Human meconium contains significant amounts of alkaline sphingomyelinase, neutral ceramidase and sphingolipid metabolites

Running title: Meconium sphingolipids

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Abbreviations

Alk-SMase, alkaline sphingomyelinase; NEC, necrotizing enterocolitis; PAF, platelet activating factor; SM, sphingomyelin

Abstract

Intestinal alkaline sphingomyelinase (Alk-SMase) and neutral ceramidase may catalyse the hydrolysis of endogenous sphingomyelin (SM) and milk SM in human-milk fed infants. The enzymes generate sphingolipid metabolites that may influence gut maturation. Alk-SMase also inactivates platelet activating factor (PAF) that is
involved in the pathogenesis of necrotizing enteritis (NEC). We examined whether the two enzymes are expressed in both preterm and term infants and analyzed alk-SMase, neutral ceramidase, SM and sphingolipid metabolites in meconium.
Meconium was collected from 46 preterm (gestational ages 23-36 weeks) and 38 term infants (gestational ages 37-42 weeks) and analysed for Alk-SMase using ^{14}C-choline labelled SM and for neutral ceramidase using ^{14}C-octanoyl-sphingosine as substrates. Molecular species of SM, ceramide and sphingosine, were analysed by HPLC mass spectroscopy.
Meconium contained significant levels of Alk-SMase and ceramidase at all gestational ages. It also contained 16-24 carbon molecular species of SM, palmitoyl- and stearoyl-sphingosine, and sphingosine. There were positive correlations between levels of SM and ceramide, and between ceramide and sphingosine levels.
In conclusion Alk-SMase and ceramidase are expressed in the gut of both preterm and term newborn infants and may generate bioactive sphingolipid messengers.

Introduction

Sphingolipids are important constituents of cell membranes, particularly plasma and lysosomal membranes, and the enteral mucosal brush border. Sphingomyelin (SM) consists of a long chain sphingoid base, linked with an amide bond to a long chain fatty acid and the polar head group phosphocholine. Sphingomyelinases (SMase) catalyse the hydrolysis of SM to phosphocholine and ceramide and ceramidases the hydrolysis of ceramide to free fatty acids and sphingoid bases primarily sphingosine (Figure 1) (1). Different SMases and ceramidases are designated by their pH optimum as acid, neutral and alkaline (2, 3). Mucosal alkaline sphingomyelinase (Alk-SMase) and neutral ceramidase are key enzymes of SM and ceramide metabolism in the gut (1, 4-6). Ceramide and sphingosine, and sphingosine-1-phosphate (S-1-P) that is formed after the absorption of sphingosine into mucosal cells, are signalling substances with multiple effects acting through several activation pathways (7-10).

The human-milk fed infant ingests about 150 mg SM per day, since SM is a major polar milk lipid accounting for 40% of the milk fat globule membrane (11, 12). Furthermore the mucosal brush border contains significant amounts of SM, ceramide and glycosphingolipids which are alternative substrates for intestinal sphingolipid hydrolysing enzymes. Unlike dietary glycerolipids, sphingolipids are not degraded by pancreatic enzymes. Sphingomyelin is sequentially hydrolyzed by Alk-SMase and neutral ceramidase acting at the brush border of the intestinal epithelium and in the gut lumen (6, 12). Alk-SMase is a tissue specific enzyme that is expressed in the gut with highest levels in the middle and lower small intestine, and in humans also in bile (1, 13, 14). The enzyme was cloned and identified as a novel member of the nucleotide phosphodiesterase (NPP) family (14). Furthermore, it was shown that Alk-SMase inactivates the potent proinflammatory lipid messenger platelet activating factor (PAF; 1-alken-2-acetyl-glycerophosphocholine) (15). Platelet activating factor can be produced in the intestine and has been ascribed a pathogenic role in neonatal necrotizing enterocolitis (NEC) (16).

Neutral ceramidase of the gut (5) has been purified and characterized in both rat (17) and human tissue (L Ohlsson et al, Abstract OP-G-283, EUGW, Copenhagen 2005), and was found to have extensive homology to neutral ceramidase of other tissues. The crucial role of neutral ceramidase in ceramide digestion in the gut was recently confirmed by studies on gene targeted mice lacking active neutral ceramidase 2 (18).

Both Alk-SMase and intestinal ceramidase are highly glycosylated and resistant to proteases, and remain active in the gut lumen (14, 17, 19, 20). They are released in active form by bile salts and, in the case of Alk-SMase by tryptic cleavage of an anchoring C-terminal fragment (21). These features made it possible to use ileostomy content as starting material for purification of both enzymes (14, 17, 20, 22), and to use faecal levels as a measure of Alk-SMase expression (23).

In the fetal rat the gut epithelium undergoes rapid transformation with formation of mature villus cells, and distinct villus and crypt structures around day 18 of gestation i.e. soon before birth at day 22 (24). Expression of Alk-SMase coincides with this differentiation (25) In human fetal life differentiation of the epithelium is extended over a longer time period. Brush border enzymes characteristic of the mature villus cells develop progressively from the 13th gestational week (26). However, no previous study has measured activity of Alk-SMase and neutral ceramidase, and sphingolipid metabolites in human newborns.

This study examines the levels of Alk-SMase, neutral ceramidase and key sphingolipid metabolites in meconium of newborn preterm and term infants, for three reasons: First, milk SM or its bioactive metabolites may influence intestinal maturation (27). Second it is important to know whether the ability to inactivate PAF via Alk-SMase is developed in the most preterm infants that are most susceptible to NEC. Third the analyses concern enzymes and metabolites that may trigger sphingolipid signalling during the lung epithelial and alveolar cell apoptotosis and inflammation that characterizes the meconium aspiration syndrome (28).

Methods

Subjects

The study population consisted of 46 preterm infants (gestational ages 23 to 36 weeks) treated in the Neonatal Intensive Care Unit, Lund University Hospital, Sweden, and 38 healthy term infants in the Maternity Unit, Department of Obstetrics and Gynecology. Infants were stratified in five groups according to gestational age: 23-25 weeks, 26-28 weeks, 29-32 weeks, 33-36 weeks and 37-42 weeks. Patient characteristics are given in Table 1. A majority of the preterm infants needed surfactant and mechanical ventilation. All preterm infants, except for one infant, who had gastrochisis, received oral feedings with human milk (donor or mother´s own), usually within the first hours of life (29). Five of the fullterm infants received milk replacement formulations. All the others were breastfed.

Sample collection and preparation.

