

A Cross-Sectional Study of Human Serum Sphingolipids, Diet and Physiologic Parameters

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ABSTRACT Sphinganine and sphingosine, the two sphingoid base backbones of sphingolipids, are highly bioactive compounds that are of increasing interest to nutritionists because they occur in food and their metabolism can be altered by fungal toxins that contaminate some foods. Nonetheless, no studies of diet and sphinganine or sphingosine concentrations in serum have yet been reported. Here we describe a cross-sectional study of 265 residents of Linxian, People's Republic of China, which examines potential demographic, physiologic and dietary correlates of serum sphinganine and sphingosine in this population. Median concentrations of serum sphinganine and sphingosine were compared among strata for 29 different variables. For sphinganine, no significant differences were found. For sphingosine, significant differences were seen among strata of age, menstruation status, serum cholesterol, carotenoids, retinol, tocopherols, fresh and dried vegetable and fresh fruit consumption. Using multivariate linear regression with stepwise selection, we found that the significant predictors for serum sphingosine included total tocopherols, age, serum selenium and retinol, with a final $R^2 = 0.22$; for sphinganine, tooth loss was the sole correlate, with $R^2 = 0.015$. Analyses using ranked sphingolipid data or principal components analysis, to simplify the food variables, did not materially alter these results. This study represents the largest report of human serum sphingolipid concentrations to date and provides insight into potential explanatory variables that can be incorporated into future studies. *J. Nutr.* 131: 2748–2752, 2001.

KEY WORDS: • sphinganine • sphingosine • humans • serum • fumonisin

Sphingolipids were discovered over 100 years ago but have only recently been recognized for their importance in nutrition (1) and as mediators of the effects of mycotoxins such as fumonisins (2). Sphingolipids are a structurally diverse group of compounds composed of a long-chain sphingoid base backbone and an amino group, which is often substituted with a long-chain fatty acid. They are found primarily in cell membranes and are known to play roles in cell-cell and cell-matrix interactions, and as second messengers (3). The complex sphingolipid precursor sphinganine (Sa) and degradation product sphingosine² (So) have been studied widely in animals exposed to fumonisins (4) and have been proposed as biomarkers of fumonisin exposure in humans (5). Sphingosine is also the precursor to the second messenger and potent mitogen sphingosine 1-phosphate (6,7).

Sphingolipids are not thought to be required in the diet for normal growth and development because most sphingolipids are synthesized *de novo* (1). Although not required in the diet,

dietary sphingolipids have been shown to cross the colonic membrane and to exert biological activity, most notably the inhibition of colon carcinogenesis (8,9).

Compared with other lipids, a paucity of data exists regarding normal human serum concentrations of Sa and So despite growing interest in their role in human health. Three studies with 14, 15 and 183 subjects, respectively, reported human urine sphingolipid levels (10–12). Two studies with 33 and 183 subjects reported sphingolipid concentrations in human serum (12,13). None of these studies directly correlated diet or other parameters with individual sphingolipid concentrations.

Our group recently completed a study examining the association between serum sphingolipid concentrations and subsequent incidence of esophageal cancer in a Chinese population at high risk for esophageal squamous cell carcinoma and gastric cardia adenocarcinoma (14). No significant associations between serum sphingolipid concentrations and esophageal cancer risk were found. We now use these same subjects in a cross-sectional study to assess correlations between serum Sa and So and various demographic characteristics, physiologic parameters, dietary intake of certain foods and serum concentrations of carotenoids, tocopherols, retinol and cholesterol in a group of 265 individuals.

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² Sphingosine refers to D-erythro-1,3-dihydroxy, 2-aminooctadec-4-ene. Sphinganine has the same structure but lacks the 4,5-*trans* double bond.

