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# Plasma Sphingomyelin Level as a Risk Factor for Coronary Artery Disease

Xian-cheng Jiang, Furcy Paultre, Thomas A. Pearson, Roberta G. Reed, Charles K. Francis, Min Lin, Lars Berglund, Alan R. Tall

**Abstract**—Only a fraction of the clinical complications of atherosclerosis are explained by known risk factors. Animal studies have shown that plasma sphingomyelin (SM) levels are closely related to the development of atherosclerosis. SM carried into the arterial wall on atherogenic lipoproteins may be locally hydrolyzed by sphingomyelinase, promoting lipoprotein aggregation and macrophage foam cell formation. A novel, high-throughput, enzymatic method to measure plasma SM levels has been developed. Plasma SM levels were related to the presence of coronary artery disease (CAD) in a biethnic angiographic case-control study (279 cases and 277 controls). Plasma SM levels were higher in CAD patients than in control subjects ( $60 \pm 29$  versus  $49 \pm 21$  mg/dL, respectively;  $P < 0.0001$ ). Moreover, the ratio of SM to SM+phosphatidylcholine (PC) was also significantly higher in cases than in controls ( $0.33 \pm 0.13$  versus  $0.29 \pm 0.10$ , respectively;  $P < 0.0001$ ). Similar relationships were observed in African Americans and whites. Plasma SM levels showed a significant correlation with remnant cholesterol levels ( $r = 0.51$ ,  $P < 0.0001$ ). By use of multivariate logistic regression analysis, plasma SM levels and the SM/(SM+PC) ratio were found to have independent predictive value for CAD after adjusting for other risk factors, including remnants. The odds ratio (OR) for CAD was significantly higher for the third and fourth quartiles of plasma SM levels (OR 2.81 [95% CI 1.66 to 4.80] and OR 2.33 [95% CI 1.38 to 3.92], respectively) as well as the SM/(SM+PC) ratio (OR 1.95 [95% CI 1.10 to 3.45] and OR 2.33 [95% CI 1.34 to 4.05], respectively). The findings indicate that human plasma SM levels are positively and independently related to CAD. Plasma SM levels could be a marker for atherogenic remnant lipoprotein accumulation and may predict lipoprotein susceptibility to arterial wall sphingomyelinase. (*Arterioscler Thromb Vasc Biol.* 2000;20:2614-2618.)

**Key Words:** sphingomyelin ■ risk factors ■ coronary artery disease

The association of lipid abnormalities and coronary atherosclerosis is well established. Case-control and prospective epidemiological studies have shown a direct correlation between coronary artery disease (CAD) and serum levels of total cholesterol and LDL cholesterol (LDL-C) and an inverse relationship between CAD and HDL cholesterol (HDL-C) levels.<sup>1</sup> However, compared with plasma cholesterol measurements, very little attention has been given to the relationship between phospholipids and CAD.<sup>2,3</sup>

Atherogenesis is initiated by the interaction of cholesterol-rich lipoproteins, such as LDL, with the arterial wall.<sup>4,5</sup> The uptake of lipoprotein cholesterol by macrophages, leading to foam cell formation, is a central event in the initiation and progression of atherosclerosis.<sup>6</sup> However, native LDL is incapable of generating foam cells from macrophages. Thus, it is thought that LDL is modified in the arterial wall by processes such as oxidation, leading to macrophage chemotaxis and the uptake of modified LDL by macrophage foam cells.<sup>7</sup> Retention of lipoproteins on the subendothelial matrix, followed by aggregation, has also emerged as a central

pathogenic process in macrophage foam cell formation and atherogenesis.<sup>8</sup> Lipoprotein aggregation in the vessel wall may result from enzymatic modification of LDL, induced by locally produced sphingomyelinase (SMase).<sup>9</sup>

It has long been known that sphingomyelin (SM) accumulates in human and animal atheroma and that the major source is plasma lipoproteins.<sup>10</sup> Plasma SM levels are increased in human familial hyperlipidemias, especially in familial hypercholesterolemia.<sup>11</sup> The concentration of SM relative to total phospholipids (principally phosphatidylcholine [PC] and SM), ie, SM/(SM+PC), is an important determinant of the susceptibility of lipoprotein SM to SMase.<sup>8,12</sup> These findings suggest that plasma SM levels and the relative SM concentration might be risk factors for atherosclerosis. However, plasma SM levels have never been systematically assessed as a risk factor for atherosclerosis in humans. This is partly due to the difficulties inherent in the classic method for SM measurement, which involves lipid extraction and thin-layer chromatography.<sup>13,14</sup> To overcome this difficulty, we have developed a novel enzymatic method for plasma SM deter-

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mination and have used this method to test the hypothesis that plasma SM levels are associated with CAD in an angiographic case-control study.

