs in an Analysis of the DM/HC Control Pigs (n=17)

Gene Category	Gene Name	Fold-change CORs	P Value	Fold-Change AAs	P Value	Fold Ratio (AA vs COR)	P Value
Inflammatory cell marker	<i>CD97</i>	1.5	0.121	−1.5	0.025	−2.2	0.013
Cytokine	<i>CHI3L1</i>	18.1	0.001	4.0	0.060	−4.5	0.042
Inflammatory enzyme	<i>TIMP1</i>	1.6	0.037	−1.1	0.586	−1.7	0.024
Metabolic regulator	<i>IRS1</i>	2.2	0.077	−1.2	0.358	−2.7	0.029
	<i>PPARG</i>	3.8	0.001	18.7	<0.001	5.0	0.022
	<i>SLC27A4</i>	3.2	<0.001	1.4	0.154	−2.3	0.029
Prostaglandin biosynthesis	<i>PTGS1</i>	2.4	0.050	−1.2	0.411	−3.0	0.015
Transmembrane transport	<i>MFSD1</i>	3.0	<0.001	1.3	0.330	−2.3	0.021

DM indicates diabetes mellitus; HC, hypercholesterolemia; COR, coronary arteries; AA, abdominal aortas.

**Table 2.** Influence of DM/HC Induction on Gene Expression for Selected Genes Similarly Affected by DM/HC in Both the CORs and AAs in an Analysis of the DM/HC Control Pigs (n=17)

Gene Category	Gene Name	Fold-Change CORs	P Value	Fold-Change AAs	P Value
Inflammatory cell markers	<i>CD4</i>	25.1	<0.001	10.3	<0.001
	<i>CD48</i>	19.7	<0.001	15.5	<0.001
	<i>CD68</i>	16.9	<0.001	14.1	<0.001
	<i>BIN2</i>	7.1	<0.001	6.0	0.002
	<i>IL2RA</i>	6.0	0.005	2.6	0.01
	<i>CD163</i>	4.8	<0.001	3.2	0.009
	<i>CD36</i>	4.5	<0.001	11.8	<0.001
Cytokines, chemokines, or chemokine receptors	<i>CCL5</i>	7.2	<0.001	3.0	0.02
	<i>CCL3</i>	2.5	0.01	4.6	0.003
	<i>IL18</i>	4.1	<0.001	6.5	<0.001
	<i>IL6</i>	2.5	0.007	3.9	0.006
	<i>TNF</i>	2.1	0.005	2.7	0.005
	<i>CCR1</i>	11.2	0.004	10.0	0.003
Inflammatory cell chemotaxis	<i>ITGB2</i>	14.7	<0.001	10.7	<0.001
	<i>VCAM1</i>	5.3	<0.001	5.8	<0.001
	<i>PTAFR</i>	5.8	<0.001	3.8	0.003
Inflammatory enzymes	<i>MMP9</i>	71.1	0.002	112.4	<0.001
	<i>CTSS</i>	16.1	<0.001	15.4	<0.001
	<i>Lp-PLA2</i>	15.7	<0.001	11.6	<0.001
	<i>PLAUR</i>	6.7	<0.001	3.2	<0.001
Oxidative stress	<i>CYBB</i>	19.8	<0.001	13.9	<0.001
	<i>NCF1</i>	10.5	<0.001	6.9	0.005
	<i>HMOX1</i>	6.4	<0.001	3.4	0.006
	<i>UCP2</i>	6.2	<0.001	4.4	0.004