SUBJECTS AND METHODS

Study population. Participants in this study were all residents of Linxian, a county in Henan Province, People's Republic of China. The individual participants in this study were selected as part of a nested case-control study of esophageal cancer conducted within a large nutrition intervention trial (15). In the original trial, 29,584 cancer-free individuals, aged 40–69 y, were recruited from the general population of four Linxian communes in 1985. All participants were interviewed to assess lifestyle, dietary patterns and medical history and were given a physical examination. For this nested study, ~100 esophageal cancer cases and 200 controls were frequency matched on sex and age strata (≤ 50 , >50 –59 or ≥ 60 y) for inclusion. A small number of selected samples were excluded because serum samples were unavailable for sphingolipid or serum nutrient analysis. A total study population of 265 individuals (136 women and 129 men) was used for all analyses. Sphingolipid levels were not different between controls and eventual cases; thus, they have been combined for the purposes of this study. This study was approved by the Institutional Review Boards of the U.S. National Cancer Institute and the Cancer Institute of the Chinese Academy of Medical Sciences.

Serum measurements. As part of the original nutrition intervention trial baseline examination, blood samples were collected in 1985 from all consenting participants (15). Serum was separated from whole blood by centrifugation ($1000 \times g$, 15 min) and stored at -80°C until divided into aliquots for analysis. Sample preparation and HPLC analyses for serum sphingolipid levels were carried out as previously described with minor modifications (16). Serum was washed from vials with methanol and 200 pmol of sphinganine analog (C20-Sa) was added as an internal standard (17). Samples were extracted with chloroform and saponified with potassium hydroxide in methanol. Extracted sphingoid bases were then derivatized with *o*-phthalaldehyde. Samples were separated by HPLC on a C18 column using a methanol gradient and fluorescence detector. The absolute quantities of Sa and So were determined by comparing the areas under the peaks to the area under the C20-Sa internal standard peak. All samples were analyzed in duplicate. Quality control samples were run in most HPLC batches. The quality control samples consisted of aliquots of a pooled blood sample collected from a Linxian blood bank in 1995. The sample's identity was unknown to the laboratory analysts.

Serum cholesterol and serum selenium were measured using established methods (18). Serum total carotenoids [β -carotene + β -cryptoxanthin + lutein/zeaxanthin (α -carotene and lycopene were generally below the limit of detection for our method)], retinol and total tocopherols (α -tocopherol + γ -tocopherol) were measured as previously described (19).

Variable definitions. Alcohol use was defined as any consumption of alcohol in the previous 12 mo. Tobacco use was defined as ever using tobacco for ≥ 6 mo. Age was divided into three strata (≤ 50 , >50 –59 and ≥ 60 y). Other continuous explanatory variables (height, weight and serum measurements) were dichotomized by splitting at the median. Food variables were dichotomized as none vs. any consumption in the previous 12 mo. If all participants were consumers, the number of meals per year was calculated and the variable was dichotomized by splitting at the median.

Statistical methods. Statistical analyses were completed using the SAS/STAT statistical software package (20) and the S-Plus programming language (21). All *P*-values refer to two-sided tests. Statistical significance was set at $P < 0.05$.

Difficulties in HPLC analysis of Sa led to an unknown interfering peak in the Sa measurements. No such interference was seen in the So measurements. The Sa interference showed a clear time trend over the 60-d HPLC analysis period. Locally weighted robust regression (*lowess*) was used to estimate and remove this time trend. Because this process gives an adjusted variable with mean zero, the unadjusted overall means for Sa and So were added to the individual adjusted values. These adjusted values showed essentially no time trend in the HPLC analysis. For some analyses, serum sphingolipid concentrations were replaced with rank values. Ties were represented by the mean of the ranks.

For each individual, the average of the two measurements for Sa or So was used. To improve normality, each average sphingolipid value was transformed by natural logarithms before further analysis. Statistical comparisons among strata were made using the nonparametric Wilcoxon rank-sum test for all categories except age for which the Kruskal-Wallis test was used to compare the three strata. Spearman's rank correlation and multiple linear regression were also used to examine some variables.

