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Lipidomic Profiling of Plasma and Erythrocytes From Septic Patients Reveals Potential Biomarker Candidates

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ABSTRACT

BACKGROUND: Sepsis remains the primary cause of death from infection, despite advances in modern medicine. The identification of reliable diagnostic biomarkers for the early detection of this disease is critical and may reduce the mortality rate as it could allow early treatment. The purpose of this study was to describe the changes in the plasma and red cells blood lipidome profiling of patients diagnosed with sepsis and septic shock with the aim to identify potentially useful metabolic markers.

METHODS: Lipids from plasma and erythrocytes from septic patients (n = 20) and healthy controls (n = 20) were evaluated by electrospray ionization quadrupole time-of-flight mass spectrometry, and the fatty acid composition of the phospholipids fraction of erythrocytes was determined by gas chromatography. The data were treated with multivariate data analysis, including principal component analysis and (orthogonal) partial least squares discriminant analysis.

RESULTS: Potential biomarkers including lysophosphatidylcholines (lyso-PCs) and sphingomyelin (SMs) with specific fatty acid chains were identified. Both Lyso-PCs and SMs were downregulated, whereas the saturated and unsaturated phosphatidylcholines (PCs) were upregulated in the plasma and erythrocytes of septic patients. An increase in oleic acid (C18:1 *n*-9) accompanied by a decrease in the unsaturation index as well as in the levels on *n*-3 polyunsaturated fatty acids was observed in erythrocytes phospholipids patients as compared with healthy controls.

CONCLUSIONS: These results suggest that lipidome profiling has great potential in discovering potential clinical biomarkers for sepsis and helping to understand its underlying mechanisms.

KEYWORDS: Biomarkers, lipidomic, sepsis, lysophosphatidylcholine, sphingomyelin

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Background

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection, and septic shock is defined as a subset of sepsis in which underlying circulatory, cellular, and metabolic abnormalities are profound enough to substantially increase mortality.[1]

Although the true incidence remains uncertain, conservative estimates indicate that sepsis is a leading cause of mortality and critical illness worldwide contributing to up to 5.3 million deaths worldwide per annum.[2] A study conducted in a general hospital in southern Brazil noted that 30% of admitted patients had sepsis and mortality was 66.5%.[3] Silva et al[4] reported the results of a prospective multicenter intensive care unit (ICU) screening study conducted in Brazil more than 9 months in 2001, in which they found an incidence density of 57 per 1000 patient-days corresponding to 30.5% screened ICU admissions. In a multicenter study involving 75 ICUs in all regions of Brazil 3128 patients were identified and 521 (16.7%) were diagnosed as having infection, sepsis, or septic shock. The overall mortality in 28 days was 46.6%.[5]

Sepsis, similar to other systemic inflammatory response syndromes, is characterized by increased secretion of stress hormones (eg, catecholamines and cortisol), cytokine overproduction, complement activation, and mitochondrial dysfunction with decreased availability of adenosine triphosphate. Sepsis-related inflammation causes microcirculatory dysfunction, inadequate tissue oxygen supply, and subcellular and cellular dysfunction.[6,7] Patients with organic dysfunction and hemodynamic instability present a high mortality rate from sepsis, and the application of adequate guideline-based therapy is related to a significant decrease in mortality. Kumar et al found that a delay of more than 1 hour in initiating antimicrobial use for unstable patients is related to higher mortality and so early diagnosis makes timely implementation of adequate therapy feasible.[8] However, antimicrobial use in the

absence of infection has its adverse effects, including the development of multidrug resistant microorganisms[9]; therefore, it is important to differentiate sepsis from other causes of systemic inflammation.

Previous studies have also demonstrated an increase in circulating phospholipase A2 type II (snp-PLA2) in patients with severe infection.[10–16] Group IIA sPLA2 is an acute-phase protein that is expressed in various tissues and cells in response to a variety pro-inflammatory cytokines and it serves to amplify the inflammatory signal and mediates the various phenomena that are seen in the inflammatory process.[14] Members of the sPLA2 family of enzymes generate important bioactive lipid mediators that include lysophosphospholipids and arachidonic acid and which can be converted to eicosanoids. Eicosanoids modulate cell growth and differentiation, immunity, inflammation, platelet aggregation, and many other functions. Eicosanoids produced from arachidonic acid by COX and LOX, respectively, are 2-series prostaglandins (PGs) and 4-series leukotrienes that act as mediators of inflammatory processes.[17]

Elevated plasma-free fatty acid (FA) levels,[18,19] changes of polyunsaturated FA (PUFA) metabolism,[20,21] decreased lysophosphatidylcholine (lyso-PC) levels, and increased ceramide (Cer) species rates in plasma are commonly associated with sepsis.[22–24]

Biomarkers have been used in a variety of disease processes and can help aid in diagnosing bacterial infections or even in the severity of sepsis. None of the currently tested new markers has sufficient specificity or sensitivity to perform as diagnostic tools. Procalcitonin and C-reactive protein have been most widely used but even these have limited ability to predict outcomes and lack accuracy to distinguish sepsis from other inflammatory conditions.[25] Profiles of lipids as accessed by lipidomics investigations may provide a chance for early diagnosis of diseases and increase the possibility of successful treatment. Mass spectrometry (MS) plays a prominent role in the lipid analysis. Although the initial cost of the equipment is high and laboratory expertise in the development, validation, and maintenance of MS-based assays may be limited, it still can be cost-effective for laboratories to develop MS tests to avoid send-out costs on higher-volume tests.[26] The advancement of this technology along with the development of new applications will accelerate the incorporation of MS into more areas of medicine.

Altered lipid profiles, along with the progress of diseases, together with genomics and proteomics, should provide new insights allowing a better understanding of the pathogenic mechanisms and to design new therapeutic strategies. Because sepsis and septic shock are accompanied by severe metabolic alterations, we hypothesize that a systematic characterization of lipids metabolites combined with multivariate data analysis should identify potential biomarkers. Herein we report on the gas chromatography (GC) and electrospray ionization quadrupole time-of-flight MS (ESI-MS q-ToF) monitoring of lipid profiles in plasma and erythrocyte membranes in the search of biomarkers that could diagnosis alterations in lipid dynamics in sepsis and septic shock.

Methods

Participants

This study has been approved by the Ethics Committee of the São Francisco University (CAEE 51356315.5.0000.5514). Written informed consent was obtained from the persons legally responsible for the patients according to the Declaration of Helsinki. This was a prospective study conducted in an adult medical ICU of the Hospital Universitário São Francisco na Providência de Deus (Bragança Paulista, SP, Brazil). A total In total, 65 patients admitted to the ICU during the study over a period of 16 months (2014 and 2015) were screened, 20 were included in the study (Figure 1) with sepsis (n = 9) and septic shock (n = 11). Exclusion criteria were age under 18 years, congenital lipid metabolism disorders, severe hemorrhagic disturbance, renal insufficiency, immunosuppressive therapy, and neoplasia. Blood samples were collected prior to initiation of enteral or parenteral therapy. The serum samples for healthy volunteers were collected at UNIFAG-USF (Unidade Integrada de Farmacologia e Gastroenterologia, Universidade São Francisco, Bragança Paulista, SP, Brazil) and revealed no clinically relevant abnormalities. Table 1 summarizes the major characteristics of all subjects.

Lipidomic analysis

Peripheral blood samples were drawn from patients within 36 hours after their admission to the ICU. Plasma and leukocytes were removed after centrifugation. Erythrocytes were washed and centrifuged twice. The samples were stored at −80°C until analysis. The blood samples of the control group (healthy volunteers) were subjected to the same procedure. Lipids from plasma and erythrocytes were extracted with chloroform-methanol (2:1) and an aqueous solution of KCl.[27] The lower lipid phase was collected and dried under nitrogen.

