Plasma ceramide and lysophosphatidylcholine inversely correlate with mortality in sepsis patients

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Abstract **Recent data indicate that ceramide (Cer) and lysophosphatidylcholine (LPC) regulate immune cell functions. Since these bioactive lipids are generated in blood plasma by inflammatory lipases, we hypothesized that they may be involved in the process of acute systemic sepsis. In order to provide support for this hypothesis, we analyzed the plasma levels of Cer and LPC by quantitative tandem mass spectrometry in 102 sepsis patients starting with the day at which the sepsis criteria were fulfilled for the first time, as well as on day 4 and day 11. The values were compared with 56 healthy controls and correlated with sepsis-related mortality within 30 days of study entry. Most Cer species were increased in sepsis patients, while all LPC species were markedly decreased. In addition, we determined the molar ratios with their precursor molecules sphingomyelin (SPM) and phosphatidylcholine (PC), which reflect the enzymatic reactions responsible for their formation. Species-specific as well as total Cer-SPM ratios were increased, whereas LPC-PC ratios were decreased in sepsis patients. The increased Cer-SPM ratios as well as the decreased LPC-PC ratios showed a strong predictive power for sepsis-related mortality. Together with existing data from in vitro experiments and animal models, the results provide the first ex vivo indication for the role of Cer and lysophospholipids in systemic inflammation in humans.**—Drobnik, W., G. Liebisch, F-X. Audebert, D. Fröhlich, T. Glück, P. Vogel, G. Rothe, and G. Schmitz. **Plasma ceramide and lysophosphatidylcholine inversely correlate with mortality in sepsis patients.** ***J. Lipid Res.*** **2003.** 44: **754–761.**

Supplementary key words outcome • lipoproteins • sphingomyelinase • phospholipase • SAPS score • prognostic marker

Sepsis is a systemic response to infection and the most common cause of death in noncoronary intensive care units. The current pathophysiological concepts of the sepsis syndrome suggest that a local immune response is followed by a systemic release of proinflammatory as well as antiinflammatory mediators, leading to a systemic disturbance of inflammatory balance that leads to progressive endothelial dysfunction, loss of blood pressure control, deterioration of the coagulation system, and ultimately multiple organ dysfunctions. Beside cytokines, well-established inflammatory lipid mediators like platelet-activating factor and eicosanoids have been associated with the development of the sepsis syndrome (1). Recent data indicate that other groups of bioactive lipids similarly transfer significant effects to immune and inflammatory cells and may therefore be regarded as candidate mediators in the septic process. Thus, for the lysophospholipid lysophosphatidylcholine (LPC), a role as regulator of immune functions is emerging (2). LPC produced by the action of the proinflammatory phospholipase A_2 on phosphatidylcholine (PC) promotes inflammatory effects, including increased endothelial expression of adhesion molecules and growth factors (3, 4), monocyte chemotaxis (5), and macrophage activation (6). Moreover, LPC has been implicated in the pathogenesis of atherosclerosis and autoimmune disease (7, 8). Ceramide (Cer), the initial breakdown product of sphingomyelin (SPM), is another example of a bioactive lipid with the potential to regulate immune cell function. Cer is a well-known intracellular second messenger, and its concentration in mononuclear cells of sepsis patients has been identified as a possible marker to predict multiorgan dysfunction (9). In addition, Cer may also act as an extracellular agonist. Thus, Cer shows some structural similarity with bacterial lipopolysaccharide (LPS), which is known to elicit strong proinflammatory responses that can cause a fatal sepsis syndrome in humans (10). We have recently shown that Cer

Manuscript received 9 October 2002 and in revised form 3 January 2003.

Published, JLR Papers in Press, January 16, 2003.
DOI 10.1194/jlr.M200401-JLR200

Abbreviations: AUC, area under the curve; Cer, ceramide; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; ROC, receiver operating curve; SPM, sphingomyelin.

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is a ligand for the LPS receptor CD14 and induces its clustering with other membrane proteins to form a multireceptor signaling complex (11). This Cer-induced receptor cluster is partially overlapping with that formed after LPS stimulation, indicating that Cer may modulate immune cell function, in part, differentially to LPS via the CD14 pathway. Since Cer and LPC are present in blood plasma, it is conceivable that they provide a regulatory environment for patrolling immune cells, and their plasma levels show some association with functionally related disorders. Indeed, in a pilot study with a small number of subjects, we observed increased levels of certain Cer species in sepsis patients (11). In the current study, we provide data on plasma levels of Cer and LPC from 102 sepsis patients. In addition to the absolute concentrations, we also analyzed the molar ratios with their respective precursor molecules. These ratios reflecting the enzymatic reactions responsible for the generation of both lipids were found to have a highly predictive power in respect to sepsis-related mortality. Together with existing data from in vitro experiments and animal models, our data support a role for Cer and LPC in the pathophysiology of the sepsis syndrome.