To examine multivariate models of serum sphingolipid predictors, the forward stepwise regression procedure within SAS/STAT PROC REG was used. All variables, except menstruation status, which was excluded because it is sex-specific, were examined as potential explanatory variables for Sa and So as concentrations or ranks. Entrance into the model required a regression coefficient with a *P*-value < 0.15 , whereas retention in the model required a *P*-value < 0.05 . Stepwise regression begins with an empty model and adds variables one at a time while reevaluating each variable at each step of the regression for inclusion/exclusion in the model (20).

A data reduction, using principal components analysis (22,23), was used to simplify the representation of the 15 dietary variables because of the high degree of correlation among these variables. SAS/STAT PROC PRINCOMP was used to carry out this analysis (20). Eigenvalues of the correlation matrix were inspected and the minimum number of principal components sufficient to explain $>50\%$ of the variance in the original data was selected. Individual subject values for each of the principal components were calculated and used in place of the original 15 dietary variables in a stepwise regression analysis as described above.

RESULTS

The current study was undertaken subsequent to a nested case-control study of serum Sa and So and risk of esophageal cancer. Approximately one third of the subjects went on to develop esophageal cancer 1–6 y after serum was collected. Serum sphingolipid levels did not differ between controls and eventual cases (14). The collection of the serum before diagnosis as well as the lack of association between sphingolipid levels and esophageal cancer incidence allowed us to combine the groups for the purposes of this study.

Median serum concentrations for the entire study population were 47.3 nmol/L (unadjusted) and 52.2 nmol/L (*lowess* adjusted) for Sa and 61.8 nmol/L (unadjusted) and 64.0 nmol/L (*lowess* adjusted) for So. In Africa, mean serum concentrations ranged from 19.7–66.8 and 74.7–215 nmol/L for Sa and So, respectively, in three different populations (12). In addition to Sa and So, several other serum parameters were also measured. Median values of these other serum parameters were as follows: cholesterol 3.78 mmol/L; total carotenoids 0.96 $\mu\text{mol/L}$; retinol 1.17 $\mu\text{mol/L}$; total tocopherols 20.11 $\mu\text{mol/L}$; and selenium 8.99 $\mu\text{mol/L}$.

Table 1 presents unadjusted and *lowess*-adjusted median serum Sa and So concentrations of 29 stratified parameters. No significant differences were found among strata in the Sa measurements. Significant differences were found in the unadjusted So measurements for age, menstruation status, serum cholesterol, retinol, carotenoids, tocopherols, and fresh vegetable and fresh fruit consumption in the summer season. For the *lowess*-adjusted So values, significant differences were seen for the same nonfood items, fresh fruit consumption in the summer season and dried vegetable consumption throughout the year.

Because menstruation and age were highly correlated ($r = 0.72$, $P < 0.0001$, in this data set), we examined the correlation among sphingolipid levels, age and menstruation status using multiple linear regression. When age was included in the model, menstruation status was no longer significantly associated ($P = 0.59$) with serum So levels.

TABLE 1

Median serum sphinganine and sphingosine concentrations in serum by 29 stratified variables¹

