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Plasma sphingolipids and lung cancer: A population-based, nested case-control study

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Abstract

BACKGROUND—Sphingosine-1-phosphate (S1P) and ceramides are bioactive signaling sphingolipids that regulate pathways that are central to cancer pathogenesis.

METHODS—A nested case-control study was implemented to test if pre-diagnostic circulating concentrations of S1P and ceramides were associated with future lung cancer risk. In the community-based CLUE II cohort study in Washington County, Maryland, the study consisted of 100 incident lung cancer cases, each matched to two cancer-free controls on age, sex, race, and cigarette smoking status. Plasma stored at −70° C at the beginning of follow-up in 1989 was assayed for sphingolipids using liquid chromatography-mass spectrometry methodology (LC/MS/MS).

RESULTS—Compared to controls, geometric mean plasma concentrations of S1P and total ceramides were 2.9% (p = 0.10) and 5.1% (p = 0.02), respectively, greater in lung cancer cases. For S1P, the odds ratios (ORs) and 95% confidence intervals (CI) for lung cancer risk were 2.7 (1.2–5.9), 2.7 (1.1–6.4), and 1.9 (0.8–4.5) for the second, third, and highest fourths, respectively, compared to the lowest fourth (overall p-value 0.006). Compared to those with total ceramide concentrations in the lowest fourth, the ORs (and 95% CI) for lung cancer risk were 1.6 (0.7–3.3), 1.5 (0.7–3.4), and 2.1 (0.9–4.7) for the second, third, and highest fourths, respectively (p-for-trend 0.01).

CONCLUSION—Higher concentrations of S1P and total ceramide in plasma were associated with increased future risk of lung cancer.

IMPACT—These novel findings suggest that perturbation of sphingolipid metabolism and S1P generation may either contribute to the etiology of lung cancer or be a marker of latent lung cancer.

Keywords

lung carcinoma; ceramides; sphingosine -1-phosphate; sphingolipids; epidemiology

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CONFLICTS OF INTEREST
The authors report no conflicts of interest.

INTRODUCTION

Sphingolipids are a family of membrane lipids that have structural roles in the regulation of the fluidity and subdomain structure of the lipid bilayers. Furthermore, bioactive sphingolipids, such as sphingosine-1-phosphate (S1P) and ceramides, are signaling molecules involved in the activation of pathways that are directly relevant to carcinogenesis (1). S1P promotes inflammation, cell survival, tumor growth and metastasis, whereas ceramides have anti-cancer properties by promoting apoptosis and senescence (1–4). Ceramides are a family of species defined by chain length and number of double bonds, so that individual ceramides with different fatty acid chain lengths (C_{14}-C_{26}-ceramides) may have distinct functions that would affect a role in pro- or anti-carcinogenesis (3).

Bioactive sphingolipids are clearly relevant to lung physiology. The relationship of the bioactive sphingolipid pathway to pulmonary disease has been studied in cellular, tissue, and animal models (5–7), including lung cancer models (8,9). Serum S1P was observed to mediate lung metastasis of bladder and melanoma tumors in preclinical models (10). Despite the important roles that S1P and ceramides play in pathways that are central to cancer pathogenesis, they have yet to be investigated with respect to lung cancer risk in epidemiologic studies. To address this lack of evidence, the present study was carried out to test whether pre-diagnostic circulating concentrations of S1P and ceramides in stored plasma were associated with future risk of lung cancer in a population-based prospective study.

METHODS

The CLUE II cohort study was used to test the question of whether plasma concentrations of the sphingolipids S1P and ceramides prior to cancer are associated with future lung cancer risk. Established in 1989, the CLUE II cohort was named for its campaign slogan, "*Give Us a Clue to Cancer and Heart Disease*." The cohort was established during May through October 1989, when 25,081 people who had a Washington County address and were greater than 18 years of age agreed to participate in CLUE II. Demographic characteristics, smoking status, and number of cigarette smoked per day were obtained using a brief questionnaire. At that time, participants also provided blood samples (20 ml) for use in biomedical research, with samples drawn into a 20 mL Vacutainer tube containing heparin and immediately refrigerated until centrifugation. Centrifugation usually took place within 6 hours, and always within 24 hours, of the blood draw. Once centrifuged, aliquots of plasma were separated and stored at −70° C. Ascertainment of lung cancer cases was achieved through linkage with the Washington County Cancer Registry, the Maryland State Cancer Registry, and death certificates.

Case and control selection

Within the community-based CLUE II cohort study, we conducted a nested case-control study of 100 primary incident lung cancer cases, each matched to two cancer-free controls. The lung cancer cases were those whose first ever cancer diagnosis was a primary lung cancer that was diagnosed between January 1, 1990 and June 30, 1997. For each case, two controls were selected who had no history of cancer, were alive at the time of the case's lung cancer diagnosis, and who matched the case by age (+/− two years), race (all were Caucasian), gender, and smoking status (never, former, current). Cases and their matched controls who were current or former smokers were also matched on the number of cigarettes smoked per day (cpd). First, the lung cancer cases were classified into three groups based on the usual number of cigarettes they had smoked per day: ≤19, 20–39, and ≥40. Lung cancer cases who smoked ≤19 cpd were matched to controls within ±5 cpd. Lung cancer cases who smoked 20–39 cpd were matched within ±10 cpd. Lung cancer cases who smoked ≥40 cpd

were matched to controls within ±20 cpd; for example, potential controls for a lung cancer case who smoked 80 cpd could range from smokers of 60 cpd to 100 cpd. The reason that the allowable ranges expanded with heavier smoking was because the pool of potential controls was smaller for heavier smoking categories.