The separation of the phospholipids was performed using solid phase extraction with aminopropyl silica cartridges (Bond Elut NH2 cartridge; Agilent Technologies, Inc., Santa Clara, CA, USA).[28] The lipid extracts were diluted in 300 µL of methanol:chloroform (2:1) and 100 µL of this solution was rediluted in 400 µL of acetonitrile:chloroform (3:1), then 1 µL was injected into a MS using an LC (Agilent 1290) without a column and with a flow of 0.5 mL min^{-1} of acetonitrile:H_2O (1:1). The MS experiments were performed on 6550 iFunnel q-ToF (Agilent Technologies) coupled with a Dual Agilent Jet Stream ESI source (Dual-AJS-ESI). The positive ion mode was selected for the collection of the mass spectra using the following conditions: gas temperature at 290°C, drying gas flow at 11 L min^{-1}, nebulizer at 45 psi, sheath gas temperature at 350°C, sheath gas flow 12 L min^{-1} VCap 3000, nozzle voltage 320 V, fragmentor 100 V, and OCT 1 RFV pp 750 V.

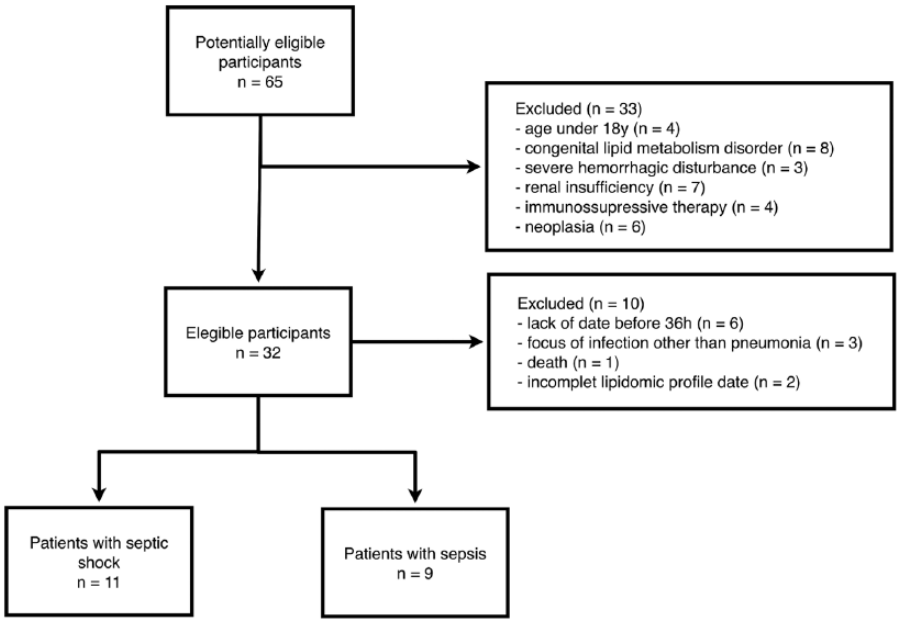

Figure 1. Patient selection flowchart.

Table 1. Demographic data and major clinical characteristics of septic patients and healthy volunteers.

	SEPTIC PATIENTS	HEALTHY VOLUNTEERS
N	20	20
Sex (M/F)	11:9	10:10
Age, y	55.7 ± 18.1	58.1 ± 11.2
BMI, kg/m^2	23.4 ± 4.8	21.8 ± 3.7
Albumin, g/L	2.8 ± 0.3	4.3 ± 0.5
C-reactive protein, mg/dL*	236.8 ± 82.4*	0.38 ± 0.24
Sepsis, No. (%)	9 (45)	—
Septic shock, No. (%)	11 (55)	—
APACHE II	14.8 ± 6.4	—
SAPS III	48.9 ± 31.7	
SOFA score	6.3 ± 4.1	—
Primary site of infection		—
Lungs (pneumonia)	15 (75)	
Urinary tract	3 (15)	
Abdomen	2 (10)	

Abbreviations: APACHE, Acute Physiology and Chronic Health Evaluation; BMI, body mass index; SAPS III, Simplified Acute Physiology Score III; SOFA, Sequential Organ Failure Assessment.
Data presented as mean ± SD.
*$P < .001$ compared with healthy volunteers.

Agilent Mass Hunter Qualitative Analysis software version B.07.00 was used to acquire and process the data. The ESI(+)-MS data were exported in Comma-Separated Values (CSV) files and statistical analyses were performed using MetaboAnalyst 2.0.

The FA composition of the phospholipids fraction of erythrocytes was determined by GC. The extracts were converted into FA methyl esters using BF_3 methanol[29] and a GC (Tech, Inc., Apple Valley, MN, USA) with a flame ionization detector equipped with a polar CP-Sil 88 column was used.[30] Fatty acid identification was