MATERIALS AND METHODS

Patients

Approval for this study was obtained from the local institutional review board, and informed consent was obtained from all patients included in the study. All patients who were admitted to the intensive care units of the University Hospital of Regensburg between October 1999 and July 2001 were evaluated prospectively. One hundred two patients were included in the study when they fulfilled the criteria of sepsis. According to the Consensus Conference Criteria (12), sepsis was diagnosed based on the presence of at least three systemic inflammatory response syndrome criteria with the simultaneous identification of focal infection by clinical, radiological, or microbiological criteria. Microbiological cultures were done according to standard techniques (13). Cultures were considered "positive" in the case of *a*) bacteremia, as determined by growth of an organism in at least one blood culture bottle, except for coagulase-negative staphylococci (these organisms needed to grow in more than two bottles drawn at different time points to be considered relevant). *b*) Growth of organisms from normally sterile body sites/fluids/cavities representing the focus of clinically-diagnosed infection. *c*) Intra-abdominal infection due to perforated bowel, which was always classified as mixed gram-positive/gram-negative infection, regardless of which organisms were eventually recovered by culture. *d*) Pulmonary infection with infiltrates on chest X-ray, and growth of a pulmonary pathogen from sputum or tracheal aspirate together with abundant white blood cells and abundant organisms on gram-stain exhibiting the same staining characteristics as the isolate from culture, or a positive culture from bronchoalveolar lavage fluid, or a positive legionella antigen test from urine. Enterococci, coagulase-negative staphylococci, and *Candida spp.* were not considered pulmonary pathogens. *e*) Growth of pathogens in cultures taken from wounds with draining pus, fasciitis, cellulitis, sinusitis, or pelvic infections. Coagulase-negative staphylococci were not considered pathogens in this respect. The isolated organisms were classified as gram-negative, gram-positive, viral, or fungal according to standard criteria (13). Microbiological cultures were considered negative if no relevant organism was identified despite appropriate sampling of specimens and the presence of the above-mentioned criteria for sepsis (e.g., if the patient had received antibiotic therapy before specimens were sent for culture).

The patients were assigned to the group of survivors or nonsurvivors, depending on mortality within 30 days after study entry, as obtained form hospital records. The Simplified Acute Physiology Core II (SAPS II) was used to classify the severity of sepsis. The first blood sample was drawn as soon as possible after patients fulfilled the criteria of sepsis for the first time (day 1). Subsequent samples were taken 3 days and 10 days thereafter. All blood samples were immediately delivered to the central laboratory and, after centrifugation, plasma was stored at −80°C within 2 h after blood drawing. Blood samples and clinical records were missing from one (1.5%), two (3%), and ten (15.8%) surviving patients at day 1, day 4, and day 11, respectively. The control group consisted of 56 healthy blood donors.

Determination of lipids

Bioactive lipids were determined as follows: reagents, methanol, and chloroform were all HPLC grade and were from Merck (Darmstadt, Germany); ammonium acetate and ammonium formiate were of the highest analytical grade available and purchased from Fluka (Buchs, Switzerland). Cer, PC, SPM, and LPC standards were all from Avati Polar Lipids (Alabaster, AL) with purity higher than 99%. Quantification of lipids by tandem mass spectrometry: 20 μl of citrate plasma was extracted according to the protocol by Bligh and Dyer (14). Cer species were quantified by electrospray ionization tandem mass spectrometry according to the principle described previously, with some modifications (15). Briefly, Cer was analyzed by direct-flow injection analysis with a Waters Alliance 2790 (Milford, MA), providing a constant flow of methanol 20 mM ammonium formiate coupled to a triple quadrupole mass spectrometer (Quattro LC, Micromass, UK) equipped with an electrospray ion source operated in positive ion mode. The Cer specific daughter ion of *m/z* 264 was used, and C17:0-Cer was utilized as a not-naturally-occurring internal standard. Quantification was achieved by addition of naturally occurring Cer species. LPC was quantified as described in a separate manuscript (16). Briefly, LPC species were monitored by the phosphocholine-specific parent scan of 184 *m/z* using not-naturally-occurring C13:0-LPC and C19:0-LPC as internal standards. For the quantification of SPM and PC, a parent scan of 184 *m/z* was applied and the not-naturally-occurring PC species C28:0-PC and C44:0-PC were used as internal standards. Overlapping PC and SPM isotope species were corrected by theoretically calculated isotope distribution. The quantification of the phosphocholine-containing lipids was achieved by calibration lines generated by the standard addition of different naturally occurring species varying in chain length and degree of unsaturation. The triple quadrupole mass spectrometer was operated with the following settings: capillary 3.5 kV, cone 41 V for PC-SPM and LPC, 40 V for Cer, collision energy 24 V, collision gas pressure 1.3 10^{-3} Torr argon for phospholipids, 0.8 10^{-4} Torr for Cer. Mass resolution was above unit resolution. Data analysis was performed with MassLynx software, including the NeoLynx tool (Micromass), by averaging the scans at half-peak height of the total ion count. NeoLynx results were exported to Excel spreadsheets and further processed by self-programmed Excel macros. The intra-day and inter-day coefficients of variation (CV) for Cer, LPC, SPM, and PC analysis were <5% and <12%, respectively.

