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BIOMARCADORES DO METABOLISMO LIPÍDICO ASSOCIADOS AO DIAGNÓSTICO E PROGNÓSTICO DA SEPSE

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EPÍGRAFE

"*Existe um momento na vida de cada pessoa*
que é possível sonhar e realizar nossos sonhos…
e esse momento tão fugaz chama-se presente
e tem a duração do tempo que passa."

Mário Quintana

RESUMO

O diagnóstico precoce da sepse ainda representa um grande desafio para o sistema de saúde. As alterações do metabolismo intermediário nesses pacientes em resposta a fase aguda devido ao estresse gerado pelo quadro clínico levam, dentre outros processos, as alterações no metabolismo lipídico. A identificação dessas biomoléculas como candidatos a biomarcadores é importante tanto para o diagnóstico precoce como para o prognóstico frente a essa situação crítica e complexa. A proposta do presente trabalho foi (i) identificar potenciais biomarcadores lipídicos para diagnóstico de sepse e choque séptico por análise comparativa aos voluntários saudáveis, (ii) avaliar o perfil das alterações lipidômicas e os possíveis candidatos a biomarcadores para a infecção bacteriana e, portanto, capazes de diferenciar pacientes com sepse e com Síndrome da Resposta Inflamatória Sistêmica (SIRS) de causa não infecciosa e ainda (iii) avaliar o uso dos biomarcadores no prognóstico dos pacientes. O estudo, de coorte prospectiva e observacional, incluiu pacientes adultos com sepse, choque séptico e SIRS internados na Unidade de Terapia Intensiva do Hospital Universitário São Francisco na Providência de Deus no período de agosto de 2016 a agosto de 2018. As análises foram realizadas por cromatografia líquida acoplada à espectrometria de massas (CL-MS) associada a análise multivariada e a cromatografia à gás (GC). Na primeira fase do estudo foram triados sessenta e cinco pacientes, destes 30,76 % (n=20) foram incluídos na pesquisa, sendo 11 com diagnóstico de choque séptico e 9 com sepse. Os dados mostraram que não há diferenças entre pacientes com sepse e com choque séptico, entretanto, ambos mostraram uma assinatura molecular específica, caracterizada por níveis reduzidos de lisofosfatidilcolina e esfingomielina, capaz de diferenciá-los dos voluntários saudáveis. Na segunda fase do estudo, 21 pacientes com sepse e 21 com SRIS foram incluídos no estudo. O foco da infecção nos pacientes com sepse foi, em sua maioria, abdominal (43%) e pulmonar (33%). A taxa de mortalidade para ambos, sepse e SRIS foi de 33.3 %. A análise lipidômica mostrou aumento nos níveis plasmáticos de derivados de carnitina resultante de distúrbios da beta-oxidação mitocondrial de ácidos graxos. L-Octanoilcarnitina (*upregulation*) e ésteres de ácidos graxos ramificados de ácidos graxos hidroxilados (FAHFA 36:4) (*dowregulation*) são apontados como principais biomarcadores para diferenciar sepse de SIRS de causa não infecciosa. Ainda, o modelo de predição (*Random Forest*) confirmou a importância da L-Octanoilcarnitina para predizer o risco de óbito nos pacientes, medidas importantes que poderão ser adotadas para a otimização do diagnóstico e no acompanhamento da evolução de pacientes críticos.

Descritores: Sepse. Síndrome da Resposta Inflamatória Sistêmica. Biomarcador. Lipidômica.

ABSTRACT

Early diagnosis of sepsis continues to be a great challenge for the health system to face. Alterations in the intermediate metabolism of sepsis patients in response to the acute stage and the stress generated by the clinical condition lead to alterations in the lipidic metabolism, among other processes. Identifying the respective biomolecules as possible biomarkers is important not only to enable early diagnosis but also to determine the prognosis in that critical and complex situation. This work proposed to (i) identify potential lipid biomarkers for sepsis and septic shock diagnosis by means of comparative analyses with healthy volunteers; (ii) evaluate the profile of the lipidomic alterations and possible candidates as biomarkers for bacterial infection that would enable a distinction between patients with sepsis and those with Systemic Inflammatory Response Syndrome (SIRS) with non-infectious causes; and, furthermore. (iii) to assess the use of biomarkers in patient prognosis. The study, a prospective and observational cohort, included adult patients with sepsis, septic shock and SIRS admitted to the Intensive Therapy Unit of the São Francisco University Hospital in the period from August 2016 to August 2018. The analyses were performed using liquid chromatography coupled to (CL-MS) mass spectrometry and multivariate statistical analysis and gas chromatography. In the first stage the study screened sixty-five patients, 30.76% (n=20) of whom were included in the study; 11 diagnosed with septic shock and 9 with sepsis. Data revealed no differences between sepsis patients and septic shock patients. However, each group showed a specific molecular signature characterized by reduced levels of lysophosphatidylcholine and sphingomyelin, capable of distinguishing them from the health volunteers. In the second stage, 21 patients with sepsis and 21 with SIRS were included. The foci of infection in the sepsis patients were mainly abdominal (43%) and pulmonary (33%). Mortality rate for both sepsis and SIRS patients was 33.3%. Lipidomic analysis revealed increased plasmatic levels of carinitine derivatives resulting from disturbances in the mitochondrial beta-oxidation of fatty acids. L-octanoylcarnitine (upregulation) and branched fatty acid esters of hydroxy fatty acids (FAHFA 36:4) (downregulation) are indicated as being the main biomarkers for distinguishing sepsis from SIRS associated to non-infectious causes. Furthermore, the Random Forest prediction model confirmed the importance of L-octanoylcarnitin in predicting patients' risk of death, which could enable important measures to be taken to optimize the diagnosis and the accompaniment of critical patient's evolution.

Key words: Sepsis. Systemic Inflammatory Response Syndrome. Biomarker. Lipidomic.

SUMÁRIO

1. INTRODUÇÃO

A sepse se caracteriza pela presença de sinais de disfunção orgânica, com manifestações clínicas decorrentes dos órgãos em disfunção. A definição de sepse foi alterada no último Consenso Internacional de Definição de Sepse, em 2016 (*The third international consensus definitions for sepsis and septic shock*; *Sepsis-3*), sendo este o terceiro desde a primeira publicação realizada em 1992 (BONE et al., 1992). Pela nova publicação sepse foi definida como uma disfunção orgânica causada pela resposta desregulada do organismo a uma infecção (SINGER et al., 2016).

A sepse é um problema de saúde pública mundialmente, com alta morbimortalidade e gera altos custos para o sistema de saúde. Um recente estudo evidenciou que, em 2017, cerca de 48,9 milhões de casos de sepse e 11 milhões de mortes foram registrados em todo o mundo, representando 19,7% de todas as causas de morte do mundo (RUDD et al., 2020). Em um estudo multicêntrico realizado no Brasil, envolvendo 229 instituições e 794 pacientes, foi observado a prevalência de 29,6% dos leitos de UTI ocupados por pacientes sépticos. A letalidade encontrada foi de 55%, valor acima da reportada em países desenvolvidos (MACHADO et al., 2017).

Todos os esforços devem ser feitos para diagnosticar a sepse em seus estágios iniciais quando a intervenção tem maior possibilidade de evitar o óbito. Embora não façam mais parte da definição de sepse, os sinais de resposta inflamatória são relevantes para o diagnóstico de infecção, considerando que são sinais de alerta que sinalizam a presença da infecção. Estes sinais, frequentes nos protocolos clínicos, indicam a necessidade de avaliação do paciente em quadro grave e com necessidade de intervenção imediata (BREKKE et al., 2019). A taquicardia é geralmente reflexa à redução da resistência vascular, objetivando garantir o débito cardíaco. A taquipnéia advém do aumento da produção de CO_2, do estímulo direto do centro respiratório por citocinas ou, quando há insuficiência respiratória, surge em consequência da hipoxemia (HOTCHKISS et al., 2016).

A intensidade da resposta inflamatória reflete a magnitude do processo fisiopatológico e está relacionada ao desenvolvimento de disfunções orgânicas e a um aumento na gravidade e mortalidade dos pacientes com sepse (HOTCHKISS et al., 2016). Para o diagnóstico de infecção são utilizados parâmetros clínicos e laboratoriais e para os

pacientes com infecção presumida ou confirmada na presença de disfunção orgânica deve ser tratada com urgência (SINGER et al., 2016). A problemática encontra-se na diferenciação da disfunção orgânica secundária a infecção ou devido a outros motivos.

O uso de critério de Síndrome da Resposta Inflamatória Sistêmica (SRIS) para diagnóstico de infecção tem graves limitações uma vez que sinais de resposta inflamatória podem estar presentes em diversas outras situações clínicas. Tais sinais são comuns não só aos processos infecciosos, mas também aqueles derivados de agressão ao organismo por outras causas, o que compromete sua especificidade. A diferenciação entre SRIS e infecção é um grande desafio quando se trata de diagnosticar infecção num paciente politraumatizado, em pós-operatório, grande queimado ou portador de pancreatite.

No que diz respeito à sensibilidade e relativa inespecificidade desses critérios, até o momento, não se dispõe de marcadores clínicos ou biológicos que possam efetivamente diferenciar sepse com acurácia bastante elevada.

O diagnóstico de sepse tem sido sugerido de acordo com as manifestações clínicas e testes laboratoriais de rotina, podendo ser posteriormente confirmado pelo isolamento do agente etiológico. No entanto, nem sempre é possível estabelecer a confirmação precoce do quadro séptico com base somente em tais critérios, o que pode levar ao início tardio das intervenções terapêuticas. A utilização de biomarcadores moleculares tem se mostrado promissora na obtenção de resultados presuntivos rápidos e de alta acurácia.

O biomarcador, considerado um parâmetro biológico mensurável, pode ser usado para vários propósitos, dependendo da finalidade do estudo. A aplicação pode ter como finalidade elucidar a relação causa-efeito e dose-efeito na avaliação de risco à saúde; para fins de diagnóstico clínico; e para fins de monitorização biológica, realizada de maneira sistemática e periódica (WORLD HEALTH ORGANIZATION, 1993). Há uma necessidade de identificar e validar para cada sistema orgânico estes parâmetros característicos que são indicativos de indução de disfunção orgânica, alteração clínica e toxicidade patológica, além de estabelecer a especificidade e sensibilidade de cada biomarcador e seu método para determinação (WORLD HEALTH ORGANIZATION 1993).

Estudos sobre marcadores para diagnóstico e prognóstico da sepse são extremamente relevantes na atualidade. O desenvolvimento de novas tecnologias de análise nesse campo vem aumentando vertiginosamente, o que poderá contribuir com a redução do uso desnecessário de antibióticos e da mortalidade de pacientes por sepse. Nesse domínio, destacam-se os estudos lipidômicos, aplicáveis para a compreensão de diferentes condições infecciosas, os quais, apesar dos resultados ainda muito incipientes na investigação da sepse, já demonstram grande potencialidade para se tornarem ferramentas úteis no diagnóstico, terapêutica e prognóstico (SU et al., 2014; LUDWIG e HUMMON, 2017; LIU et al., 2019).

Assim, com a intenção de identificar moléculas lipídicas diferenciais de resposta a um insulto infeccioso visando o diagnóstico precoce e específico da sepse para que o tratamento seja instituído de forma mais rápida e eficaz, a presente tese descreve a identificação de biomarcadores através da combinação de análises lipidômicas e tratamento multivariados dos dados obtidos após as análises experimentais.

As análises lipidômicas foram realizadas por cromatografia à gás (CG) e cromatografia líquida acoplada a espectrometria de massa (CL-EM), técnicas clássicas de alta sensibilidade que permitem a avaliação rápida e precisa de diferentes classes de lipídeos para aplicação clínica (ZHANG et al., 2018). A cromatografia à gás e a cromatografia líquida fornecem informações detalhadas, permitindo quantificar compostos individuais da amostra. Foi usado o sistema Waters Acquity de Cromatografia Líquida de Ultra Performance (UPLC) acoplado a um analisador Tempo-de-Voo (TOF) Waters Micromass LCT equipado com uma interface electrospray (ESI). TOF são analisadores de alta relação custo benefício devido à alta velocidade, simplicidade e análise de massas não discriminatória, com custos relativamente baixos (GUILHAUS et al., 2000). Após as análises, o tratamento de dados foi realizado através do software MassLynx, (Waters Corporation).

O tratamento por análises multivariadas foi usado com o objetivo de diferenciar em classes um conjunto complexo de dados, reduzindo sua dimensionalidade e maximizando a variância entre as classes. (GORROCHATEGUI et al., 2016). A identificação dos metabólitos selecionados como discriminantes foi feita através de estudos de fragmentação e a identidade das moléculas foi obtida em banco de dados como o Metlin (SMITH et al., 2005), LipidMaps (FAHY et al., 2007) ou HMDB (WISHART et al., 2007).

A estrutura da tese está dividida em três capítulos, sendo que no primeiro é apresentado um referencial teórico sobre a sepse, abordando as características clínicas, epidemiológicas e o diagnóstico a partir biomarcadores usados atualmente na prática clínica. Também são discutidos as alterações lipídicas e novos biomarcadores provenientes de distúrbios no metabolismo lipídico de pacientes com sepse. O artigo de revisão foi publicado e apresentado no capítulo 1 (MECATTI et al., Lipidomic profile and candidate biomarkers in septic patients. *Lipids in Health and Disease*, 19, 68, 2020).

A partir dessa revisão foi possível identificar alguns artigos na literatura que trazem a descrição de biomarcadores lipídicos, elementos importantes que foram usados para a construção da idéia principal da tese. No capítulo 2 é apresentado o artigo que aborda as alterações de algumas classes de lipídeos e a identificação de moléculas diferenciais presentes no plasma e eritrócitos de pacientes com sepse comparado ao grupo saudável (MECATTI et al., Lipidomic Profiling of Plasma and Erythrocytes From Septic Patients Reveals Potential Biomarker Candidates. *Biomarker Insights*, 13, 1, 2018).