		Level	Sphinganine		Sphingosine	
			Unadjusted	Adjusted ²	Unadjusted	Adjusted
					<i>nmol/L</i>	
Overall			47.3	52.2	62.3	64.0
Sex	Female	51%	51.6	51.9	64.2	65.1
	Male	49%	46.9	52.4	61.0	63.2
Age, y		≤50	48.0	51.6	56.5	59.0
		51–59	50.4	51.7	61.6	61.3
		≥60	46.8	52.9	72.5***	74.3***
Use of alcohol ³		No (79%)	46.3	52.1	63.9	65.0
		Yes (21%)	79.2	52.4	60.3	63.4
Use of cigarettes ⁴		No (66%)	48.3	51.9	64.2	64.4
		Yes (34%)	42.3	52.4	61.0	63.2
Tooth loss ⁵		No (20%)	56.1	50.1	58.9	61.7
		Yes (80%)	46.5	52.6	63.9	65.1
Height, m		Low (1.53)	48.0	53.0	67.6	66.4
		High (1.64)	46.9	51.0	60.4	61.6
Weight, kg		Low (49)	45.0	52.4	63.1	65.0
		High (59)	49.7	52.0	61.8	63.2
Body mass index, kg/m ²		Low (20.0)	46.6	52.6	64.3	65.4
		High (23.0)	48.6	51.4	60.4	63.2
Menstruating		No (71%)	51.6	53.0	71.6	67.9
		Yes (29%)	48.0	47.8	58.8**	57.6**
Serum cholesterol, mmol/L		Low (3.21)	48.6	50.7	55.4	57.6
		High (4.34)	46.9	52.7	68.0***	69.4***
Serum carotenoids, μmol/L		Low (0.69)	44.1	51.5	55.5	57.4
		High (1.31)	53.4	52.5	70.1***	69.8***
Serum retinol, μmol/L		Low (0.92)	47.3	51.0	56.2	58.0
		High (1.36)	47.3	52.7	67.6**	69.5**
Serum tocopherols, μmol/L		Low (17.00)	46.3	51.2	54.3	56.1
		High (24.13)	48.6	53.0	71.5***	70.4***
Serum selenium, μmol/L		Low (7.60)	52.0	51.6	64.7	66.2
		High (10.39)	44.4	52.8	58.1	61.3
Foods						
Persimmon bread		None (96%)	47.1	52.3	63.8	64.6
		Any (4%)	61.6	51.8	49.6	51.7
Moldy bread		None (82%)	47.0	52.1	64.0	65.4
		Any (18%)	53.9	52.5	56.3	57.7
Fresh vegetables	Summer ⁶	Low (360)	46.5	52.5	58.8	62.0
		High (540)	49.0	52.1	67.3*	66.6
Fresh vegetables	Winter ⁶	Low (180)	52.4	53.2	62.1	65.1
		High (360)	46.9	51.4	63.4	63.2
Dried vegetables	Summer	None (96%)	47.0	52.2	61.6	63.2
		Any (4%)	76.8	51.2	80.4	80.4*
Dried vegetables	Winter	None (24%)	50.0	51.4	56.3	57.6
		Any (76%)	47.2	52.4	64.5	66.3*
Pickled vegetables	Summer	None (99%)	47.4	52.1	62.3	64.0
		Any (1%)	44.1	58.0	62.5	67.0
Pickled vegetables	Winter	None (94%)	47.1	52.3	61.8	63.3
		Any (6%)	49.2	51.4	68.7	70.1
Fresh fruit	Summer	None (46%)	46.8	52.0	69.0	67.5
		Any (54%)	48.3	52.5	55.9**	60.3**
Fresh fruit	Winter	None (85%)	47.1	51.7	62.1	64.2
		Any (15%)	63.9	55.6	63.9	63.6
Dried fruit	Summer	None (76%)	47.0	52.5	64.0	65.0
		Any (24%)	51.6	51.4	59.0	62.0
Dried fruit	Winter	None (81%)	47.2	52.1	63.7	65.1
		Any (19%)	48.0	52.7	57.0	61.6
Meat		None (16%)	64.6	52.6	57.1	60.3
		Any (84%)	45.6	52.2	63.9	65.1
Eggs		None (28%)	48.0	52.2	68.8	66.4
		Any (72%)	47.0	52.2	60.5	63.1
Foods cooked in oil		Low (5)	41.6	52.2	63.0	66.1
		High (12)	49.7	52.2	61.8	62.2

¹ Each variable was divided into the specified categories and the median concentration for the category is reported. For each variable, the percentage of respondents in each category (categorical variables) or the median value for the subcategory (continuous variables) is given. Food variables were examined as continuous variables only when all respondents were consumers (meals per year given in parentheses). Medians were compared among strata using the Wilcoxon rank-sum test, except for age, whose 3 strata were compared using the Kruskal-Wallis test. * Significantly different at $P < 0.05$; ** significantly different at $P < 0.01$; *** significantly different at $P < 0.001$.