Assays

For each of the 100 lung cancer cases and 200 matched cancer-free controls, 200 microliters of plasma were aliquotted and shipped to the Lipidomics Shared Resource Laboratory at the Medical University of South Carolina. Sphingolipids, including S1P, C_{14}-C_{26}-ceramides, and sphingosine, were assayed using a liquid chromatography-mass spectrometry (LC/MS/MS)-based lipidomics approach (11). Each plasma sample was assayed in duplicate. Laboratory personnel were blinded to the case-control status of the samples. The plasma samples were organized in case-control sets comprised of one case and two matched controls. Assays for each set were performed on the same day.

Thirty quality control samples were randomly interspersed with the samples in groups of three to mimic the typical case-control set of three. Based on the assay results from these quality control samples, the intra-set and inter-set coefficients of variation were estimated to be 12% and 19%, respectively, for total ceramides and 9% and 15%, respectively, for S1P.

This research was approved by the Institutional Review Board of the Medical University of South Carolina.

Statistical analysis

Total ceramide was defined as the sum of the following individual ceramide species: C_{14}, C_{16}, C_{18}, $C_{18:1}$, C_{20}, $C_{20:1}$, C_{22}, $C_{22:1}$, C_{24}, $C_{24:1}$, C_{26}, and $C_{26:1}$. The distributions of S1P and ceramides were examined and transformed by the natural logarithm to improve symmetry and meet assumptions for statistical analysis. Analyses were performed on the log scale, and results then back transformed to the original scale. Mean concentrations of each sphingolipid were compared in cases versus controls using a linear mixed effects model, accounting for the duplicate assay results per subject, case-control matching, and batch effects (date of experiment).

Conditional logistic regression, which accounted for the matched study design, was used to estimate matched odds ratios and 95% confidence intervals for the association between plasma concentrations of ceramides and S1P with lung cancer risk. The quartiles of the distribution of each sphingolipid among the controls were used to determine the cut-off points to classify the sphingolipid values into fourths. Based on the coefficients from the described linear mixed models, we corrected patients' duplicate concentrations for estimated batch effects, and used average corrected concentrations among the 200 controls to identify quartiles of the control distribution. Using the lowest quartile as the referent category, the matched odds ratios (ORs) and 95% confidence intervals (CIs) for each of the three higher quartiles were estimated. We graphically assessed the assumption of linearity on the logit scale by examining loess-smoothed plots of the log-odds of being a case versus log sphingolipid concentration. For markers suitably linear on the logit scale, the strength of a linear dose-response trend was assessed by fitting a conditional logistic regression model with log sphingolipid concentration as a continuous variable. For markers with a non-linear association, the strength of the association was assessed based on a likelihood ratio test evaluating the collective significance of all relevant model parameters. ORs presented for continuous variables are for an increase in log concentration of 0.1 log μM, the approximate increase in log concentration corresponding to a one quartile increment based on log concentrations in the control population, and are equivalent to a fold increase of 1.1 on the

original scale. To control for potential residual confounding by cigarette smoking, cigarette smoking intensity was added as a continuous variable to the conditional logistic regression model, as well as other potential confounders that were not matched on, such as education and body mass index.

The follow-up period for the present study extended 7.5 years, including lung cancer cases diagnosed between January 1, 1990 and June 30, 1997. To assess whether the association between circulating sphingolipid concentrations and lung cancer risk varied according to the time-to-diagnosis, the median follow-up date (6/30/1994) was used to distinguish “early” versus “late” lung cancer diagnoses. Due to smaller strata sizes in these stratified analyses, the associations were examined by thirds instead of fourths using the same conditional logistic regression models described above. The tertiles of the control distribution in each stratum were used to define the cutoff points for these analyses stratified by cases’ diagnosis date.

All statistical tests were two-sided and a two-tailed p-value of < 0.05 was considered to be statistically significant. Analyses were performed using SAS v9.3 software.

RESULTS

Lung cancer cases and controls were matched on age, gender and smoking status and did not differ significantly with regard to other selected demographic characteristics (Table 1, **second column**). The mean age for lung cancer cases and controls at study baseline were 63.9 and 63.7 years, respectively. Table 1 also summarizes the distribution of baseline case and control characteristics according to quartile of S1P and total ceramides. These results showed similar case-control distributions of S1P and ceramides for age, number of cigarettes per day, education, and body mass index. For the matched factor of gender, for S1P there was a higher proportion of males cases than controls in the upper two quartiles, with a less consistent pattern for total ceramides. Case-control variability across quartiles was present in both former and current smokers, but for both S1P and total ceramides no consistent pattern was present across these smoking categories. The presence of variability but lack of a consistent pattern across categories was also observed for cholesterol status. A more discernible pattern in S1P was present for marital status, with the proportion of cases married lower than controls in the lowest quartile, approximately equal in the middle quartiles, and higher in the highest quartile.