O terceiro capítulo descreve as principais vias lipídicas alteradas na sepse, as moléculas identificadas capazes de diferenciar SIRS de sepse assim como os possíveis marcadores para o prognóstico, com uma acurácia de 75%. A presença do agente infeccioso resulta em uma assinatura lipídica característica com potencial de auxiliar o diagnóstico da sepse. O estudo descreve aumento dos níveis de derivados de carnitina, resultante do distúrbio da oxidação mitocondrial de ácidos graxos, e a identificação da L-octanoilcarnitina como possível candidato a biomarcador para o diagnóstico e prognóstico de pacientes com sepse. O capítulo descrito na forma de artigo foi recentemente publicado (MECATTI et al., Potential Lipid Signatures for Diagnosis and Prognosis of Sepsis and Systemic Inflammatory Response Syndrome. *Metabolites* 10, 359, 2020).

2. OBJETIVOS

2.1 OBJETIVO GERAL

Identificar metabólitos lipídicos circulantes como possíveis candidatos a biomarcadores para corroborar no diagnóstico e prognóstico de pacientes com sepse.

2.2 OBJETIVOS ESPECIFICOS

- ✓ Identificar moléculas lipídicas diferenciais em sangue (plasma e eritrócitos) de pacientes com sepse e choque séptico por análise comparativa aos voluntários saudáveis (Capitulo II)

- ✓ Avaliar a assinatura lipidômica e identificar candidatos a biomarcadores capazes de identificar a infeção e, portanto, diferenciar pacientes com sepse e com SIRS - causa não infecciosa (Capitulo III)

- ✓ Avaliar o uso dos biomarcadores no prognóstico dos pacientes com sepse e SIRS-causa não infecciosa (Capitulo III)

3. ARTIGOS PUBLICADOS

3.1 CAPÍTULO I

MECATTI GC, MESSIAS MCF, CARVALHO PO. Lipidomic profile and candidate biomarkers in septic patients. *Lipids in Health and Disease* v. 19, p. 68-77, 2020. *doi.org/10.1186/s12944-020-01246-2*

O artigo descreve uma revisão realizada nas principais bases de dados eletrônicas (*PubMed, MEDLINE, Scopus e Web of Science*), referente ao período 2000-2020, que versa sobre biomarcadores usados atualmente na prática clínica, entre eles, procalcitonina e proteína C reativa; e também novas abordagens para o uso de biomarcadores lipídicos. A proteína C reativa (PCR) e a procalcitonina (PCT) são os marcadores biológicos mais estudados e aplicados na clínica como ferramentas auxiliares no diagnóstico e prognóstico de pacientes com suspeita de infecções bacterianas. Isto se deve, em grande parte, a facilidade de mensuração e acessibilidade aos serviços de saúde. Entretanto, diferentes estudos mostram que esses marcadores têm limitações relativas à sensibilidade e à especificidade, o que dificulta o amplo uso na identificação de pacientes com diagnóstico de sepse e/ou SRIS. Dentre os mecanismos relacionados à patogênese da sepse estão as alterações causadas pelo metabolismo lipídico e metabolismo oxidativo. A revisão descreve essas alterações com foco na molécula de lisofosfatidilcolina e a possibilidade de usar esse metabólito como biomarcadores da sepse.

Mecatti et al. Lipids in Health and Disease (2020) 19:68
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Lipids in Health and Disease

REVIEW Open Access

Lipidomic profile and candidate biomarkers in septic patients

Giovana Colozza Mecatti*, Márcia Cristina Fernandes Messias and Patrícia de Oliveira Carvalho

Abstract

Sepsis is a severe disease with a high mortality rate. Identification and treatment in the initial hours of the disease improve outcomes. Some biomarkers like procalcitonin and C-reactive protein are used for diagnosis and to access sepsis prognosis and they can help in clinical decision-making, but none has sufficient specificity or sensitivity to be routinely employed in clinical practice. This review seeks to evaluate lipid metabolism alterations in patients with sepsis and the possibility of using the respective metabolites as biomarkers of the disease. A search of the main electronic biomedical databases was conducted for the 20-year period ending in February 2020, focused on primary research articles on biomarkers in sepsis. The keywords included sepsis, septic shock, biomarker, metabolomic, lipidomic and lysophosphatidylcoline.
. It concludes that altered lipid profiles, along with the progress of the disease should provide new insights, enabling a better understanding of the pathogenic mechanisms and making it possible to design new early diagnosis and therapeutic procedures for sepsis.

Keywords: Sepsis, Septic shock, Biomarker, Metabolomic, Lipidomic, Lysophosphatidylcoline

Background

Sepsis is a major healthcare problem and affects millions of people around the world. Patients who develop the illness have high mortality rates (at least one in four) [1, 2]. Identification and treatment in the initial hours of the disease considerably improve outcomes [3]. Sepsis is a situation in which affected individuals develop an inflammatory response to an infection that harms their own organs and culminates in organ dysfunction [4]. Patients present signs of systemic inflammatory response syndrome and sometimes it is difficult for clinicians to define whether it is due to infection or other causes [5]. In that situation, the use of biomarkers could help with early diagnosis and improved risk stratification and clinical decision making [6–8].

Some biomarkers have been evaluated for use in sepsis diagnosis but none have sufficient specificity or sensitivity to be routinely employed in clinical practice. Procalcitonin (PCT) and C-reactive protein (CRP) have been the most widely used, but even they have limited ability to distinguish sepsis from other inflammatory conditions or to predict outcomes [9]. Altered lipid metabolism and its pro/anti-inflammatory lipid mediators play key roles in sepsis pathophysiology.

The use of multi-'omics' (association of at least two 'omic' variables: genomic, lipidomic, proteomic or metabolomic) may lead to an understanding of the pathophysiology of the disease and to the development of appropriate therapeutics. For example, in the treatment cascade of an endotoxin, the performance of a drug could alter its neutralization, influencing clearance, inflammation, bacterial load and mortality [10].

The objective of this review is to evaluate the changes of lipid metabolism in patients with sepsis and the possibility of using the respective metabolites as biomarkers of this disease. A search of the main electronic biomedical databases (PubMed, MEDLINE, Scopus, and Web of Science) was conducted for the 20-year period ending in

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February 2020, focused on primary research articles on biomarkers in sepsis. The keywords searched for in the abstracts and titles included “sepsis”, “septic shock”, “biomarker,” “metabolomic”, “lipidomic”, and “lysophosphatidylcoline”. The search identified 16 references with the words lipidomic x sepsis x septic shock, 34 references with the words metabolomic x sepsis x septic shock, and 13 references with the words lysophosphatidylcoline x sepsis x septic shock.

Review

Sepsis as a healthcare problem

Sepsis is a major healthcare problem and affects millions of people around the world. The mortality rate among patients that develop the illness is high (at least one in four) [1, 2]. The actual incidence of the disease in the world is uncertain. However, Fleischmann et al. made a systematic bibliographic survey and based on a statistical extrapolation of the results obtained, they suggest estimates of 31.5 million cases ofinfection, 19.4 million cases of sepsis and 5.3 million deaths per year [11].

The incidence of sepsis is increasing annually. Analyses of the occurrence of sepsis over a 22-year period (1979 to 2000), using data of a nationally representative sample of hospitals in the United States, identified more than 10 million cases in 750 million hospitalizations [1]. A large observational study in a European setting revealed an estimated mean sepsis incidence of 212.7 cases per 100,000 inhabitants with an annual incidence increase of 7.3%. In Brazil, sepsis incidence was 36.3 per 1000 patient-days and sepsis mortality was 55.7% [12].

Sepsis is a syndrome involving factors of both pathogen and host such as age, comorbidities, environment and race. It is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection; a situation in which affected individuals develop an inflammatory response to an infection injuring their own organs and culminating in organ dysfunction. Septic shock is defined as a subset of sepsis in which underlying circulatory and cellular metabolism abnormalities are profound enough to substantially increase mortality. The patients present persistent hypotension requiring vasopressors to maintain mean arterial pressure ≥ 65 mmHg and hyperlactatemia in the absence of hypovolemia. In septic shock, mortality is more elevated (> 40%) and early identification and treatment in the initial hours of the disease improve outcomes [3].

Sepsis, similar to other systemic inflammatory response syndromes, is characterized by increased secretion of stress hormones (e.g. catecholamines and cortisol) and cytokines, complement system activation and mitochondrial dysfunction with decreased availability of ATP. Sepsis-related inflammation causes microcirculatory dysfunction, inadequate tissue oxygen supply and subcellular and cellular dysfunction [13]. Initially, in response to infection, the innate immunity is activated when microorganisms contact receptors localized in cell surfaces (toll-like receptors - TLR). Binding TLR stimulates intracellular signaling and, in turn, production of proinflammatory (TNF- α, IL-1) and anti-inflammatory molecules (IL-10) [14, 15]. There is an alteration to the pro-oxidant-antioxidant balance. Also, there is an increase in the concentration of inflammatory cytokines (TNF-α and IL-8) and a decrease in the plasma activity of superoxide dismutase (SOD) and catalase (CAT) [16]. TNF-α and IL-8 exert cardiac depression by reducing myocardial shortening [17], further jeopardizing the patient’s hemodynamics. Pro-inflammatory cytokines lead to larger adhesion molecules in neutrophils and endothelial cells. Activated neutrophils promote microorganism kill and injure endothelial cells too, increasing vascular permeability [18]. Cytokines foster coagulation, stimulating thrombin formation in the microvascular bed and contributing to organ failure. In addition, consumption of coagulation proteins promotes bleeding [15]. Organ failure may be explained by microvascular occlusion, disruption of oxygenation with tissue exudate and production of reactive oxygen types [19]. In addition, there is evidence that in sepsis, alterations occur in mitochondrial function, with a decrease in the supply of tissue oxygen thereby contributing to organic dysfunction and increased production of free radicals, impacting on cellular metabolism and inflammatory processes [20, 21]. The increase in the production of reactive oxygen species (ROS) leads to organic dysfunctions caused by cellular and endothelial lesion due to protein modification and lipid peroxidation [22].

Effects of infection and inflammation on lipid and lipoprotein metabolism

Septic patients present alterations in lipid metabolism such as hypertriglyceridemia, a decrease in HDL and LDL-cholesterol and insulin resistance [23, 24]. Lipoprotein concentration can be reduced to 50% in patients with sepsis and those reductions seem to be related to the severity of the disease [25]. The primary decline is found in HDL and slow recovery occurs in HDL and LDL fractions. The decrease of HDL is not found in patients with trauma or other critical illnesses [26]. During sepsis, HDL is elevated in the HDL-acute phase attained in the presence of serum amyloid A, one of the three major acute phase proteins [27], and in depleted cholesterol and apolipoprotein A-1 [28] conditions. Inhibition of lipoprotein lipase, upregulated hepatic triglyceride production stimulated by hyperglycemia and hyperinsulinemia, the action of cytokines and the disruption of the synthesis-utilization balance are probably responsible for those alterations [29].

The changes in lipid metabolism during sepsis serve as a protective response against infection.

Lipopolysaccharide (LPS) is a constituent of Gram-negative bacteria that is involved in the inflammatory response to sepsis [30] and the presence of LPS in patients' blood is a clear indicator of sepsis. However, detection of LPS in aqueous blood is complicated by the molecule's amphiphilic biochemistry, which drives it to associate with host carrier lipoproteins [31] and other molecules such as LPS-binding protein (LBP), high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) and bactericidal/permeability-increasing protein [32]. Lipoproteins are known to be involved in the response of immunity neutralizing LPS, reducing cellular adhesion and reducing inducible nitric oxide synthase expression [33]. The structural changes in HDL may have a protective function and have metabolic consequences [34]. Chylomicron and very low-density lipoprotein neutralize the biological effects of endotoxin, and HDL particles control infection and the systemic inflammatory response [29].

Inflammation is modulated by lipid mediators derived from long chain polyunsaturated fatty acids (PUFA) with 20 or 22 carbons (n-6 or n-3 families). Those lipid mediators (eicosanoids and docosanoids) lead to metabolic changes that alter the plasma FA profile [22]. Patients with sepsis present low concentrations of n-6 and n-3 PUFAs and a high n-6/n-3 ratio and that is associated with high mortality [35–38]. An increase in oleic acid (C18:1 *n*-9) accompanied by a decrease in the unsaturation index as well as in the levels of *n*-3 PUFA was observed in erythrocyte phospholipids of septic patients as compared to healthy controls [39]. Arachidonic acid metabolism is also markedly affected in patients with sepsis. A reduced LPS-induced release of AA and the COX-associated AA metabolites, 11-HETE, PGE2, and TXB2 was apparent in septic patients [40]. Also, decreased lysophosphatidylcholine (LPC) levels and increased ceramide (Cer) species rates in plasma are commonly associated with sepsis [37–41]. An investigation of sepsis from peritonitis using a swine model monitored changes in hemodynamic, blood chemistry, and inflammatory markers. Mass spectrometry-based targeted quantitative analyses of blood samples were performed and found marked decreased in PC and LPC species [42]. Those results were supported by our group in a clinical study which observed important alterations in lipid metabolism in patients with sepsis, specifically including LPCs and sphingomyelin (SMs). Both LPCs and SMs were downregulated, whereas the saturated and unsaturated PCs were upregulated in the plasma and erythrocytes of septic patients [39]. Previous studies have also demonstrated an increase in circulating phospholipase A2 type II (snp-PLA2) in patients with severe infection [43, 44]. Group IIA sPLA2 is an acute-phase protein that is expressed in various tissues and cells in response to pro-inflammatory cytokines and it serves to amplify the systemic inflammatory response [45]. Members of the sPLA2 family of enzymes generate bioactive lipid mediators that include lysophosphospholipids and arachidonic acid and which can be converted to eicosanoids. Eicosanoids modulate cell growth and differentiation, inflammation, immunity, platelet aggregation and many other functions. Eicosanoids produced from arachidonic acid by COX and LOX, respectively, are 2-series PG and 4-series LT that act as mediators of inflammatory processes [46].