The study was approved by the Lund University Research Ethics Committee, written informed parental consent was obtained before inclusion in the study. Meconium was collected soon after birth and frozen in test tubes. Before determination, samples were weighed and suspended in 0.15 M NaCl containing 1 mM benzamidine to a concentration about 0.25 g/ml. The samples were stirred vigorously and then centrifuged at 3000 rpm for 10 min. The supernatant was saved for biochemical analysis and lipid extraction.

Enzyme assays

Sphingomyelinase assay. The activities of different types of SMase activity were analyzed as described (30). The buffers used were 50 mM Tris-maleate buffer pH 5.0 containing 0.15 M NaCl and 0.12% Triton X100 for acid SMase, 50 mM Tris-HCl buffer pH

7.5 containing 4 mM Mg^{2+} and 0.12% Triton X100 for neutral SMase, and 50 mM Tris-HCl pH 9.0, containing 2 mM EDTA, 0.15 M NaCl, and 6 mM taurocholate for Alk-SMase. For each determination, 10 μl sample was mixed with 90 μl buffer containing 80 pmole [^{14}C]SM (8000 dpm) and incubated at 37 ^{o}C for 30 min. The reaction was terminated by adding 0.4 ml of chloroform/methanol (2/1, v/v) followed by centrifugation at 10,000 g for 5 sec. An aliquot of the upper phase was taken and the production of ^{14}C-phosphocholine was determined by liquid scintillation.

Neutral ceramidase was assayed as described (17). ^{14}C-octanoyl-sphingosine was used as substrate and the incubation buffer was 50 mM Tris-maleate pH 7.0 containing 10 mM sodium taurocholate. The assay was started by adding 10 μl of sample containing the enzyme with 90 μl of the assay buffer with substrate, and the samples were incubated at 37^{o} C for one h. The reaction was interrupted by adding 0.6 ml methanol-chloroform-heptane in proportions of 28:25:20 (vol/vol/vol) and 0.2 ml 0.05 M K2CO3-K2B2O2, pH 10. After mixing the tubes were centrifuged at 10000 rpm for 10 s. An aliquot of 200 μl of upper phase was taken for liquid scintillation counting and the rate of hydrolysis calculated from the proportion of the radioactive substrate that had been hydrolysed (31).

Analysis of sphingomyelin, ceramide and sphingosine

Ceramide was determined by liquid chromatography tandem mass spectrometry (LC-MS-MS) as described (32). 18-d-erythro-sphingosine, C-22-SM, C-23 and C-24-SM were analysed by the same method. The fragments analyzed were for 18-d-erythro-sphingosine m/z 300.1/264.4 (collision energy [CE] 28 volts), for C-22-SM m/z 787.9/183.8 (CE 39 volts), for C-23-SM m/z 801.9/183.8 (CE 39 volts) and for C-24-SM m/z 815.9/183.8 (CE 39 volts). Weighed standards C-16- and C-18-ceramide were used for preparation of calibration curves. For C-22, C-23 and C-24-SM milk SM containing a mixture of several molecular species of SM was used as standard. Thus the content of each individual C-22-24 SM species in the standard sample was calculated from the total SM mass and the known fatty acid composition of milk SM (33).

C-16-SM (palmitoyl-SM) is abundant among intestinal epithelial and bile SM species that also contains 18 carbon d-erythro-sphingosine, whereas longer fatty acids (more than 20 carbons) are abundant in SM species containing 4-hydroxysphinganine as sphingoid base and in glycolipids (34). Comparison of C-16-SM and C-16-ceramide levels would therefore be expected to best reflect SM hydrolysis and analyses were focused on C-16 and C-18 species of ceramide, for which authentic synthetic standards are also available. Since SM of most tissues contains high proportions of longer chain SM species we analysed, however, also meconium for C-22:0, C-23:0 and C-24:0 SM species, to see if the fatty acid composition reflects intestinal tissue SM rather than bile SM.

Statistical analysis

Apart from the individual values shown in some figures, most of the data are presented as Mean $\pm$ standard errors. The correlation was performed by linear regression analysis using a soft program GraphPad Prism 4. $P < 0.05$ is considered statistically significant.

Results

Alkaline sphingomyelinase and neutral ceramidase in meconium

Alk-SMase was the predominant SMase at all gestational ages, being much higher than the other SMase activities, i.e. acid and neutral SMase. Values for the activity of the different SMases in all meconium samples, expressed as pmole/h/mg meconium (means(SEM)) were 30160(3010) (means(SEM)) for A
Alk-SMase, 7.43(0.92) for acid SMase and 5.03(0.47) for neutral SMase. Significant Alk-SMase activity was found in all meconium samples, i.e. also in those from the most preterm

children. If anything the activity per gram of meconium was higher in samples from preterm than in samples from term infants (Fig. 2 A). Since Alk-SMase and neutral intestinal ceramidase catalyse the sequential degradation of SM to sphingosine and free fatty acid, we asked whether the ceramidase and the Alk-SMase are simultaneously expressed. As shown in figure 2 B, ceramidase was present in all meconium samples. The relation of ceramidase activity to gestational age was similar to that of Alk-SMase, i.e. the enzyme was present also in the most preterm infants and the activity per weight meconium not lower than in the term infants. There was a strong positive correlation between the levels of the two enzymes (Figure 3). However, with the assay methods used the activity of Alk-SMase was found to be about 10 times higher than that of neutral ceramidase.

Since most of the preterm infants had received treatment with antenatal corticosteroids, antibiotics and surfactant it was not possible to examine possible effects from these interventions on Alk-SMase and ceramidase levels. The premature child with gastrochisis were among those that had low alk-SMase values. The five term infants that received milk replacement formulations did not differ significantly from the breastfed children in any of the parameters.

Sphingolipids in meconium
Levels of C-16- and C-18-SM and of the corresponding ceramide species are summarized in figure 4. The levels of C-16-species of both SM and ceramide exceeded the levels of C-18-species. The average C-16-ceramide/C-16-SM w/w ratio was 0.6 and the average mol/mol ratio 0.8, indicating that hydrolysis of C-16-SM had occurred. Individual data for C-16-SM and C-16-ceramide in relation to gestational age are shown in Figure 5A and 5B. Both compounds were present in all samples examined but individual values for each compound or for the ratio C-16-ceramide/C-16-SM did not correlate to gestational age or gender.

(C-18-D-erythro-)sphingosine levels are shown in Figure 5 C. The sphingosine levels were lower than the ceramide levels and did not correlate to gestational age or gender, but sphingosine was found in all samples examined. When the correlations between related metabolites were examined, we found that the sum of C-16- plus C-18-SM was positively correlated with that of C-16 plus C-18-ceramide and the levels of these ceramides is positively related to that of sphingosine (Fig. 6 A and B).

Meconium also contained long chain (C-22:0, -23:0 and –24:0) SM species (data not shown).