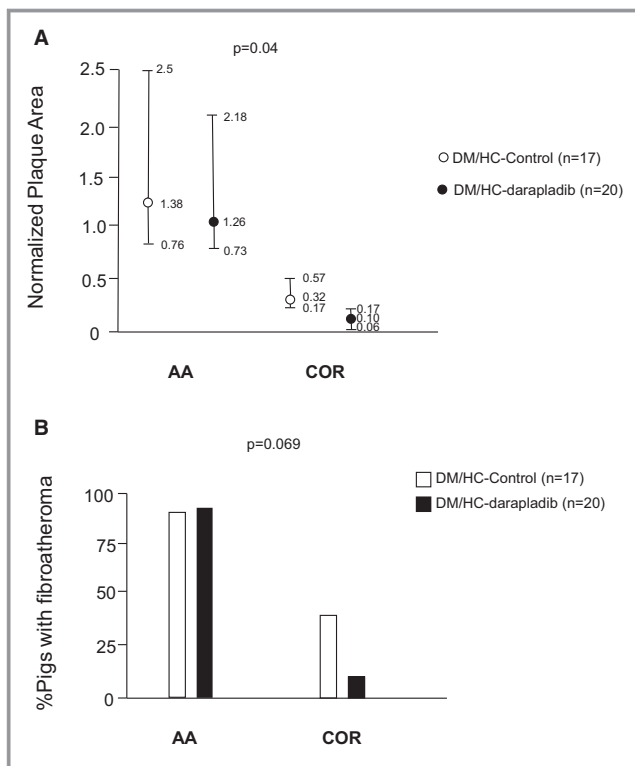
DM indicates diabetes mellitus; HC, hypercholesterolemia; COR, coronary arteries; AA, abdominal aortas.

disease severity. Interestingly, treatment with darapladib significantly affected development of AA intraplaque hemorrhage (1 of 20 darapladib pigs versus 6 of 17 control pigs had intraplaque hemorrhage,  $P=0.033$ ). No CORs demonstrated intraplaque hemorrhage in either group.

### Lp-PLA<sub>2</sub> Inhibition Has a Similar Effect on Atherosclerotic Gene Expression in the AAs and the CORs

In previous work, darapladib treatment was shown to significantly affect 24 of the 87 genes studied in the CORs, including important inflammatory genes associated with macrophage function (*CD68*, Lp-PLA<sub>2</sub>, cathepsin S), T-helper type 1 lymphocyte function (*CXCR3*), and monocyte and T-cell function (*CD18*, *BIN2*).<sup>7</sup> In the current study, the effect of darapladib treatment on atherosclerotic gene expression

showed similar changes in 85 of 87 genes analyzed for both the AAs and the CORs (Table S3). One gene that was differentially affected by darapladib was *CCR2*, a gene encoding the receptor for monocyte chemoattractant protein 1 (MCP-1), important for the recruitment of monocytes to areas of active inflammatory plaque.<sup>14</sup> Its expression was significantly decreased by 1.8-fold in CORs versus a 1.2-fold decrease in the AAs with darapladib treatment ( $P=0.032$ ). The other gene with differential expression was *ADAMDEC1*, a gene encoding for a secreted protein belonging to the disintegrin metalloproteinase family important for dendritic cell maturation and shown to have increased expression in unstable human carotid plaques.<sup>10</sup> Its expression was increased in the CORs by 1.2-fold versus a decrease of 3.3-fold in the AAs with darapladib treatment ( $P=0.040$ ). Despite similar effects on gene expression patterns in the CORs and AAs, Lp-PLA<sub>2</sub> inhibition had a significantly greater effect on



**Figure 3.** Inhibition of lipoprotein-associated phospholipase  $A_2$  has site specific effects in an analysis comparing DM/HC control pigs (n=17) to DM/HC darapladib pigs (n=20). A, Darapladib-induced attenuation of normalized plaque area was significantly greater in the CORs by a factor of 2.9 compared with the AAs. Values represent the geometric means  $\pm$  95% CIs. B, Darapladib-induced attenuation of fibroatheroma development trended toward a site-specific differential effect in the CORs compared with the AAs. All  $P$  values test for a significant interaction between the respective measure of darapladib treatment and vascular site. AA indicates abdominal aorta; COR, coronary artery; DM, diabetes mellitus; HC, hypercholesterolemia.

attenuation of plaque development in the CORs, suggesting that the inflammatory cascade is of greater importance in the CORs compared with the AAs.

### Glucose Levels Are Correlated to AA Lesion Area but Not COR Lesion Area

To study the relationships between the normalized plaque area and the severity of hyperglycemia and hypercholesterolemia, the normalized plaque area was plotted against total cumulative plasma glucose levels and total cumulative plasma cholesterol levels for each vascular site. For the range of total cumulative plasma glucose and cholesterol levels observed in the study, the AA normalized plaque area significantly correlated with the severity of hyperglycemia (Figure 5A) while the COR normalized plaque area did not (Figure 5B). Neither AA nor COR normalized plaque area

significantly correlated with severity of dyslipidemia (Figure 5C and 5D).