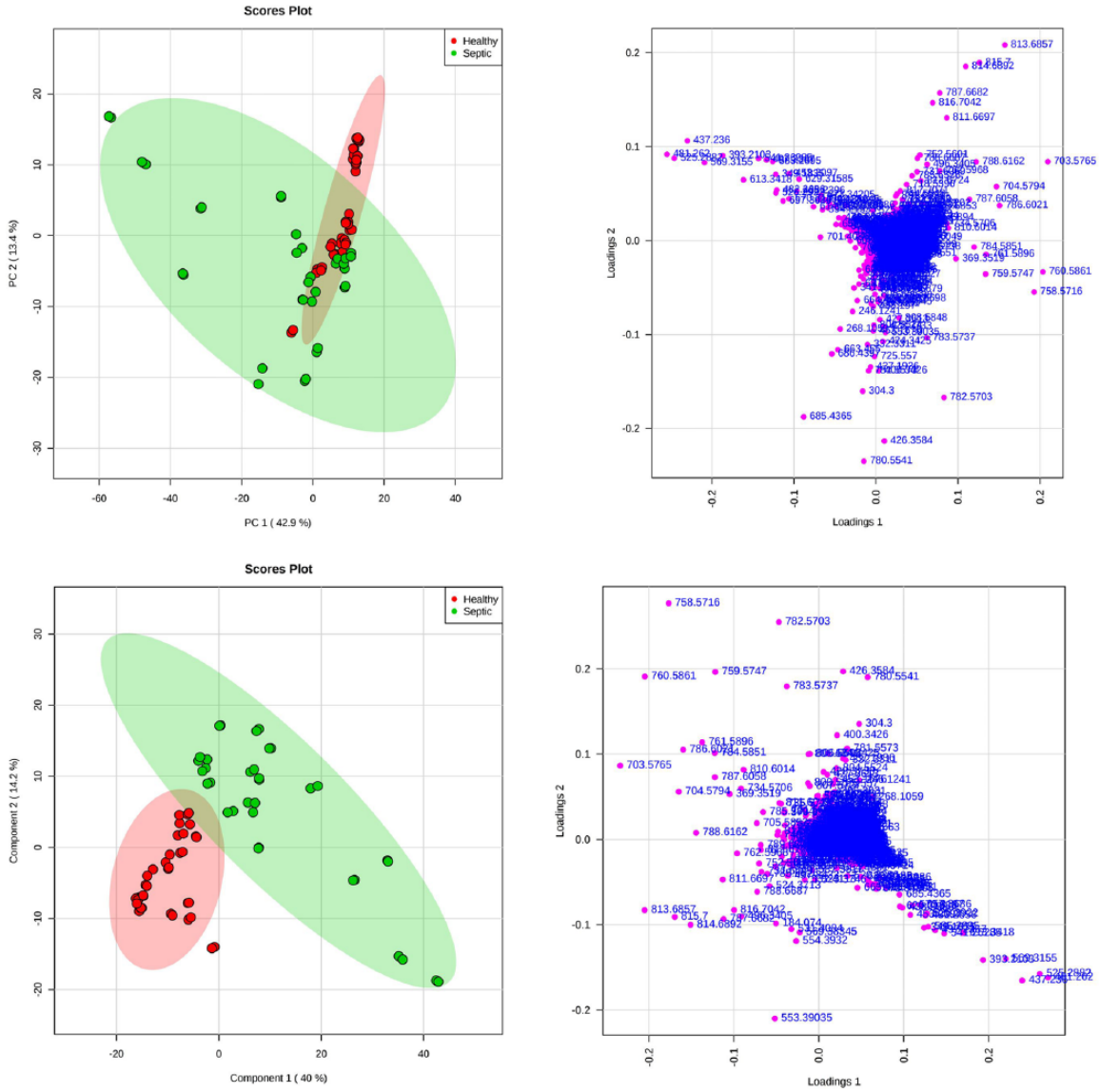

Figure 2. Top left: PCA scores plot of PC1 (first principal component) vs PC2 (second principal component) showing the separation between healthy volunteers (red) and septic patients (green). Top right: Loadings plot for PC1 and PC2 showing the metabolite ions (*m/z*) that were major contributors to the separation of groups observed in PCA scores plot. Bottom left: PLS-DA discrimination of MS spectra from healthy volunteers (red) and septic patients (green). Bottom right: Loadings plot for PC1 and PC2 showing the metabolite ions (*m/z*) that were major contributors to the separation of groups observed in PLS-DA scores plot. Analysis without previous variable selection. PCA indicates principal component analysis; PLS-DA, partial least squares discriminant analysis.

made by comparing retention times with authentic standards (Sigma-Aldrich, St. Louis, MO, USA) injected under the same conditions. Fatty acid composition was determined by comparing the retention times with authentic standards (Sigma-Aldrich) and calculating the relative percentages.

Statistical analysis

For the statistical analysis, each molecular feature (ion) was normalized by sum, and unsupervised segregation was evaluated using statistical Web platform MetaboAnalyst. Principal component analysis (PCA) was performed using Pareto and the results were used to show the lipids that most strongly influence the discrimination between groups. To enhance data discrimination, the data were also analyzed using the (orthogonal) partial least squares discriminant analysis ((O)PLS-DA) method. Biomarkers were selected according to their variable importance in projection (VIP) values. In addition, an independent *t* test ($P \leq .05$) was used to evaluate whether different biomarker candidates were statistically significant between groups. The differences of FA composition between groups were analyzed by 1-way analysis of variance, followed by the Tukey test and $P < .05$ was considered to be statistically significant.

Results

To access data quality of lipid matrix data, we first performed an unsupervised multivariate method (PCA) because it may show sample outliers and/or reveal hidden biases (Figure 2). Our previous results showed 3 possible subgroups in septic patients, which are further correlated with primary site of

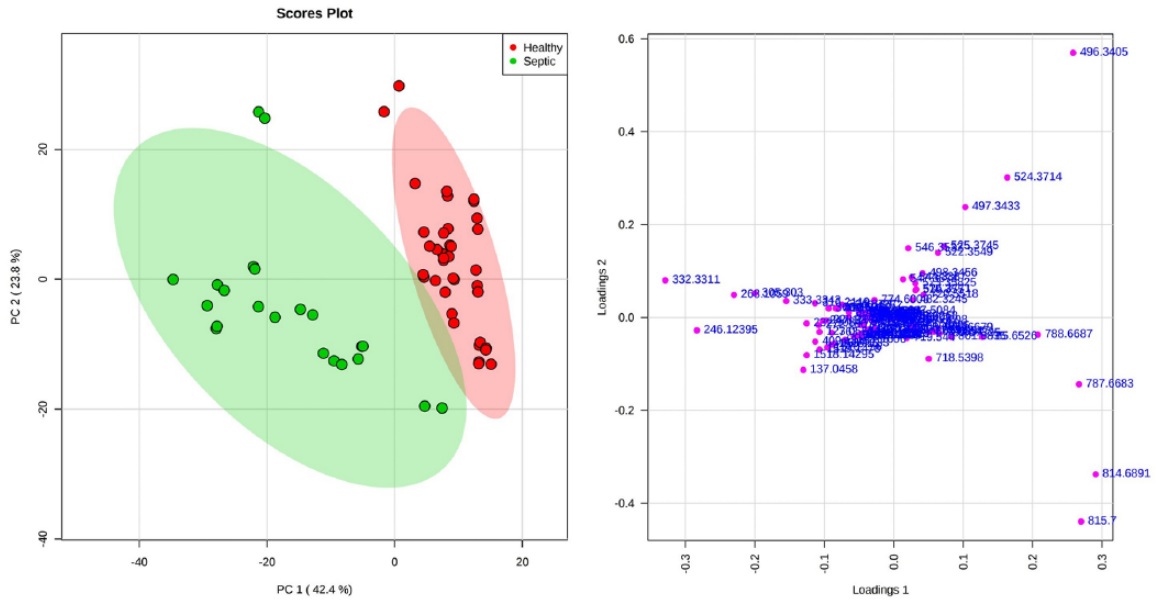

Figure 3. Left: PCA scores plot of PC1 (first principal component) vs PC2 (second principal component) showing the separation between healthy volunteers (red) and septic patients (green). Right: Loadings plot for PC1 and PC2 showing the metabolite ions (*m/z*) that were major contributors to the separation of groups observed in PCA scores plot. Analysis after previous variable selection with the data of septic patient from pneumonia infection was used in the multivariate variable selection (MVS) to improve statistical results. PCA indicates principal component analysis.

infection. Because of their difference, we selected only septic patient from pneumonia infection as there were few number of other septic patients. For the statistical data analysis, the data of septic patient from pneumonia infection were used in the multivariate variable selection (MVS) to improve statistical results. After variable selection, the explain variance increased to 65% in the first 2 PCs showing the improvement of data analysis (Figure 3). To access the major changes in lipid analysis between septic patients and healthy volunteers, we therefore also performed supervised statistical methods: a PLS-DA (Figure 4), an (O)PLS-DA (Figure 5), and cross-validation and permutation tests from PLS-DA (Figure 6). Both protocols show great robustness as indicated by their low *P* values in permutation tests ($P < 5e^{-4}$).

Possible sepsis biomarkers were revealed via VIP variables with high statistical significance. Figure 4 shows top 15 significant features of the metabolite markers based the VIP projection. Potential metabolites of significant contribution are listed in Table 2. Results from PLS-DA and (O)PLS-DA were also quite similar showing minimal or no response (*Y*) uncorrelated variation in the data after variable selection. The major changes in the lipid profiles between septic patients and healthy volunteers were seen for the phosphosphingolipids and glycerophosphocholine classes (Figure 7). The abundances of the di-, monounsaturated, and/or saturated phosphosphingolipid ions of *m/z* 703, 717, 757, 785, 787, 789, 799, 801, 813, and 815 in septic patients were significantly decreased (Table 2). The abundances of the lyso-PC ions of *m/z* 482, 496, 518, 520, 522, 524, 542, 544, and 546 also decreased, whereas the saturated and unsaturated phosphatidylcholine (PC) ions of *m/z* 744, 758, 760, 780, and 782 also increased in septic patients. In addition, a cardiolipin ion of *m/z* 1518 and a phosphatidylserine (PS) ion of *m/z* 846 were found as upregulated lipids by the statistical analysis (Table 2). Figure 8 shows 2 representative examples of ESI(+)-MS (q-ToF) of plasma lipid extract from healthy and septic patients.