Total cholesterol, triglycerides, and HDL cholesterol (HDL-C) were determined using routine enzymatic methods on a Bayer ADVIA 1650 analyzer. LDL and VLDL were calculated according to the Friedewald equation.

Statistical analysis

The data are expressed as mean ± SE. The Mann-Whitney unpaired test was used to examine differences between patient and control groups. The performance of plasma bioactive lipids in predicting sepsis-related mortality was compared with that of the SAPS II score by using a receiver operating curve (ROC) approach. ROC curves represent graphically the principle of a spectrum of sensitivity-specificity in which all possible decision thresholds are included. In all tests, $P < 0.05$ was considered significant. The SPSS 10 for Windows (SPSS, Inc.) program was used for statistical analysis.

RESULTS

Patients

One hundred two patients were included in the study the first time they fulfilled the criteria of sepsis. In regard to the detected microorganisms, the following distribution was observed: gram-positive bacteria, 32 patients; gram-negative bacteria, 28 patients; mixed gram-positive and gram-negative bacteria, 13 patients; fungi, 13 patients; viral, 2 patients; others, 2 patients. Twelve patients were culture-negative but fulfilled the criteria for sepsis (e.g., if the patient had received antibiotic therapy before specimens were sent for culture).

Within 30 days, 39 patients (38.2%) died because of sepsis-related reasons. Of these 39 nonsurvivors, 8 patients died within the first three days, 13 patients died within 10 days, and 19 patients died between day 11 and day 30. The baseline demographics of survivors and nonsurvivors are shown in **Table 1**. Total cholesterol, HDL-C, and LDL-C in sepsis patients were markedly reduced compared with a healthy population. In addition, HDL-C and LDL-C tended to be lower in nonsurvivors compared with survivors, although this did not reach statistical significance.

TABLE 1. Patient demographics at study entry

Variable	Survivors (n = 63)	Nonsurvivors (n = 39)
Age, years, mean	53.8 (range: 20–91)	54.9 (range: 17–80)
Gender F/M	20/43	11/28
Patients with at least three out of the following four SIRS criteria	63	39
Rectal temperature >38°C or <36°C	45	32
White blood cell count >12/nl or <4/nl	56	33
Heart rate >90 beats/min	55	36
Respiratory rate >20/min or assisted ventilation	63	38
Lipid values		
Cholesterol (mg/dl)	91 ± 5	85 ± 9
Triglycerides (mg/dl)	207 ± 20	236 ± 28
VLDL cholesterol (mg/dl)	41 ± 4	47 ± 6
LDL cholesterol (mg/dl)	38 ± 4	29 ± 6
HDL cholesterol (mg/dl)	12 ± 1	9 ± 1

SIRS, systemic inflammatory response syndrome. Except for age and lipoprotein values, data are numbers of patients. Lipid values are expressed as mean ± SE. Normal values: rectal temperature, 36.5–37.4°C; white blood cell count, 4.8–10.8/nl; heart rate, 60–75 beats/min; cholesterol, <200 mg/dl; triglycerides, <200 mg/dl; VLDL cholesterol, <40 mg/dl; LDL cholesterol, <150 mg dl; and HDL cholesterol, 35–68 mg/dl.

Cer and LPC plasma concentrations in sepsis patients and healthy controls

As shown in **Fig. 1A**, eight different Cer species with a concentration above 0.1 μmol/l could be detected in plasma of healthy controls, with C24:0 and C24:1 as the major species. Compared with these control values, plasma samples of sepsis patients showed significant dif-