Biomarkers in sepsis

With the present systemic inflammatory response syndrome signals, it is sometimes difficult for clinicians to define whether it is due to infection or other causes [5]. In that situation, the use of biomarkers could help with early diagnosis, improving risk stratification and clinical decision-making [6–8].

Some biomarkers have been evaluated for use in sepsis. Most of them have been tested clinically, primarily as prognostic markers in sepsis. There are hundreds of biomarkers which could potentially be used for diagnosis and prognosis in septic patients [47]. They are classified as cytokine/chemokine biomarkers, cell marker biomarkers, receptor biomarkers, coagulation biomarkers, biomarkers related to vascular endothelial damage, biomarkers related to vasodilation, biomarkers of organ dysfunction and acute phase protein biomarkers [9]. Also, thirty-four biomarkers have been identified for use specifically in the diagnosis of sepsis but only five of them (CD11b, CD64, IL-12, IP-10 and PLA2-II) have reported sensitivity and specificity values greater than 90%.

A study with proteomic analysis, conducted with patients with sepsis and septic shock with a pulmonary focus, showed alterations in the proteins expressed in surviving and non-surviving sepsis patients alike. Of a total of 179, after excluding albumin and immunoglobulins, 48 were found to have been altered (16 specific proteins for survivors and 20 for non-survivors). Among the alterations in the concentrations of the proteins found were those associated to cytoskeletal organization, cell movement, energy metabolism, inflammation, coagulation and bleeding. The results also showed negative regulation of apolipoproteins like ApoA2, ApoA4, ApoC1, ApoC2, ApoC3, Apod and Pon1 [48].

So, due to their low specificity or sensitivity the use of these biomarkers is limited in routine clinical practice. Procalcitonin (PCT) and C-reactive protein (CRP) have been most widely used, but even they have limited ability to distinguish sepsis from other inflammatory conditions or to predict outcomes. Procalcitonin (PCT) is a

propeptide of calcitonin produced in low concentrations by the thyroid, gastrointestinal tract and lungs in healthy individuals. In the presence of bacterial infections, pro-inflammatory mediators induce an upregulated production and, with treatment, levels decrease by 50% per day [49]. The use of PCT to guide antimicrobial therapy has low to moderate quality in minimizing endpoints like mortality, mechanical ventilation, clinical severity and reinfection [50].

Liu et al. conducted a meta-analysis with 86 articles and a total of 10,438 subjects included. They found descriptions of 60 biomarkers and the most common were procalcitonin, C-reactive protein, interleukin 6, soluble triggering receptor expressed on myeloid cells-1, presepsin, lipopolysaccharide binding protein and CD64. Plasma PCT, Strem-1 and presepsin had moderate diagnostic utility for indicating systemic response caused by infection rather than other causes [51]. C-reactive protein (CRP) and procalcitonin are the most commonly used biomarkers. However, CRP has low specificity [52]. Procalcitonin is more specific [53] than CPR, but it remains difficult for it to differentiate sepsis from other non-infection causes of inflammation [54].

A recent comprehensive review of the available experimental evidence has shown that different biomarkers have clearly been demonstrated as indicating varying injury mechanisms and can be used in early diagnosis for sepsis-induced acute kidney injury [55].

Lipid biomarker

Lipids are regulators of cellular function and their metabolism is altered in patients with sepsis. Based on that, lipidomics can be used to understand the pathophysiological mechanisms involved in the diagnosis and the response to therapeutic measures [56]. Lipidomics is the analysis of lipid metabolism and is accessed by spectrophotometric techniques [57] and chromatography [58].

LPC has been suggested to serve as a more useful prognostic marker for sepsis [37, 59, 60]. Park et al. performed a study comparing quantitative analyses of LPC 16:0 by using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and found a sensitivity and a selectivity of medical diagnosis of sepsis estimated to be 97.9 and 95.5% on comparing analyses of sera from patients with severe sepsis and septic shock (n = 143), pneumonia patients (n = 12), and healthy individuals (n = 31) [61]. Lysophospholipids are membrane-derived phospholipids that can arise from homeostatic lipid metabolism or as a response to stimulus-induced cellular activation. Sources of plasma LPC include hydrolysis of PC by secretory phospholipase A2 (sPLA2) or lecithin:cholesterol acyltransferase (LCAT). LPC, in turn, is hydrolyzed to LPA in the plasma by autotaxin. LPA can also be synthesized from PA by sPLA2 [62]. In the phospholipid remodeling pathway, LPC is converted to PC via reacylation by acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT) in various tissues [63]. The schematic representation of the biosynthesis of LPC is represented in Fig. 1.

Total LPC concentration, as well as the concentration of the main LPC species, was markedly reduced in sepsis patients compared to controls and the difference in LPC-PC ratio was higher in survivors compared to non-survivors [37].

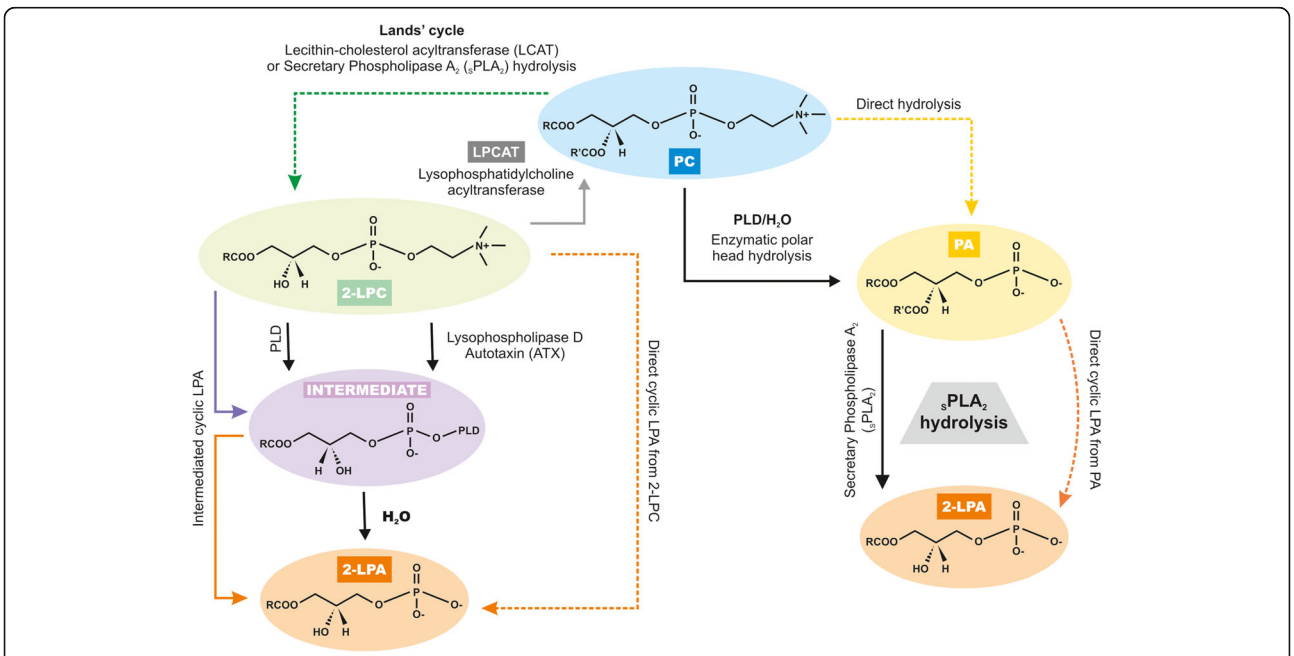

Fig. 1 Metabolic pathways for LPC biosynthesis

Table 1 Summary of Studies with evaluation of lipid biomarkers

Study	Methods	Participants	Interventions	Results
Drobnik et al., 2003 [37]	Prospective Experimental	102 patients with sepsis and 56 control	Analyses by mass spectrometry. The samples were collected as soon as sepsis criteria were met and mortality analyzed at 30 days.	Most Cer species were increased in sepsis patients, while all LPC species were markedly decreased. 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. Total cholesterol, HDL-C, and LDL-C in sepsis patients were markedly reduced compared with a healthy population.
Cho et al., 2012 [59]	Prospective Not randomized	Patients meeting sepsis criteria (105) and control – healthy blood donors (21)	Blood samples collected on the first day. Samples were analyzed using the ANZWELL LPC Assay commercial kit (Alfresa Pharma Corporation, Osaka, Japan).	Mean of serum concentration of LPC was significantly lower in patients with sepsis than in healthy individuals. No differences were observed between survivors and non-survivors in septic patients.
Schmerler et al., 2012 [69]	Prospective Not randomized	161 patients (74 with SIRS, 69 with sepsis and 18 control – patients in ICU without SIRS)	Samples of blood samples were collected within the first 24 h of admission of patients with SIRS. For patients with sepsis, samples were collected at 24 h after the onset of organ dysfunction. Analyzes were performed by mass spectrometry.	Acylcarnitine (C10:1) and Phosphatidylcholine (PCaaC32:0) were significantly higher in patients with sepsis compared to patients with non-infectious SIRS.
Cho et al., 2014 [65]	Prospective Not randomized	A total of 56 patients with community-acquired pneumonia (CAP)	Blood samples were collected from patients with CAP on days 1 and 7 and analyzed for their plasma LPC concentrations. Blood samples were analyzed using an Anzwell LPC Assay Kit commercial (Alfresa Pharma, Osaka, Japan).	24 (42.9%) of patients required intubation and 15 (26.8%) died. The mean LPC concentrations on days 1 and 7 were significantly lower in the non-survivors. LPC levels < 29.6 μmol/L at day 1 were associated with outcomes such as the need for mechanical ventilation, vasopressors, ICU admission and hospital mortality.
Park et al., 2014 [67]	Prospective, observational	A total of 74 patients with confirmed diagnosis of infection with at least two criteria of SIRS, within 24 h of admission in ICU	Blood sample analyzed on day 1 and day 7. Blood samples were analyzed using an Anzwell LPC Assay Kit commercial (Alfresa Pharma, Osaka, Japan).	The LPC concentration on day 7 was significantly lower in non-survivors. A decreased LPC concentration on day 7 and sustained high concentration of procalcitonin on day 7 were related to 28-day mortality. LPC concentrations increased over time in patients with appropriate antibiotics.
Liang et al., 2016 [70]	Prospective	ICU patients with sepsis induced lung injury - SLI (80) and healthy volunteers (82)	Plasma samples were collected in the morning at ICU with 10 h of fasting and analyzed by chromatography/mass spectrometry.	Significant changes were found in 7 metabolites, with an increase in concentration in SLI patients in 5 of them and a decrease in 2. Lipid metabolites include PE (P-19: 1 (12Z) / 0: 0), PE (22: 2 13Z, 16Z) / 15: 0), PC (17: 0/0: 0), LPC (P-16: 0), PE (20: 3 (8Z, 11Z, 14Z) / 0: 16: 0/0: 0) and PC (17: 1 (10Z) / 0: 0). PE (P-19: 1 (12Z) / 0: 0) showed sensitivity of 98.1% and specificity of 97.3%. Three lipids (PE (P-19: 1 (12Z) / 0: 0), PE (22: 2 (13Z, 16Z) / 15: 0), PC (17: 0/0: 0)) were selected to form a group of biomarkers to improve risk discrimination among SIL patients and healthy cases.
Ferrario et al., 2016 [64]	Retrospective	Plasma of 20 patients with severe septic shock (SOFA score > 8) enrolled in a multicenter Study (Albumin Italian Outcome Sepsis Study)	Plasma samples were analyzed by spectrometry that included quantitative measurements of acylcarnitines, aminoacids, biogenicamines, glycerophospholipids, sphingolipids, and sugars.	Unsaturated long-chain phosphatidylcholines and LPC species were associated to the event at 28-days and 90-days in combination with clinical variables such as cardiovascular SOFA score (28-day mortality model) or

Table 1 Summary of Studies with evaluation of lipid biomarkers *(Continued)*

Study	Methods	Participants	Interventions	Results
				renal replacement therapy (90-day mortality model).
Mecatti et al., 2018 [39]	Prospective Not randomized	Septic patients (n = 20) and healthy controls (*n* = 20)	Samples were collected in the first 36 h of admission to the ICU and analyzed by gas chromatography and mass spectrometry.	LPCs and SMs were downregulated, whereas the saturated and unsaturated phosphatidylcholines (PCs) were upregulated in the plasma and erythrocytes of septic patients.
Park et al., 2019 [61]	Prospective Controlled	Patients with severe sepsis and septic shock (*n* = 143), with pneumonia (*n* = 12), and healthy individuals (*n* = 31)	Quantitative analyses of LPC 16:0 were performed in samples of sera using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry on a parylene-matrix chip.	Sensitivity of 97.9% and selectivity of 95.5% in sepsis diagnosis as compared to healthy individuals and patients with pneumonia.
Ferrario et al., 2019 [42]	Experimental Controlled	Swine model (*n* = 9)	Induced peritonitis was performed in a swine model, and changes in hemodynamic, blood chemistry, and inflammatory markers were monitored. Quantitative mass spectrometry-based targeted metabolomic analyses were performed.	Marked decrease in phosphatidylcholines and LPC species, altered alanine-glucose cycle and inter-organ amino acid metabolism.
Arshad et al., 2019 [66]	Prospective Controlled	Patients with community-acquired pneumonia (*n* = 29) and with chronic obstructive pulmonary disease exacerbation with infection (*n* = 13) and control group (*n* = 33)	105 phospholipids, 40 acylcarnitines, and 4 ceramides, as well as acid sphingomyelinase activity were analysed in plasma using a triple-quadrupole mass spectrometer.	Phospholipid concentrations were greatly decreased in community-acquired pneumonia and normalized in the course of clinical improvement. The changes in COPD were less pronounced, but also differed qualitatively.