Table 3 presents the major FA detected by GC for the erythrocyte phospholipids of septic patients and healthy volunteers. Data are given as percentage of the phospholipid fatty acyl species. The FA pattern in septic patients showed a marked increase in the sum of monounsaturated fatty acid (MUFA), that is, mainly oleic acid (18:1 *n*-9) increases accompanied by a decrease in total *n*-3 PUFA, whereas saturated and *n*-6 PUFA remains substantially unaltered. These trends lead to a 16% increase in the MUFA/*n*-6 ratio and to a 24% decrease in the unsaturation index. Figure 9 shows the percentage of different subclasses of FA in the erythrocyte phospholipid fraction.

Discussion

Glycerophosphocholine role in sepsis

Our results show that the major changes in glycerophosphocholine species between septic and healthy patients were in monoacyl (lyso-PC) and diacylglycerophosphocholine (PC) as indicated by the PLS-DA analysis of the ESI-MS lipid profile data (Figure 4). We observed therefore an upregulation in PC and a downregulation in lyso-PC species in lipid extracts of both plasma and erythrocytes. The lyso-PC results from the action of phospholipase A2, which liberates arachidonic acid from PC. The action of lyso-PC on immunoregulatory cells is very diverse and they participate in many induced inflammation signaling pathway.[31] Erythrocyte membrane

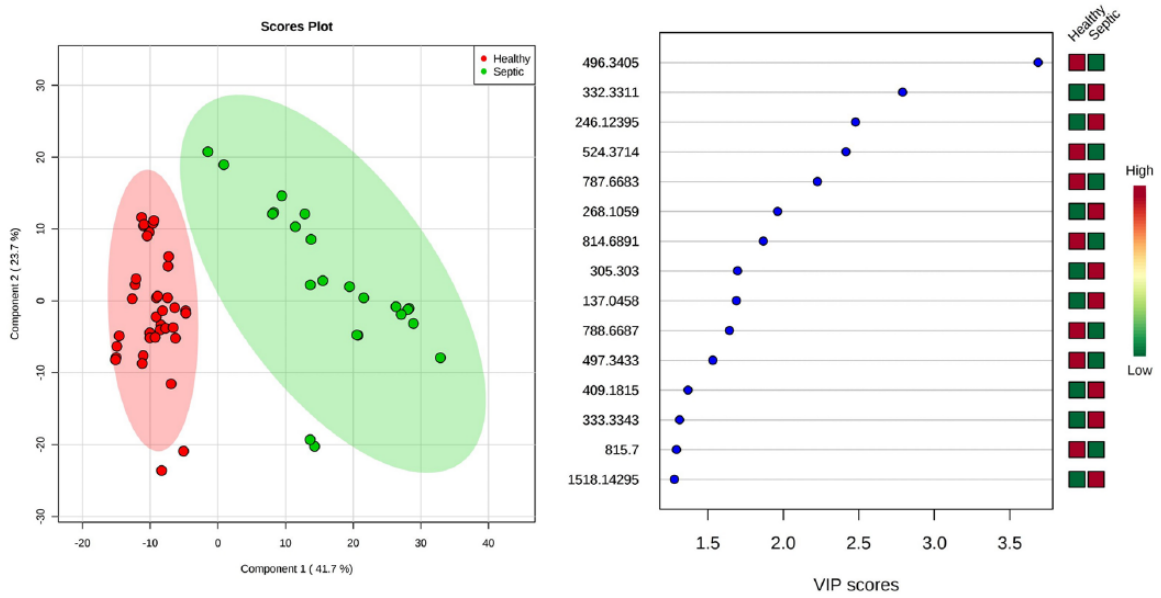

Figure 4. Left: Scores from PLS-DA discrimination of ESI-MS data from healthy volunteers (red) and septic patients (green). Right: Important metabolite ions selected on the basis of VIP score. The colored boxes on the right indicate relative bin integrals from healthy volunteers and septic patients. VIP score is a weighted sum of squares of PLS-DA loadings taking into account the amount of explained *Y*-variation in each dimension. See Figures 2 and 6 for the loading plots, permutation, and cross-validation tests. ESI-MS, electrospray ionization mass spectrometry; PLS-DA, partial least squares discriminant analysis; VIP, variable importance in projection.

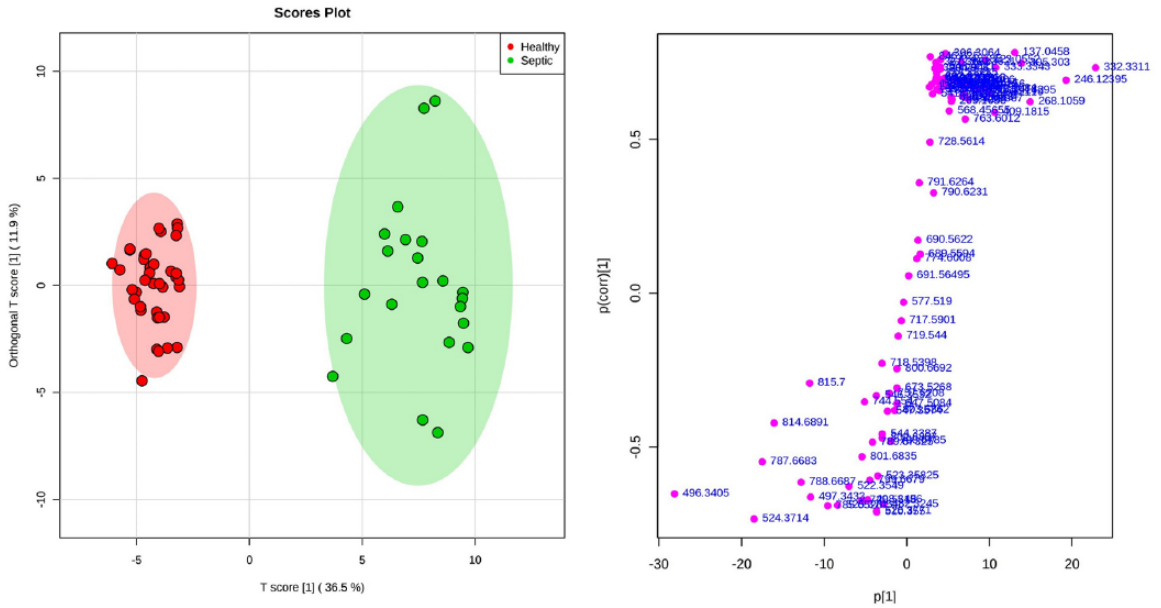

Figure 5. Left: (O)PLS-DA scores plot form comparison of the metabolite profiles of healthy volunteers (red) and septic patients (green). 36.5% and 11.9% are the scores of the *T* score and orthogonal *T* score, respectively. Right: Loadings plot for feature importance showing the metabolite ions (*m*/*z*) that were contributors to separation groups observed in (O)PLS-DA score plot. Analysis after previous variable selection. (O)PLS-DA indicates (orthogonal) partial least squares discriminant analysis.