Geometric mean plasma concentrations of S1P and total ceramide were 2.9% ($p = 0.10$) and 5.1% ($p = 0.02$), respectively, greater in lung cancer cases compared to controls (Table 2). For each individual ceramide species assayed the geometric mean plasma concentration was greater in lung cancer cases compared to controls, with percentage differences that ranged from 2.9% to 7.7%; the case-control differences were statistically significant for the $C_{18:1}$, $C_{22:1}$, C_{24}, and C_{26} ceramides. Not counting the measure of total ceramides, which was the summed total of individual ceramide species, there were 17 independent sphingolipids assayed. Regardless of the statistical significance, for 16 of these the mean of the cases was greater than the controls (sign test p-value 0.0003).

For S1P, the ORs (and 95% CIs) for lung cancer risk were 2.7 (1.2–5.9), 2.7 (1.1–6.4), and 1.9 (0.8–4.5) for the second, third, and highest fourths, respectively, compared to the lowest fourth (Table 3). When analyzed as a continuous variable, the association between S1P concentrations and lung cancer risk showed a strong increasing trend at concentrations lower than 0.7 μM, OR = 3.0 (95% CI: 1.3 – 6.8) that leveled off at concentrations higher than 0.7 μM, OR = 0.9 (95% CI: 0.7 – 1.3), an association that overall was highly statistically significant (p-value 0.006) (Figure 1). Compared to those with total ceramide concentrations

in the lowest fourth, the ORs (and 95% CI) for lung cancer risk were 1.6 (0.7–3.3), 1.5 (0.7–3.4), and 2.1 (0.9–4.7) for the second, third, and highest fourths, respectively (p-for-trend 0.01). When analyzed as a continuous variable, the OR for total ceramide concentration was 1.2 (95% CI: 1.1 – 1.5). The results for most of the specific ceramide species were also consistent with a dose-response trend. Of the 12 species, the p-values for the dose-response trends were <0.05 for six, 0.05–0.10 for four, and 0.11–0.20 for two. Of these, the strongest association was for C_{24}-ceramide, with ORs (and 95% CI) by fourth of 1.0 (referent), 2.3 (1.0–5.2), 2.3 (1.0–5.4), and 3.1 (1.4–7.2) (p-for-trend 0.009). The results described above were from matched analyses that accounted for the control of age, sex, and smoking in the design of the study; additional adjustments for potential residual confounding by age and number of cigarettes per day, as well as education, BMI, and cholesterol did not alter the tenor of the results (Table 3). When the potential influence of sphingolipid values that were outliers on these overall results was assessed by excluding values that were >1.5 times the inter-quartile range below the first quartile or above the third quartile, the results presented were not materially altered.

In analyses stratified by the median follow-up date to assess whether the association between circulating sphingolipid concentrations and lung cancer risk varied according to the time to diagnosis, the case-control differences in means showed little difference for S1P, but the increased risk associated with increased plasma concentrations of ceramides seen in the overall study was primarily concentrated early in the follow-up period (Table 4). For example, for total ceramides, lung cancer cases had 8.5% higher concentrations than controls (p = 0.016) early in the follow-up period compared to only 1.8% difference (p = 0.50) later in the follow-up period. The analysis of ORs by tertiles reinforced these findings for total ceramides, with matched ORs by increasing thirds of 1.0, 1.9, and 2.9 early in the follow-up period (p-for-trend 0.013) compared to 1.0, 1.4, and 1.2 late in the follow-up period (p-for-trend 0.40). For S1P, although the mean case-control difference was relatively constant across the two time periods (3.8% early vs. 2.1% late), the ORs by tertiles were more in the direction of increased risk in the early follow-up period (ORs = 1.0, 2.5, and 1.5) than later in the follow-up period (ORs = 1.0, 0.8, and 1.2) (data not shown).

In exploratory subgroup analyses, the case-control percentage difference in mean S1P concentrations by histologic type were 1.1% for adenocarcinoma (n=35 case-control sets), 4.6% for squamous cell carcinoma (n=17 case-control sets), and 3.7% for all other histologic types combined (n=48 case-control sets). The corresponding results for total ceramides were 7.0% for adenocarcinoma, 3.4% for squamous cell carcinoma, and 4.3% for other types. Thus, the pattern of lung cancer cases having higher circulating concentrations of S1P and total ceramides than controls was preserved across the histologic type groups, even though none of these differences was statistically significant.