CRP C-reactive protein, *ICU* intensive care unit, *LPC* lysophosphatidilcholine, *PCT* procalcitonin, *SIRS* systemic inflammatory response syndrome, *SLI* sepsis-induced lung injury

Cho et al., analyzed LPC concentration in blood samples on the first day after diagnosis of septic patients and compared them with a control group of healthy blood donors. The mean serum LPC concentration was significantly lower than in the healthy controls. No difference in serum LPC concentration was evident between survivors and non- survivors and no correlation was found with severity of the disease [59]. On the other hand a preliminary retrospective investigation of the analyses Ferrario et al. conducted of the plasma of 20 patients with septic shock found that decreases of unsaturated long-chain PC and LPC species were associated to the event at 28-days and 90-days, in combination with clinical variables such as cardiovascular SOFA score (28-day mortality model) or renal replacement therapy (90-day mortality model) [64].

In another study, authors evaluated serum LPC concentrations in patients in an emergency department with community-acquired pneumonia and correlated them to scores of clinical prediction indicators (pneumonia severity index (PSI) and CURB-65 score) and the concentration of procalcitonin. Samples of days 1 and 7 were analyzed. The mean LPC concentrations on days 1 and 7 were significantly lower in the non-survivors. Day 1 LPC concentrations were inversely correlated with the PSI and CURB- 65 scores. Day 1 LPC cut-off levels< 29.6 μmol/L were associated with the need for mechanical ventilation, vasopressors, intensive care unit admission, and hospital mortality [65]. Arshad et al. measured 105 phospholipids, 40 acylcarnitines, and 4 ceramides, as well as acid sphingomyelinase activity, in plasma from patients with community-acquired pneumonia, chronic obstructive pulmonary disease (COPD) exacerbation with infection and a control group, and found that Phospholipid concentrations were greatly decreased in community-acquired pneumonia and normalized in the course of clinical improvement. They also observed that changes in COPD were less pronounced, but also differed qualitatively [66].

The relation between serial LPC measurements with 28-day mortality was analyzed in a tertiary ICU in patients with sepsis and septic shock. Serum LPC, white blood cell, C-reactive protein and procalcitonin levels were measured at baseline (day 1 of admission) and day 7. The LPC concentration on day 7 was significantly lower in non-survivors compared to survivors and a decreased LPC concentration on day 7 and a sustained high concentration of procalcitonin on day 7 were useful for predicting the 28-day mortality. LPC concentrations increased over time in patients with appropriate antibiotics, but not in those with inappropriate antibiotics [67].

Other lipids have also been pointed out as possible sepsis markers. Ahn et al. evaluated alteration of the lipid profile of mice with sepsis induced by cecal bacterial peritonitis after ligature by cecal puncture. They observed that among 147 lipid species in the plasma, 13 subgroups (FA, LPA, LPC, LPE, PA, PC, PE, PI, MG, DG, TG, SM and Cer) had alterations in sepsis. The group also evaluated the response to administration of LPC and LPA with altered lipid profile in response [68].

Schmerler et al. demonstrate that acylcarnitines and glycerophosphatidylcholines may be helpful for differentiating infectious from non-infectious systemic inflammation due to their significantly higher concentration in sepsis patients [69]. Three lipids (PC(17:0/0:0, PE(P-19:1(12Z)/0:0), PE(22:2(13Z,16Z)/15:0),)) were selected to form a biomarker group to improve risk discrimination between the sepsis-induced lung injury patients and healthy cases [70].

Additional study design details in humans are listed in Table 1.

Conclusion

In concluding this review, we can say that altered lipid profiles, along with the progress of diseases, should provide new insights that will enable a better understanding of the physiopathology of sepsis, contributing new possibilities for effective diagnoses and therapies. The current review deals with the lipid molecules that are up-regulated or down-regulated during the early stages of sepsis, as shown in the data presented in the present review and in earlier work of our research group [39]. Based on those aspects, we suggest that replenishing the protective molecules that are down-regulated in sepsis while withdrawing the elevated deleterious factors may lead to the discovery of new therapies for improving survival in septic patients; a goal that has been elusive for decades. In view of the complexity of the sepsis response, it is unlikely that a single ideal biomarker will ever be found. A combination of several sepsis biomarkers may be more effective, but that requires further evaluation.

Abbreviations

11-HETE: 11-hydroxy-5,8,12,14-eicosatetraenoic acid; AA: Arachidonic acid; Apo: Apolipoprotein; CAT: Catalase; Cer: Ceramide; COX: Cyclooxygenase; CRP: C-reactive protein; DG: Diacylglyceride; DHA: Docosahexaenoic acid; FA: Fatty acid; HDL: High-density lipoprotein; IL: Interleukin; IP-10: Interferon gamma-induced protein 10; LDL: Low-density lipoprotein; LPA: Lysophosphate; LPC: Lysophosphatidylcholine; LPE: Lysophosphatidylethanolamine; PCT: Procalcitonin; PGE2: Prostaglandin E2; LOX: Lipoxygenase; LPS: Lipopolysaccharide; LT: Leukotriene; MG: Monoacylglyceride; PA: Phosphatidic acid; PC: Phosphatidylcholine; PE: Phosphatidiylethanolamine; PI: Phosphatidylinositol; PLA2-II: Phospholipase A2; PSI: Pneumonia severity index; PUFA: Polyunsaturated fatty acids; ROS: Reactive oxygen species; S1P: Sphingosine-1 phosphate; SM: Sphingomyelin; Snp-PLA2: Phospholipase A2 type II; Strem-1: Soluble triggering receptor expressed on myeloid cells-1; SOD: Superoxide dismutase; TG: Triacylglyceride; TLR: Toll-like receptors; TNF-alfa: Tumor necrosis factor alfa; TXB2: Thromboxane B2; VLDL: Very low-density lipoprotein

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Consent to publication
Not applicable.

Authors' contributions
GCM, MCFM, POC contributed to the study concept and design, GCM and POC performed in the acquisition and tabulation of data, and drafted the manuscript. All authors read and approved the final manuscript.

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Not applicable.

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Not applicable.

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

3.2 CAPÍTULO II

MECATTI GC, MESSIAS MCF, SANT´ANNA RMP, ANGOLINI CF, CUNHA IBD, EBERLIN MN, CARVALHO PO. Lipidomic Profiling of Plasma and Erythrocytes from Septic Patients Reveals Potential Biomarker Candidates. *Biomarker Insights*, v. 13, p. 1-13, 2018. *doi.org/10.1177/1177271918765137.*

O trabalho descreve o perfil de compostos lipídicos detectados no plasma e em eritrócitos de pacientes com sepse (n=9) e choque séptico (n=11), comparado aos voluntários saudáveis (n=20), com o objetivo de identificar potenciais marcadores metabólicos. As análises foram realizadas por cromatografia líquida acoplada a espectrometria de massas (CL-EM) e por cromatografia à gás (CG). O estudo foi aprovado pelo Comitê de Ética da Universidade de São Francisco (CAEE 51356315.5.0000.5514). Foram identificadas diferentes moléculas diferenciais incluindo lisofosfatidilcolinas (LFCs) e esfingomielinas (EMs) com cadeias específicas de ácidos graxos como as principais envolvidas na patogênese da sepse. Ambos LFCs e EMs estavam com níveis reduzidos, enquanto que as fosfatidilcolinas saturadas e insaturadas estavam com níveis aumentados no plasma e nos eritrócitos de pacientes sépticos. Um aumento nos níveis de ácido oleico (18:1 *n*-9) acompanhado por uma diminuição dos ácidos graxos poliinsaturados (AGPI) da família *n*-3 foi observado nos fosfolipídios de pacientes com sepse. A redução dos níveis de AGPI *n*-3 parece ter relação com o aumento de estresse oxidativo e na síntese de mediadores lipídicos envolvidos na inflamação, vasomotricidade e permeabilidade capilar. Os resultados sugerem que os metabólitos lipídicos têm grande potencial como biomarcadores clínicos para a sepse além de contribuir no entendimento dos mecanismos metabólicos envolvidos. O trabalho descreve a identificação de uma ampla variedade de lipídeos diferenciais com potencial para abrir novos caminhos para a medicina diagnóstica.

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.

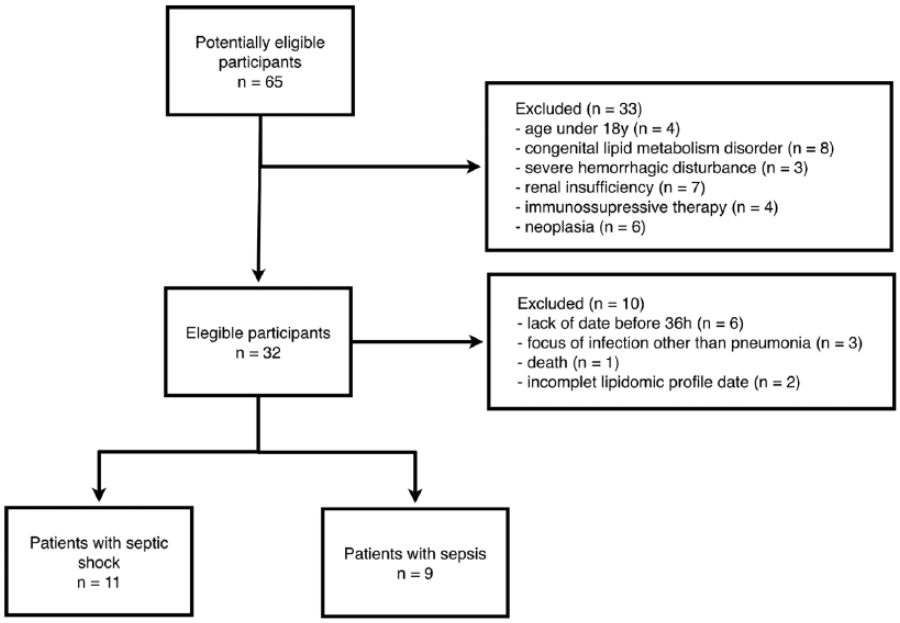

Figure 1. Patient selection flowchart.

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

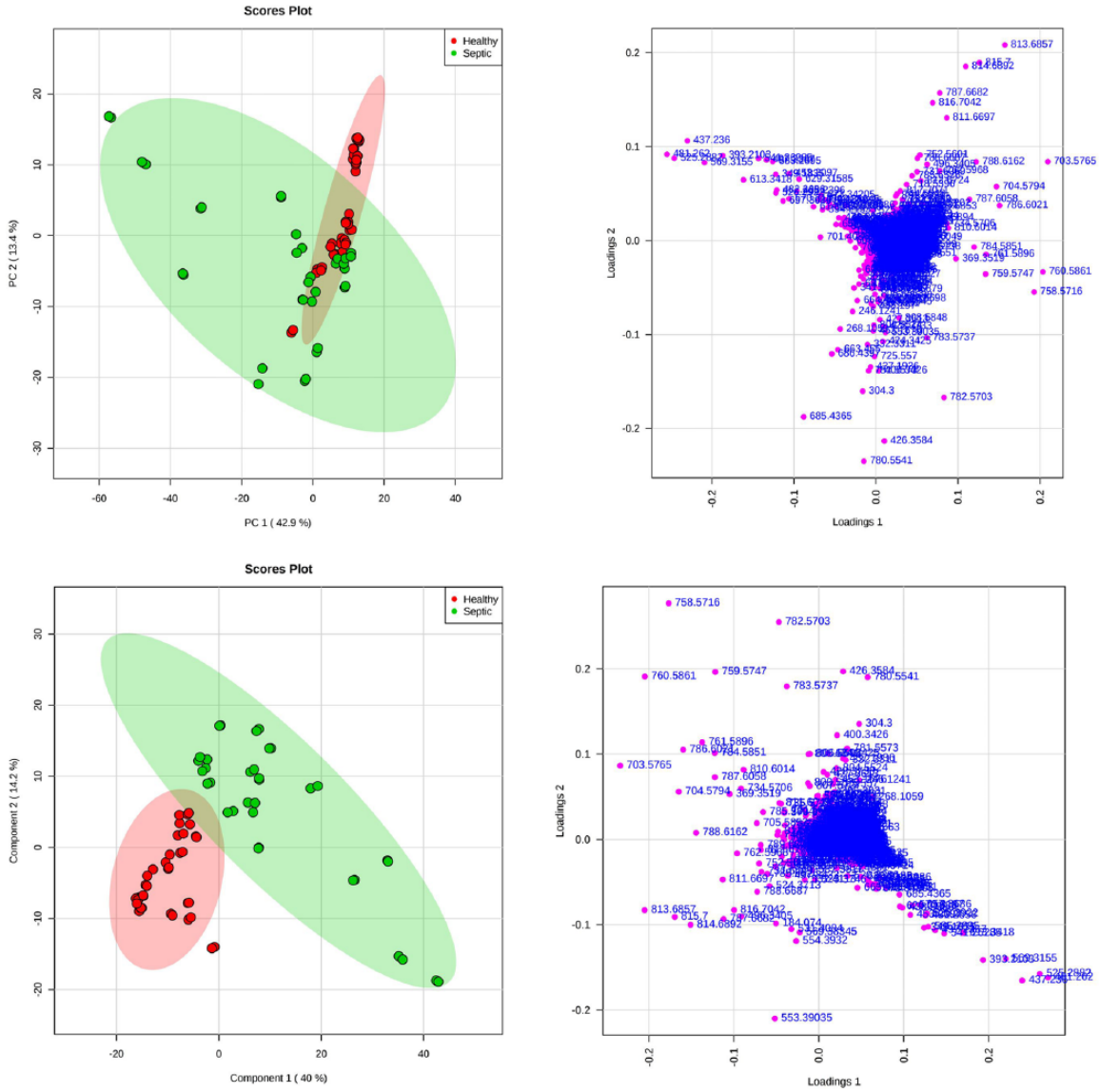

Figure 2. Top left: PCA scores plot of PC1 (first principal component) vs PC2 (second principal component) showing the separation between healthy volunteers (red) and septic patients (green). Top right: Loadings plot for PC1 and PC2 showing the metabolite ions (*m/z*) that were major contributors to 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