## Methods

### Subjects

Subjects were recruited from a patient population scheduled for diagnostic coronary arteriography at either Harlem Hospital Center in New York, NY, or the Mary Imogene Bassett Hospital in Cooperstown, NY. All consecutive patients scheduled for coronary arteriography at the 2 sites between June 1993 and April 1997 were approached. In the present study, 556 patients (341 men and 215 women) ethnically self-identified as African Americans ( $n=229$ ) or whites ( $n=327$ ) were enrolled. The mean age was 54.9 and 54.6 years for African American men and women and 56.8 and 56.5 years for white men and women, respectively. Exclusion criteria were as follows: age  $>70$  years, recent (within 6 months) myocardial infarction or thrombolysis, a history of percutaneous transluminal coronary angioplasty, surgery during the previous 6 weeks, a known communicable disease such as hepatitis or AIDS, or current lipid-lowering medication. Information on diabetes mellitus, hypertension, and smoking was obtained by a standardized questionnaire on entry into the study. Among white subjects, 25.6%, 27.3%, and 20.4% of the men and 20.5%, 25.6%, and 20.3% of the women were smokers, had hypertension, and had diabetes, respectively. The corresponding numbers for African Americans were 51.1%, 67.4%, and 24.2% for the men and 42.0%, 78.2%, and 35.6% for the women. The present study was approved by the Institutional Review Boards at Harlem Hospital, Bassett Healthcare, and Columbia University College of Physicians and Surgeons.

### Plasma SM Measurement

Enzymatic measurement of plasma SM levels was carried out by using a novel 4-step procedure. In the first step, bacterial SMase hydrolyzed SM to phosphorylcholine and *n*-acylsphingosine. Thereafter, the addition of alkaline phosphatase generated choline from phosphorylcholine. The newly formed choline was used to generate hydrogen peroxide in a reaction catalyzed by choline oxidase. Finally, with peroxidase as a catalyst, hydrogen peroxide was used together with phenol and 4-aminopyrine to generate a red quinone pigment, with an optimal absorption at 505 nm. The plasma SM levels were measured in a blinded fashion. The linear range of plasma SM in this assay was between 10 and 120  $\mu\text{g/dL}$ . The interassay coefficient of variation of the SM assay was  $2.8 \pm 0.3\%$ . PC levels did not influence SM measurement (data not shown). The detailed procedure will be published elsewhere.

To validate our novel SM assay, we compared the results with those obtained by the classic method.<sup>13,14</sup> The 2 methods were well correlated ( $r=0.91$ ,  $P<0.01$ ;  $n=60$ ).

### Plasma PC Measurement

The total choline-containing phospholipid in plasma was assayed by an enzymatic method (Wako Pure Chemical Industries Ltd). PC concentration was obtained by subtracting SM from total phospholipid concentration.

### Lipoprotein and Inflammatory Marker Measurements

Serum total cholesterol, triglycerides, and HDL-C were determined by using standard enzymatic procedures. HDL-C levels were measured after precipitation of apoB-containing lipoproteins with dextran sulfate,<sup>15</sup> and LDL-C was calculated by using the Friedewald formula.<sup>16</sup> C-reactive protein (CRP) was measured by a sensitive ELISA,<sup>17</sup> and fibrinogen levels were estimated by the clot-rate method of Clauss.<sup>18</sup> Remnant cholesterol was determined by the method of Nakajima et al.<sup>19</sup> Briefly, remnant lipoprotein was isolated on the basis of the removal of apoA-I-containing particles (HDL) and most apoB-containing particles (LDL, nascent VLDL, and nascent chylomicrons) by use of an immunoseparation technique, which has been shown to leave remnants of both intestinal and hepatic origin in the unbound fraction. The cholesterol concentration

in the unbound fraction was determined by a standard enzymatic assay.

### Angiographic Definition of CAD

Coronary angiograms were read by 2 experienced readers who were blinded to patient identity, the clinical diagnosis, and the lipoprotein results. The readers recorded the location and extent of luminal narrowing for 15 segments of the major coronary arteries.<sup>20</sup> The presence of CAD (ie, case) was defined as the presence of at least 50% stenosis in any 1 of 15 coronary artery segments.