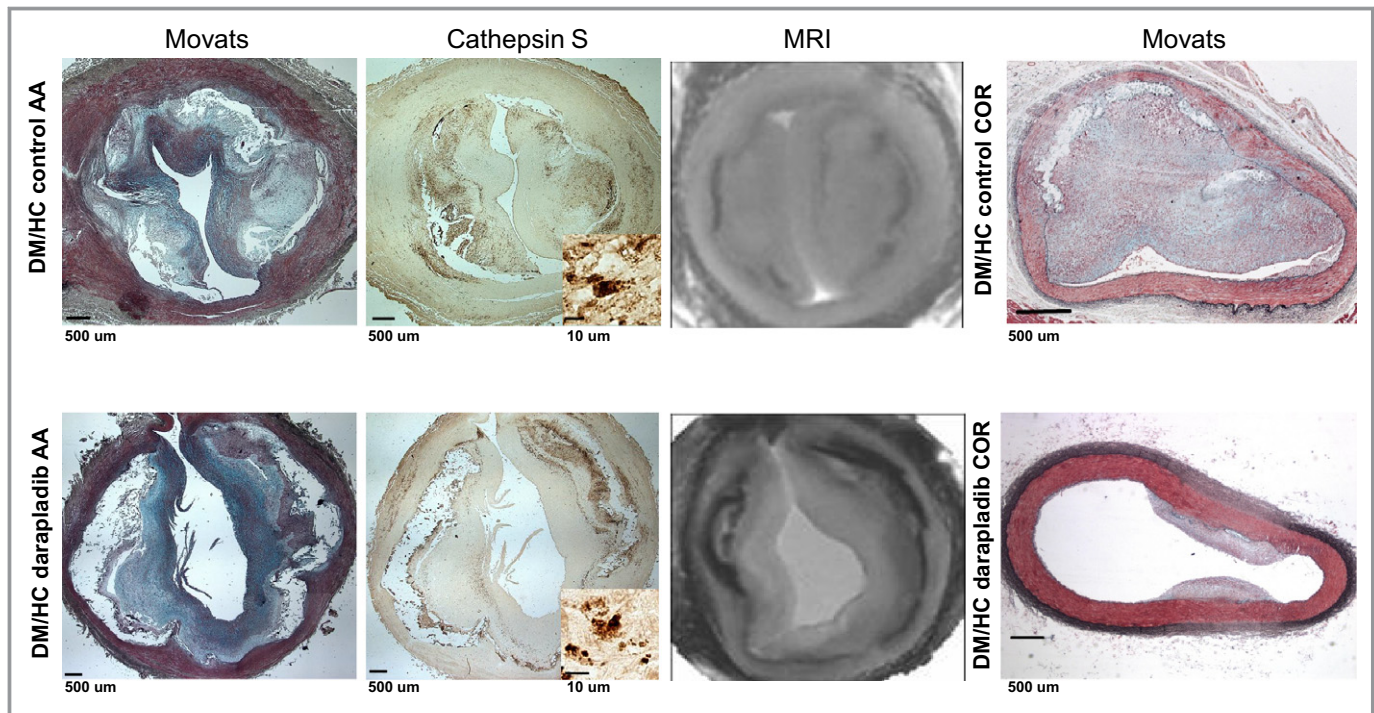
## Discussion

In this study, we have shown that DM/HC induction results in variable development of atherosclerosis in CORs and AAs. Plaque severity and inflammatory gene expression differed between the 2 vascular beds, with more inflammatory gene expression in the CORs yet greater plaque severity in the AAs. There was no correlation of severity of disease response to DM/HC induction between the 2 sites and response to Lp-PLA<sub>2</sub> inhibition was site specific. Even though darapladib treatment reduced Lp-PLA<sub>2</sub> activity in the proximal iliac arteries/distal AA<sup>7</sup> and had a similar effect on inflammatory genes at both arterial sites, Lp-PLA<sub>2</sub> inhibition was effective in reducing plaque area in the CORs but not the AAs. Taken together, these results suggest a differential role of inflammatory pathways between vascular sites. Furthermore, these findings demonstrate the difficulty of using noncardiac vasculature as a surrogate marker for coronary heart disease in the detection and management of atherosclerosis as well as the development of novel targeted anti-inflammatory therapeutics.