Discussion

This study shows that Alk-SMase and neutral ceramidase are present in significant amounts in meconium of newborn human infants. Both enzymes were expressed even in the most premature children born at 23 to 26 gestational weeks. Slightly higher values per gram meconium were actually found in the samples from the preterm infants, which may reflect the amount of enzymes produced as well as dilution factors. The levels are comparable with those found in adult faeces, in faeces from three week old pigs, and in duodenal and jejunal content from a few measurements in newborns (35).

The findings are in line with those of studies on other brush border enzymes as alkaline phosphatase and lactase that are expressed from 17 gestational weeks (26), i.e. when mature villi differentiate in humans. By 20 weeks of gestation, the anatomic differentiation of the fetal gut has progressed to the extent that it resembles that of a newborn (26). Thus the presence of both neutral ceramidase and Alk-SMase probably reflects the formation of mature epithelium expressing brush border enzymes. This interpretation is in line with our finding in fetal rat that Alk-SMase was promptly expressed around day 20 as the appearance of mature differentiated epithelium occurs before the delivery at day 22 (36). As the Alk-SMase increased, the acid SMase decreased promptly in the rat. In the present study acid and neutral

SMase activities were far lower than that of Alk-SMase, which may reflect either a similar down regulation of acid SMase in humans or differences in stability between the SMases. Whereas Alk-SMase is protease resistant and survives in active form in intestinal and faecal environment, this is not the case for acid and neutral SMase (37). The ratios between acid or neutral and Alk-SMase may therefore be higher in intestinal epithelium than is reflected in meconium. Intestinal neutral ceramidase is as stable to proteases as alk-SMase (17). The strong correlation between Alk-SMase and neutral ceramidase (Figure 3) thus most likely reflects co-expression of both enzymes. They may enter the meconium both by sloughing of mucosal cells and by release from intact villus cells by bile salts or trypsin (14, 17, 21).

Corticosteroid treatment is known to enhance intestinal maturation and expression of brush border enzymes and transporters (26). Since most of the premature infants had been exposed to antenatal treatment with corticosteroids, Alk-SMase and ceramidase expression may thus have been influenced by this treatment.

Three pieces of evidence indicate that Alk-SMase and neutral ceramidase are key enzymes in digestion of SM (1, 5, 6, 14, 17, 21). First, enzymes hydrolysing SM are absent in pancreatic juice (5). Second, SM digestion occurs mainly in the middle and lower small intestine where Alk-SMase and neutral ceramidase are preferentially located (1, 31). Third, Alk-SMase is specifically dependent on certain bile salts, primarily taurocholate and taurochenodeoxycholate (14). Human milk bile salt-stimulated lipase (BSSL) has some ceramidase activity (38), but is active primarily in the proximal intestine and is not active against ceramide in the presence of other competing substrates as glycerolipids (39). Furthermore gene targeted mice lacking BSSL exhibited normal ceramidase activity except in the most upper part of the gut (39) whereas neutral ceramidase 2 gene knockout mice exhibited a decreased ceramide digestion with increased levels of ceramide in faeces (18).

By the action of Alk-SMase and neutral ceramidase on SM of the polar lipids in the milk fat globule membrane, the human milk-fed infant may thus utilize both the phosphocholine and the hydrophobic components of SM. Furthermore the sphingolipid metabolites that these enzymes generate may influence cellular growth and differentiation in the fetal and neonatal gut. It is recognized that mothers' milk is important for the normal maturation of the infant gut mucosa but the factors involved are only partly known. Motouri et al (27) fed newborn rat pups milk replacement formulas with and without SM, resulting in differences in lactase activity, distribution of vacuolated cells and Auerbach nerve plexus area, indicating that SM intake influenced neonatal gut maturation. Our data indicate that if this effect requires hydrolysis of SM it may be exerted both in premature and full term children that are fed mothers' milk.

A novel function of Alk-SMase was recently discovered. Platelet activating factor (PAF), a strong proinflammatory lipid messenger (1-alken.2-acetyl-3-phosphocholine glycerol), was found to be hydrolysed and inactivated by Alk-SMase (15). Since PAF is known to be present in meconium, and found in increased amounts in infants with necrotizing enteritis (NEC) (16), the finding of Alk-SMase in meconium of preterm infants provides a potential novel antiinflammatory mechanism that may counteract NEC.

Milk feeding is associated with a lower risk for NEC (40). The reasons are unknown. Carlson et al (41) reported a lower incidence of NEC in infants fed a preterm formula with egg phospholipids and emphasized the supply of polyunsaturated fatty acids, in particular of arachidonic acid as a precursor of eicosanoids. Since about 70% of egg phospholipids are phosphatidylcholine and 3% SM the supply of choline may be important as well. Combined with our data the findings emphasize that polar milk lipids are potential protective agents against NEC. Such an action may be exerted by the intact polar lipids, or by metabolites with anti-inflammatory action (e.g. ceramide and sphingosine) or the ability to stimulate

regeneration or survival of mucosal epithelial cells (S-1-P, prostaglandins, lyso-phosphatidic acid).

Since Alk-SMase and ceramidase may generate bioactive sphingolipid metabolites from endogenous SM during fetal development we examined the levels of key SM and ceramide species and of sphingosine. Meconium contained both C-16, C-18 SM and SM with very long chain fatty acids. High levels of C-22-C-24 SM indicated that much of the meconium SM originates from intestinal tissue rather than bile. Meconium also contained C-16- and C-18-ceramide and free sphingosine. Palmitic acid, and sphingosine as a base, are abundant in SM of intestinal epithelial cells and bile whereas longer fatty acids are abundant in glycolipids and in SM containing 4-hydroxysphinganine (42). The C-16-ceramide/C-16-SM ratio is therefore likely to indicate the degree of hydrolysis of C-16-SM and was found to be much higher than is normally found in tissues. There was a positive correlation between C-16 and C-18 SM and ceramide species which further supports that these ceramides in meconium are derived mainly from hydrolysis of SM. Obviously the C-16 and C-18 ceramides are not likely to be the only ceramide species in meconium. Hydrolysis of long chain SM and of glycosphingolipids, as cerebroside, hematoside and blood group active fucolipids known to be present in meconium (43-45) may generate molecular ceramide species containing very long chain and hydroxylated fatty acids.