### Statistical Analysis

Comparisons between groups were made by the Wilcoxon test, because plasma SM levels and SM/(SM+PC) ratios are not normally distributed. The Fisher exact test was used to calculate the probability value for the odds ratios (ORs) of the association of univariate categorical data with case-control status. Stepwise logistic regression was used to assess association with case-control status for multivariate models. SAS was used for all calculations.

## Results

Regarding established risk factors, mean total and LDL cholesterol levels were higher among patients with CAD compared with patients without CAD for both African Americans and whites. Also, mean triglyceride levels were higher among patients with CAD in both ethnic groups. Fibrinogen levels were significantly higher among patients with CAD in both ethnic groups, whereas there was no difference in CRP levels between cases and controls. A complete summary of the results for lipid and inflammatory parameters will be published separately (R.G.R., unpublished data, 2000).

For all subjects, patients with CAD had significantly higher plasma SM concentrations than did controls (Table 1). When analyzing the 2 ethnic groups separately, the plasma SM concentration was significantly increased in African Americans and whites with CAD ( $P=0.012$  and  $P=0.0001$ , respectively; Table 1). As seen in the Figure, the distribution of plasma SM levels was skewed in cases and controls. However, a consistent pattern was seen over the entire range of SM values: CAD cases were found more commonly at higher SM levels than were controls (panel A of Figure).

To evaluate whether the increased plasma SM levels among cases reflected an overall increase in phospholipid levels or an increased proportion of SM among total plasma choline-containing phospholipids, we compared the SM/(SM+PC) ratio (ie, relative concentration of SM) in case and control groups. The SM/(SM+PC) ratio was significantly higher for cases than for controls among all subjects as well as among African Americans and whites when the 2 groups were analyzed separately (Table 1). However, the difference in the SM/(SM+PC) ratio ( $\approx 14\%$ ) between cases and controls was smaller than the difference in total SM ( $\approx 22\%$ ), indicating that the ratio only partly accounted for the increase in plasma SM concentrations. Panel B of the Figure shows the SM/(SM+PC) ratio distribution in all subjects. Again, the distribution was skewed, but cases were found to have higher levels than controls over the entire range of SM/(SM+PC) values.

To investigate the possibility that plasma SM could act as a marker of atherogenic lipoprotein remnants, we measured remnant lipoprotein cholesterol levels. There were moderate but significant correlations between plasma SM levels and remnant lipoprotein cholesterol levels ( $r=0.451$ ,  $P<0.0001$ ) and between

**TABLE 1. SM Concentration and SM/(SM+PC) Ratio in Case and Control Samples**

	n	SM Values				SM/(SM+PC) Values			
		SM, mg/dL	Median	IQR	P*	SM/(SM+PC)	Median	IQR	P*
<b>All</b>									
Control	277	49±21	44	24		0.29±0.10	0.27	0.12	
Case	279	60±29	52	21	<0.0001	0.33±0.13	0.30	0.13	<0.0001
<b>Whites</b>									
Control	146	50±22	45	20		0.29±0.11	0.27	0.13	
Case	181	63±31	54	26	<0.0001	0.33±0.14	0.30	0.14	0.0026
<b>African Americans</b>									
Control	131	48±20	43	22		0.28±0.09	0.27	0.10	
Case	98	55±23	50	22	0.0118	0.32±0.10	0.31	0.12	0.0006

IQR indicates interquartile range.  
\*Wilcoxon test.

the SM/(SM+PC) ratio and remnant cholesterol levels ( $r=0.34, P<0.0001$ ).

