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# Changes in the phospholipid composition of the arterial cell can result in severe atherosclerotic lesions

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**Abstract**

The oxysterol concentration in the plasma and the phospholipid composition of vascular tissue obtained by coronary artery bypass grafting (CABG) were compared with plasma and vascular tissue from age and sex matched controls. The plasma from CABG patients had a higher concentration of oxysterols than was present in the controls. Human endothelial cells were cultured for 72 hours in a medium containing plasma obtained from CABG patients, from controls or from the same controls to which 5 oxysterols were added to make the total oxysterol level equivalent to that in the CABG plasma and then pulsed with calcium ($^{45}Ca^{2+}$) for one hr. A significantly higher influx of $^{45}Ca^{2+}$ was noted in the endothelial cells cultured in the plasma obtained from CABG patients and from the controls with 5 added oxysterols, but not in those cultured without added oxysterols indicating that oxysterols increased calcium influx into endothelial cells. A phospholipid analysis indicated that the arterial tissue from CABG patients had 48.2% sphingomyelin in its phospholipid fraction compared to 10% in arterial tissue from umbilical cords. The saphenous vein obtained during CABG surgery from the same patient had only 24% sphingomyelin in its phospholipid fraction and unlike the coronary arteries had no atherosclerotic lesions. The higher level of oxysterol in the plasma of patients suffering from severe atherosclerosis could increase the concentration of sphingomyelin in the arterial cell membrane and thereby increase calcium influx required for producing the calcific type VII lesions in the coronary arteries. 

*Keywords:* Oxysterols; Sphingomyelin; Calcium; Stenosis; Arterial bifurcations; Coronary heart disease

## 1. Introduction

Heart disease due to the development of atherosclerotic lesions in the coronary arteries is now the leading cause of death in human populations on all six continents [1]. Atherosclerotic lesions cause stenosis (narrowing) of the coronary arteries making less blood available for the heart to pump to the body. That atherosclerotic lesions develop in the arteries has been known for over 100 years but why lesions develop in the arteries and not in the veins of the same person remains unknown.

Stary [2] classified atherosclerotic lesions into eight types according to their order of development: type I isolated macrophage foam cells, type II multiple foam cell layers, type III preatheroma intermediate lesion, type IV atheroma, type V fibroatheroma, type VI fissured with ulcerated hemorrhagic thrombotic lesion, type VII calcific lesion and type VIII fibrotic lesion. Type I lesions were the first detectable accumulations of lipids associated with cell reactions in the intima. Although this lesion type was found by Stary [3] to be most frequent in infants and children, such lesions were also found in adults, particularly those who had little atherosclerosis in locations in arteries that were only moderately susceptible. Stary [2] stated "when 50% or more of the cross-sectional area of a lesion consists of mineral, it may be called a type VII or calcific lesion." He also found that the first evidence of calcification of arterial tissue occurs in the intimal muscle cells where the calcium remains embedded among the lipid core (plaque) causing stenosis [3]. When the cells die and disintegrate, mineralized cells then become part of the extracellular accumulation in the lipid core. Other investigators have also found calcium in atherosclerotic lesions [4–6].

Carpenter et al. [7,8] compared the lipid composition of human atherosclerotic lesions at different stages of devel-

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opment with normal aorta. Each category of lesion was more opulent than the normal artery in all the lipids measured and in oxidized lipids (oxysterols and hydroxyoctadecadienoic acids). A degree of overlap existed among the compositions of the various categories of lesion. The 27-hydroxycholesterol (27OHC) and 7$\beta$-hydroxycholesterol (7$\beta$OHC) levels were extremely low or undetectable in the normal artery but became significantly higher in each of the categories of lesions [7,8]. Fatty streaks showed the highest concentration of 7$\beta$OHC relative to cholesterol. Levels of the enzymatic product of 27OHC were significantly more abundant in advanced lesions than in intermediate lesions or fatty streaks. Chisolm et al. [9] treated LDL with cupric sulfate *in vitro* and found essentially the same compounds that Carpenter found in the atherosclerotic lesion, thus establishing as others have [10–19] that atherosclerotic lesions originated from oxLDL.