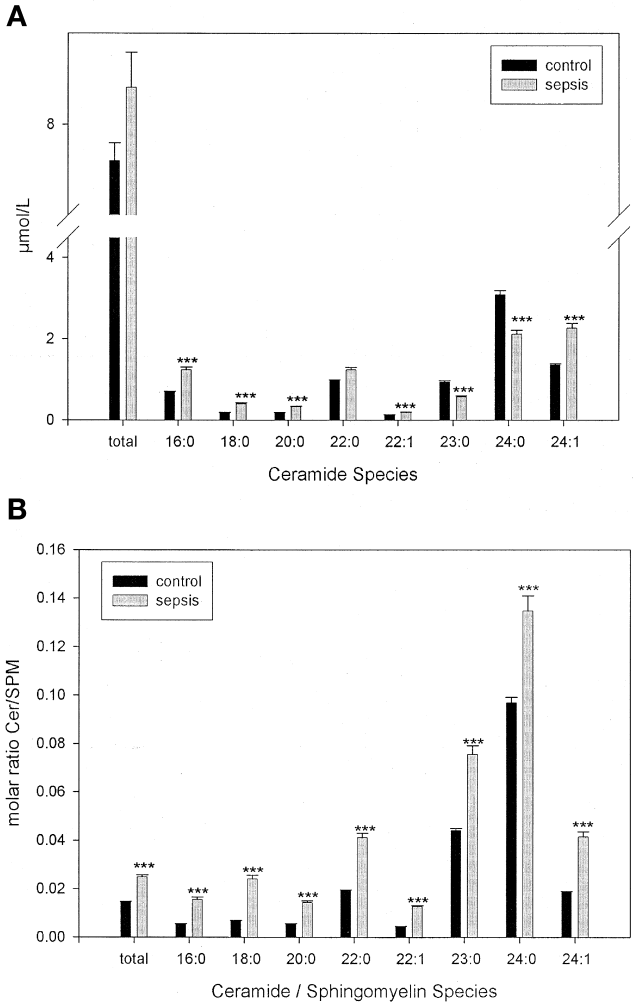

Fig. 1. Plasma concentrations of ceramide (Cer) species and Cer-sphingomyelin (SPM) molar ratios in sepsis patients compared with healthy controls. Plasma concentrations of Cer and SPM species were determined in sepsis patients at the day of study entry (day 1, black bars) as well as in healthy controls (gray bars) by quantitative tandem mass spectrometry. Bar graphs in A represent total or species-specific Cer concentrations in μmol/l. Species are identified by the number of C-atoms in the N-linked acyl chain followed by the number of double bonds. B: Shows the total or species-specific molar ratios between Cer and SPM. The species-specific ratios are calculated from Cer and SPM species with identical N-linked acyl chains, as indicated in the figure. The data are expressed as mean ± SE from 101 sepsis patients and 56 healthy controls. *** $P < 0.001$ control versus sepsis; Mann-Whitney unpaired test.