DISCUSSION

The present study was carried out to measure the association between circulating concentrations of the bioactive sphingolipids S1P and ceramide in relation to the future risk of developing lung cancer. These associations were measured in the context of a nested case-control study in a well-established population-based cohort, comprised of 100 incident lung cancer cases matched to 200 controls on age, race, sex, and smoking history. The results revealed that higher circulating concentrations of both S1P and ceramides were associated with increased risk of developing lung cancer. For S1P, the mean concentrations were only slightly and not significantly greater in the cases than controls, but the odds ratio analysis as a continuous variable showed a strong trend at lower concentrations that leveled off at higher concentrations, an association that overall was highly statistically significant (p-value 0.006). This was reflected in the OR analysis by quartiles, which showed any

category above the lowest fourth of the distribution associated with a 1.9-fold or greater risk of lung cancer. The second and third quartiles, but not the fourth, were associated with significantly increased risk.

For total ceramide, the case-control difference in means was statistically significant, but differed by only 5%. Total ceramide exhibited a statistically significant dose-response trend, with lung cancer risk increasing according to increasing ceramide concentrations. The results for most of the specific ceramide species were also consistent with a dose-response trend. Of these, the strongest association was for the most abundant ceramide species in circulation, C_{24}- ceramide, with an odds ratio of 3.1 (95% CI 1.4–7.2) for the highest-versus-lowest quartile. The fact that C_{24}-ceramide accounted for 47% of the total ceramide measured in controls and was strongly associated with lung cancer risk suggests that C_{24}-ceramide was an important driver of the overall association observed for total ceramide.

The source of sphingolipids in circulation is not known. If the associations observed in the present study are genuine, it is uncertain whether they arose due to 1) the S1P and ceramide measurements acting as markers of systemic concentrations, and hence markers of lung cancer susceptibility, or rather 2) increased sphingolipid production by the lung tumor, in which case the measurements may be serving as markers of preclinical disease. When the data were evaluated by time to diagnosis, the results revealed associations that tended to stronger earlier in the follow-up period than later in the follow-up period. This was especially true for ceramides. These results are thus more consistent with the notion that the observed associations between the sphingolipids and lung cancer may more likely be due to a role as tumor markers, rather than susceptibility markers. Clearly, more evidence is needed to more definitively clarify this.

Based on S1P's pro-growth and pro-survival properties noted in general (2, 12, 13) and specifically in the lung (10), the *a priori* hypothesis was that plasma concentrations of S1P would be associated with increased lung cancer risk. The results were in accordance with this hypothesis.

On the other hand, the role of ceramides in cell signaling is known to act in a pro-apoptotic, anti-tumor growth capacity (3, 14,15) including in the lung (1,16,17), leading to the *a priori* hypothesis that plasma concentrations of ceramides would be inversely associated with lung cancer risk. The results for ceramides ran counter to expectation, however, as higher concentrations were associated with increased lung cancer risk. The reasons for this observation are uncertain, but could possibly be due to a link between perturbation in sphingolipid metabolism and lung carcinogenesis. For example, precancerous or cancerous cells may produce an increased pool of ceramide, generating increased amounts for conversion to S1P. Under this hypothesis cancer risk increases as the balance shifts toward S1P, with dysregulation of S1P metabolism resulting in up-regulation of both S1P and ceramides. Ceramide plays a central role in sphingolipid metabolism, and can be converted to S1P via a pathway that involves ceramidase-catalyzed conversion to sphingosine, which in turn is converted to S1P by sphingosine kinase (13). For example, elevated circulating ceramides could result from their decreased tumor levels via exosomal shedding from tumors.

The observation in the present study that 16 of the 17 sphingolipids assayed were higher in cases than controls ($p=0.0003$) is consistent with a potential role for perturbation in S1P metabolism, marked by overall up-regulation of bioactive sphingolipids, contributing to the pattern of associations observed in the present study. Accordingly, recent data suggest that ceramides might have distinct and opposing functions based on their fatty acid chain lengths: whereas C_{18}-ceramide was tumor suppressive, C_{16}-ceramide increased proliferation

in head and neck cancers (18). On the other hand, ceramide synthase 5-generated C_{16}-ceramide mediated radiation-induced apoptosis, whereas ceramide synthase 2-generated C_{24}-ceramide caused resistance to radiation-induced apoptosis (19). Cigarette smoking, the major cause of lung cancer, could be a source of the pathway perturbation, as in the animal model exposure to cigarette smoke leads to accumulation of ceramide in the lung (5, 20). This notion of dysregulation of the S1P metabolism contributing to disease etiology has been previously invoked with respect to emphysema where, consistent with our findings, elevations of both S1P and ceramide have been observed in disease (21). This provides precedent for up-regulation in S1P metabolism and signaling contributing to the etiology of pulmonary disease, but the emphysema model is premised on a mechanism of ceramide-caused apoptosis creating tissue destruction as part of the etiology of emphysema (6), a concept that is not transferable to the cancer model. Alternatively, the circulating concentrations resulting from dysregulation of the S1P pathway may be acting as tumor markers rather than playing an etiologic role.