Our data suggest that the DM/HC state may have resulted in increased expression of more inflammatory genes in the CORs compared with the AAs with functions important in activated lymphocyte and macrophage functioning (*CD97*) and cytokine signaling (*CHI3L1*). Despite this, CORs developed less severe lesions compared with the AAs. This discordance between plaque severity and inflammatory response in the AAs was unexpected but corroborates previously published human data. Clinical studies have shown that systemic inflammation defined by CRP levels is greater in patients with COR atherosclerosis and evidence of plaque rupture, whereas the largest study to assess peripheral plaque rupture in the iliofemoral arteries showed no such association with CRP levels.<sup>15,16</sup> By showing that AA lesion area correlates with levels of plasma glucose while COR lesion area does not, it appears that disease severity in the AAs is directly related to hyperglycemia while in the CORs this is not the case. A potential explanation is that the presence of hyperglycemia indirectly drives atherosclerosis development and progression in the CORs via an effect on inflammation. Perhaps other potent risk factors for peripheral atherosclerosis not present in this study such as smoking are important for the link between inflammation and plaque severity in the AAs.

The underlying mechanism causing these fundamental differences in the development of atherosclerosis and the differential role of Lp-PLA<sub>2</sub> in the inflammatory cascade



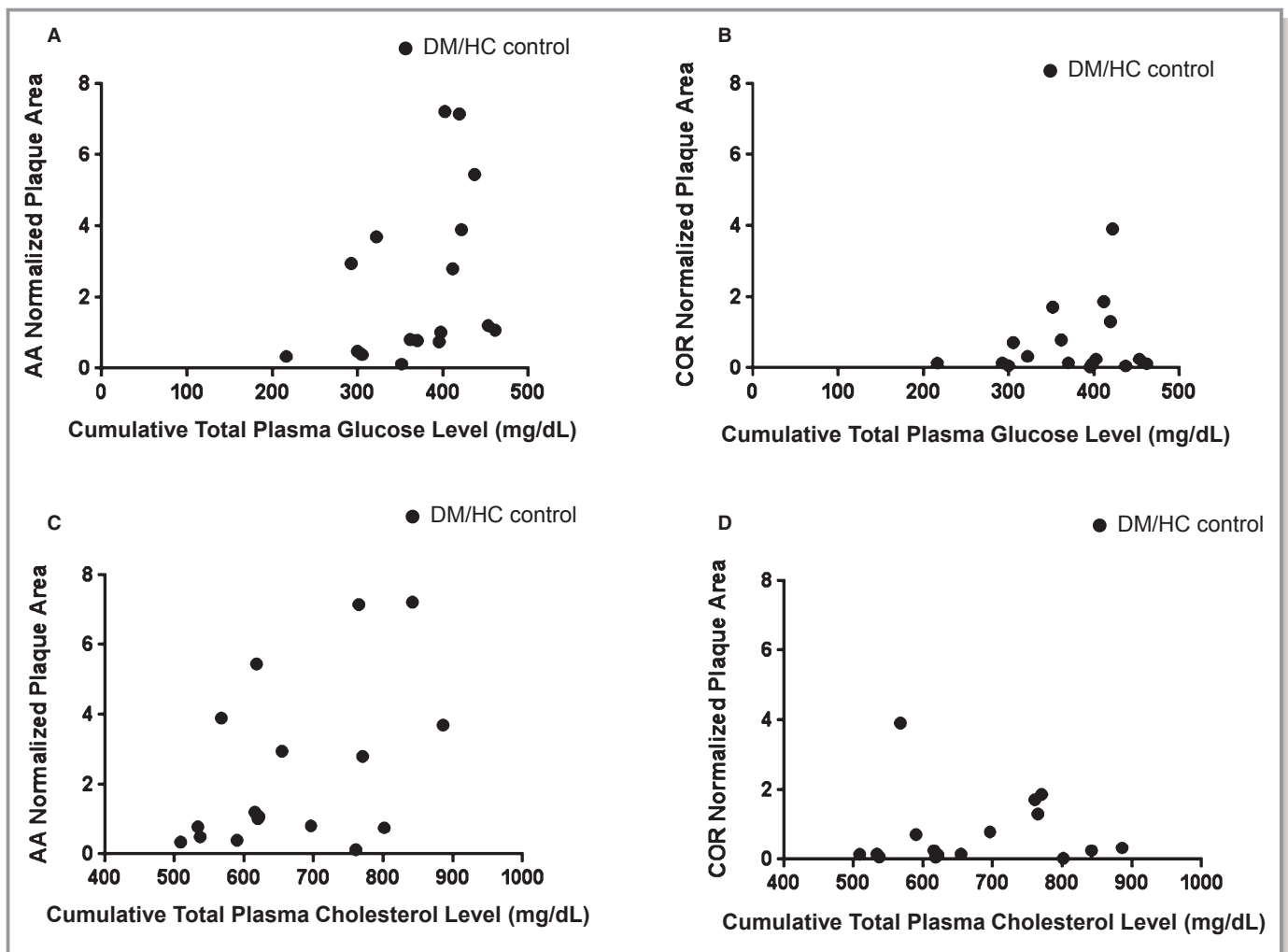


**Figure 4.** Illustrative example of the lack of plaque attenuation in the AA from inhibition of lipoprotein-associated phospholipase  $A_2$  compared with a significant effect on plaque attenuation in the COR. Movat's pentachrome staining of representative sections from a DM/HC control AA and a DM/HC darapladib AA reveals little effect of darapladib on lesion progression at this vascular site. Cathepsin S staining shows no significant effect of darapladib on macrophage plaque infiltration. MRI imaging confirms the findings found by histology. This is in contrast to the representative COR sections showing significantly less disease severity in the DM/HC darapladib coronary section compared with the DM/HC control coronary section. AA indicates abdominal aorta; COR, coronary artery; DM/HC, diabetes/hypercholesterolemia.