All meconium samples also contained sphingosine. Sphingosine is derived from delta-4-desaturation of dihydrosphingosine in ceramide. Free sphingosine in meconium must therefore be formed by hydrolysis of ceramide. Accordingly we found a positive correlation between the levels of ceramide (C-16 and C-18) and of sphingosine. The level of sphingosine was lower than that of ceramide, probably due to absorption and metabolism of sphingosine by the gut (4). Yet, the amount may be highly significant, considering that ceramide and sphingosine are lipid messengers that exert biological effects at low concentrations. Thus ceramide and sphingosine are present in meconium and may be generated by Alk-SMase and ceramidase. Further studies of their biological effects during the gut maturation and differentiation are therefore highly motivated. On the basis of our findings, Alk-SMase, ceramidase and the sphingolipid metabolites should also be added to the list of compounds that might exert biological effects in the lungs during meconium aspiration (46).

In conclusion, in this paper we studied the levels of Alk-SMase and neutral ceramidase in meconium at different gestational ages and found that both enzymes are expressed early. We also found that meconium contains significant amounts of SM, ceramide, and free sphingosine. We postulate that the two enzymes are important in the metabolism of milk and mucosal sphingolipids and generate sphingolipid metabolites with important biological effects during intestinal maturation.

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Legends to figures

Figure 1
Formation of metabolites of sphingomyelin
The figure illustrates the formation of sphingolipid metabolites via the action of sphingomyelinase, ceramidase and sphingosine kinase.

Figure 2
Alkaline sphingomyelinase and neutral ceramidase at different gestational ages
The figure shows individual values for alk-SMase (Figure 2A) and neutral ceramidase (Figure 2B) in meconium from newborns with different gestational age. There was no significant correlation between gestational age and enzyme levels.

Figure 3
Correlation between alkaline sphingomyelinase and neutral ceramidase
The figure shows the correlation between alk-SMase and neutral ceramidase in all meconium samples examined. The correlation was highly significant (r^2=0.3045, $p<0.0001$).

Figure 4
Palmitoyl- and stearoyl species of SM and ceramide, and sphingosine in meconium
The figure shows means(SEM) for concentration in meconium of C-16-SM, C-16-ceramide, C-18-SM, C-18-ceramide and sphingosine (sph). Values are given as mass per wet weight meconium. The average molar ratio of C-16-ceramide/C-16-SM was 0.79.

Figure 5
Sphingomyelin, ceramide and sphingosine in meconium
Figure 5A shows individual values for C-16-SM. In this figure 4 outlying values are not shown: 283.3 at 23 weeks, 178.8 at 24 weeks, 311.6 at 26 weeks and 414.6 at 28 weeks. Figure 5B shows values for C-16-ceramide and Figure 6C for sphingosine. In Figure 5C 4 outlying values are not shown: 15.5 at 25 weeks, 23.1 at 28 weeks, 44.4 at 29 weeks and 26.8 at 34 weeks. There was no significant correlation between SM, or any of the two metabolites to gestational age.

Figure 6
Correlations between sphingomyelin, ceramide and sphingosine levels in meconium
Figure 6 A shows the correlation between SM (sum of C-16 and C-18-SM) and of ceramide (sum of C-16- and C-18-ceramide). The r^2=0.2691 and $p<0.001$. Figure 6 B shows the correlation between C-16 plus C-18-ceramide and sphingosine. The r^2=0.3749 and $p<0.0001$. Thus the levels of ceramide and sphingosine correlated strongly to the levels of the substrates from which these metabolites are formed.

Fig. 1

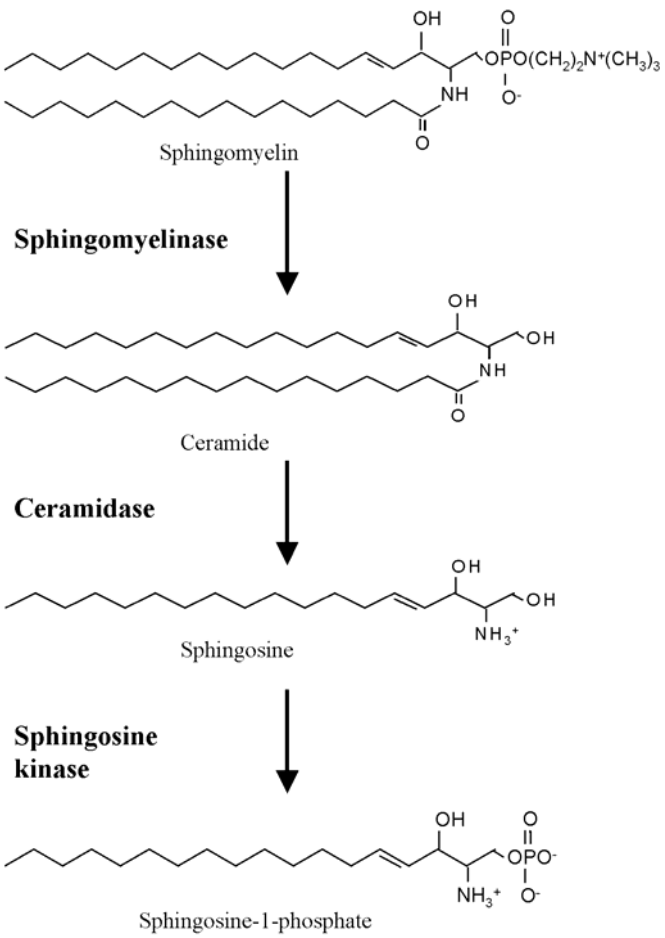

OH
O
OPO(CH2)2N+(CH3)3
O-
NH
O
Sphingomyelin
Sphingomyelinase
OH
OH
NH
O
Ceramide
Ceramidase
OH
OH
NH3+
Sphingosine
Sphingosine kinase
OH
O
OPO-
NH3+
O-
Sphingosine-1-phosphate