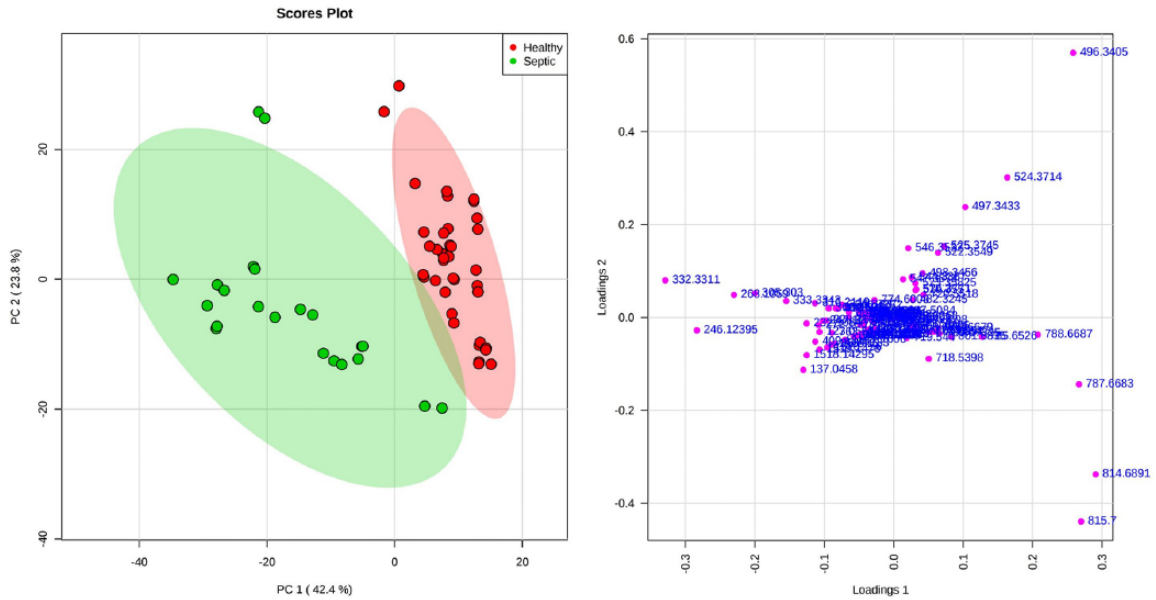

Figure 3. Left: PCA scores plot of PC1 (first principal component) vs PC2 (second principal component) showing the separation between healthy volunteers (red) and septic patients (green). Right: Loadings plot for PC1 and PC2 showing the metabolite ions (*m/z*) that were major contributors to the separation 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

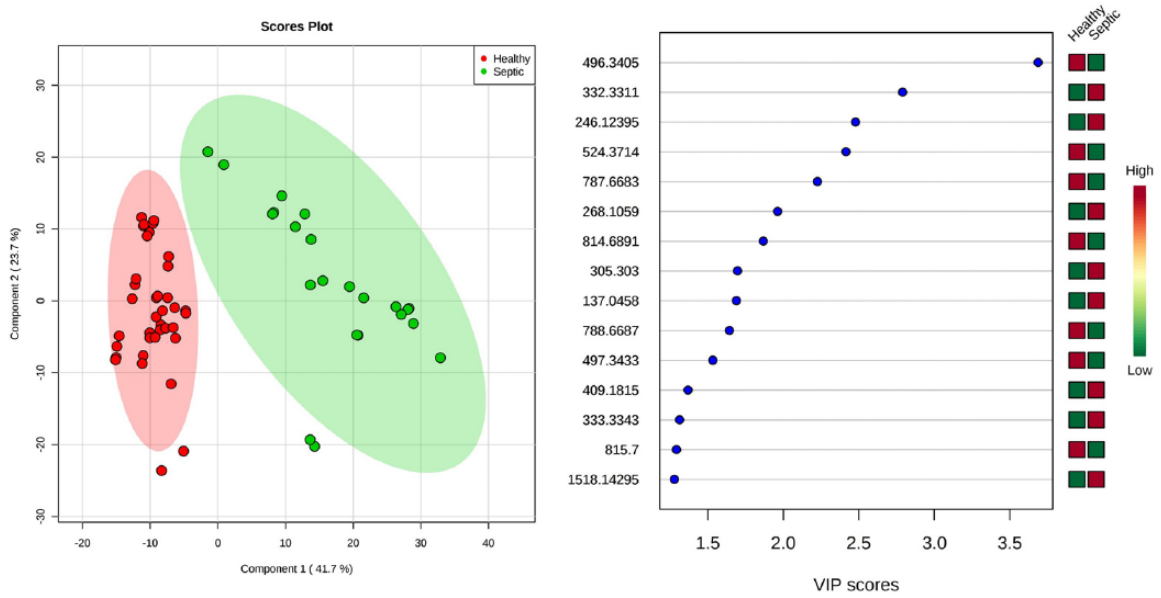

Figure 4. Left: Scores from PLS-DA discrimination of ESI-MS data from healthy volunteers (red) and septic patients (green). Right: Important metabolite ions selected on the basis of VIP score. The colored boxes on the right indicate relative bin integrals from healthy volunteers and septic patients. 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.

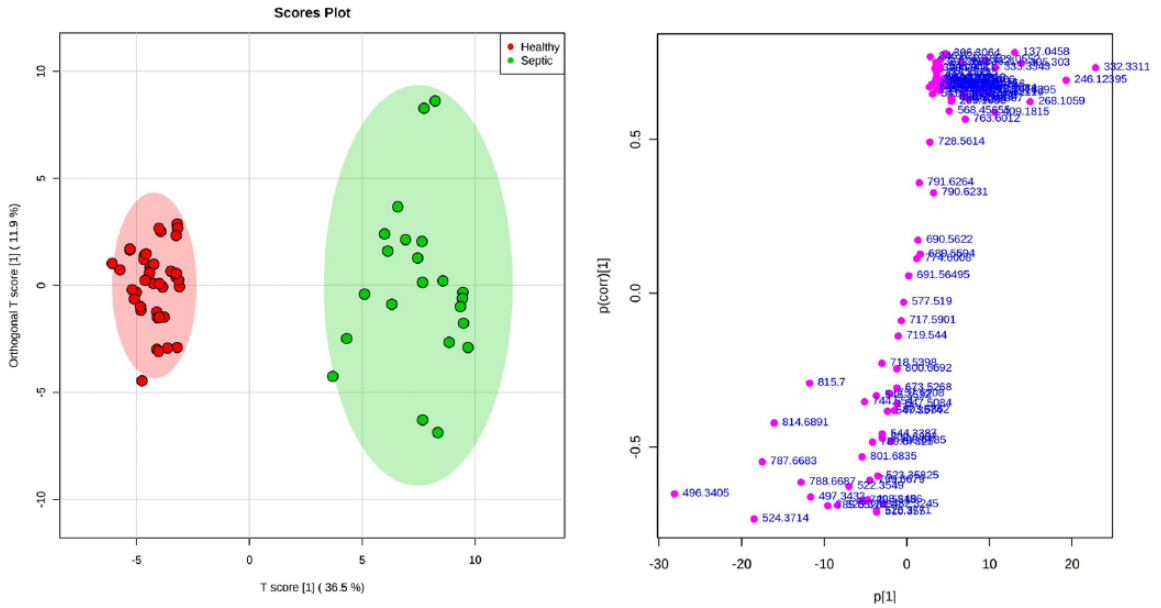

Figure 5. Left: (O)PLS-DA scores plot form comparison of the metabolite profiles of healthy volunteers (red) and septic patients (green). 36.5% and 11.9% are the scores of the *T* score and orthogonal *T* score, respectively. Right: Loadings plot for feature importance showing the metabolite ions (*m*/*z*) 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

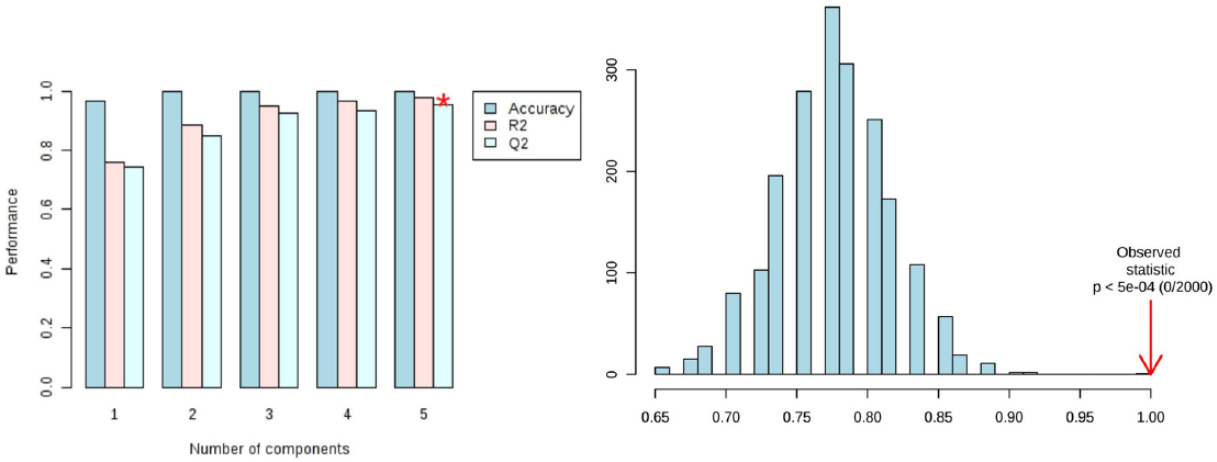

Figure 6. Left: Cross-validation showing the 3 performance measures (prediction accuracy, R^2, and Q^2) using different numbers of components. * indicates the best values of the currently selected measures Q^2 (0.86). Right: the result of permutation test statistics summarized by a histogram ($P < 5e^{-4}$).

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.

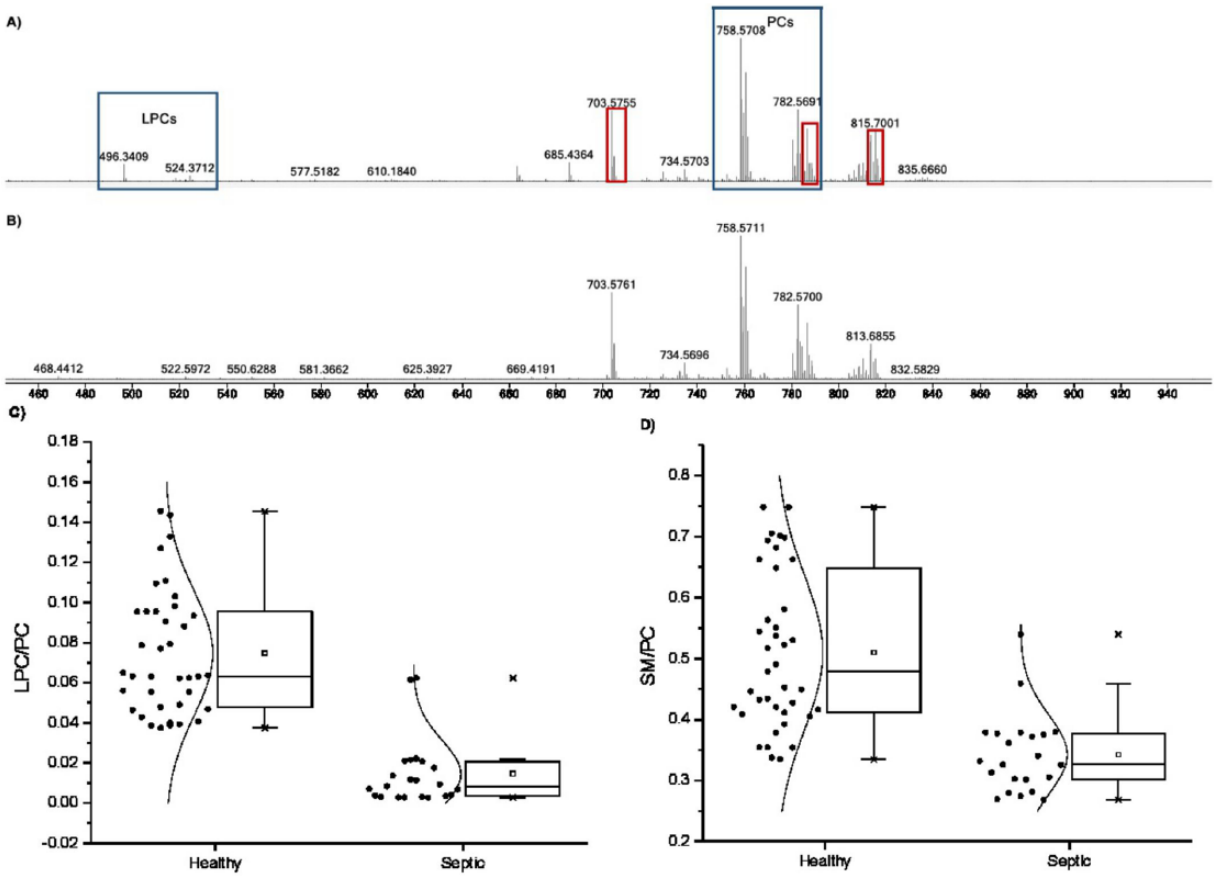

Figure 7. Typical ESI(+)-MS of erythrocyte membrane showing lipid profiles for (A) healthy and (B) septic patients. (C) Lyso-PC/PC and SM/PC ratios for healthy and septic patients. Lyso-PC/PC ratio for healthy and septic patients was determined dividing the combined pool of lyso-PC (16:0, 18:3, 18:2, 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.