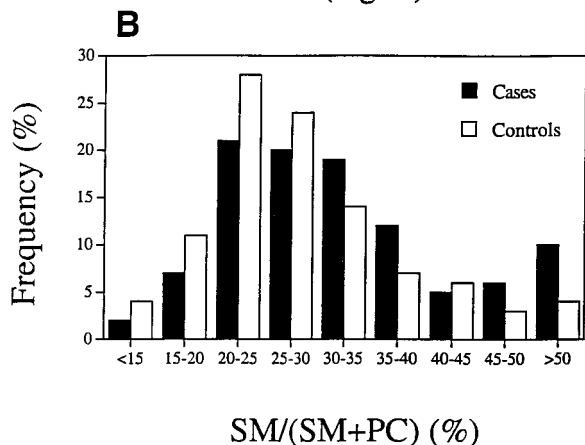
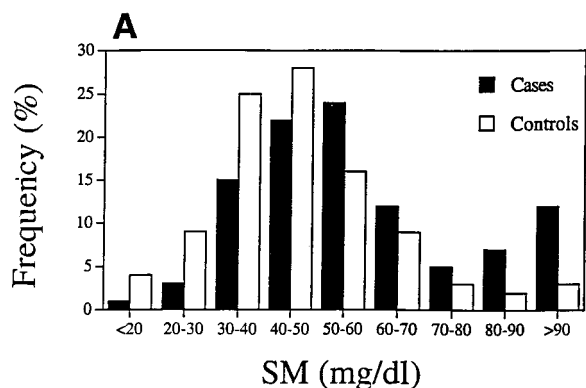
To evaluate the risk associated with increasing plasma SM concentration, we calculated ORs for each quartile relative to the first. Because African Americans and whites had similar mean and median values for plasma SM levels and SM/(SM+PC) ratios, we grouped all subjects together in this analysis. The OR for CAD for the third and fourth quartiles was significantly higher than the first quartile for both measurements (Table 2).

To evaluate whether the plasma concentration of SM and the SM/(SM+PC) ratio was associated with CAD indepen-

dent of other known risk factors, we carried out stepwise multivariate logistic regression controlling for age, diabetes, smoking, hypertension, LDL-C, HDL-C, logarithmically transformed triglycerides, remnant cholesterol, fibrinogen, and CRP. The OR for CAD increased with increasing quartiles of SM levels and SM/(SM+PC) ratios. As shown in Table 3, the OR for CAD in the third and fourth quartiles of plasma SM levels was significantly higher than in the first quartile ( $P=0.0001$  and  $P=0.0015$ , respectively), indicating that plasma SM level was an independent risk factor for CAD in this case-control study. In addition, the OR for CAD for the relative concentration of SM, ie, the SM/(SM+PC) ratio, was significantly higher for the third and fourth quartiles compared with the first quartile ( $P=0.0218$  and  $P=0.0028$ , respectively), indicating that the relative concentration of SM was also associated with CAD, independent of age, diabetes, smoking, hypertension, LDL-C, HDL-C, logarithmically transformed triglycerides, remnant cholesterol, fibrinogen, and CRP (Table 3).

**Discussion**

Although traditional measurements have focused on plasma total and LDL cholesterol as indicators of atherogenesis, a



Distribution of absolute and relative concentration of plasma SM in cases and controls. A, Absolute concentration. B, Relative concentration: SM/(SM+PC) ratio.

**TABLE 2. ORs of Association of Univariate Categorical Data With Case-Control Status**

Quartile	Control, n	Case, n	P	OR	Lower CI	Upper CI
<b>SM</b>						
1	89	50	...	1	...	...
2	75	64	0.33	1.30	0.80	2.11
3	59	80	<0.0001	2.83	1.74	4.60
4	54	85	0.0001	2.59	1.60	4.19
<b>SM/(SM+PC)</b>						
1	89	50	...	1	...	...
2	75	64	0.11	1.52	0.94	2.46
3	59	80	0.0005	2.41	1.49	3.91
4	54	85	0.0004	2.80	1.72	4.56

First quartile SM levels are 39.2 mg/dL, and SM/(SM+PC) ratios are 0.228. Second quartile SM levels are 49.2 mg/dL, and SM/(SM+PC) ratios are 0.285. Third quartile SM levels are 63.5 mg/dL, and SM/(SM+PC) ratios are 0.350. Fourth quartile SM levels are >63.5 mg/dL, and SM/(SM+PC) ratios are >0.350.

**TABLE 3. Multivariate Results From Stepwise Logistic Regression Controlling for Age, Diabetes, Smoking, Hypertension, LDL-C, HDL-C, Remnant Cholesterol, Logarithmically Transformed Triglycerides, Fibrinogen, and CRP**

Quartile	OR	P	Lower CI	Upper CI
SM				
3	2.81	0.0001	1.66	4.80
4	2.33	0.0015	1.38	3.92
SM/(SM+PC)				
3	1.95	0.0218	1.10	3.45
4	2.33	0.0028	1.34	4.05

Second quartile was eliminated from model by stepwise logistic regression processing.

body of in vitro and in vivo results<sup>11,12,21</sup> has suggested that plasma SM levels could also be related to atherosclerosis. However, this has never been systematically assessed, partly because of the difficulties of measuring SM in large numbers of samples. To overcome this problem, we have developed a simple enzymatic assay to permit the measurement of plasma SM concentrations. In the present study, we demonstrate for the first time that plasma SM levels were higher in cases with CAD than in controls, and this difference was found for African Americans and whites. The increase in plasma SM was in part selective, reflected as an increase in SM concentration relative to other phospholipids, ie, the SM/(SM+PC) ratio, and the relative SM concentration was also independently related to CAD case-control status. Although these findings are biologically plausible,<sup>9,22</sup> it will be important to confirm them in other samples and in a prospective study design.