Fig. 2

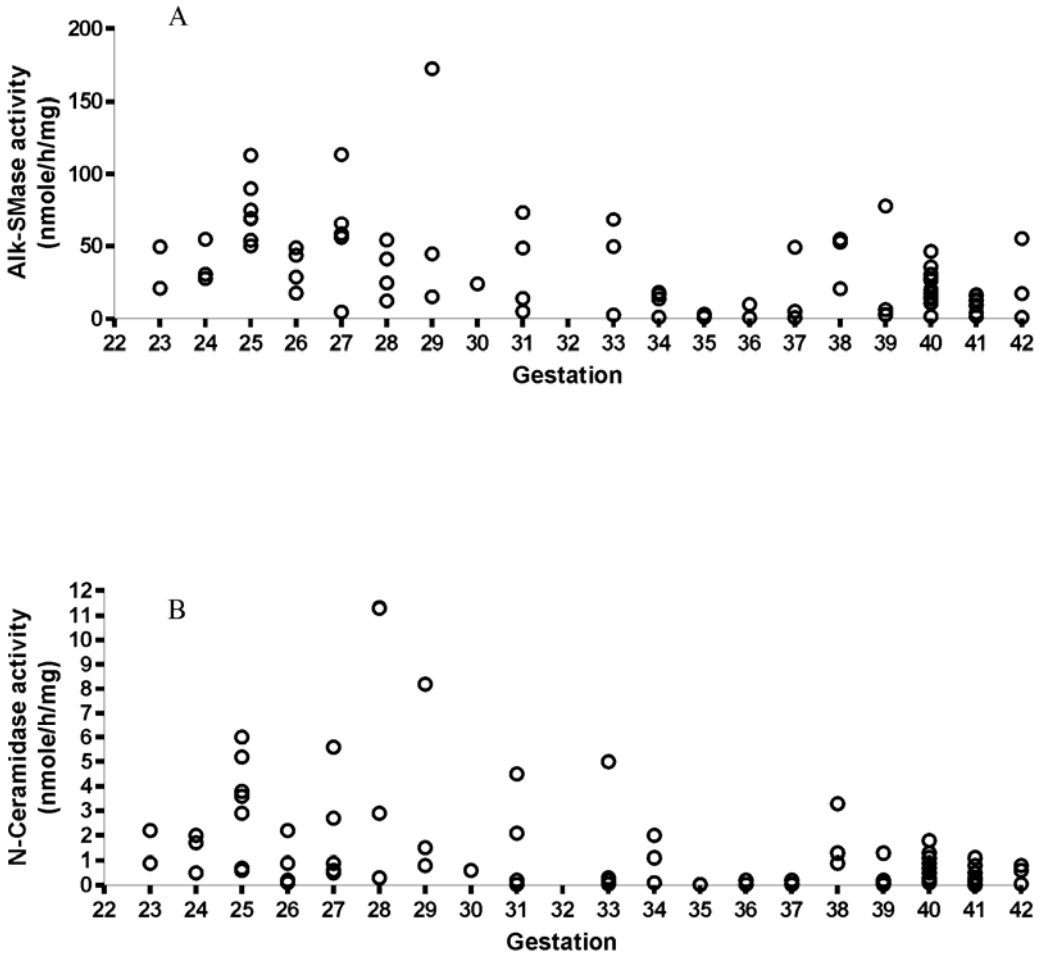

A
Alk-SMase activity (nmole/h/mg)
200
150
100
50
0
22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42
Gestation
B
N-Ceramidase activity (nmole/h/mg)
12
11
10
9
8
7
6
5
4
3
2
1
0
22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42
Gestation

Fig. 3

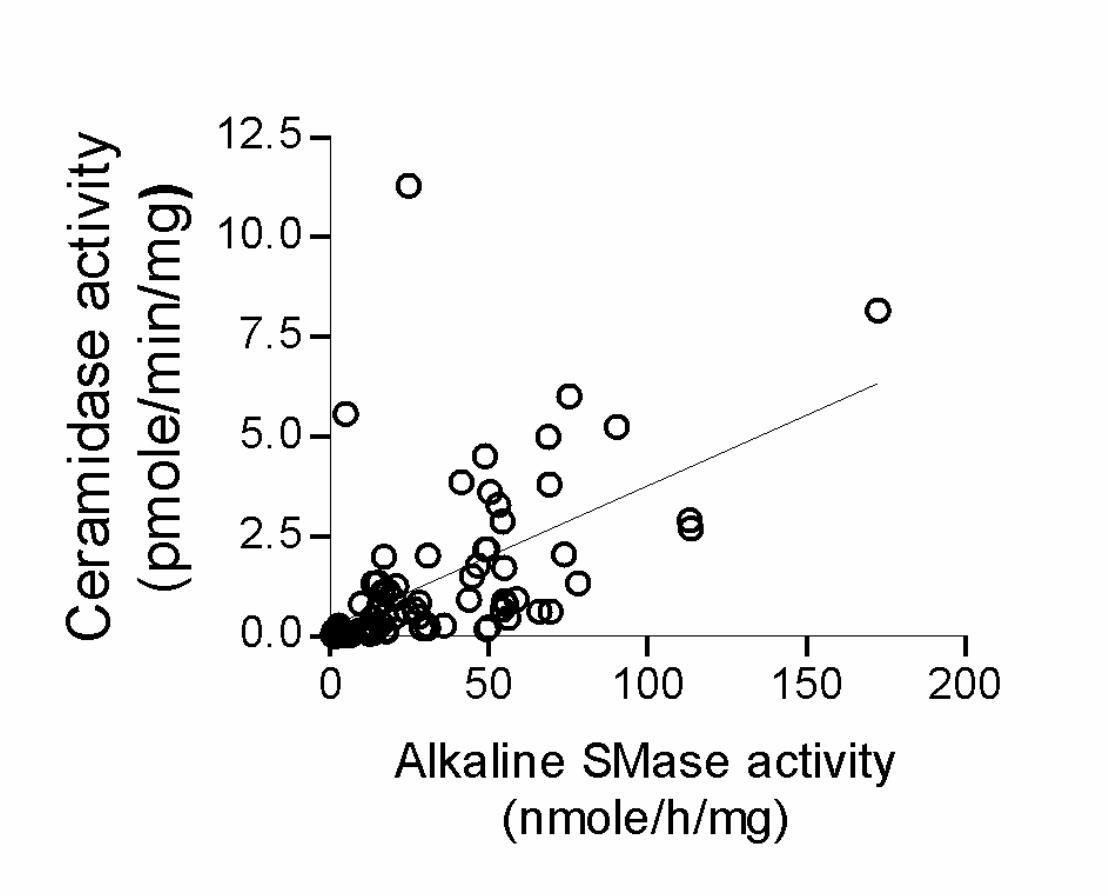

Ceramidase activity
(pmole/min/mg)
12.5
10.0
7.5
5.0
2.5
0.0
0
50
100
150
200
Alkaline SMase activity
(nmole/h/mg)