between the 2 sites is unclear but possibly relates to differences in flow hemodynamics as well as embryologic origin of the vasculature. Using a similar model, the Stone group has shown that local differences in endothelial shear stress in the coronary vasculature led to changes in inflammatory gene expression and the development of thin fibrous cap atheromas. Areas of low endothelial shear stress in the coronary vasculature, which typically occur at bifurcations or areas of curvature, are at increased risk of developing focal atherosclerotic lesions.<sup>17</sup> These disturbances in laminar flow have been shown to change expression profiles of endothelial cells in healthy pigs and correlate to areas of high susceptibility to atherosclerosis.<sup>18</sup> Other work has illustrated differences in wall shear stress based on the anatomic location of the vascular bed in reference to the distance from the aortic root as well as the diameter of the blood vessels and the location of branching segments.<sup>19,20</sup> As such, the local variation in hemodynamic forces and shear stress in the CORs and the AAs caused by differences in vessel diameter, tortuosity and branching may account for some of the unique site-specific features observed in this study. The embryologic origin of smooth muscle cells at different sites has also been shown to affect the development of atherosclerosis and may relate to some of the observed differences in this study. Haimovici et al

showed that a relatively atherosclerosis-resistant thoracic aorta transplanted to an atherosclerosis-susceptible distal AA location remains resistant to atherosclerosis. The atherosclerosis-susceptible distal AA transplanted to the thoracic aorta would still develop disease.<sup>21</sup> In healthy Rapacz familial hypercholesterolemic swine, Bahls et al showed that athero-protected brachial arteries differ from the athero-susceptible femoral arteries in gene expression profiles despite similar patterns of blood flow.<sup>22</sup>

Given these findings of fundamental differences in atherosclerotic plaque development between vascular sites in DM/HC pigs with human-like lesions, similar observations in human and animal studies can be better appreciated. An autopsy study of 100 individuals comparing lesions at the coronary, carotid, and superficial femoral arteries showed important histologic differences in plaque morphology between the sites.<sup>23</sup> There is also evidence that gene expression profiles of human carotid arteries, femoral arteries, and aortas vary significantly,<sup>24</sup> and animal studies have also shown that experimental COR atherosclerosis is associated with more inflammatory gene expression than AA atherosclerosis.<sup>3,25</sup> The present study supports findings showing the site-specific efficacy of novel targeted anti-inflammatory pharmacologic agents.<sup>26</sup> These differences in