In previous *in vitro* studies with endothelial and smooth muscle cells in tissue culture, we found that oxysterols increased both sphingomyelin synthesis [20] and calcium uptake [21–23]. These studies showed that: (1) 27OHC enhanced markedly $^{45}Ca^{2+}$ uptake, and the enhancement was not diminished by nifedipine; (2) 27OHC decreased cholesterol concentration in the cell membrane by inhibiting cholesterol uptake and synthesis, and [$^{14}$C]cholesterol uptake was not LDL-receptor dependent; (3) 27OHC induced a shift of [$^{14}$C]acetate from cholesterol into phospholipid synthesis, but the radioactive incorporation into triglyceride and the cholesterol ester was inhibited by 27OHC; and (4) 27OHC inserted itself into the cell membrane. At a higher than normal level [20] 27OHC induced increased [$^{3}$H]choline incorporation into sphingomyelin accompanied by decreased radioactivity in phosphatidylcholine but did not alter [$^{3}$H]choline content in phosphocholine and cytidine choline 5′-diphosphate. The increased radioactivity in sphingomyelin induced by 27OHC was detected first, followed by detection of the enhanced $Ca^{2+}$ uptake and cytosolic free $Ca^{2+}$.

In the present study, we used the plasma, saphenous vein and arterial tissue obtained by coronary artery bypass grafting (CABG) to demonstrate that changes in the phospholipid composition of the arterial tissue can result in calcification severe enough to result in type VII lesions.

## 2. Methods

### *2.1. Arterial tissue source*

Available for this study were 17 men and 15 women in whom cardiac catheterization indicated 80 ± 8% stenosis of their coronary arteries. Written informed consent for CABG surgery and for accompanying blood tests had been obtained from each patient as well as for matching controls. The research review boards of both Carle Foundation Hospital and the University of Illinois approved the protocol. The controls were age and sex matched persons free of apparent coronary heart disease.

### *2.2. Analysis of free oxysterols in the plasma*

Ten ml of fasting blood prior to CABG surgery and 10 ml of fasting blood from the controls were collected in tubes treated with 0.01% heparin and 25 $\mu$g butylated hydroxytoluene (BHT) to prevent autoxidation of the lipids in the plasma. The tubes were immediately cooled on wet ice and centrifuged at 4°C. The plasma from both CABG surgery and from controls were kept frozen at −80°C until analysis for oxysterols.

The frozen sample was thawed to room temperature. To one ml of plasma in duplicate 150 ng of 25-hydroxycholesterol was added to serve as an internal standard in order to quantitate the free oxysterols. The lipids were extracted using 7.5 ml of chloroform: methanol (1:1, v/v) in the presence of 75 $\mu$g of BHT as an antioxidant and freed from solvent under vacuum in a rotary evaporator until dryness. The lipid residue was dissolved in 500 $\mu$l acetonitrile and vortexed after which 500 $\mu$l of water was added and vortexed again.

The lipid in acetonitrile-water was loaded on a reverse phase C-18 Sep-Pak cartridge (pre-washed with 10 ml acetonitrile then with 10 ml of distilled water). The sample was rinsed with 3 ml of ethanol:water (70:30, v/v) at a rate of 1 ml/min using a syringe pump and then the sample was eluted with 6 ml of acetonitrile. The eluted sample was then dried under argon for HPLC injection. The purification of oxysterol was carried out on a reverse phase HPLC on a Waters Liquid chromatograph model M-600 H pump and a Waters 484 absorbance detector using a 10 cm supelcosil LC-18, 5 $\mu$m column with ID 10 mm and a mobile phase acetonitrile:isopropanol:water (45:45:10, v/v/v) at a flow rate of 1.8 ml/min and UV detector at 210 nm. The fraction of oxysterols (2–12 min) was collected for quantitative analysis by GC. The retention time of the unoxidized cholesterol was 25.3 min under the reverse HPLC method. Prior to GC the oxysterols were derivatized to trimethylsilyl ethers (TMS) by addition of 500 $\mu$l of N,N-dimethylformamide (Fisher Scientific, Fair Lawn, NJ, USA) and 500 $\mu$l of bistrimethylsilyl-trifluoroacetamide (Supelco, Inc., Bellefonte, PA, USA) in teflon screw capped tubes under argon and allowed to stand for 1 hr at 60°C. The TMS reagent was removed under a stream of argon and the dry residue was redissolved in 50 $\mu$l iso-octane, and 1–2 $\mu$l were injected into a GC. Gas chromatography was performed on a Hewlett-Packard HP 5890II coupled with Hewlett-Packard HP 5971 quadrupole mass spectrometer. Identity of all peaks was confirmed by use of authentic standards to compare retention times and spectra. Mass spectrometer was acquiring data in a SIM mode—with simultaneous detection of 10 ions: m/z 129, 131, 201, 384, 403, 456, 461, 472, 474, 546. For identification purposes following ions and their intensities were used for each oxysterol:

Table 1
Oxysterols in the plasma from CABG patients compared to controls

| | WOMEN UNDER 60 YEARS | | MEN UNDER 60 YEARS | | WOMEN OVER 60 YEARS | | MEN OVER 60 YEARS | |
|---|---|---|---|---|---|---|---|---|
| | Controls | Patients | Controls | Patients | Controls | Patients | Controls | Patients |
| Number | 7 | 7 | 8 | 8 | 8 | 8 | 9 | 9 |
| 7α-OHC | 22.5 ± 5.7 | 27.2 ± 8.8 | 21.3 ± 5.4 | 29.1 ± 4.6[b] | 26.6 ± 4.2 | 26.8 ± 8.1 | 22.1 ± 3.6 | 26.3 ± 6.9 |
| 7β-OHC | 17.7 ± 4.4 | 18.6 ± 2.7 | 14.4 ± 2.8 | 21.5 ± 4.4[b] | 18.6 ± 6.5 | 23.4 ± 3.9 | 18.1 ± 5.2 | 21.5 ± 6.6 |
| βepoxy-C | 25.2 ± 6.9 | 42.4 ± 9.5[b] | 24.0 ± 4.6 | 34.6 ± 7.4[b] | 31.4 ± 9.3 | 36.4 ± 8.6 | 29.1 ± 8.5 | 37.8 ± 8.6[a] |
| αepoxy-C | 16.5 ± 4.6 | 20.7 ± 3.1 | 17.7 ± 2.5 | 19.1 ± 1.7 | 20.8 ± 5.9 | 21.5 ± 6.2 | 18.8 ± 3.7 | 21.8 ± 5.5 |
| Triol-C | 26.1 ± 4.8 | 36.9 ± 17.2 | 20.6 ± 7.5 | 34.6 ± 6.3[b] | 29.2 ± 8.3 | 38.1 ± 3.9[a] | 29.3 ± 9.3 | 35.7 ± 9.4 |
| 7keto-C | 27.4 ± 6.5 | 35.9 ± 12.2 | 23.0 ± 3.6 | 31.9 ± 7.1[b] | 30.8 ± 5.5 | 39.1 ± 6.2[a] | 29.3 ± 8.4 | 34.5 ± 8.6 |
| 27-OHC | 20.6 ± 3.9 | 23.5 ± 6.9 | 19.4 ± 4.8 | 20.9 ± 5.0 | 20.3 ± 4.9 | 25.8 ± 2.4[a] | 20.4 ± 2.1 | 24.2 ± 2.1[b] |
| Total | 156.3 ± 24.2 | 200.8 ± 31.1[a] | 141.0 ± 18.7 | 191.7 ± 20.2[b] | 177.9 ± 24.2 | 211.3 ± 13.4[b] | 167.2 ± 23.5 | 201.8 ± 25.3[b] |

7α-OHC: 7α-hydroxycholesterol, 7β-OHC: 7β-hydroxycholesterol, βepoxy-C: β-epoxycholesterol, αepoxy-C: α-epoxycholesterol, triol-C: cholestane-3β,5α,6β-triol, 7keto-C: 7-ketocholesterol, 27-OHC: 27-hydroxycholesterol. Results are expressed as ng oxysterols/ml plasma and given as mean ± SD.
[a] Means within the same line with a superscript letter are statistically different at levels of $P < 0.05$.
[b] $P < 0.01$, compared with controls.