² See methods for correction procedure.

³ Any alcohol consumption in previous 12 mo.

⁴ Ever smoked cigarettes for ≥ 6 mo.

⁵ Any tooth loss.

⁶ Summer includes Summer and Autumn while Winter includes Winter and Spring.

TABLE 2

Results of stepwise linear regression with sphingolipids as continuous and ranked variables¹

Sphingolipids as continuous variables				
Analyte	Correlate	P-value	Partial R ²	Model R ²
Sphinganine	Lost teeth	0.046	—	0.015
Sphingosine	Total tocopherol	<0.0001	0.146	0.146
	Age	0.0005	0.0387	0.185
	Selenium	0.0081	0.0217	0.206
	Retinol	0.035	0.0134	0.220
Sphingolipids as ranked variables				
Analyte	Correlate	P-value	Partial R ²	Model R ²
Sphinganine	Meat	0.045	0.0152	0.0152
	Fresh fruit (summer)	0.045	0.0151	0.0302
Sphingosine	Total tocopherol	<0.0001	0.168	0.168
	Age	0.0002	0.0423	0.210
	Retinol	0.0015	0.0301	0.240
	Selenium	0.0066	0.0213	0.261

¹ Entrance in to the model required $P \leq 0.15$, whereas retention in the model required $P \leq 0.05$. Final model R² values are given in bold.

To determine multivariate relationships between demographic and food intake variables and serum sphingolipid levels, stepwise multiple linear regression with all variables except menstruation status was used with the *lowess*-adjusted serum sphingolipid levels, both as serum concentrations and as ranks. The results of these analyses, with sphingolipid values and ranks separately, are presented in Table 2. When serum sphingolipid concentrations were used, only tooth loss was sufficiently correlated with Sa to be retained in the model. The R² for this model was very low, only 0.015. The model for serum So retained four parameters, i.e., total tocopherol, age, serum selenium and serum retinol. The total R² for this model was 0.22, with a majority of the variability explained by the correlation with total serum tocopherols, R² = 0.15.

When rank values were substituted for serum concentrations in the stepwise regression analysis, similar results were found (Table 2). For serum Sa, two food variables, meat consumption and fresh fruit in the summer, replaced lost teeth as explanatory variables, but the overall model R² was still a low 0.03 and individual P-values were of borderline significance. For So, the same four variables were retained and the overall model R² rose to 0.26.

Dietary variables had a high degree of correlation with each other (data not shown). To simplify these variables, we used principal components analysis to reduce the number of variables used to represent the diet (22,23). We found that the 15 dietary variables could be reduced to 5 principal components, which represented 58% of the variance in the dietary data. When individual participant values for these 5 principal components were substituted into the stepwise regression model for the 15 original dietary factors, we found results identical to those of the first regression analyses presented above. Dietary factors still had little effect on interindividual variation in serum sphingolipid concentrations.

DISCUSSION

All participants in this study were residents of Linxian, People's Republic of China, an area with some of the highest

rates of esophageal and gastric cardia cancer in the world (24). Most people in Linxian are functional vegetarians, consuming little or no animal products, and have diets borderline or inadequate in many nutrients (25). The concentrations of Sa and So were measured in this group as biomarkers of exposure to the fungal toxin fumonisin for a case-control study of this exposure and esophageal cancer, which was nested in a larger nutrition intervention trial in Linxian. This nested case-control study showed no association between serum sphingolipid concentrations and esophageal cancer incidence (14). To further understand the physiology of Sa and So, we conducted a cross-sectional study of 265 participants to assess physiologic and dietary correlates of serum Sa and So levels. Little prior information was available as a guide in the selection of potential correlates with serum Sa and So. Therefore, no a priori hypotheses regarding the potential associations were used, and the results are presented not as a predictive model but as information that may be useful to guide future studies.