A strength of the study was the careful case-control matching that ensured cases and controls were similar for age, race, gender, and smoking status, thereby controlling for these important potential confounding factors. The tight matching for cigarette smoking was especially important because cigarette smoking is not only the predominant cause of lung cancer but the characteristics of smokers and nonsmokers differ in many respects, making it challenging to adequately control for smoking through statistical adjustment. The prospective study design, with plasma samples collected and stored prior to the cases' diagnosis of lung cancer, avoids the complications to inferences that can arise when samples are not collected until after the cancer is diagnosed, due to the potential influences of clinical cancer and its treatment.

The limitations of the study should be taken into account when drawing inferences from these results. One limitation is that the sample size of 100 lung cancer cases was not large enough to allow for meaningful subgroup comparisons that could help to shed further light on these findings. Further, due to resource constraints, the lung cancer cases selected for inclusion in the present study were purposefully concentrated within the first 7.5 years of follow-up of a study with >22 years of follow-up. Solid inferences may therefore be drawn about the findings within this follow-up interval, but this design feature leaves as an open question whether or not the observed findings generalize to longer periods of follow-up. As a result, the results may be more applicable to sphingolipids as an early marker of disease rather than a susceptibility factor for disease. For these reasons and because there is presently no comparable study with which to compare these novel findings, these results should be considered hypothesis-generating until larger studies with longer duration of follow-up are carried out to verify these observations.

In summary, the results indicated that compared to low concentrations, higher circulating concentrations of S1P and total ceramide in plasma were associated with increased future risk of lung cancer. These associations were evident even after matching for age, sex, race, and smoking, raising the possibility that perturbation in the S1P pathway may be a marker of lung cancer risk. The novelty of this study question in combination with the relatively strong associations observed suggests that this is a promising line of inquiry. If the associations observed in the present study are true, it is uncertain whether they arose because S1P and ceramides were acting as a susceptibility factor or a biomarker of latent lung cancer. Regardless of the mechanistic role of S1P and ceramides, these findings represents a step in a new direction in the study of the epidemiology of lung cancer with regard to altered sphingolipid metabolism, with further study in a large-scale investigation warranted to further characterize this association.

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ABBREVIATIONS

S1P	sphingosine -1-phosphate
BMI	body mass index

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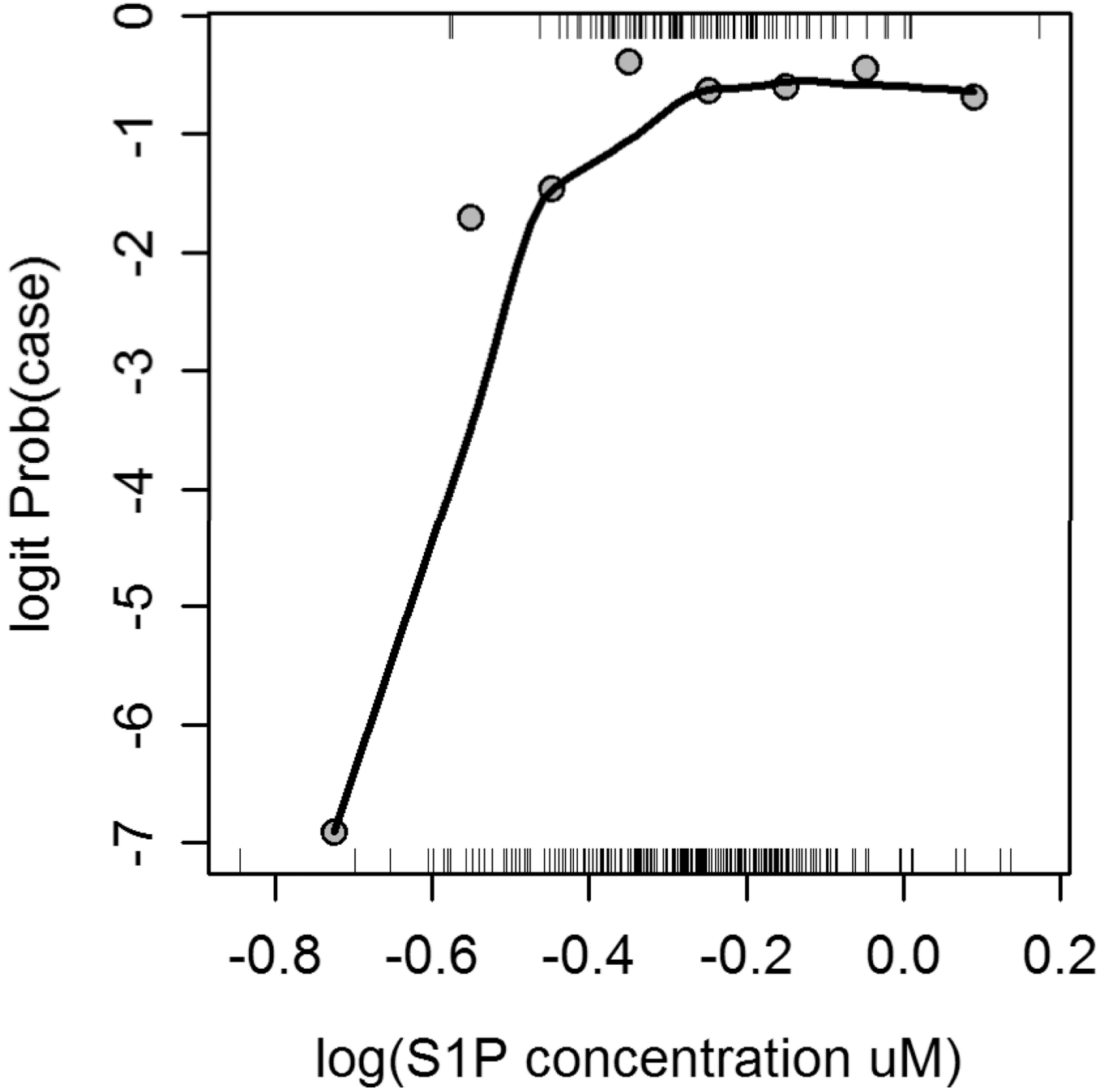