**Figure 5.** Normalized plaque area directly correlates with cumulative total plasma glucose levels in the AA but not the COR in an analysis of the DM/HC control animals ( $n=17$ ). A, AA normalized plaque area significantly correlates with cumulative total plasma glucose levels,  $r=0.56$ ,  $P=0.022$ . B, COR normalized plaque area does not significantly correlate with cumulative total plasma glucose levels,  $r=0.09$ ,  $P=0.74$ . C, AA normalized plaque area does not significantly correlate with cumulative total plasma cholesterol levels,  $r=0.41$ ,  $P=0.10$ . D, COR normalized plaque area does not significantly correlate with cumulative total plasma cholesterol levels,  $r=0.19$ ,  $P=0.46$ . AA indicates abdominal aorta; DM/HC, diabetes/hypercholesterolemia; COR, coronary artery.

variability of disease, plaque severity, gene expression, and response to targeted therapies are consistent with our findings in DM/HC pigs and may be explained by a differential role of inflammatory pathways between vascular sites.

The present study lends insight into the mechanism of differences between Lp-PLA<sub>2</sub> associations with atherosclerosis in the CORs and AAs observed in human studies. While multiple epidemiologic studies show that Lp-PLA<sub>2</sub> is independently associated with risk of coronary heart disease,<sup>27</sup> several studies have also shown that Lp-PLA<sub>2</sub> levels do not correlate with the degree of atherosclerosis in the AA. In the Rotterdam Study, the odds ratio of the presence of aortic-iliac-femoral atherosclerosis according to

tertiles of Lp-PLA<sub>2</sub> activity lost statistical significance after adjusting for total cholesterol and HDL levels.<sup>28</sup> In the Veterans Affairs Diabetes Trial, Lp-PLA<sub>2</sub> mass after adjustment for standard risk factors predicted progression of coronary calcium on computed tomography (CT) but did not predict progression of AA calcium.<sup>29</sup> Similarly, in the Dallas Heart Study, Lp-PLA<sub>2</sub> mass was independently associated with COR calcium on CT but not AA plaque and aortic wall thickness on MRI.<sup>30</sup> The Lp-PLA<sub>2</sub> inhibitor rilapladib clinically showed no significant effect of treatment on FDG-PET uptake at the carotid and ascending aorta sites,<sup>31</sup> and most recently, in a randomized clinical trial of darapladib in stable coronary heart disease, there was a nominally significant reduction in the risk of 2 secondary composite end points

of major coronary events and total coronary events, while darapladib had no effect on stroke.<sup>8</sup>

These translational findings are relevant to several aspects of atherosclerosis research. Because our findings predict that novel anti-inflammatory therapeutics targeting specific steps in the inflammatory cascade<sup>32</sup> may have differential site-specific efficacy, this work highlights the importance of assessing drug responses at the vascular site of interest. Indeed, the use of surrogate end points such as carotid intima-media thickness<sup>33</sup> to assess the efficacy of new drugs to prevent coronary events may not reflect the potential therapeutic effects at the targeted vascular site, again highlighting the importance of assessing atherosclerosis in multiple places.

There are potential limitations in this study. Several of these limitations relate to the gene expression comparisons. First, measuring gene expression of whole arteries prevents conclusions from being drawn about which specific cell types are responsible for the findings. However, our conclusions are based on the overall signal of inflammation within the arterial wall, which is ideally defined by gene expression profiles. Unlike our previous publication assessing gene expression in different vascular beds at different time points,<sup>3</sup> this study looked at gene expression at a single time point late in the development of advanced atherosclerosis and thus may miss the activation of genes important for earlier stages of lesion development. A further limitation of the gene expression comparisons is the number of separate genes evaluated. These multiple comparisons may result in a small number of unadjusted *P*-value estimates occurring by chance rather than as an indication of an unusual event. Additional limitations are associated with the translation of the study model to human disease. The type 1 diabetes mellitus model used in this study may not be translatable to factors leading to atherosclerosis related to insulin resistance. Finally, a second phase III clinical trial testing darapladib in patients with a recent acute coronary syndrome did not find a benefit on reducing major coronary events,<sup>34</sup> so these findings in pigs may not translate to humans.

In summary, despite the identical systemic exposure to hypercholesterolemia and hyperglycemia, the development of atherosclerosis varies between vascular sites. By analyzing differences in plaque severity, gene expression profiles, and the response to a targeted anti-inflammatory, there is evidence that inflammatory pathways using Lp-PLA<sub>2</sub> may play a more significant role in COR atherosclerosis compared with AA atherosclerosis.

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