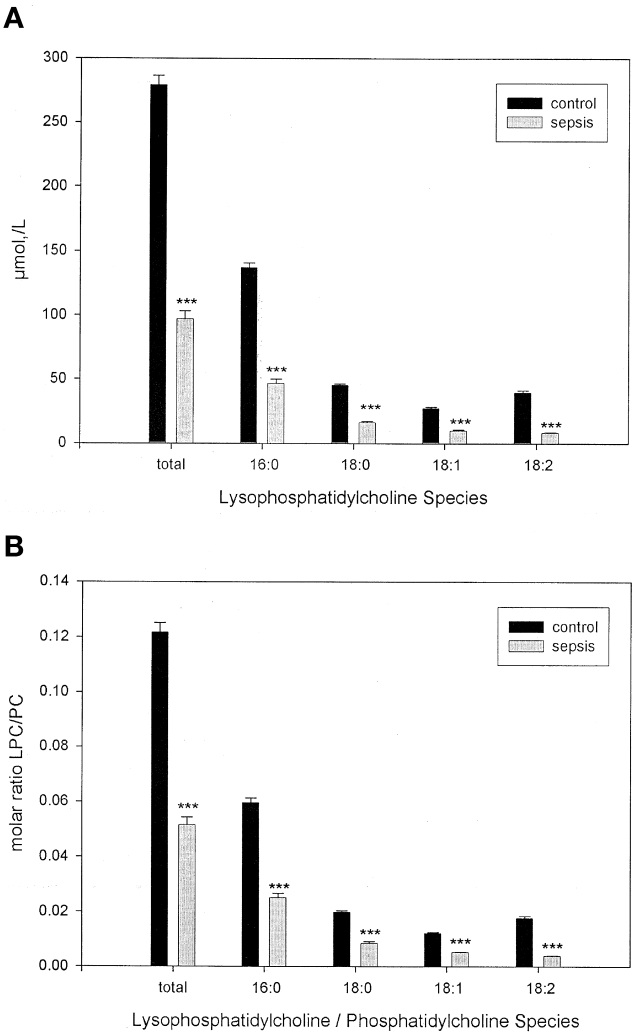

Fig. 2. Plasma concentrations of lysophosphatidylcholine (LPC) species and lysohosphatidylcholine-PC molar ratios in sepsis patients compared with healthy controls. Plasma concentrations of LPC and phosphatidylholine (PC) species were determined in sepsis patients at the day of study entry (day 1, black bars), as well as in healthy controls (gray bars) by quantitative tandem mass spectrometry. Bars graphs in A represent the concentration of total LPC and the concentration of the quantitatively major LPC species (~90% of total) in µmol/l. Species are identified by the number of C-atoms in the O-linked acyl chain followed by the number of double bonds. B: Shows the total or species-specific molar ratios between LPC and PC. In the case of species-specific ratios, saturated LPC species (C16:0 and C18:0) were related to total PC concentration, while unsaturated LPC species (C18:1 and C18:2) were related to the concentration of unsaturated PC. The data are expressed as mean ± SE from 101 sepsis patients and 56 healthy controls. *** $P <$ 0.001 control versus sepsis; Mann-Whitney unpaired test.

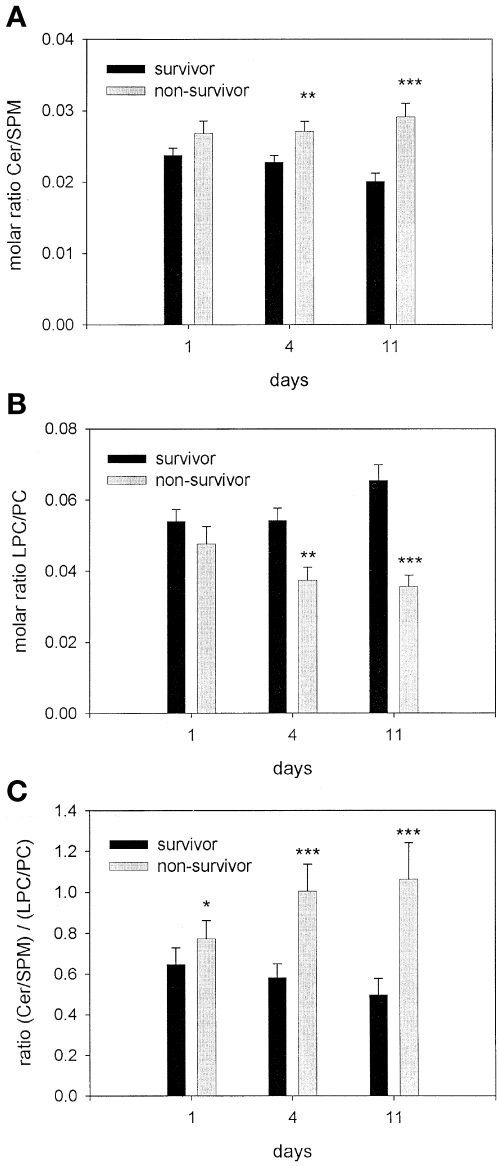

Fig. 3. Molar ratios of Cer-SPM and LPC-PC in survivors compared with nonsurvivors of sepsis. Plasma concentrations of total Cer, SPM, lysophosphatidylholine, and PC were determined in sepsis patients at the day 1, day 4, and day 11. A–C: Represent the molar ratios of total Cer-SPM, total lysophosphatidylcholine phosphatidylcholine (LPC-PC), and (Cer-SPM)-(LPC-PC) in survivors (black bars) and nonsurvivors (gray bars), respectively. The data are expressed as mean ± SE from 62 survivors versus 39 nonsurvivors at day 1; 61 survivors versus 31 nonsurvivors at day 4; and 53 survivors versus 19 nonsurvivors at day 11. *** $P < 0.001$, ** $P < 0.005$, * $P <$ 0.05 survivors versus nonsurvivors; Mann-Whitney unpaired test.

ferences. Whereas the concentration of most Cer species including C24:1 was significantly increased in sepsis patients, C24:0-Cer and C23:0-Cer levels were found to be decreased compared with controls (Fig. 1A). As a consequence of these inverse changes, the increase of total Cers in sepsis patients (representing the sum of all species) did not reach statistical significance. In contrast, the molar ratios between Cer and SPM species with the same N-linked acyl group, reflecting the enzymatic reaction responsible for Cer formation, were significantly increased in sepsis patients (Fig. 1B). Similarly, the molar ratio of total plasma Cer and total plasma SPM was markedly elevated in septic compared with control patients (Fig. 1B).