Figure 1.
Loess-smoothed plot of the log odds of lung cancer by log S1P concentration (uM). Rug plots show concentrations for cases (top) and controls (bottom).

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Table 1

The distribution of baseline characteristics of lung cancer cases and controls according to quartiles of S1P and total ceramides, Washington County, MD (1989).

	Total	S1P				Total Ceramides			
		Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Total (N)									
Case	100	14	32	30	24	18	26	24	32
Control	200	50	50	50	50	50	50	50	50
Age (years) at baseline, Mean (SD)[a]									
Case	63.9	68.3	62.9	64.5	61.9	65.9	64.9	62.6	62.9
Control	63.7	63.9	64.9	61.2	64.7	66.4	62.2	64.0	62.0
Gender, % Male [a]									
Case	54.0	64	44	53	63	61	54	50	53
Control	54.0	62	58	42	54	52	66	54	44
Cigarette Smoking Status, % [a]									
Never									
Case	7.0	0	6	17	0	0	12	0	13
Control	8.0	4	8	6	14	2	8	12	10
Former									
Case	49.0	64	53	30	58	50	38	54	53
Control	49.0	58	44	36	58	60	56	46	34
Current									
Case	44.0	36	41	53	42	50	50	46	34
Control	43.0	38	48	58	28	38	36	42	56
Cigarette/Day, Mean (SD) [a]									
Former									
Case	30.4	26.8	27.4	33.3	34.5	36.7	36.0	23.8	28.3
Control	28.7	26.1	33.0	29.4	27.9	28.9	25.4	31.0	31.2
Current									

	Total	S1P				Total Ceramides			
		Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Case	24.2	21.0	24.6	26.9	21.0	17.2	27.7	20.0	30.0
Control	24.3	25.0	24.1	24.3	23.6	19.8	23.6	22.6	29.0
	Group Total	**S1P**				**Total Ceramides**			
		Q1	**Q2**	**Q3**	**Q4**	**Q1**	**Q2**	**Q3**	**Q4**
Marital Status, % Married									
Case	69.6	57	72	62	79	61	65	78	69
Control	64.5	68	68	60	62	50	72	72	64
<12 Years of School (years), %									
Case	40.4	43	34	40	48	44	46	39	34
Control	36.0	32	34	34	44	38	32	38	36
Body Mass Index, Mean									
Case	26.1	24.4	27.3	26.2	25.6	25.7	27.1	25.4	26.1
Control	26.4	26.4	25.7	26.3	27.1	27.0	26.5	26.0	26.0
Cholesterol, %									
Case									
Low	38.1	43	34	28	55	61	27	36	35
Borderline	32.0	21	34	45	18	22	50	27	26
High – Not Treated	19.6	29	25	10	18	11	15	14	32
High – Treated	10.3	7	6	17	9	6	8	23	6
Control									
Low	32.7	44	30	18	38	40	33	30	28
Borderline	39.7	32	42	50	36	32	49	36	42
High – Not Treated	24.1	18	28	29	22	22	16	28	30
High – Treated	3.5	6	0	4	4	6	2	6	0

[a]Matching factor

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Table 2

Geometric mean (μM) plasma sphingolipid concentrations in lung cancer cases (n=100) and matched controls (n=200), Washington County, MD (1990–1997).[a]

Sphingolipid	Geometric Means (μM)			
	Cases	Controls	% Difference[b]	P-value
S1P	0.78	0.75	+2.9	0.10
Total Ceramides	5.48	5.21	+5.1	0.021
Ceramide species				
C_{14}-ceramide	0.064	0.066	+2.9	0.16
C_{16}-ceramide	0.43	0.41	+3.7	0.14
C_{18}-ceramide	0.15	0.15	+3.8	0.29
$C_{18:1}$-ceramide	0.046	0.043	+6.8	0.043
C_{20}-ceramide	0.40	0.37	+7.7	0.06
$C_{20:1}$-ceramide	0.035	0.033	+4.7	0.17
C_{22}-ceramide	0.36	0.35	+3.9	0.07
$C_{22:1}$-ceramide	0.100	0.096	+4.3	0.048
C_{24}-ceramide	2.64	2.48	+6.2	0.016
$C_{24:1}$-ceramide	1.00	0.96	+3.8	0.07
C_{26}-ceramide	0.101	0.096	+5.6	0.049
$C_{26:1}$-ceramide	0.054	0.052	+4.0	0.12
Other sphingolipids				
DihydroC_{16}-ceramide	0.049	0.047	+3.5	0.32
Dihydrosphingosine	0.022	0.022	−0.7	0.84
DihydroS1P	0.11	0.11	+3.0	0.12
Sphingosine	0.031	0.030	+4.2	0.22