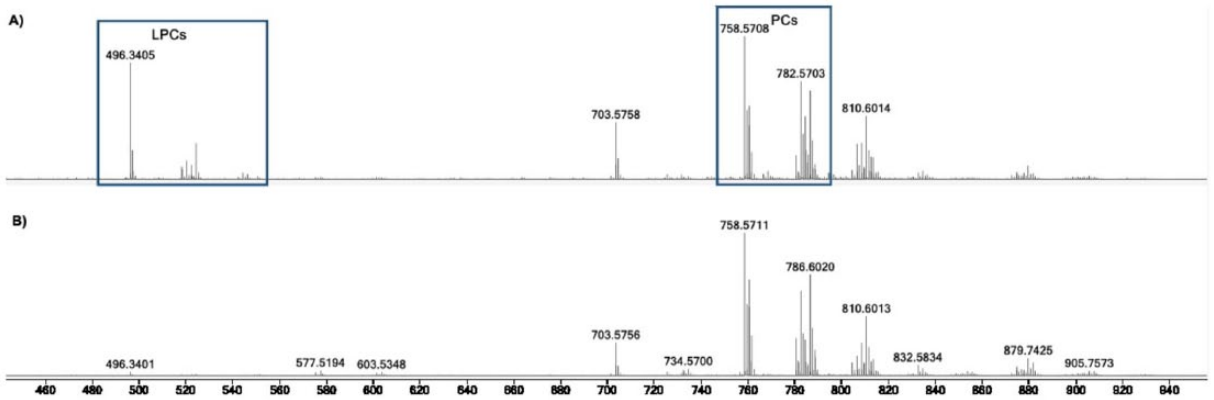

Figure 8. Two representatives examples of ESI(+)-MS of plasma lipid extract from (A) healthy and (B) septic patients. ESI(+)-MS indicates electrospray ionization mass spectrometry; PC, phosphatidylcholine.

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).

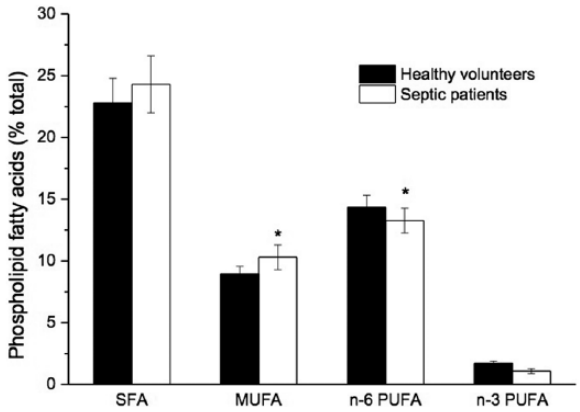

Figure 9. Percentages of different subclasses of fatty acids in the erythrocyte phospholipid fraction. MUFA indicates monounsaturated fatty acids; *n*-3 PUFA, *n*-3 polyunsaturated fatty acids; *n*-6 PUFA, *n*-6 polyunsaturated fatty acids; SFA, saturated fatty acid. *$P < .05$ compared with healthy 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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3.3 CAPÍTULO III

MECATTI GC, SÁNCHEZ-VINCES S, FERNANDES AMAP, MESSIAS MCF, DE SANTIS GKD, PORCARI AM, MARSON FAL, CARVALHO PO. Potential Lipid Signatures for Diagnosis and Prognosis of Sepsis and Systemic Inflammatory Response Syndrome. *Metabolites*, v. 10, p. 359 - 376, 2020. *doi:10.3390/metabo10090359.*

O artigo descreve a assinatura lipidômica e a identificação de potenciais moléculas capazes de diferenciar pacientes com sepse daqueles com Síndrome da Resposta Inflamatória Sistêmica (SIRS). O sangue (plasma) de 21 pacientes com sepse e 21 com SRIS foi avaliado por cromatografia líquida acoplada à espectrometria de massas (CL-EM) usando análise multivariada de dados e medida de predição (*Random Forest*). O estudo foi aprovado pelo Comitê de Ética da Universidade de São Francisco (CAEE 51356315.5.0000.5514). A análise lipidômica foi capaz de promover com êxito a identificação da infecção nos pacientes com sepse os quais mostraram aumento nos níveis plasmáticos de glicerofosfolipídeos, ceramidas e em especial, derivados de carnitina metabólitos de desregulação da beta-oxidação mitocondrial de ácidos graxos. L-Octanoilcarnitina (*upregulation*) e ésteres de ácidos graxos ramificados de ácidos graxos hidroxilados (FAHFA 36:4) (*dowregulation*) são apontados como potenciais biomarcadores para diferenciar sepse de SIRS. A importância da L-Octanoilcarnitina foi confirmada para predizer o risco de óbito nos pacientes. Os resultados apontam que essas moléculas poderão ser usadas para corroborar no diagnóstico e prognóstico da sepse.

metabolites

MDPI

Article

Potential Lipid Signatures for Diagnosis and Prognosis of Sepsis and Systemic Inflammatory Response Syndrome

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Abstract: Systemic inflammatory response syndrome (SIRS) and sepsis are two conditions which are difficult to differentiate clinically and which are strongly impacted for prompt intervention. This study identified potential lipid signatures that are able to differentiate SIRS from sepsis and to predict prognosis. Forty-two patients, including 21 patients with sepsis and 21 patients with SIRS, were involved in the study. Liquid chromatography coupled to mass spectrometry and multivariate statistical methods were used to determine lipids present in patient plasma. The obtained lipid signatures revealed 355 features for the negative ion mode and 297 for the positive ion mode, which were relevant for differential diagnosis of sepsis and SIRS. These lipids were also tested as prognosis predictors. Lastly, L-octanoylcarnitine was found to be the most promising lipid signature for both the diagnosis and prognosis of critically ill patients, with accuracies of 75% for both purposes. In short, we presented the determination of lipid signatures as a potential tool for differential diagnosis of sepsis and SIRS and prognosis of these patients.

Keywords: sepsis; SIRS; lipidomics; multivariate analysis

1. Introduction

The definition of sepsis, as introduced in 2016, updated several concepts and brought some new ones. Now, sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection that includes immune as well as nonimmune responses [1]. All over the world, nearly 6 million people die of sepsis annually [2]. Systemic inflammatory response syndrome (SIRS) is a condition in which the patient presents two of the following signs: tachycardia, fever or hypothermia, leukocytosis or leukopenia and tachypnea. It may also occur in response to various forms of aggression such as infection, trauma or surgery. Almost all septic patients have SIRS, but not all SIRS patients are septic. As an exception to this theory, it has been suggested that there are subgroups of hospitalized elderly patients who do not meet criteria for SIRS on presentation but progress to severe infection and multiple organ dysfunction and death. For this reason, SIRS could be an element of confusion for the diagnosis, management plan or evolution assessment and, eventually, patient prognosis prediction [3].

An insufficient ability to predict sepsis prognosis continues to be an important issue, despite the increasing use of clinical tools [4], severity scores (e.g., the Acute Physiology and Chronic Health Evaluation II (APACHE II) [5], the Simplified Acute Physiology Score III (SAPS III) [6], Sequential Organ Failure Assessment (SOFA), qSOFA (quick SOFA)) and biomarkers (e.g., procalcitonin (PCT), presepsin and C-reactive protein (CRP) [7]). Since sepsis has a clinical diagnosis of great biological complexity, not much progress has been made towards an effective predictive approach to sepsis in terms of specific diagnosis or prognosis [8]. The characterization of molecular mechanisms of events associated with sepsis, such as organ failure, treatment response evolution and death, are also not well understood [9].

SIRS suffers from a lack of precision in defining the factors that produce a disease (infectious or not), its evolution and the patient's outcome [1]. Satisfying the two minimum criteria for a SIRS diagnosis is relatively common in some infectious or non-infectious diseases (i.e., pancreatitis or trauma) [10]. However, the importance of assessing the presence of these factors has generated different results and, at the very least, they are intriguing due to the evident selectivity in terms of when and where they are most useful [11].

Recent omics techniques have facilitated high-throughput profiling of pathology-related signatures and biomarkers in biological fluids [12]. Lipidomics is one of the most recent, rapidly developing and promising approaches [13]. Lipidomics studies the state of the lipid molecular phenotype, reflecting the functional "landscape" of lipid activity in cells and tissues [14]. For this reason, clinical lipidomics offers the possibility of elucidating the strategic roles of lipids in disease and the immune system [15], identifying biomarkers and developing new therapeutics [16]. Recent studies have shown the potential diagnostic [17] and prognostic [18] roles of lipidomics in sepsis patients. Some promising results from the existing literature have evaluated differences between control and sepsis patients [17,19] and between patients who survived sepsis and those who did not [20]. A less frequent comparison covers the difference between sepsis and SIRS by evaluating the potential for the diagnosis or prognosis of either [20,21]. The terms outcome and prognosis have been used here as if they were synonyms and are understood to be the final survival report of each patient [22].

In this prospective study, the lipid profiles obtained from plasma samples of patients diagnosed with sepsis were compared with patients diagnosed with non-septic SIRS in order to identify sepsis-specific biomarkers. These lipids with potential for differential diagnosis were assessed as prognostic biomarkers and their putative biological roles were summarized.

2. Results

2.1. Subject and Clinical Data

Table 1 shows a statistical comparison of the two groups for the baseline characteristics of the participants involved in this study. No significant differences were found between the demographic characteristics of the groups. Other prognostic scores did not present a statistically significant difference. Almost no comorbidities were present in the SIRS group; this can be explained by the epidemiological characteristics of this group of patients as they were almost all victims of poly-trauma. A higher frequency of comorbidities is expected in patients in the sepsis group [23], leading to a statistically significant difference for systemic hypertension and for diabetes mellitus. The other comorbidities were less frequent in our patients with sepsis, so there were no statistically significant differences, even in the absence of the comorbidities in the SIRS group. No significant differences were found in the frequency of organ dysfunctions between the groups, since both diagnoses can lead to the occurrence of these dysfunctions. None of these variables had a statistically significant effect on the multiple linear regression model for diagnostic classification (Supplementary Table S3). All of the non-survivor patients in the study died during their intensive care unit (ICU) stay.

Table 1. Baseline characteristics of the study population.

	Sepsis			SIRS			Sepsis vs. SIRS
	N	**Mean**	**SD**	**N**	**Mean**	**SD**	***p*-Value**
Age	21	55.52	19.79	21	48.00	17.44	0.20
BMI	21	25.10	4.92	21	24.96	3.49	0.92
SAPSIII	21	56.95	17.17	21	53.05	14.72	0.43
Risk of death (%)	21	38.33	29.39	21	29.03	24.01	0.27
SOFA score	21	5.14	2.95	21	6.43	3.11	0.18
Comorbidities							
Systemic hypertension	7	0.33	-	1	0.05	-	0.05
Diabetes mellitus	5	0.24	-	0	0.00	-	0.05
Dyslipidemia	0	0.00	-	0	0.00	-	1.00
Coronary insufficiency	1	0.48	-	0	0.00	-	1.00
COPD	4	0.19	-	0	0.00	-	0.11
Neoplasm	3	0.14	-	0	0.00	-	0.23
Organ dysfunction							
by patient	21	2.05	-	21	2.05	-	0.82
AP < 90 mmHg	10	0.48	-	16	0.76	-	0.11
Lactate > 20 mg/dL	11	0.52	-	13	0.62	-	0.76
AKI	5	0.24	-	6	0.29	-	1.00
Total bilirubin > 2 mg/dL	3	0.14	-	1	0.05	-	0.61
INR > 1.6	8	0.38	-	1	0.05	-	0.02
Platelets < 150,000/mm^3	1	0.05	-	4	19.05	-	0.34
PaO_2/FiO_2 ratio < 300	5	0.24	-	1	4.76	-	0.18
Site of infection							
Pneumonia	7	0.33	-	-	-	-	-
Abdominal	9	0.43	-	-	-	-	-
UTI	1	0.05	-	-	-	-	-
Others	4	0.19	-	-	-	-	-
Outcome (death)							
ICU length of stay	21	7.91	5.99	21	10.81	6.90	0.15
Total outcome	7	0.33	-	7	0.33	-	1.00

N: number of patients measured for each characteristic; Mean: average value for quantitative characteristics and proportion value for qualitative; SD: standard deviation; SIRS: systemic inflammatory response syndrome; BMI: body mass index; SAPSIII: Simplified Acute Physiology Score; SOFA: Sequential Organ Failure Assessment; COPD: chronic obstructive pulmonary disease; AP: arterial pressure; mmHg: millimeters of mercury; mg/dL: milligrams per deciliter; mm^3: cubic millimeter; AKI: acute kidney injury; INR: international normalized ratio; FIO_2: fraction of inspired oxygen; PaO_2: partial pressure of oxygen; UTI: urinary tract infection. ICU: intensive care unit.

2.2. Analysis of Plasma Samples

In this study, 42 samples were assessed: 21 plasma samples from male patients diagnosed with sepsis and 21 plasma samples from male patients diagnosed with SIRS. After applying quality control (QC) and non-QC filters and making corrections, final numbers of 733 features for negative ion mode and 1703 features for positive ion mode were obtained. The obtained lipidome data were assessed using principal component analysis (PCA) for both negative (Figure 1A) and positive ionization modes (Figure 1B). In negative mode, both groups presented very close individual profiles which impeded complete separation of groups. In positive mode, there is a total overlap of the groups. Supplementary Figure S1 shows PCA for samples and QC, where high-quality data depict QC samples in clusters tighter than those observed for biological samples [24].

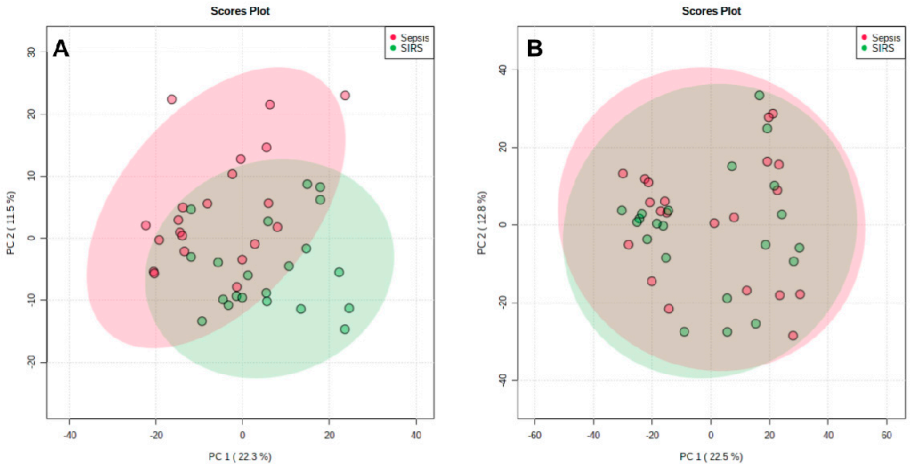

Figure 1. Principal component analysis (PCA) score plot between the first 2 principal components (PC) for negative ionization mode (**A**) and positive ionization mode (**B**). Areas of 95% confidence are highlighted in red and green. Variance explanation (%) for each PC is indicated.