Fig. 4

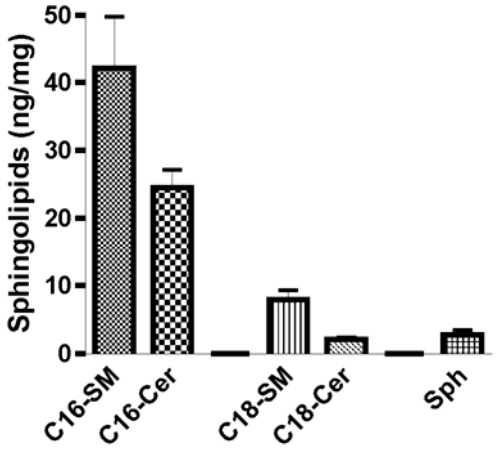

Sphingolipids (ng/mg)
50
40
30
20
10
0
C16-SM
C16-Cer
C18-SM
C18-Cer
Sph

Fig. 5

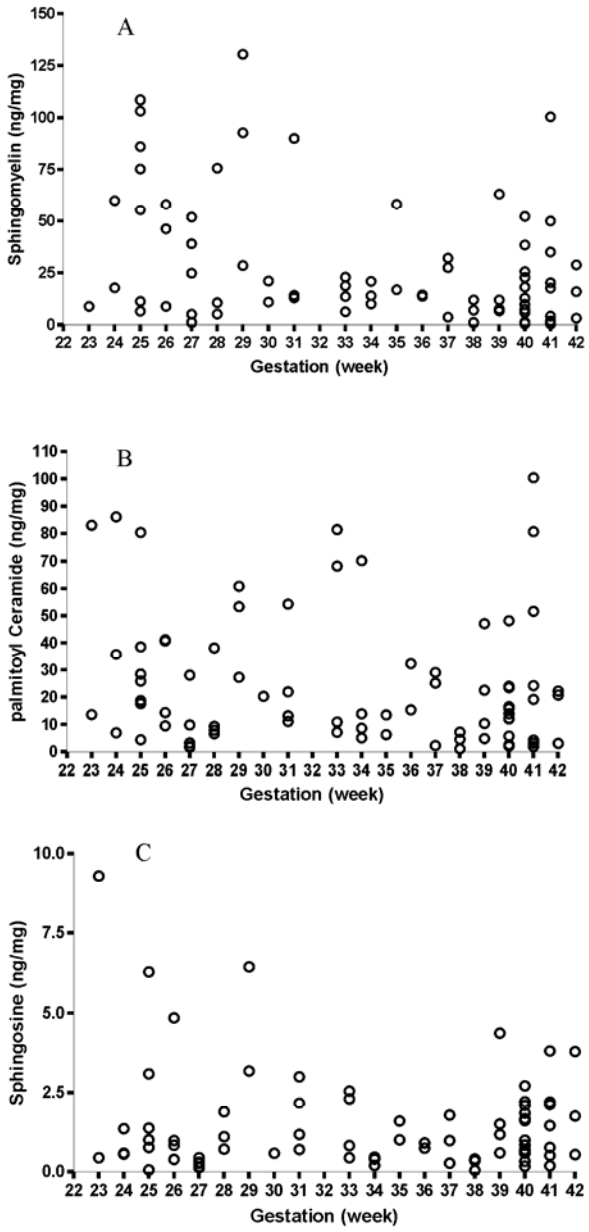

A
Sphingomyelin (ng/mg)
Gestation (week)
B
palmitoyl Ceramide (ng/mg)
Gestation (week)
C
Sphingosine (ng/mg)
Gestation (week)

Fig. 6

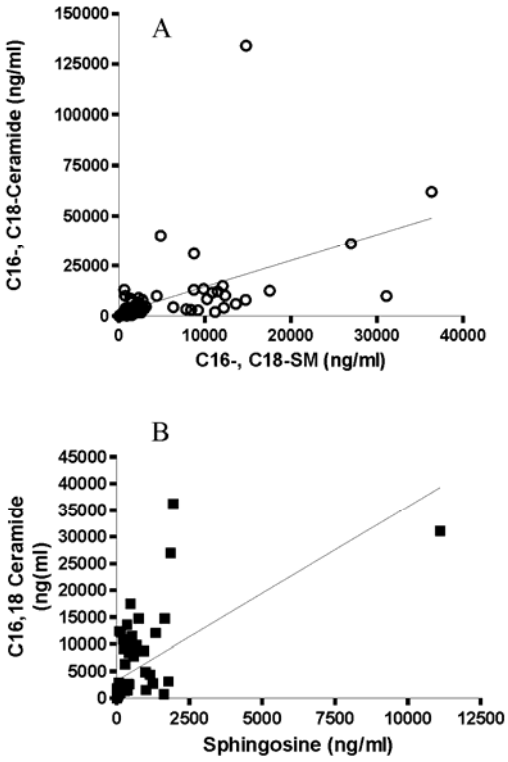

A
C16-, C18-Ceramide (ng/ml)
150000
125000
100000
75000
50000
25000
0
0
10000
20000
30000
40000
C16-, C18-SM (ng/ml)
B
C16,18 Ceramide (ng(ml)
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
0
2500
5000
7500
10000
12500
Sphingosine (ng/ml)

Table 1. Characteristics of study population, values are median, ranges and percentages.

	Group 1 (n=12)	Group 2 (n=13)	Group 3 (n=8)	Group 4 (n=13)	Group 5 (n=38)
Gestational age, w	25 (23-25)	27 (26-28)	30.5 (29-31)	34 (33-36)	40 (37-42)
Birth weight, g	700 (475-1000)	1126 (396-1460)	1493 (976-2500)	2290 (1645-3175)	3640 (2150-4400)
5 min Apgar score	7 (4-10)	7.5 (5-9)	8.5 (4-10)	8 (6-10)	10 (8-10)
Male gender, n (%)	8 (66.7)	9 (69.2)	2 (25)	6 (46.2)	21 (55.3)
Antenatal steroids, n (%)	12 (100)	11 (85)	6 (75)	8 (62)	2 (5)
Surfactant treatment, n (%)	11 (92)	10 (77)	3 (38)	1 (8)	0
Antibiotics, n (%)	12 (100)	13 (100)	8 (100)	9 (69)	0
Postnatal age at meconium sampling, h	92 (48-175)	100 (24-208)	38.5 (24-111)	15 (3-180)	16.5 (7-83)