[a] Based on a mixed model with the sphingolipid as a continuous dependent variable and adjusted for assay date

[b] Calculated as [(case mean – control mean)/control mean] × 100

Table 3

Matched odds ratios (and 95% confidence intervals) for lung cancer according to quartiles of plasma sphingolipid concentration, Washington County, MD.

	Matched analysis					Additionally adjusted for age, number of cigarettes per day, education, BMI, cholesterol				
Sphingolipid	**Q1**[1]	**Q2**	**Q3**	**Q4**	**P_{trend}**[2]	**Q1**[1]	**Q2**	**Q3**	**Q4**	**P_{trend}**[2]
S1P	1.0	2.7 (1.2 – 5.9)	2.7 (1.1 – 6.4)	1.9 (0.8 – 4.5)	0.006[3]	1.0	2.7(1.2 – 6.2)	2.9 (1.2 – 7.2)	1.8 (0.7 – 4.5)	0.003[3]
Total Ceramides	1.0	1.6 (0.7 – 3.3)	1.5 (0.7 – 3.4)	2.1 (0.9 – 4.7)	0.011	1.0	1.7 (0.8 – 3.7)	1.5 (0.6 – 3.4)	2.1 (0.9 – 4.9)	0.018
Ceramide Species										
C_{14}-ceramide	1.0	2.0 (0.9 – 4.4)	1.7 (0.8 – 4.0)	2.1 (0.9 – 5.1)	0.13	1.0	1.9 (0.8 – 4.5)	1.7 (0.7 – 4.2)	2.1 (0.9 – 5.2)	0.18
C_{16}-ceramide	1.0	1.1 (0.5 – 2.4)	1.3 (0.6 – 2.8)	2.0 (0.8 – 4.9)	0.07	1.0	1.0 (0.5 – 2.3)	1.1 (0.5 – 2.5)	1.9 (0.8 – 4.6)	0.13
C_{18}-ceramide	1.0	1.4 (0.6 – 3.2)	1.5 (0.7 – 3.5)	2.0 (0.8 – 4.7)	0.18	1.0	1.6 (0.7 – 3.6)	1.6 (0.7 – 3.7)	2.0 (0.8 – 4.9)	0.25
$C_{18:1}$-ceramide	1.0	0.8 (0.3 – 1.9)	1.1 (0.4 – 2.5)	2.0 (0.9 – 4.7)	0.011	1.0	0.8 (0.3 – 2.1)	1.0 (0.4 – 2.4)	1.8 (0.8 – 4.4)	0.023
C_{20}-ceramide	1.0	2.0 (0.9 – 4.6)	1.3 (0.6 – 3.1)	2.7 (1.1 – 6.6)	0.026	1.0	1.9 (0.8 – 4.6)	1.3 (0.5 – 3.1)	2.8 (1.1 – 7.0)	0.052
$C_{20:1}$-ceramide	1.0	1.5 (0.7 – 3.3)	1.8 (0.8 – 4.1)	2.3 (0.9 – 5.8)	0.09	1.0	1.4 (0.6 – 3.2)	1.8 (0.7 – 4.2)	2.2 (0.9 – 5.4)	0.16
C_{22}-ceramide	1.0	1.5 (0.7 – 3.3)	1.3 (0.6 – 3.1)	1.9 (0.8 – 4.2)	0.043	1.0	1.5 (0.7 – 3.5)	1.2 (0.5 – 2.8)	2.0 (0.9 – 4.6)	0.06
$C_{22:1}$-ceramide	1.0	1.5 (0.7 – 3.3)	0.7 (0.3 – 1.8)	1.9 (0.8 – 4.2)	0.020	1.0	1.5 (0.7 – 3.4)	0.6 (0.2 – 1.6)	1.7 (0.7 – 4.1)	0.038
C_{24}-ceramide	1.0	2.3 (1.0 – 5.2)	2.3 (1.0 – 5.4)	3.1 (1.4 – 7.2)	0.009	1.0	2.6 (1.1 – 6.1)	2.5 (1.0 – 6.0)	3.4 (1.4 – 8.2)	0.016
$C_{24:1}$-ceramide	1.0	1.7 (0.8 – 3.5)	1.3 (0.6 – 2.9)	1.9 (0.8 – 4.3)	0.052	1.0	1.7 (0.8 – 3.7)	1.3 (0.6 – 3.0)	1.9 (0.8 – 4.5)	0.07
C_{26}-ceramide	1.0	2.1 (1.0 – 4.6)	2.1 (1.0 – 4.4)	2.0 (0.9 – 4.6)	0.030	1.0	2.2 (1.0 – 5.0)	2.2 (1.0 – 4.9)	2.0 (0.8 – 4.7)	0.041
$C_{26:1}$-ceramide	1.0	2.5 (1.1 – 5.4)	1.6 (0.7 – 3.6)	2.9 (1.3 – 6.5)	0.10	1.0	2.9 (1.3 – 6.6)	1.7 (0.7 – 4.2)	3.0 (1.3 – 7.1)	0.11
Other sphingolipids										
DihydroC_{16}-ceramide	1.0	0.7 (0.3 – 1.6)	1.0 (0.5 – 2.2)	1.2 (0.6 – 2.8)	0.28	1.0	0.7 (0.3 – 1.5)	1.1 (0.5 – 2.3)	1.1 (0.5 – 2.6)	0.45
Dihydrosphingosine	1.0	0.8 (0.4 – 1.6)	0.8 (0.4 – 1.6)	1.3 (0.6 – 2.7)	0.82	1.0	0.8 (0.4 – 1.7)	0.7 (0.3 – 1.5)	1.2 (0.6 – 2.8)	0.74
DihydroS1P	1.0	3.1 (1.3 – 7.5)	2.8 (1.1 – 7.0)	2.6 (1.0 – 6.7)	0.08	1.0	3.3 (1.3 – 8.2)	2.4 (1.0 – 6.1)	2.3 (0.9 – 6.1)	0.22
Sphingosine	1.0	1.8 (0.8 – 3.8)	1.8 (0.8 – 4.2)	1.8 (0.8 – 4.2)	0.20	1.0	1.4 (0.6 – 3.2)	1.7 (0.7 – 4.0)	1.8 (0.7 – 4.3)	0.18