Other descriptive analyses, such as volcano plot and heatmap of clustered intensities, were performed for all the features. The results are represented in Figure 2 for both negative and positive modes. These descriptive results show that, despite the difficulty in differentiating groups by PCA, it is still possible to determine features with differential abundances.

2.2.1. Analysis of Lipid Signatures for Diagnosis

In order to identify the most relevant features in the task of correctly classifying the samples by diagnosis (sepsis or SIRS), a selection of the lipid signatures was made with prediction models using the random forest (RF) method implemented in MetaboanalystR. The final model for the negative mode (accuracy = 84.7%, area under the curve (AUC) = 0.935) selected 355 features as relevant. The final model for the positive mode (accuracy = 75.7%, AUC = 0.868) selected 297 features as relevant. Receiver Operating Characteristic (ROC) curves for these models are provided as Supplementary Figures S2 and S3.

Matching the obtained list of features from the RF model for negative and positive mode with Lipidmaps and Human Metabolome DataBase (HMDB) databases resulted in the annotation of 33 significant features as possible biomarkers for discriminant diagnosis between sepsis and SIRS (Table 2). Annotated lipids such as L-palmitoylcarnitine, gamma-linolenyl carnitine, linoleyl carnitine and the omega 6 polyunsaturated fatty acid arachidonic acid were found in higher abundance in the sepsis patient's plasma and were significant contributors to differentiation among sepsis and SIRS. The predictive importance of these putatively identified lipids was evaluated in subsequent analyses.

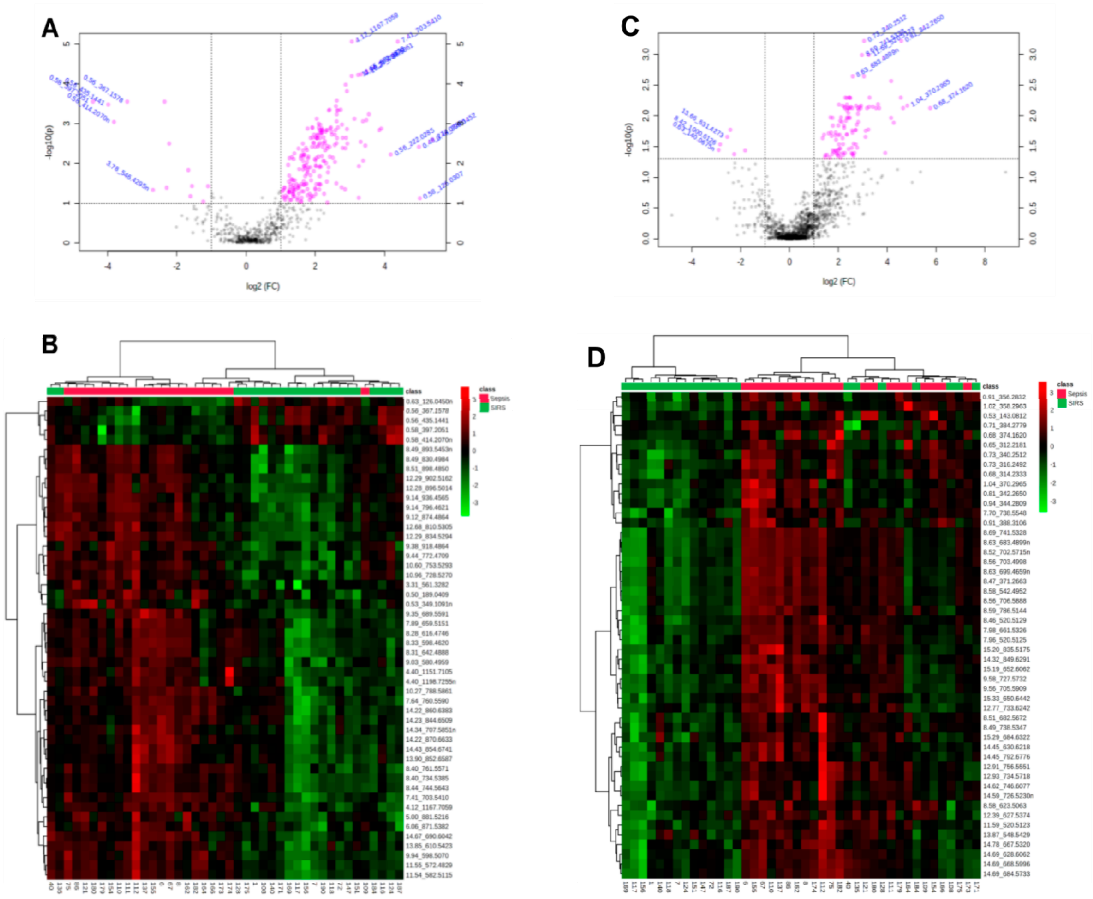

Figure 2. Volcano plot of features for negative ionization mode (**A**) and positive ionization mode (**C**). Heat map of clustered differential features and samples for negative mode (**B**) and positive mode (**D**). In the volcano plot, highlighted features with adjusted *p*-value of 0.05 and log (fold change) of 1. Heatmap depicts top 50 features with lowest adjusted *p*-values.

Table 2. Relevant ions selected by random forest (RF) models (positive and negative ion mode) to the diagnostic classification of plasma from sepsis and SIRS diagnosed patients.

Measured *m/z*	Ion Mode	Adducts	Lipid Assignment	Proposed Formula	Mass Error (ppm)	Abundance Sepsis	Abundance SIRS
129.0555	-	M-H_2O-H [1−]	Mevalonic acid [a]	$C_6H_{12}O_4$	−1.69	1385.62 (722.52)	1277.46 (743.76)
132.0657	+	M+H [1+]	2-amino-4-oxopentanoic acid [a]	$C_5H_9NO_3$	1.51	991.28 (466.89)	1013.26 (580.62)
133.0854	+	M+H [1+]	6-hydroxyhexanoic acid [a]	$C_6H_{12}O_3$	−3.76	679.72 (866.08)	782.53 (914.21)
238.1169	+	M+H [1+]	S-aminomethyldihydrolipoamide [a]	$C_9H_{20}N_2OS_2$	0.39	1114.93 (320.52)	1135.13 (536.29)
282.1251	-	M−2H [2−]	Leukotriene F4 [b]	$C_{28}H_{44}N_2O_8S$	−2.54	1154.50 (1659.83)	1066.37 (662.07)
288.2181	+	M+H [1+]	L-octanoylcarnitine [b]	$C_{15}H_{29}NO_4$	4.16	1452.21 (1113.79)	659.59 (430.61)
293.2119	-	M−H_2O-H [1−]	13-L-hydroperoxylinoleic acid [b]	$C_{18}H_{32}O_4$	−1.08	891.92 (644.89)	751.29 (539.68)
295.2277	-	M−H [1−]	13S-hydroxyoctadecadienoic acid [b]	$C_{18}H_{32}O_3$	−0.68	909.30 (645.14)	492.81 (355.81)

Table 2. *Cont.*

Measured *m/z*	Ion Mode	Adducts	Lipid Assignment	Proposed Formula	Mass Error (ppm)	Abundance Sepsis	Abundance SIRS
303.2333	-	M−H [1−]	Arachidonic acid [b]	$C_{20}H_{32}O_2$	0.99	817.75 (713.45)	1104.24 (653.40)
326.2670	+	M+H−H_2O [+1]	N-palmitoyl serine [a]	$C_{19}H_{37}NO_4$	−5.64	2286.56 (4267.87)	3535.59 (5678.59)
327.2332	-	M−H [1−]	Docosahexaenoic acid [a]	$C_{22}H_{32}O_2$	0.61	812.39 (508.09)	780.96 (365.68)
331.2280	+	M+H [1+]	17-hydroxyprogesterone [b]	$C_{21}H_{30}O_3$	3.62	1142.74 (400.95)	1133.59 (530.18)
335.2218	+	M+H [1+]	PGE2 1,15-lactone [a]	$C_{20}H_{30}O_4$	0.30	709.67 (263.66)	561.39 (187.29)
353.2326	+	M+H [+1]	Prostaglandin E2 [b]	$C_{20}H_{32}O_5$	1.13	1405.19 (1305.15)	698.16 (637.36)
367.1578	-	M−H [1−]	Dehydroepiandrosterone sulfate [b]	$C_{19}H_{28}O_5S$	−1.90	614.23 (463.79)	2599.17 (1954.71)
397.2051	-	M−H_2O-H [−1]	7-[(2,4,6-trihydroxy-2,5,5,8a-tetramethyl-decahydronaphthalen-1-yl)methoxy]-2H-chromen-2-one [a]	$C_{24}H_{32}O_6$	7.28	436.61 (267.23)	1948.12 (1895.83)
400.3438	+	M+H [1+]	L-palmitoylcarnitine [b]	$C_{23}H_{45}NO_4$	4.25	1275.23 (1117.99)	684.14 (547.16)
422.3260	+	M+H [1+]	Gamma-linolenyl carnitine [a]	$C_{25}H_{43}NO_4$	−1.18	994.63 (388.35)	832.62 (254.73)
424.3432	+	M+H [1+]	Linoleyl carnitine [b]	$C_{25}H_{45}NO_4$	2.59	1087.23 (1266.79)	598.35 (520.68)
426.3589	+	M+ACN+H [+1]	Tetrahydropersin [a]	$C_{23}H_{44}O_4$	2.89	1146.89 (1099.49)	568.64 (508.05)
464.3016	-	M-H [1−]	Glycocholic acid [a]	$C_{26}H_{43}NO_6$	−0.43	1506.15 (4407.32)	639.15 (1149.97)
477.2132	+	M+H [1+]	2-methoxyestrone 3-glucuronide [a]	$C_{25}H_{32}O_9$	2.72	1057.75 (267.34)	1088.04 (224.43)
510.3940	+	M+H [1+]	LysoPC (O-18:0) [b]	$C_8H_{20}NO_6PR$	4.31	932.19 (572.29)	776.95 (514.12)
557.4584	–	M−H [−1]	FAHFA 36:4 [a]	$C_{36}H_{62}O_4$	1.62	760.47 (665.67)	1528.53 (1306.92)
582.5110	–	M+FA−H [−1]	Cer (d16:1/18:0) [a]	$C_{34}H_{67}NO_3$	1.38	1931.34 (2267.96)	593.15 (508.32)
610.5423	–	M+FA−H [−1]	Cer (d36:1) [a]	$C_{36}H_{71}NO_3$	1.32	1497.42 (786.64)	707.04 (471.59)
753.5293	–	M+FA−H [−1]	PG (O−32:0) [a]	$C_{38}H_{77}O_9P$	0.87	1374.76 (687.85)	458.97 (455.32)
760.5590	–	M+FA−H [−1]	AS 1-5 [a]	$C_{40}H_{77}NO_9$	1.34	1273.41 (530.64)	489.87 (320.17)
762.5650	–	M−H [−1]	PS (O−35:0) [a]	$C_{41}H_{82}NO_9P$	−0.64	1541.92 (1105.94)	674.73 (559.37)
834.5294	–	M−H [1−]	PS (16:0/16:0) [b]	$C_{38}H_{74}NO_{10}P$	−0.41	1351.20 (525.54)	733.77 (538.39)
856.5141	–	M+Na−2H [−1]	PS (40:6) [b]	$C_{46}H_{78}NO_{10}P$	3.69	1575.69 (1472.24)	463.90 (483.53)
908.6356	–	M+Na−2H [−1]	PS (43:1) [b]	$C_{49}H_{94}NO_{10}P$	−0.63	1575.01 (1318.04)	1016.98 (1203.54)
932.6353	–	M+FA−H [−1]	PC (44:7) [a]	$C_{52}H_{90}NO_8P$	−3.75	1854.09 (1614.94)	621.01 (482.13)

m/z: mass-to-charge ratio; LysoPC: Lysophosphatidylcholine; PC: phosphatidylcholine; PG: phosphatidylglycerol; PGE2: prostaglandin E2; FAHFA: branched fatty acid esters of hydroxy fatty acids. Corrected abundance expressed as mean (standard deviation); a: Level 2 and b: Level 3 of annotation (see Methods).

Figure 3 shows the metabolic pathways most associated with the lipids found to be relevant. A large impact on the pathway is related to the importance of the compound within the metabolic network evaluated; a higher log (p) (or lower *p*-value) indicates the over-representation of the evaluated pathway in relation to the list of compounds consulted. Only 22 compounds were found in the HMDB database. Supplementary Table S2 shows information on the matched lipids and statistics of the enriched pathways.

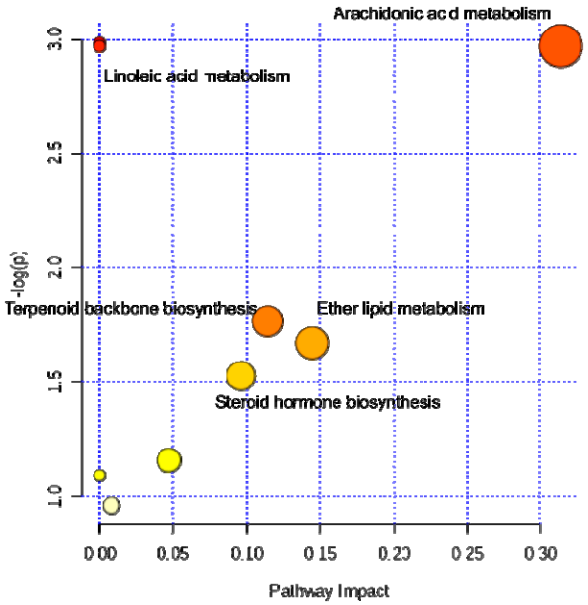

Figure 3. Summary of pathway analysis.