phospholipids reflect systemic changes caused by inflammatory response and oxidative stress in septic patients. Their role in inflammation process is known to be very complex and is not completely understood, whereas their plasma composition can be directly influenced by diet.[32] Generally, lyso-PC is upregulated at sites of inflammation, but in sepsis, an acute systemic inflammatory condition, decreased levels of lyso-PC/PC ratios were observed (Figure 7). Such reduced ratio has been correlated with sepsis mortality.[22] This correlation was also corroborated by Yan et al[33] who found that therapeutic administration of lyso-PC after induction of sepsis effectively inhibited lethality in mouse models. In addition, Dinkla et al[16] showed that lyso-PC formation increases in in vitro studies when erythrocytes of healthy patients were treated with plasma of septic patients. These trends may be contradictory due to pro-inflammatory effects of lyso-PC, but their decrease could

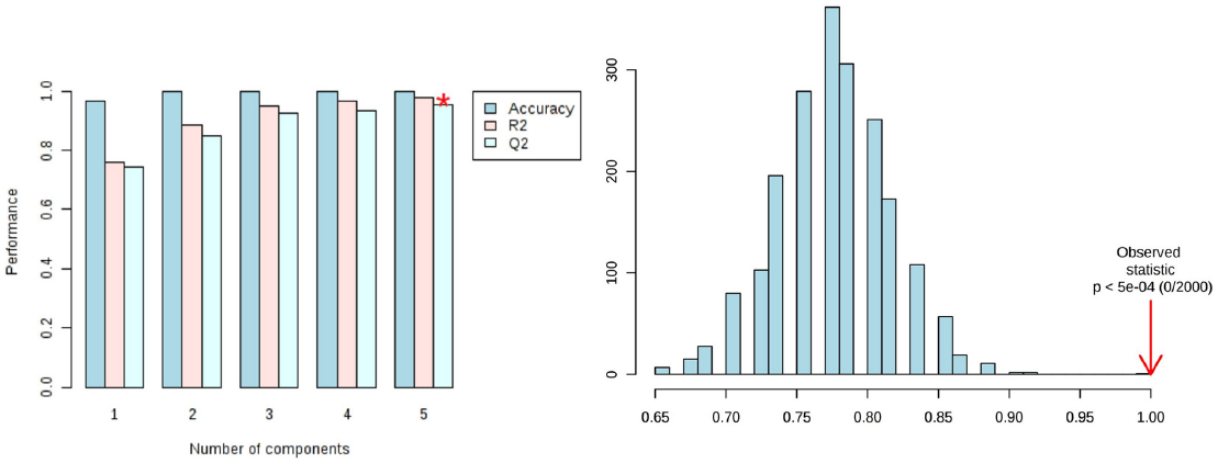

Figure 6. Left: Cross-validation showing the 3 performance measures (prediction accuracy, R^2, and Q^2) using different numbers of components. * indicates the best values of the currently selected measures Q^2 (0.86). Right: the result of permutation test statistics summarized by a histogram ($P < 5e^{-4}$).

be a later response to inflammation due to anti-inflammatory lyso-PA production.

A metabolomic study performed by Kamisoglu et al[24] has found that 5 lyso-PC species decreased significantly in the experimental and clinical studies of sepsis. The lyso-PC concentration on day 7 was significantly lower in nonsurvivors and lyso-PC concentrations increased over time in patients treated with appropriate antibiotics but not in those treated with inappropriate antibiotics.[34] The authors found that serial measurements of lyso-PC helped in the prediction of 28-day mortality in ICU patients with severe sepsis or septic shock.

The lysophosphatidic acid (lyso-PA) production occurs by action of autotaxin, a plasma lysophospholipase D[35] which acts in lyso-PC hydrolysis and promotes lyso-PA's anti-inflammatory action on macrophages.[36] Via ESI-MS, we failed to detect any sign of lyso-PA, maybe because they are locally formed and rapidly degraded in vivo.[37–39] Finally, we observed a PS increase in septic patients, and this increase could be related to lyso-PA production because they also induce PS exposure of erythrocytes during endotoxemia.[16]

We also noted that Drobnik et al[22] have shown a decreased lyso-PC/PC and increased Cer/SM ratios in septic patients as compared with healthy control subjects. These findings corroborate the strong predictive factors for sepsis-related mortality for such ratios. Highly increased PCs seem to be sepsis specific because they are not detectable in systemic inflammatory response syndrome samples without infection compared with ICU control subjects.[40]

Phosphosphingolipids

Inflammation triggers the acid sphingomyelinase (SMase) which catalyzes the hydrolysis of SM, a major component of cell membranes, into phosphocholine and Cer.[41] These changes alter membrane curvature and decrease plasma membrane integrity enhancing PS exposure and erythrocyte clearance, contributing to anemia. Erythrocytes do not possess SMase activity of their own, but they can be exposed to secreted SMases,[23,42] herein we observed an SM concentration decrease in septic patients (Table 2, Figure 7), but the corresponding formation of Cer was not observed in the lipid extracts of both plasma and erythrocytes. These findings agreed with those from Dinkla et al[16] who observed that erythrocytes are very sensitive to Cer-induced changes in membrane organization suggesting that, in vivo, these changes quickly triggered erythrocyte clearance.[43]

FA profile

The FA profile of plasma phospholipids seemed very interesting because these molecules carry the most important part of PUFA which serve as precursors for signaling molecules (eicosanoids and docosanoids).[44] The phospholipids FA profile is also less affected by fat intake than other plasma lipids, ie, triacylglycerols or nonesterified FAs. The changes of FA profile from erythrocytes phospholipids in septic patients were associated mainly by an increase in oleic acid levels (C18:1 *n*-9) accompanied by a proportional decrease in *n*-3 PUFA and *n*-6 PUFA levels (Table 3). Oleic acid is produced by stearoyl-CoA desaturase (SCD1), which is an enzyme localized in the endoplasmic reticulum that converts palmitoyl-CoA (C16:0) and stearoyl-CoA (C18:0) to palmitoleoyl-CoA (16:1) and oleoyl-CoA (18:1), respectively, with stearoyl-CoA being the main substrate.[45] These MUFAs are the key components of triglycerides and membrane phospholipids. The higher percentage of oleic acid could reflect an adipose-stimulated lipolysis which has been observed in septic shock patients. Such high percentages have been associated with a rising plasma nonesterified FA concentrations, hypoalbuminemia, and reduction in energy supply to the organs.[18,19,46] The elevation of plasma nonesterified FA levels has been reported to produce important myocardial damage, arrhythmias, and reduction in heart rate variability in septic patients.[18] The decrease in energy supply to the organs contributes therefore to multiple organ failure and death.[47] Although the oleic acid affects different biological processes,

Table 2. The most significant lipids with contrasting abundances for septic patients and healthy volunteers.