7-α-OH-C: m/z 456 (100%), 129 (54)
7-β-OH-C: 456 (100), 129 (47)
β-epoxy-C: 129 (100), 201 (22), 384 (19), 474 (17)
α-epoxy-C: 129 (100), 210 (18), 384 (17), 474 (12)
triol-C: 129 (100), 403 (39), 456 (21), 546 (9)
7-keto-C: 129 (100), 201 (6), 472 (20)
27-OH-C: 129 (100), 456 (6), 546 (2)

### *2.3. The influence of the plasma on $^{45}Ca^{2+}$ influx into endothelial cells*

Endothelial cells (ECs) were scrapped from the arteries of umbilical cords obtained on cesarean section from 20–25-year-old women [23,24]. The ECs were cultured under 5% $CO_2$ at 37°C in minimum essential medium (MEM) (Sigma Chemical Company, St. Louis, MO, USA) supplemented with 20% calf bovine serum (CBS) (Sigma). The passages of ECs used in this study varied from 13–19. The plasma used for cell culture was heated at 55°C for 30 min to remove fibrinogen and was sterilized by passing through a 0.45 μm pore filter. The concentration of the plasma in the culture medium was 10% for both patients and controls.

These endothelial cells were further cultured in 12 well plates for 72 hrs in minimum essential medium (MEM) containing the plasma from either the fasting blood obtained prior to CABG surgery or from the controls or from the same controls with the addition of five oxysterols (7-ketocholesterol, cholestane-3β, 5α, 6β-triol, 7β-hydroxycholesterol, β-epoxy cholesterol, and 7α-hydroxycholesterol). The oxysterols were dissolved in absolute ethanol at a concentration of 10 mg/ml and stored at −20°C under $N_2$. One hour before culturing with cells these oxysterols were mixed at a ratio equivalent to their amounts in the CABG plasma. This mixture was diluted to 8 mg/ml with absolute ethanol. Then 50 ng of the mixture was added to 10 ml of the plasma from the controls. The final concentration of the oxysterols in this 10 ml plasma was almost equivalent to that of the 10 ml plasma from men under 60 yrs of age collected prior to CABG. After 72 h of culture, the cells were isolated and incubated for 1 h with 1 μCi of $^{45}Ca^{2+}$ in 1 ml MEM. The monolayers were then washed thrice with cold PBS containing 1 mM EGTA and were treated with 0.5 ml of 0.1 N NaOH. We used 0.3 ml of the digested cells for measuring radioactivity in a liquid scintillation counter and 0.15 ml for assay of protein (Bio-Rad, Hercules, CA). The oxysterols were obtained from Sigma and Research Plus Inc. (Bayonne, NJ, USA).

### *2.4. An analysis of veins obtained on CABG surgery and from umbilical arteries*

Lipids from coronary arteries and veins obtained on CABG surgery and from umbilical arteries were extracted with chloroform/methanol (2:1) and subjected to phospholipid analysis, according to the method of Skipski et al. [25]. The phospholipids were subjected to thin layer chromatography on silica gel G TLC plates with chloroform/methanol/acetic acid/water (25:15:4:2,v/v/v/v) as the developing solvent.

### *2.5. Statistical analysis*

All results are expressed as means ± SD. A students' t test was used to determine the statistical significance.

## 3. Results

Our lipid analysis of the plasma and arterial tissue obtained prior to and during CABG surgery revealed, when compared to controls, a higher concentration of oxysterols in the plasma, and a significantly higher concentration of sphingomyelin in arterial tissue. Approximately 45 ng more or 200.8 ng/ml of free oxysterols (Table 1) were present in the plasma obtained from CABG women under 60 yrs of age contrasted with controls having 156.3 ng/ml ($p <$

Table 2
$^{45}Ca^{++}$ influx into the endothelial cells cultured for 72 hrs with the plasma from CABG patients or from age- and sex-matched controls with or without added oxysterols

| $^{45}Ca^{++}$ influx (CPM/$\mu$g protein) | |
|---|---|
| Control plasma | 26 ± 5.1[a,b] |
| Patient plasma | 68 ± 7.1[a] |
| Control plasma ± oxysterols | 60 ± 6.3[b] |

[a,b] Values are mean ± S.D. of 12 separate experiments. Mean values with a common letter are significantly different at a level of $P < 0.001$.

0.05). The plasma obtained from CABG men under 60 yrs of age had 50.7 ng more free oxysterols than controls or 191.7 and 141.6 ng/ml ($p < 0.05$), respectively. The plasma obtained from CABG women over 60 yrs of age had 33.4 ng more free oxysterols than controls or 211.3 and 177.9 ng/ml, respectively, while plasma obtained from CABG men over 60 yrs of age had 34.6 ng more free oxysterols than the controls or 210.8 and 167.2 ng/ml, respectively ($p < 0.01$).