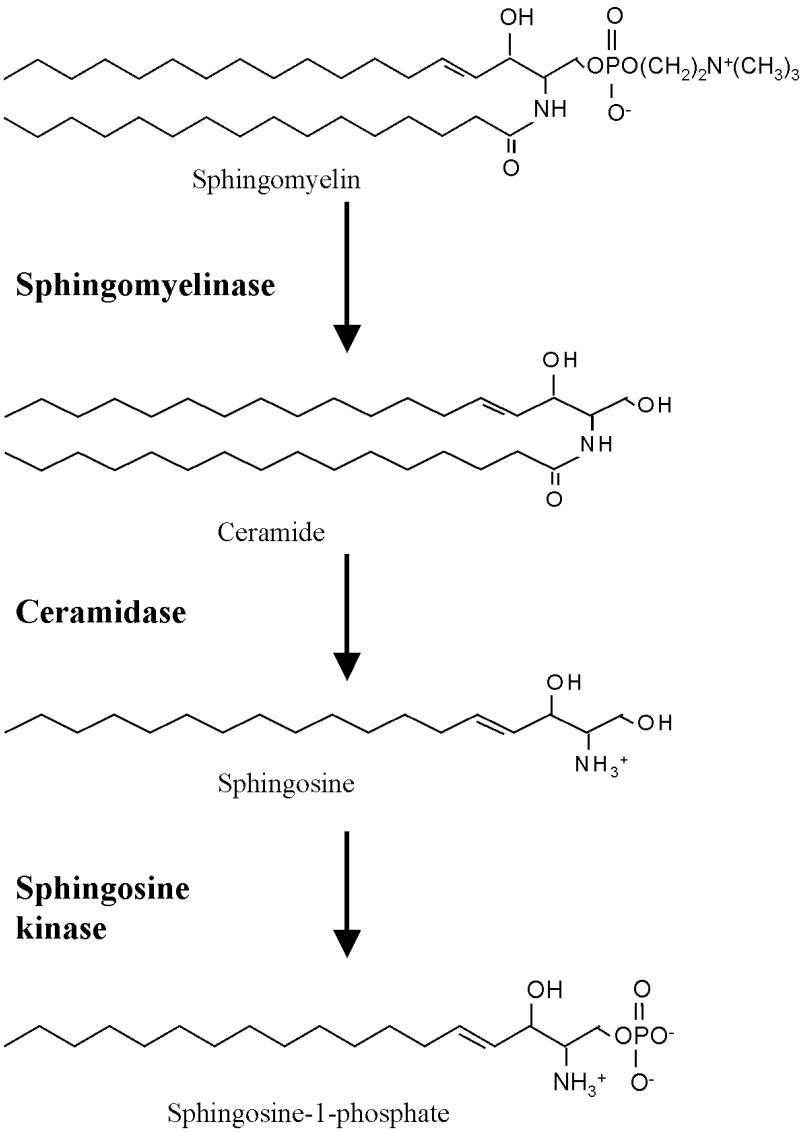

OH
O
OPO(CH2)2N+(CH3)3
O-
NH
O
Sphingomyelin
Sphingomyelinase
OH
OH
NH
O
Ceramide
Ceramidase
OH
OH
NH3+
Sphingosine
Sphingosine kinase
OH
O
OPO-
NH3+
O-
Sphingosine-1-phosphate