M/Z	LIPID	MOLECULAR FORMULA	ABSOLUTE ABUNDANCE MEAN ± SD (FDR)[a]				LIPID CLASS	TENDENCY	
			HEALTHY		SEPTIC			ERYTHROCYTES	PLASMA
			ERYTHROCYTES	PLASMA	ERYTHROCYTES	PLASMA			
703.5758	SM(d18:1/16:0)	$C_{39}H_{79}N_2O_6P$	nd	4.4E6 ± 0.7E6 (4.31E–4)	nd	3.1E6 ± 1.2E6 (4.31E–4)	Phosphosphingolipids	—	Down
717.5901	SM(d18:1/17:0)	$C_{40}H_{81}N_2O_6P$	7.9E4 ± 1.8E4 (5.34E–7)	5.0E4 ± 0.8E4 (6.98E–7)	4.5E4 ± 1.4E4 (5.34E–7)	2.3E4 ± 0.8E4 (6.98E–7)	Phosphosphingolipids	Down	Down
757.6208	SM(d18:1/20:1)	$C_{43}H_{85}N_2O_6P$	6.6E4 ± 1.9E4 (8.20E–7)	9.7E4 ± 3.5E4 (2.71E–7)	3.3E4 ± 1.0E4 (8.20E–7)	4.0E4 ± 1.6E4 (8.20E–7)	Phosphosphingolipids	Down	Down
785.6526	SM(d18:1/22:1)/ SM(d18:2/22:0)	$C_{45}H_{89}N_2O_6P$	3.8E5 ± 1.2E5 (3.17E–7)	5.0E5 ± 2.1E5 (2.71E–7)	1.7E5 ± 6.1E4 (3.17E–7)	1.6E5 ± 0.7E5 (2.71E–7)	Phosphosphingolipids	Down	Down
787.6683	SM(d18:1/22:0)/ SM(d16:1/24:0)	$C_{45}H_{91}N_2O_6P$	1.3E6 ± 6.7E5 (7.84E–7)	8.3E5 ± 4.9E5 (6.37E–6)	4.9E5 ± 2E5 (7.84E–7)	2.7E5 ± 2.5E5 (6.37E–6)	Phosphosphingolipids	Down	Down
789.6745	SM(d18:0/22:0)/ SM(d16:0/24:0)	$C_{45}H_{93}N_2O_6P$	1.2E5 ± 4.2E4 (2.08E–6)	5.9E4 ± 2.4E4 (1.64E–6)	5.3E4 ± 2.2E4 (2.08E–6)	2.1E4 ± 1.1E4 (1.64E–6)	Phosphosphingolipids	Down	Down
799.6679	SM(d18:2/23:0)	$C_{46}H_{91}N_2O_6P$	9.5E4 ± 3.5E4 (3.17E–7)	1.4E5 ± 5.5E4 (1.59E–7)	4.1E4 ± 1.5E4 (3.17E–7)	4.0E4 ± 1.7E4 (1.59E–7)	Phosphosphingolipids	Down	Down
801.6835	SM(d18:1/23:0)	$C_{46}H_{93}N_2O_6P$	1.4E5 ± 6.4E4 (1.45E–6)	1.6E5 ± 0.7E5 (2.77E–7)	5.6E4 ± 2.7E4 (1.45E–6)	4.2E4 ± 2.1E4 (2.77E–7)	Phosphosphingolipids	Down	Down
813.6856	SM(d18:2/24:0)/ SM(d18:1/24:1)	$C_{47}H_{93}N_2O_6P$	4.2E6 ± 1.3E6 (3.94E–6)	1.2E6 ± 0.6E6 (0.0010)	2.6E6 ± 0.6E6 (3.94E–6)	5.1E5 ± 2.4E5 (0.0010)	Phosphosphingolipid	Down	Down
815.7000	SM(d18:1/24:0)/ SM(d18:0/24:1)	$C_{47}H_{95}N_2O_6P$	2.8E6 ± 9.4E5 (7.44E–6)	3.2E5 ± 1.3E5 (1.53E–5)	1.4E6 ± 6.7E5 (7.44E–6)	1.1E5 ± 0.5E4 (1.53E–5)	Phosphosphingolipids	Down	Down
482.3245	Lyso-PC(15:0/0:0)	$C_{23}H_{48}NO_7P$	1.2E4 ± 0.5E4 (1.05E–6)	4.8E4 ± 1.3E4 (8.61E–8)	6.1E3 ± 1.4E3 (1.05E–6)	5.7E3 ± 1.6E3 (8.61E–8)	Glycerophosphocholine	Down	Down
496.3405	Lyso-PC(16:0/0:0)	$C_{24}H_{50}NO_7P$	1.2E6 ± 0.6E6 (3.18E–7)	9.1E6 ±1.3E6 (1.14E–6)	1.5E5 ± 2.2E5 (3.18E–7)	5.0E5 ± 1.6E5 (1.14E–6)	Glycerophosphocholine	Down	Down
518.3218	Lyso-PC(18:3/0:0)	$C_{26}H_{48}NO_7P$	9.8E4 ± 4.8E4 (5.53E–6)	1.1E6 ± 0.6E6 (8.64E–8)	3.9E4 ± 7.1E4 (5.53E–6)	9.7E4 ± 5.6E4 (8.64E–8)	Glycerophosphocholine	Down	Down
520.3389	Lyso-PC(18:2/0:0)	$C_{26}H_{50}NO_7P$	7.8E4 ± 3.3E4 (0.0334)	1.3E6 ± 0.6E6 (8.64E–8)	9.5E4 ± 1.2E5 (0.0334)	1.3E5 ± 1.1E5 (8.64E–8)	Glycerophosphocholine	—	Down
522.3549	Lyso-PC(18:1/0:0)	$C_{26}H_{52}NO_7P$	9.3 E4 ± 4.2E4 (1.31E–7)	1.1E6 ± 0.5E6 (8.64E–8)	2.1E4 ± 1.2E4 (1.31E–7)	5.9E4 ± 2.0E4 (8.64E–8)	Glycerophosphocholine	Down	Down
524.3715	Lyso-PC(18:0/0:0)	$C_{26}H_{54}NO_7P$	4.4E5 ± 1.3E5 (1.31E–7)	2.7E6 ± 0.7E6 (8.64E–8)	6.5E4 ± 8.1E4 (1.31E–7)	1.4E5 ± 0.4E5 (8.64E–8)	Glycerophosphocholine	Down	Down

Table 2. (Continued)