[1] Referent category

[2] Trend test with sphingolipid modeled as a continuous variable.

[32] Due to the piece-wise linear dose-response association of S1P with lung cancer risk (Figure 1), this p-value is based on a two degree of freedom likelihood ratio test (see text).

Table 4

Geometric mean (μM) plasma sphingolipid concentrations in lung cancer cases (n=100) and matched controls (n=200), stratified by early (1/1/1990–6/30/1994) versus late (7/1/1994–6/30/1997) diagnosis, Washington County, MD (1990–1997).[a]

	Early Diagnosis (1/1/1990–6/30/1994)					Late Diagnosis (7/1/1994–6/30/1997)		
Sphingolipid	**Cases**	**Controls**	**% Difference[b]**	**P-value**	**Cases**	**Controls**	**% Difference[b]**	**P-value**
S1P	0.77	0.75	+3.8	0.14	0.77	0.75	+2.1	0.39
Total Ceramide	5.39	4.97	+8.5	0.016	5.51	5.41	+1.8	0.50
Ceramide Species								
C_{14}-ceramide	0.064	0.062	+4.2	0.19	0.068	0.067	+1.7	0.54
C_{16}-ceramide	0.47	0.44	+7.6	0.043	0.41	0.41	−0.2	0.95
C_{18}-ceramide	0.16	0.15	+8.5	0.12	0.16	0.16	−0.7	0.89
$C_{18:1}$-ceramide	0.045	0.041	+10.2	0.043	0.046	0.044	+3.6	0.42
C_{20}-ceramide	0.38	0.33	+13.9	0.036	0.42	0.41	+1.7	0.72
$C_{20:1}$-ceramide	0.033	0.030	+9.4	0.09	0.036	0.036	+0.1	0.99
C_{22}-ceramide	0.36	0.34	+6.5	0.053	0.36	0.35	+1.4	0.59
$C_{22:1}$-ceramide	0.97	0.90	+8.1	0.029	0.10	0.10	+0.7	0.77
C_{24}-ceramide	2.59	2.36	+9.6	0.016	2.65	2.58	+2.8	0.39
$C_{24:1}$-ceramide	0.95	0.89	+6.4	0.045	1.02	1.01	+1.2	0.67
C_{26}-ceramide	0.102	0.094	+8.2	0.053	0.101	0.098	+3.0	0.43
$C_{26:1}$-ceramide	0.051	0.048	+5.9	0.10	0.057	0.055	+2.0	0.59
Other sphingolipids								
DihydroC_{16}-ceramide	0.048	0.046	+4.0	0.45	0.052	0.050	+3.1	0.51
Dihydro-sphingosine	0.018	0.018	−0.6	0.81	0.026	0.026	−0.2	0.96
Dihydro-S1P	0.11	0.10	+4.1	0.12	0.11	0.11	+1.8	0.51
Sphingosine	0.032	0.031	+3.5	0.47	0.031	0.029	+4.9	0.32

[a] Based on a mixed model with sphingolipid as a continuous dependent variable and adjusted for assay date

[b] Calculated as [(case mean – control mean)/(control mean)] × 100