Tandem mass spectrometric analysis of plasma LPC revealed the presence of 12 different species; however, four species, i.e., C16:0, C18:0, C18:1, and C18:2, account for over 90% of total plasma LPC concentration. As shown in **Fig. 2A**, total LPC concentration, as well as the concentration of the main LPC species, was markedly reduced in pa-

tients with sepsis. Similar to the Cer-SPM ratio, we also related the concentration of LPC to its precursor molecule PC on a molar basis. Since the applied tandem mass spectrometry method did not allow discrimination of the molecular masses of the acyl chains at the *sn*-1 and the *sn*-2 position of the PC molecule, it was not possible to calculate the molar ratios for directly corresponding LPC-PC species pairs. Instead, we determined the molar ratios between C16:0- or C18:0-LPC and total PC, as well as the ratios between C18:1 or C18:2 LPC and the sum of all unsaturated PCs. Saturated LPC species were related to total PC, since saturated LPC could arise from unsaturated as well as saturated PC. Similar to the absolute concentrations of total LPC or the main LPC species, we observed a significant decrease of the respective molar ratios in sepsis patients compared with controls (Fig. 2B).

Molar ratios of Cer-SPM and LPC-PC in survivors and nonsurvivors of sepsis

Since the comparison of sepsis patients and healthy controls revealed substantial differences, especially with respect to the molar ratios of Cer-SPM and LPC-PC, we further investigated whether these ratios could be used to discriminate between survivors and nonsurvivors of sepsis. Thereby, we focused on the ratios based on total Cer, SPM, LPC, and PC concentrations. **Figure 3A** shows that, although the molar ratios of Cer-SPM did not significantly differ on day 1, the ratios progressively increased at day 4 and day 11 in the group of nonsurvivors, while they declined in the surviving group. This resulted in significantly different Cer-SPM ratios in survivors compared with nonsurvivors from day 4 on. A similar finding was observed for the LPC-PC ratio, with the important difference that the LPC-PC ratio was higher in survivors compared with nonsurvivors (Fig. 3B). These oppositional changes suggested that calculating the ratio between Cer-SPM and LPC-PC may help to better discriminate between both groups. Indeed, the (Cer-SPM)-(LPC-PC) ratio was already significantly different on day 1, and the differences on day 4 and day 11 were more pronounced compared with the Cer-SPM or LPC-PC ratios (Fig. 3C). In order to exclude the possibility that the increasing discriminatory power from day 1 to day 11 was biased by mortality-induced changes in the study population, we have separately analyzed the time course of the different molar ratios for those patients who where present all 3 days. The time kinetics of the molar ratios for this subpopulation were similar to the data shown for the complete study population (data not shown).

In order to investigate the predictive value of molar Cer-SPM, LPC-PC, and (Cer-SPM)-(LPC-PC) ratios, we analyzed the corresponding ROC in prediction of sepsis-related mortality within 30 days of study entry (**Table 2**). The area under the curve (AUC) values shown in Table 1 indicate that high Cer-SPM and low LPC-PC ratios at day 4 and day 11 are significantly associated with mortality, with AUCs comparable to that of the SAPS II score. High (Cer-SPM)-(LPC-PC) ratios were already significantly associated with mortality at day 1, and the AUCs at day 4 and day 11 showed a discriminative power slightly superior to the SAPS II score (Table 2). Statistical evaluation of predictive powers is shown in **Table 3**. At optimal cutoff values, the positive and negative predictive values at day 1 are similar for the (Cer-SPM)-(LPC-PC) ratio and the SAPS II score. At day 4 and day 11, the negative predictive values of all analyzed molar-lipid ratios ranged between 0.792 and 0.968, which is at least as good as that of the SAPS II score. The positive predictive values at these time points are ~0.50.

A potential influence of the involved microorganisms on lipid alterations was investigated by comparing the lipid ratios at study entry (day 1) between the largest subgroups as defined by the involved microorganisms, e.g., patients with either gram-positive (n = 32; 18 survivor and 14 nonsurvivor) or gram-negative (n = 28; 15 survivor and 13 nonsurvivor) bacterial infections. The Cer-SPM ratios, LPC-PC ratios, and (Cer-SPM)-(LPC-PC) ratios of patients with gram-positive and gram-negative bacterial infections were 0.025 ± 0.002 versus 0.025 ± 0.002 (P = 0.885), 0.059 ± 0.005 versus 0.044 ± 0.004 (P = 0.024), and 0.532 ± 0.071 versus 0.838 ± 0.128 (P = 0.095), respectively.

TABLE 2. Association between molar lipid ratios and sepsis-related mortality within 30 days after study entry

Day	Variable	Association with Mortality AUC	95% Confidence Interval	Survivor/Nonsurvivor	P
1	SAPS II	0.676	0.512–0.739	62/39	0.003
	Cer-SPM	0.596	0.484–0.709	62/39	0.104
	LPC-PC	0.596	0.479–0.712	62/39	0.106
	(Cer-SPM)-(LysoPC/PC)	0.629	0.518–0.741	62/39	0.029
4	SAPS II	0.723	0.607–0.839	57/31	0.001
	Cer-SPM	0.687	0.573–0.801	61/31	0.003
	LPC-PC	0.695	0.579–0.811	61/31	0.002
	(Cer-SPM)-(LPC-PC)	0.743	0.635–0.851	61/31	0.000
11	SAPS II	0.795	0.649–0.886	49/16	0.001
	Cer-SPM	0.818	0.709–0.927	53/19	0.000
	LPC-PC	0.776	0.670–0.881	53/19	0.000
	(Cer-SPM)-(LysoPC-PC)	0.829	0.735–0.924	53/19	0.000