M/Z	LIPID	MOLECULAR FORMULA	ABSOLUTE ABUNDANCE MEAN ± SD (FDR)[a]				LIPID CLASS	TENDENCY	
			HEALTHY		SEPTIC			ERYTHROCYTES	PLASMA
			ERYTHROCYTES	PLASMA	ERYTHROCYTES	PLASMA			
542.3217	Lyso-PC(20:5/0:0)	$C_{28}H_{48}NO_7P$	1.1E4 ± 0.3E4 (4.31E−4)	1.1E5 ± 0.5E5 (8.64E−8)	8.8E3 ± 1.2E3 (4.31E−4)	1.5E4 ± 0.9E4 (8.64E−8)	Glycerophosphocholine	Down	Down
544.3387	Lyso-PC(20:4/0:0)	$C_{28}H_{50}NO_7P$	3.1E4 ± 1.3E4 (1.16E−6)	4.0E5 ± 1.4E5 (8.61E−8)	1.1E4 ± 0.6E4 (1.16E−6)	3.4E4 ± 1.2E4 (8.61E−8)	Glycerophosphocholine	Down	Down
546.3532	Lyso-PC(20:3/0:0)	$C_{28}H_{52}NO_7P$	5.2 E4 ± 2.0E4 (2.44E−6)	3.3E5 ± 1.1E5 (8.61E−8)	1.9E4 ± 2.2E4 (2.44E−6)	3.3E4 ± 0.8E4 (8.61E−8)	Glycerophosphocholine	Down	Down
744.5547	PC(15:0/18:2)	$C_{41}H_{78}NO_8P$	1.7E6 ± 0.04E6 (9.51E−6)	nd (0.0794)	1.0E5 ± 0.3E5 (9.51E−6)	4.5E4 ± 2.1E4 (0.0794)	Glycerophosphocholine	Down	Up
758.5715	PC(16:0/18:2)	$C_{42}H_{80}NO_8P$	1.0E7 ± 0.1E7 (0.03978)	1.2E7 ± 0.2E7 (0.03906)	1.1E7 ± 0.1E7 (0.03978)	1.6E7 ± 0.3E7 (0.03906)	Glycerophosphocholines	Up	Up
760.5861	PC(16:0/18:1)	$C_{42}H_{82}NO_8P$	nd	6.6E6 ± 1.1E6 (0.01915)	nd	8.9E6 ± 2.3E6 (0.01915)	Glycerophosphocholine	—	Up
780.5542	PC(16:0/20:5)	$C_{44}H_{78}NO_8P$	1.3E6 ± 0.7E6 (0.00316)	2.0E6 ± 0.5E6 (0.00300)	2.0E6 ± 0.7E6 (0.00316)	4.2E6 ± 1.5E6 (0.00300)	Glycerophosphocholines	Up	Up
782.5704	PC(16:0/20:4)	$C_{44}H_{80}NO_8P$	4.0E6 ± 0.7E6 (0.01478)	nd	4.7E6 ± 1.0E6 (0.01478)	nd	Glycerophosphocholines	Up	—
784.5847	PC(16:0/20:3)	$C_{44}H_{82}NO_8P$	nd	5.1E6 ± 0.8E6 (3.00E−4)	nd	3.7E6 ± 0.9E6 (3.00E−4)	Glycerophosphocholines	—	Down
788.6687	PC(16:0/20:1)	$C_{44}H_{86}NO_8P$	5.5E5 ± 1.8E5 (4.37E−7)	2.8E5 ± 1.1E5 (3.31E−6)	2.5E5 ± 1.0E5 (4.37E−7)	9.8E4 ± 7.6E4 (3.31E−6)	Glycerophosphocholines	Down	Down
1518.1429	CL(1′-[18:0/18:2]/3′-[20:0/20:0])	$C_{85}H_{162}O_{17}P_2$	3.0E4 ± 1.7E4 (1.33E−5)	3.0E4 ± 1.2E4 (0.00300)	7.7E4 ± 4.2E4 (1.33E−5)	8.8E4 ± 5.5E4 (0.00300)	Glycerophosphoglycero-phosphoglycerols	Up	Up
846.625	PS(18:0/22:1)	$C_{46}H_{88}NO_{10}P$	4.4E4 ± 0.8E4 (2.13E−5)	nd	7.3E4 ± 1.6E4 (2.13E−5)	nd	Glycerophosphoserines	Up	—

Abbreviations: CL, cardiolipin; FDR, false discovery rate; lyso-PC, lysophosphatidylcholine; nd, not detected; PC, phosphatidylcholine; PS, phosphatidylserine; SM, sphingomyelin.
[a]The FDR values were determined in a parametric *t* test.

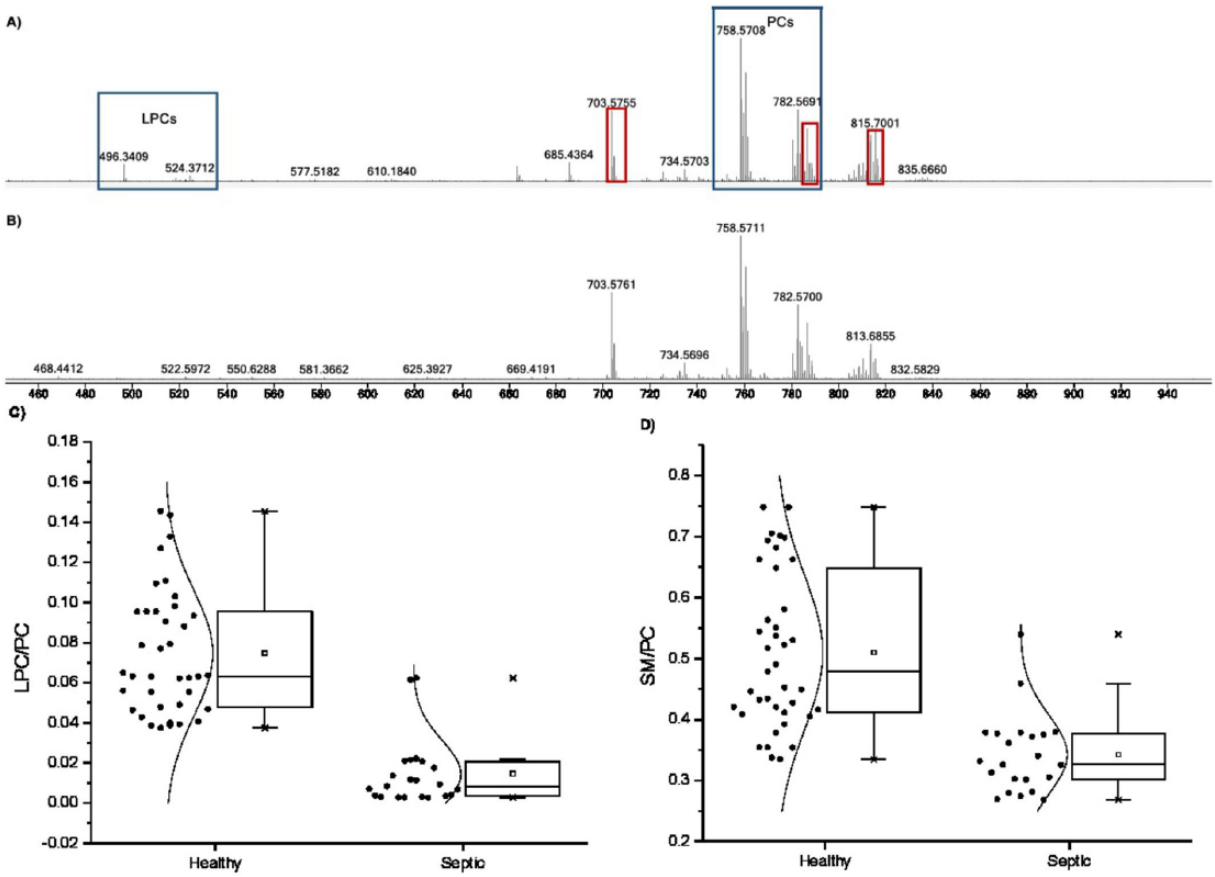

Figure 7. Typical ESI(+)-MS of erythrocyte membrane showing lipid profiles for (A) healthy and (B) septic patients. (C) Lyso-PC/PC and SM/PC ratios for healthy and septic patients. Lyso-PC/PC ratio for healthy and septic patients was determined dividing the combined pool of lyso-PC (16:0, 18:3, 18:2, 18:1, 18:0, 20:5, 20:4, and 20:3) by the combined pool of PC (16:*X*/18:*Y* – *X* + *Y* = 1-2, 16*X*/20*Y* *X* + *Y* = 1-5) from healthy and septic patients. The SM/PC ratio for healthy and septic patients was determined dividing the combine pool of SM(d18:1/16:0, d18:1/24:1, d18:1/24:0, d16:1/24:1, 16:1/24:0) by combined pool of PC from healthy and septic patients. For more detailed lipid class composition, see Table 2. ESI(+)-MS indicates electrospray ionization mass spectrometry; lyso-PC, lysophosphatidylcholine; SM, sphingomyelin.