A number of different mechanisms could explain the relationship between plasma SM and CAD case-control status. Because LDL is an atherogenic lipoprotein, SM could be a surrogate marker for LDL cholesterol levels. However, this appears unlikely because the SM relation to case-control status was independent of LDL-C levels (Table 3). SM could also be a marker for an inflammatory effect, and inflammatory markers such as CRP have been shown to be important risk factors for atherosclerosis.<sup>23</sup> However, in this case-control study, plasma SM levels did not correlate with 2 well-known inflammatory markers, fibrinogen and CRP (data not shown), and were independently related to case-control status in a multivariate analysis that included these measurements (Table 3). Thus, it is unlikely that SM is behaving as a surrogate inflammatory marker.

The hypothesis that we most favor is that plasma SM levels are determined by a unique set of metabolic determinants and that plasma SM, carried by lipoproteins, is directly involved in the atherogenic process subsequent to retention in the artery wall.<sup>8,9,24</sup> Thus, we propose that plasma SM levels are directly and causally related to atherogenesis.

Substantial evidence now supports the role of lipoprotein SM and arterial SMase in atherogenesis. SM carried into the arterial wall on atherogenic lipoproteins is acted on by an arterial wall SMase, leading to an increase in ceramide content and promoting lipoprotein aggregation.<sup>9</sup> LDL extracted from human atherosclerotic lesions is highly enriched in SM compared with plasma LDL. In addition, a significant

fraction of LDL extracted from fresh human lesions is aggregated and has a high content of ceramide, indicating that the LDL has been modified by SMase, resulting in aggregation.<sup>9</sup> The absolute and relative concentrations of plasma SM are both increased in atherosclerosis-susceptible animal models.<sup>12,20,26</sup> In vitro manipulation has shown that the relative SM concentration is an important determinant of susceptibility to SMase-induced aggregation.<sup>12,24</sup> Recently, transgenic animals with increased or decreased SMase activity in the arterial wall have been shown to have correspondingly altered atherosclerosis.<sup>26</sup>

Plasma lipoprotein SM is derived principally from biosynthesis in the liver. The rate-limiting step in SM biosynthesis is the enzyme serine:palmitoyl coenzyme A transferase, and the activity of this enzyme is increased in an atherosclerosis-susceptible animal model.<sup>12</sup> Inhibitors of serine:palmitoyl coenzyme A transferase have been described, so there might be some potential for therapeutic modulation of hepatic synthesis. Alternatively, the arterial wall SMase could represent another target for intervention.

Unlike plasma PC, SM is not degraded by plasma enzymes such as lecithin:cholesterol acyltransferase or by lipases.<sup>27,28</sup> Thus, SM removal from plasma is absolutely dependent on hepatic clearance mechanisms, such as the LDL receptor, the LDL receptor-related protein, or proteoglycan pathways. Because SM is not degraded in plasma, it becomes enriched in remnants of triglyceride-rich lipoproteins.<sup>12,20</sup> Several lines of evidence suggest that such remnants are particularly atherogenic,<sup>29</sup> but the relevant fraction of plasma lipoproteins has been difficult to measure. In part, plasma SM measurements may be acting as a marker of atherogenic remnant accumulation. This speculation is supported by the finding that plasma SM levels showed a significant, although moderate, correlation with remnant cholesterol levels. However, in multivariate analysis, plasma SM level remained as a significant predictor of CAD, even after additional adjustment for remnant lipoprotein cholesterol levels.

Although presently known risk factors have some predictive value for CAD, a major part of the variability in this process remains unexplained.<sup>30</sup> Also, therapy aimed at lowering LDL cholesterol reduces only a fraction (~30%) of the burden of atherosclerotic disease.<sup>31</sup> Although our findings that SM is a risk factor for CAD need to be confirmed in additional studies, they hold the promise of a simple test that may have independent predictive value for CAD and provide a novel therapeutic target.

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