When sphingolipid concentrations were compared among strata of other parameters, no significant differences by strata were found with serum Sa. In contrast, many correlates of serum So were found, particularly age and serum values for cholesterol, retinol, total carotenoids and total tocopherols. Multiple linear regression analysis, with Sa and So as continuous variables and as ranks, demonstrated the same pattern, with very few variables correlating with Sa, whereas multiple factors (total tocopherols, age, retinol and selenium) were associated with So. In addition, the relative strength of the associations was greater for So than Sa. These two sphingolipid bases may be influenced by different factors because Sa is an intermediate of de novo sphingolipid biosynthesis, whereas So is formed during the turnover of complex sphingolipids (1).

No previous studies have examined the association between age and serum sphingolipid base concentrations. There is, however, a growing literature on changes in sphingolipid metabolism with aging, and the possibility that these changes may contribute to age-related disease [for examples, see references (26) and (27)]. Studies with old (24 mo) vs. young (5 mo) rats have shown that aging is accompanied by an elevation in hepatic sphingomyelin turnover, a decrease in sphingomyelin synthesis and an increase in ceramidase activity, all of which might result in increased production of So (26). Our finding that serum So increases significantly with age is consistent with the findings in this model.

Cell culture studies have suggested that several serum vitamins should interact with the sphingolipid pathway. In one study, treatment of cells with retinoic acid caused an increase in ceramide but not So (28). In a different cell culture system, α -tocopherol was shown to inhibit sphingomyelinase activity (29). Sphingolipid concentrations have been previously shown to correlate with serum cholesterol in laboratory and domestic animals (30). This association may be linked to their co-occurrence in so-called "microdomains" within the plasma membrane (31). Inhibitors of cholesterol synthesis can affect sphingolipid metabolism. The converse, alteration of cholesterol homeostasis by changes in sphingolipid metabolism, has also been demonstrated after experimental exposure of laboratory animals to fumonisin B₁, which acts by disrupting sphingolipid biosynthesis (30). Serum So was different among strata for cholesterol in the univariate analysis but did not appear in our final multivariate model, possibly because of its correlation with serum tocopherol.

A small number of studies have examined associations between selenium and sphingolipids. One study reported that in areas of China in which pathologically low serum selenium levels exist, and the attendant Kaschin-Beck disease is en-

demic, serum concentrations of sphingomyelin are elevated (32). Similarly, in rats fed a low selenium diet, significant elevations in serum sphingomyelin have been noted (33). Our study also showed an inverse association between serum selenium and sphingolipid levels.

When serum Sa and So have been assayed in mice and rats exposed to fumonisin B₁, clear sex differences have been seen, with only female mice and male rats responding with elevations in serum Sa (31,32). Nonetheless, we saw no differences between males and females in serum sphingolipid concentrations. A recent study that examined human serum Sa and So in Kenya and two areas of South Africa also reported no sex differences in serum sphingolipid concentrations (12).

Only a few correlations between dietary variables and serum sphingolipid concentrations surfaced in this study, but this may be due to the relatively crude dietary assessment, our focus on only So and Sa (serum also contains other sphingolipids such as So 1-phosphate and sphingosylphosphocholine) (35,36), the limited variability in the diet of the study participants, the deficient nature of the diet or other factors. The possibility that recently ingested sphingolipids might contribute to the amounts found in serum should also be considered. Sphingolipids are not thought to be major components of most foods (or required as nutrients) (1). Nonetheless, when mice have been fed radiolabeled sphingolipids, a small portion of the ingested sphingoid bases was found in the circulation (8). Even small amounts may have health significance because when sphingolipids have been fed to mice in amounts similar to those in food, they suppressed chemically induced colon carcinogenesis (9).

In summary, this cross-sectional study of serum sphingolipid correlates in a Chinese population, using univariate and multivariate analyses, found few associations between serum concentrations of Sa and demographic characteristics, diet and physiologic parameters. In contrast, many parameters were correlated with serum So, including age, serum tocopherols, carotenoids, selenium, retinol and cholesterol.

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