Cer, ceramide; SPM, sphingomyelin; LPC, lysophosphatidylcholine; SAPS II, Simplified Acute Physiology Core II; PC, phosphatidylcholine; AUC, area under the receiver operating characteristic curves in prediction of sepsis-related mortality within 30 days after study entry. For calculation of receiver operating curve, low LPC-PC ratios and high values for all other variables were assumed to be predictive for mortality.

TABLE 3. Predictive power of molar lipid ratios for sepsis-related mortality within 30 days after study entry at optimal cutoff levels

Day	Variable	Cutoff Value	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
1	(Cer-SPM)-(LPC-PC)	≥0.6457	0.615	0.726	0.581	0.753
	SAPS II	≥51.5	0.590	0.758	0.601	0.749
4	(Cer-SPM)-(LPC-PC)	≥0.5578	0.774	0.689	0.558	0.857
	Cer-SPM	≥0.0224	0.774	0.607	0.500	0.841
	LPC/PC	≤0.0400	0.645	0.689	0.513	0.792
	SAPS II	≥51.5	0.581	0.877	0.716	0.796
11	(Cer-SPM)-(LPC-PC)	≥0.5155	0.842	0.755	0.552	0.930
	Cer-SPM	≥0.0203	0.947	0.698	0.529	0.973
	LPC/PC	≤0.0553	0.947	0.585	0.450	0.968
	SAPS II	≥43.0	0.813	0.776	0.542	0.927

Cer, ceramide; SPM, sphingomyelin; LPC, lysophosphatidylcholine; PC, phosphatidylcholine. Optimal cut off was defined as the value when the sum of specificity and sensitivity reached a maximum.

Based on these data, microorganism-specific differences may be present; however, the relatively low number of individuals in this subgroup analysis limits the interpretation of the results and also prevents a valid evaluation of the predictive values in regard to sepsis-related mortality. Thus, further data from the ongoing study will be necessary to establish potential microorganism-dependent differences.

In order to investigate whether the analyzed molar-lipid ratios of bioactive lipids correlate with specific lipoprotein profiles, we compared the Cer-SPM, LPC-PC, and (Cer-SPM)-(LPC-PC) ratios in patients with high or low concentrations of HDL-C, LDL-C, and VLDL-C, respectively. The data shown in **Table 4** demonstrate that at day 11, low HDL and LDL as well as high VLDL levels correlate with prognostic unfavorable lipid ratios.

DISCUSSION

In the present study, we have shown that plasma levels of Cer and LPC are significantly different in sepsis patients compared with healthy controls. The concentration of all main plasma LPC species, and consequently also the total LPC level, was markedly decreased in sepsis patients. In contrast, total Cer levels showed a tendency toward increased levels in sepsis patients, with an inverse behavior of the two main species, C24:1- and C24:0-Cer, according to previous results from a pilot study (11). However, the observed plasma concentrations for LPC and Cer in sepsis patients as well as healthy controls clearly exceed the concentrations that were reported to induce biological effects in artificial plasma-free conditions. Thus, a significant fraction of these plasma lipids is located within biologically inactive lipoprotein- or albumin-associated pools (17). Since the actually active fraction could not be directly detected, we additionally determined the molar ratios of Cer-SPM and LPC-PC, which reflect the enzymatic reactions responsible for the generation of both bioactive lipids. As indicated by the enhanced Cer formation in sepsis patients, the ratios between Cer and SPM species with the same N-linked acyl moiety as well as the total Cer to SPM ratio were found consistently elevated in sepsis patients. Moreover, the total Cer-to-SPM ratio, as well as that of the individual species (data not shown) significantly discriminated between surviving and nonsurviving patients. Whereas higher molar Cer-SPM ratios predicted a worse outcome, the opposite was observed for the LPC-PC ratio. The inverse behaviors of both markers led us to test the predictive value of the combined (Cer-SPM)-(LPC-PC) ratio, which, at least in the early phase of sepsis (day 1), provided additional predictive power for sepsis-related mortality compared with the single ratios. In general, the discriminatory potency of all ratios increased during the study period. This is explained by the fact that, in survivors, the ratios developed toward the levels found in

TABLE 4. Comparison of molar lipid ratios between patients with high and low HDL cholesterol, LDL cholesterol, or VLDL cholesterol