Figure 1

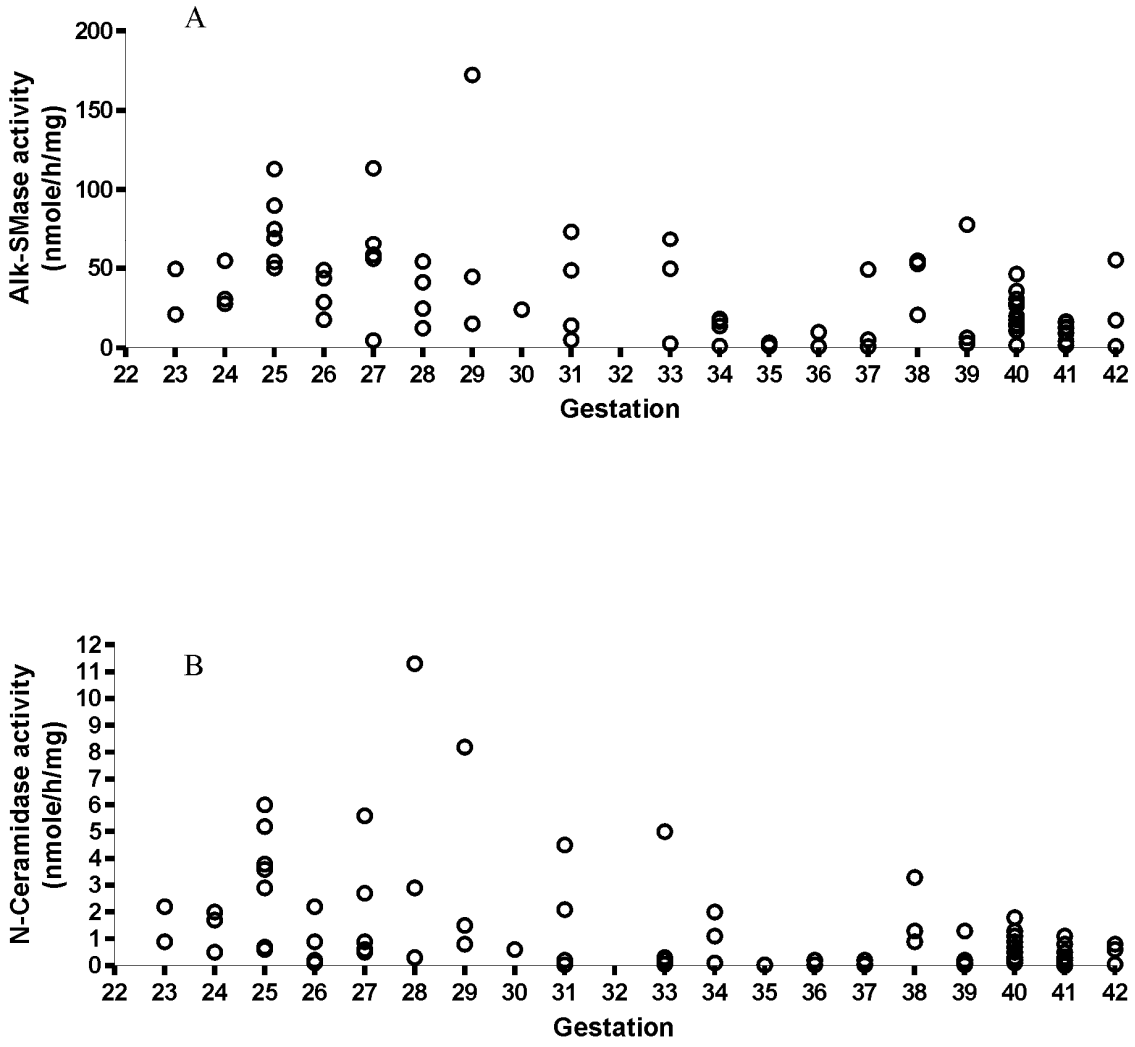


Figure 2

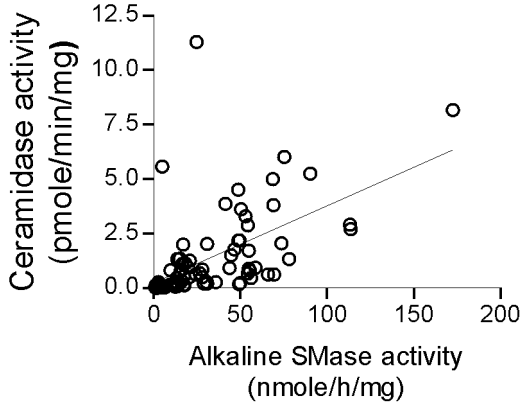


Figure 3

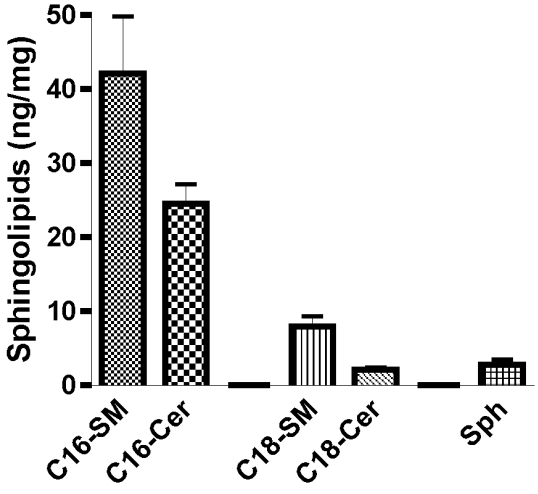
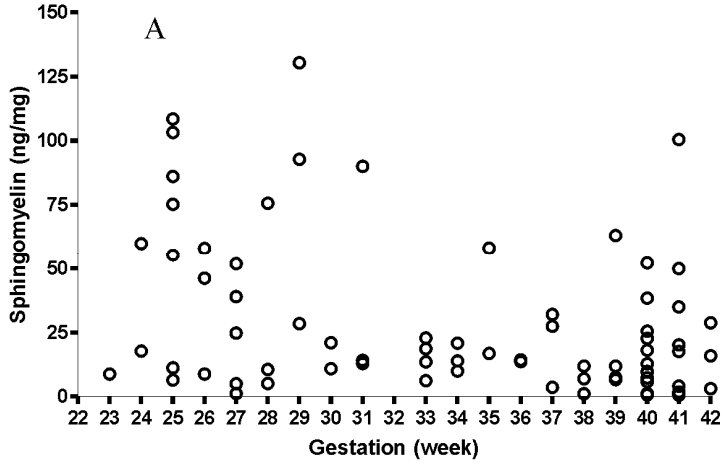
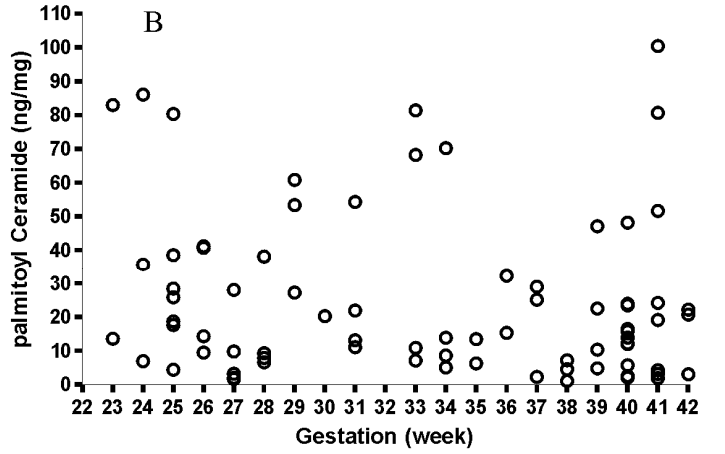


Figure 4


A
Sphingomyelin (ng/mg)
150
125
100
75
50
25
0
22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42
Gestation (week)


B
palmitoyl Ceramide (ng/mg)
110
100
90
80
70
60
50
40
30
20
10
0
22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42
Gestation (week)

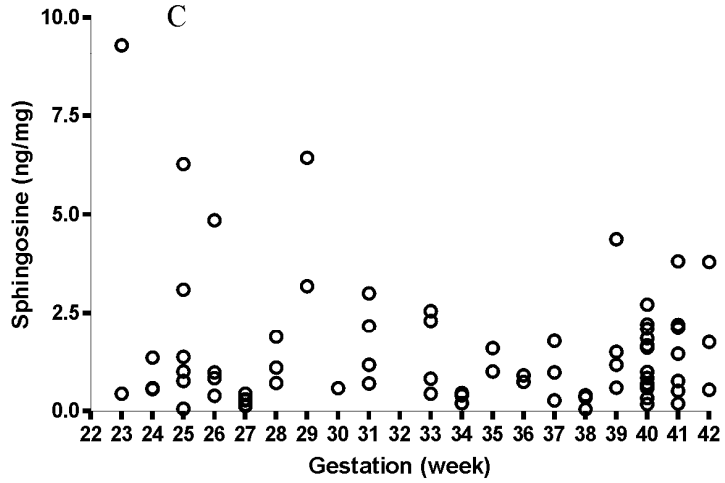

C
Sphingosine (ng/mg)
10.0
7.5
5.0
2.5
0.0
22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42
Gestation (week)

Figure 5

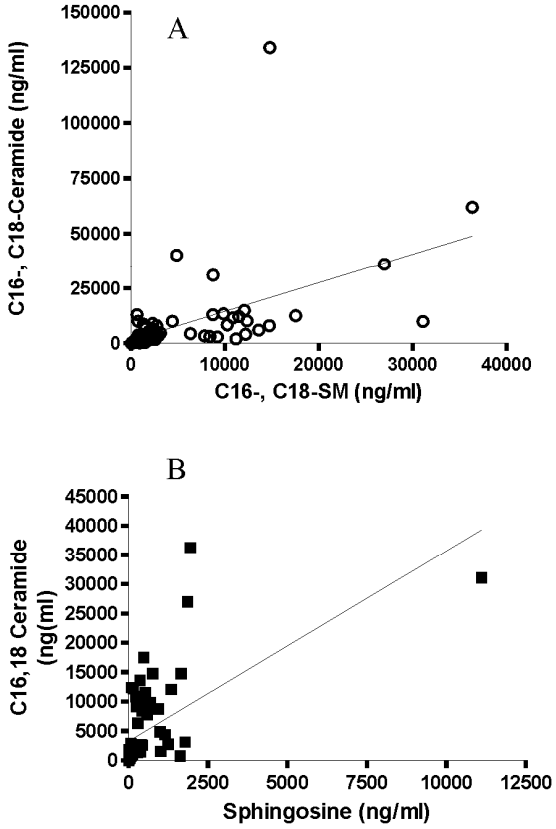

A
C16-, C18-Ceramide (ng/ml)
150000
125000
100000
75000
50000
25000
0
0
10000
20000
30000
40000
C16-, C18-SM (ng/ml)
B
C16,18 Ceramide (ng(ml)
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
0
2500
5000
7500
10000
12500
Sphingosine (ng/ml)

Figure 6