Endothelial cells cultured in CABG plasma and pulsed with $^{45}Ca^{2+}$ showed a significant increase in $^{45}Ca^{2+}$ influx (Table 2) in comparison to the cells cultured in the plasma from controls or 68 ± 7.1 and 26 ± 5.1 CPM/$\mu$g protein ($P < 0.001$), respectively. When 5 chemically synthesized oxysterols were added to the plasma of controls in an amount to match that present in plasma obtained on CABG surgery, the $^{45}Ca^{2+}$ influx did not differ significantly or 60 ± 6.3 and 68 ± 7.1 CPM/$\mu$g protein, respectively. The addition of the 5 synthesized oxysterols to plasma of the control group produced the same cytotoxicity as present in the plasma obtained from CABG surgery. These results indicated that the increased $^{45}Ca^{2+}$ influx into endothelial cells was due to the higher concentrations of oxysterols in plasma of CABG patients.

Approximately five times more sphingomyelin was found in the coronary artery tissue from CABG patients than arterial tissue from umbilical cords or 48.2% and 10.0%, respectively (Table 3). The thin and thick sections of the saphenous vein used in CABG surgery, on the other hand, did not vary significantly in sphingomyelin concentration or 24.2 and 24.3%, respectively.

Table 3
Comparison of phospholipid composition of veins and arteries obtained on CABG surgery

| Phospholipid (%) | | | | |
|---|---|---|---|---|
| | Veins | | Arteries | |
| | Thin | Thick | Umbilical Cord | CABG |
| SPH | 24.2 ± 2.2 | 24.3 ± 1.0 | 10.0 ± 0.3 | 48.2 ± 2.0 |
| PC | 35.8 ± 1.4 | 36.0 ± 1.7 | 43.5 ± 0.3 | 27.3 ± 1.6 |
| PE | 22.4 ± 1.4 | 20.7 ± 3.2 | 19.4 ± 0.5 | 12.6 ± 2.1 |
| PI + PS | 6.2 ± 2.1 | 5.9 ± 2.6 | 14.0 ± 0.3 | 6.7 ± 0.8 |
| Lyso PC | 6.1 ± 2.1 | 6.0 ± 2.6 | 3.4 ± 0.1 | 2.3 ± 1.0 |

SPH: Sphingomyelin, PC: Phosphatidylcholine, PE: Phosphatidylethanolamine, PI + PS: Phosphatidyl Inositol + Phosphatidyl serine, Lyso PC: Lysophosphatidylcholine.

Table 4
Phospholipid composition of coronary arteries from CABG patients

| | Age Range 26–37 | Age Range 51–76 |
|---|---|---|
| SPH | 47.2% | 48.4% |
| PC | 26.9 | 27.4 |
| PE | 13.9 | 12.4 |
| PS | 3.2 | 3.5 |
| Lyso PC | 3.2 | 2.1 |

SPH: Sphingomyelin, PC: Phosphatidylcholine, PE: Phosphatidylethanolamine, PS: Phosphatidylserine, Lyso PC: Lysophosphatidylcholine.

An analysis of the phospholipid composition of the coronary artery tissue indicated that the sphingomyelin concentration of this tissue from CABG patients ranging from 26–37 yrs of age was not significantly different from that of CABG patients ranging from 51–76 yrs of age or 47.2 and 48.4%, respectively (Table 4). These data indicated that a high content of sphingomyelin in arterial tissue was a risk factor to CABG patients regardless of age.

## 4. Discussion

Our study suggests that oxysterols changed the percentage composition of sphingomyelin in the phospholipid of the endothelial cell membrane with a consequential alteration of membrane permeability to calcium. To quote Ito et al. [26]: “Many reports indicate that cholesterol is tightly associated with sphingomyelin rather than other phospholipid in the plasma membrane. Such an interaction seems to be one of the key factors for the construction of the membrane microdomain rich in cholesterol and sphingomyelin that may exhibit many of the communicative cellular functions”. Shore et al. [27] established that phospholipids were synthesized by the arterial cell rather than derived from the plasma. McCandless and Zilversmit [28] further demonstrated in cholesterol-fed rabbits that sphingomyelin increased significantly in phospholipids of the atheromatous plaque. Steele observed a similar increase in sphingomyelin concentration in human atheromatous aorta and coronary vessels [29]. These changes in phospholipid composition agree with previous results which showed that phospholipid proportions in membranes are closely related to intracellular calcium content [30–37]. The results reported by Yla-Herttuala et al. support these observations [5]. They found that in a high coronary heart disease risk population, the deposition of calcium in the coronary arteries was directly proportional to an elevated sphingomyelin. Sphingomyelin may increase $Ca^{2+}$ influx mainly by additional calcium bonding to the cell membrane. Sphingomyelin located on the exterior of the plasma membrane [38] has an exposed polar head group accessible to the aqueous environment.