	Lipoprotein			Cer-SPM			LysoPC-PC			(Cer-SPM)-(LPC-PC)		
		Mean	SE	Mean	SE	*P*	Mean	SE	*P*	Mean	SE	*P*
		mg/dl		*molar ratio*			*molar ratio*			*molar ratio*		
HDL	Low, n = 38	6.6	0.5	0.0270	0.0015		0.0415	0.0039		0.9752	0.1281	
	High, n = 34	20.2	0.9	0.0174	0.0009	0.000	0.0756	0.0047	0.000	0.2808	0.0306	0.000
LDL	Low, n = 39	29.7	4.2	0.0259	0.0015		0.0449	0.0042		0.8927	0.1238	
	High, n = 33	108.1	5.8	0.0184	0.0011	0.000	0.0726	0.0052	0.000	0.3573	0.0683	0.000
VLDL	Low, n = 37	27.9	1.1	0.0182	0.0011		0.0674	0.0047		0.3828	0.0638	
	High, n = 35	72.5	5.8	0.0269	0.0016	0.000	0.0472	0.0051	0.003	0.9269	0.1360	0.000

Mann-Whitney unpaired test was used to examine differences between patient groups with high and low lipoprotein serum concentrations. *P* level of significance. All values were determined at day 11 after study entry.

healthy controls, whereas in nonsurvivors, the opposite effect was observed. Despite the already remarkable predictive power of the analyzed ratios, with further increasing numbers of patients in our ongoing sepsis study, we attempt to identify subgroups of patients for whom the described lipid parameters may provide an even greater diagnostic benefit.

However, the strong association with sepsis-related mortality suggests that increased Cer, as well as decreased LPC levels, may be involved in the complex network of processes finally leading to the unfavorable outcome in many septic patients. In support of this hypothesis, recent data suggest a link between inflammation and extracellular Cer production. Secretory sphingomyelinase (sSPMase) activity, which mediates the extracellular degradation of SPM to Cer, was shown to increase during monocyte-to-macrophage differentiation (18), as well as after stimulation of endothelial cells with inflammatory cytokines (19). Moreover, Wong et al. recently showed that, in a mouse model, the induction of an acute systemic inflammation by injection of LPS increases the serum levels of sSPMase by a pathway involving interleukin-1 production (20). The sSPMase-mediated Cer production has been associated with the development of atherosclerotic lesions (21), since accumulation of Cer in lipoproteins promotes their aggregation and retention in the subendothelial space. However, the ability of Cer to induce clustering of the LPS receptor CD14 with other membrane proteins and the recent demonstration that Cer regulates the clustering of CD40 in lymphocytes (22) strongly suggest additional immunological functions for this bioactive lipid. The current finding of increased Cer-SPM ratios in septic patients and its correlation with disease mortality extends the data from these in vitro experiments and animal models to the in vivo situation of humans with systemic inflammation.

The inverse correlation of LPC-PC ratios (as well as absolute LPC concentrations; unpublished observations) with sepsis-related mortality seems to contradict the previously described proinflammatory effects of LPC. However, LPC has also been identified as a ligand for the immunoregulatory receptor G2A, which is predominantly expressed in immature T- and B-cells (23). G2A deficient mice develop an autoimmune syndrome with abnormal expansion of lymphocytes and hyper-responsive T-cells (24). Based on these data, LPC may exert immune-suppressive functions by its binding to G2A, which could explain the observed association of low LPC levels and poor outcome in sepsis patients. Alternatively, the decreased LPC concentration may reflect its enhanced conversion to lysophosphatidic acid (LPA) by the activity of a plasmatic lysophospholipase D (25). LPA binds to different members of the edg-receptor family (edg-2, edg-4, edg-7) and is known to induce a multitude of cellular responses, including LPA-driven effects on immune cells, like the promotion of T-cell and macrophage survival, and the increased endothelial adhesion molecule expression [for review see ref. (26)]. Moreover, LPA-mediated activation of edg-2 on T-cells was shown to stimulate interleukin-2 production and inhibit cell migration, whereas binding to edg-4 had the opposite effect (27, 28). Thus, LPA may also be an attractive candidate mediator in the sepsis process, and as a prerequisite to precisely quantify this bioactive lipid in a large series of patient samples to which we are currently extending our tandem mass spectrometry-based methodology. Future work will also be directed toward the issue of whether the observed association of unfavorable bioactive lipid ratios with low levels of HDL and LDL but high concentrations of VLDL are due to subclass-specific properties of lipoproteins in promoting or inhibiting the formation of bioactive lipids.

In summary, the data provide clinical support for in vitro experiments and data from animal models pointing to a role for Cer and lysophospholipids in the complex pathophysiology of the sepsis syndrome.

The excellent technical assistance of Daniela Hant, Doreen Müller, and Maria Baumgärtner is greatly appreciated.

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