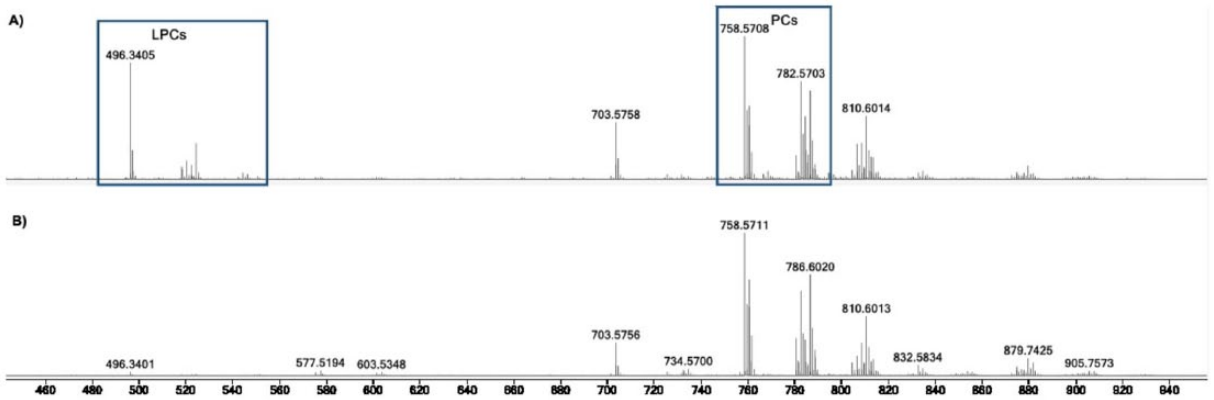

Figure 8. Two representatives examples of ESI(+)-MS of plasma lipid extract from (A) healthy and (B) septic patients. ESI(+)-MS indicates electrospray ionization mass spectrometry; PC, phosphatidylcholine.

such as decreases plasma-free FA concentration and increases CPT1A and UCP2 and AMPK levels, decreasing levels of reactive oxygen species in septic mice, its detailed mechanism of action is still not completely understood.[48]

Our results show decline in PUFAs (more specifically >20 carbons) which could result either from their degradation by peroxidation from reactive oxygen species or to a higher synthesis of inflammatory lipid mediators because these PUFAs are the precursors of eicosanoids (prostaglandins, prostacyclins, and thromboxanes) and docosanoids (protectins and resolvins) which are involved in inflammation, vasomotricity, and capillary permeability.[49]

Table 3. FA composition of the erythrocyte phospholipids of both septic patients and healthy volunteers (% relative of total FA).

	SEPTIC PATIENTS	HEALTHY VOLUNTEERS
Palmitic acid (C16:0)	31.25 ± 1.15	29.96 ± 1.23
Stearic acid (C18:0)	17.27 ± 1.33	16.68 ± 1.09
Total SFAs	48.52 ± 1.64	46.64 ± 1.45
Palmitoleic acid (C16:1 *n*-7)	2.03 ± 0.38	2.51 ± 0.84
Oleic acid (C18:1 *n*-9)	18.58 ± 0.84*	15.41 ± 1.05
Total MUFAs	20.61 ± 0.86*	17.92 ± 1.14
Linoleic acid (C18:2 *n*-6)	13.35 ± 2.59	15.14 ± 1.44
Arachidonic acid (C20:4 *n*-6)	13.20 ± 1.98	13.52 ± 1.87
Total *n*-6 PUFAs	26.54 ± 2.10	28.66 ± 1.63
Linolenic acid (C18:3 *n*-3)	0.57 ± 0.07	0.44 ± 0.13
Eicosapentaenoic acid (C20:5 *n*-3)	0.72 ± 0.23	1.02 ± 0.44
Docosapentaenoic acid (C22:5 *n*-3)	1.67 ± 0.28*	2.57 ± 0.38
Docosahexaenoic acid (C22:6 *n*-3)	1.42 ± 0.16*	2.83 ± 0.21
Total *n*-3 PUFAs	4.38 ± 0.72*	6.87 ± 0.74
n-6 PUFAs/*n*-3 PUFAs	6.05 ± 1.83	4.19 ± 2.05
MUFA/*n*-6 PUFAs	0.77 ± 0.23	0.62 ± 0.12
Unsaturation index	122	154

Abbreviations: MUFA, monounsaturated fatty acids; *n*-3 PUFA, *n*-3 polyunsaturated fatty acids; *n*-6 PUFA, *n*-6 polyunsaturated fatty acids; SFA, saturated fatty acid.
*$P < .05$ compared with healthy volunteers (Tukey test).

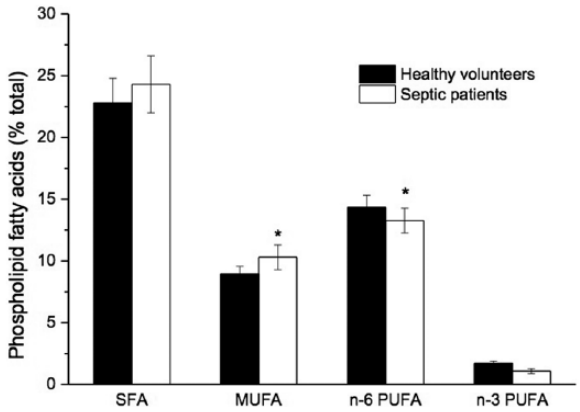

Figure 9. Percentages of different subclasses of fatty acids in the erythrocyte phospholipid fraction. MUFA indicates monounsaturated fatty acids; *n*-3 PUFA, *n*-3 polyunsaturated fatty acids; *n*-6 PUFA, *n*-6 polyunsaturated fatty acids; SFA, saturated fatty acid. *$P < .05$ compared with healthy volunteers (Tukey test).

Our results are consistent with those described by Rival et al[21] which observed high percentage of saturated fatty acids and MUFAs with low concentrations of plasma phospholipid *n*-6 and *n*-3 PUFAs in patients with septic shock. Barros et al[50] observed altered FA profiles in plasma PC in critically ill patients, mostly diagnosed with sepsis and septic shock, compared with healthy elderly subjects. Surviving ICU patients displayed higher levels of docosahexaenoic acid and total *n*-3 PUFA and a lower *n*-6/*n*-3 PUFA ratio in plasma PC than nonsurvivors.

Conclusions

A total of 29 potential biomarkers for sepsis and septic shock have been identified via ESI-MS (q-ToF) lipid profile screening. Most contrasting lipids were from the phosphosphingolipids and glycerophosphocholine classes which were observed in all samples with significant variations in abundances between septic patients and healthy controls. Septic patients also displayed erythrocyte membranes characterized by higher levels of oleic acid and lower levels of *n*-6 PUFA, hence with reduced unsaturation indexes. Combined with the above analysis, we believe that lyso-PC (16:0) and SM may both be involved in the pathogenesis of sepsis and hope that they can be developed as sensitive and specific diagnostic biomarkers candidates of sepsis, which require confirmation in further functional studies and large-sample validation. We have confirmed the metabolic alterations of some functional lipids that may support the understanding of the pathogenesis of sepsis. A limiting factor in this study is the small number of research subjects, and more studies are needed for more robust conclusions. In this study, other groups of patients, such as those with inflammatory process without organ dysfunction,

were not evaluated. Possibly, the combinations of lipidome profile with others pro- and anti-inflammatory biomarkers in a multimarker panel may help identify patients who are developing sepsis before organ dysfunction has advanced too far. Such knowledge is crucial due to the high severity and mortality of this disease and may help to in the design of clinical diagnosis, sepsis monitoring, and therapy.

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Author Contributions

GCM and POC: conception and design of research. GCM, MCFM, RMSP, CFFA and IBSC participated in acquisition, analysis and interpretation of data. CFFA, MNE and POC drafted manuscript. All authors read and approved final version of manuscript

Availability of Data and Materials

All data are available in this manuscript.

Ethical Approval and Consent to Participate

This study has been approved by the Ethics Committee of the São Francisco University (CAEE 51356315.5.0000.5514). Written informed consent was obtained from the persons legally responsible for the patients according to the Declaration of Helsinki.

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