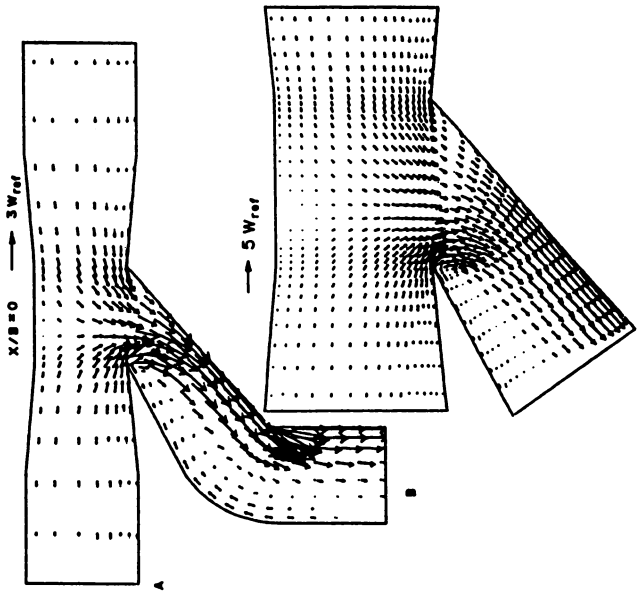


Fig. 1. Diagram of turbulent flow courtesy of Leschziner M.A. and Dimitriadis K.P. Computations of three-dimensional turbulent Flow in no-orthogonal junctions by a branch-coupling method. Computers & Fluids 17, 351–396, 1989.

The negative charge on sphingomyelin would thus be accessible for ionic bonding with $^{45}Ca^{2+}$. In an in vitro study using large unilamellar vesicles as a model membrane, we found [39] that sphingomyelin altered negatively charged lipid in the bilayer in such a way that the bilayer interacted more strongly with calcium.

In 1,200 patients in whom angiography had indicated the presence of coronary heart disease, the total cholesterol concentration did not reveal the level of stenosis [40]. However, all patients had higher concentrations of oxidation products of LDL and oxidation products of cholesterol in their plasma [41] than had person free of coronary heart disease. Moreover, an analyses of LDL and HDL cholesterol concentration in the plasma of CABG patients (data not shown) failed to reveal statistically significant differences or a correlation to the severity of stenosis [40]. Our lipid analysis of vascular tissue from CABG surgery suggested that changes occur in the composition of phospholipid of arterial tissue during the progression from type I to type VII lesions. Keaney [42] stated that the gene expression pattern in the arterial wall is subject to influence by modified forms of LDL, which altered both scavenger receptor (CD36) expression and the expression of pro-inflammatory genes [43].

The disturbed laminar flow pattern of fluids (Fig. 1) occurs near branch points [44], bifurcations, at major curves and at arterial geometries [45] that are typically associated with the earliest appearance (and subsequent progression) of atherosclerotic lesions [46]. An endothelial receptor for oxLDL, a designated lectin-like oxLDL receptor (LOX-1) [47], was recently identified [48,49]. The transient application of shear stress showed that the initial stimulation of shear stress was sufficient for induced expression of LOX-1 and that sustained application of shear stress was not required [50]. The over-expression of LOX-1 receptors at the bifurcation and the higher level of modified LDL and oxysterols in the plasma of persons needing CABG surgery could lead to a higher uptake of modified LDL, resulting in a greater delivery of oxysterols to the endothelial cells at the bifurcations. Our *in vitro* studies [20–24] indicated that a higher oxysterol concentration in the plasma enhanced sphingomyelin synthesis in the arterial cell membrane and resulted in more calcium deposition which can lead to the formation of type VII atherosclerotic lesions in the CABG patients [2].

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