2.2.2. Performance Evaluation of Diagnostic Lipid Signatures Used for Prognostic Prediction

With a more reduced but significant list of features, random forest for multivariate classification was used to assess features' performances as possible signatures for prognostic classification (Figure 4). This model had an average accuracy of 61.3% and an AUC = 0.676 (see Supplementary Figure S4). Supplementary Table S1 provides a complete list of ranked scores. Although this model presents low accuracy due to the small number of features selected for prognostic classification, its results enabled the identification of the most relevant features for further analysis.

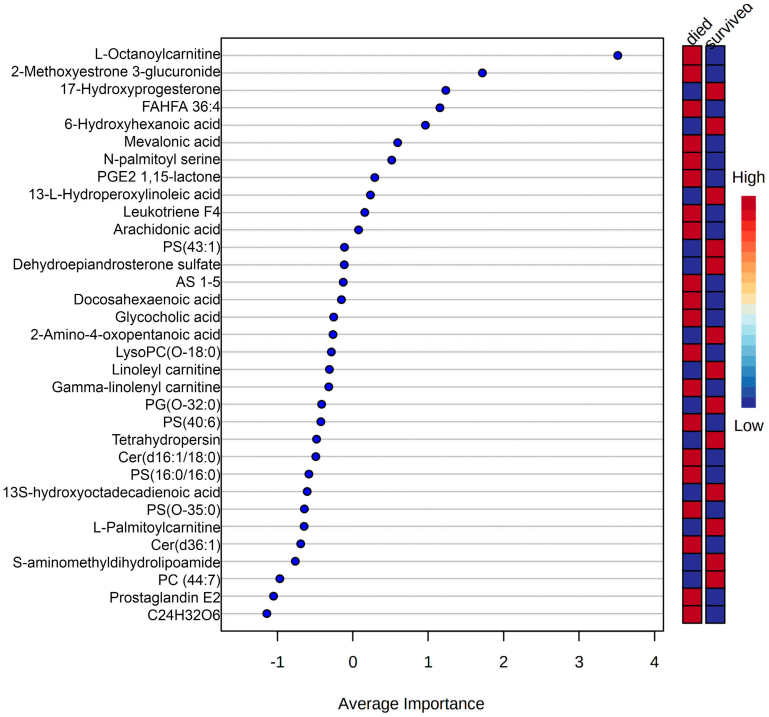

Figure 4. Prognostic classification using identified lipids from diagnosis classification model. Lipids ranked by importance for the random forest based model.

2.2.3. Performance Evaluation of L-Octanoylcarnitine as Diagnostic and Prognostic Predictor

The lipid L-Octanoylcarnitine was found to be the most relevant for the prognostic classification (samples from patients who survived and died), with a notable difference in importance in relation to the other lipids. To evaluate its individual importance in diagnostic and prognostic classification, classification prediction models were used based on random forest. To build the classification models, eight samples were randomly selected and unlabeled (four from each group for each classification) to define a validation group. As a diagnostic signature (Figure 5A), L-octanoylcarnitine obtained an AUC = 0.89, an average accuracy based on 100 cross-validations of 0.848 and accuracy for validation data prediction of 0.75 (6/8), one mismatch per class. As a prognostic signature (Figure 5B), L-octanoylcarnitine obtained an AUC = 0.713, an average accuracy based on 100 cross-validations of 0.658 and accuracy for validation data prediction was 0.75 (6/8), one mismatch per class.

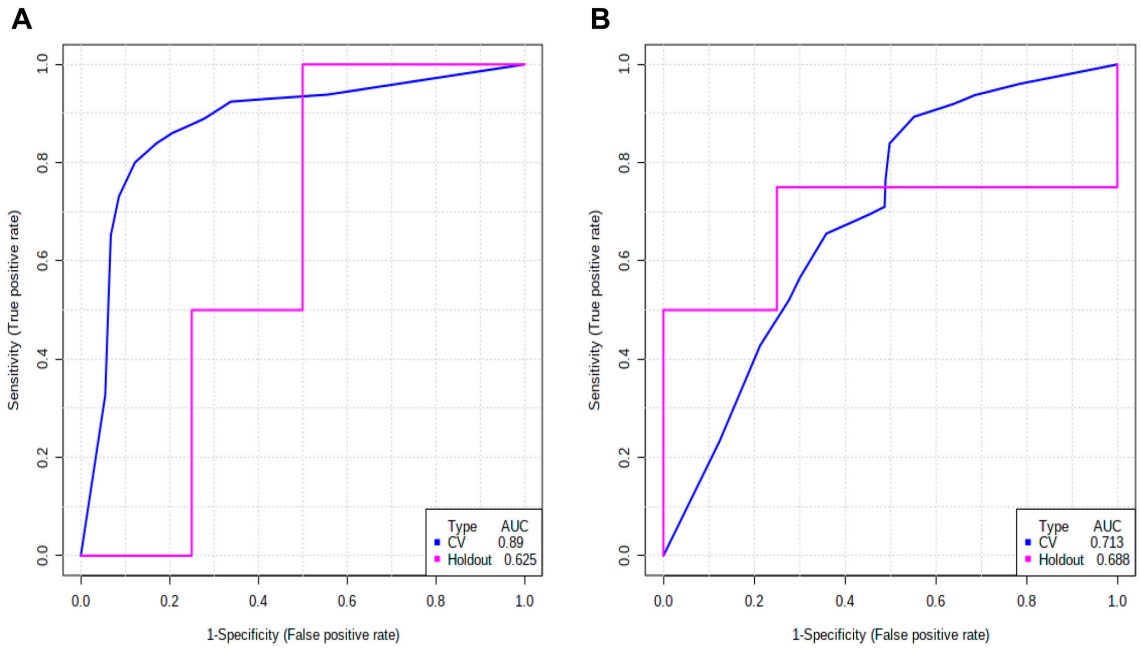

Figure 5. Classification performance of L-octanoylcarnitine for diagnosis (**A**) and prognosis (**B**). In (**A**), the receiver operating characteristic (ROC) curve shows AUC = 0.89 for training/test and ROC AUC = 0.625 for validation (holdout). (**B**) For prognostic classification, it shows the receiver operating characteristic (ROC) curve AUC = 0.713 for training/test and ROC AUC = 0.688 for validation (holdout).

The two-way ANOVA analysis identified seven significant variables for classification by diagnosis (PS (40:6), PS (16:0/16:0), Cer (d36:1), PG (O-32:0), prostaglandin E2, AS 1-5, dehydroepiandrosterone sulfate), two for classification by prognosis (arachidonic acid, docosahexaenoic acid) and two relevant for both classifications: L-octanoylcarnitine and FAHFA 36:4. Figure 6A shows a heatmap of clustered intensities of these lipids for samples grouped by diagnosis and subgrouped by prognosis. Figure 6B plots the differences in intensity by each group for the lipids found to be important for both categories.

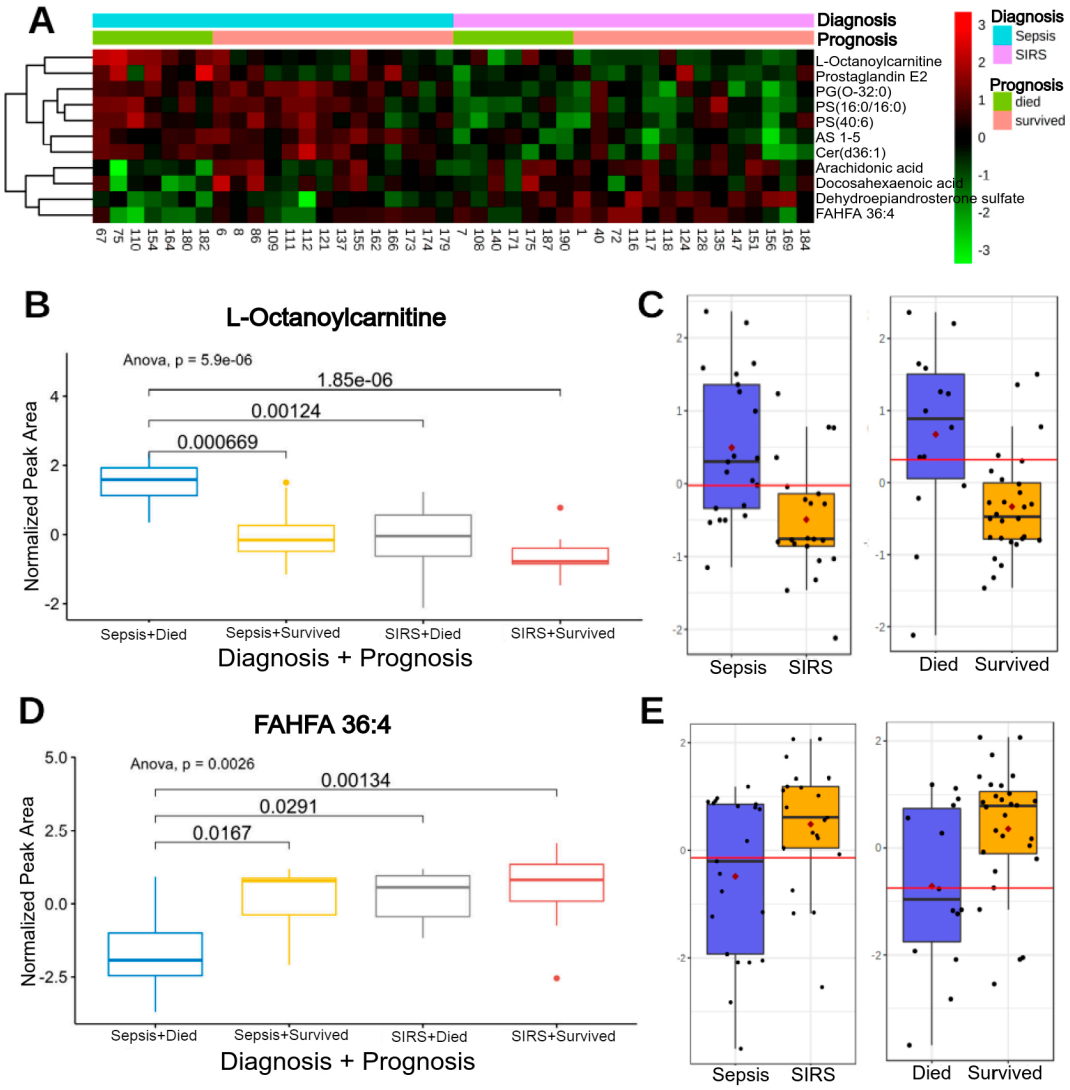

Figure 6. (**A**) Heatmap of standardized intensities of significant lipids obtained by two-way ANOVA. The upper bars indicate the group to which the samples belong (columns). Lipid clusters on the left side. (**B**,**D**) Boxplot of the 2 most relevant lipids. Boxplot of subgroups (diagnosis + prognosis) with statistical significance values obtained by ANOVA and Tukey's test. Boxplots for diagnosis and prognosis classification for (**C**) L-octanoylcarnintie and (**E**) FAHFA 36:4.

3. Discussion

Sepsis, one of the major causes of death in the world, is a serious medical condition associated with high incidence and mortality rates [25]. The discovery of differentiators of patients with a high chance of poor outcome should optimize the selection of better treatment strategies. Similarly, early discrimination between sepsis and some other similar clinical condition, such as SIRS, would make better decision making possible by preventing the progression of the disease, even before organ dysfunction. Here, we have shown that the differences in the lipidomes of patients diagnosed with sepsis or SIRS are relevant for the patients' prognoses. Although differences in the prognosis of patients with sepsis or SIRS, detected either by different omic or clinical approaches [1,17,21,26], have been previously reported; in the present study, we report the co-occurrence of variations in lipid abundance with diagnostic and prognostic potential.

Some species of glycosphingolipids (AS 1–5), glycerophospholipids (PS (16:0/16:0), PS (40:6), PG (O-32:0), PG (O-32:0)), N-acylsphingosines (Cer (d16:1/18:0), Cer (d36:1)), prostaglandins (prostaglandin E2), lineolic acids (13S-hydroxyoctadecadienoic acid), phosphatidylcholine (PC (44:7)) and acylcarnitines (L-octanoylcarnitine, L-palmitoylcarnitine) were more abundant in the sepsis diagnosed group when compared with the SIRS group, while some species of sulfated steroid (dehydroepiandrosterone sulfate,), fatty acid esters of hydroxy fatty acids (FAHFA 36:4), were more abundant in cases with SIRS when compared with the sepsis cases. When comparing the groups of survivor and non-survivor patients, no univariate adjusted statistical difference (false discovery rate (FDR) $p < 0.05$) was found, but multivariate relevance was found, especially in L-octanoylcarnitine (more abundant in non-survivor patients) and FAHFA 36:4 (more abundant in survivor patients). In addition, the last two compounds present interaction between the two groups, with abundances codependent on prognosis and diagnosis.

Glycosphingolipids (GSLs) are a subclass of sphingolipids with glycans exposed to the extracellular space. These lipids are abundant components of the cell membrane [27]. GSLs are related to many biological processes including infections by specific pathogens as binding receptors at the surface of host cells [28]. GSLs play a role in immune cell function as a signal transducer (i.e., toxins or IgM antibodies) or in binding lipid rafts to trigger chemotaxis, phagocytosis and phagolysosome formation [28] and are involved in regulatory aspects of T cell biology [29]. Some clinical uses for GSLs, such as lipid-rafts for signaling the presence of pathogens, and pharmacological reduction of GSL are being actively studied [30]. However, so far, there have been no studies that describe or associate AS 1-5, a glycosylated N-acylsphingosine, with immune response, inflammation or infection so far.

Glycerophospholipids or phosphoglycerides are lipids with hydrophobic regions composed of two fatty acids linked to glycerol. Sphingolipids are lipids with a single fatty acid linked to a fatty amine, sphingosine. Both lipids are the main components of biological membranes. A wide variety of these compounds have been reported as differentials in assessing septic mortality [21] or in differentiating stages of sepsis and SIRS [20]. These compounds present an increase in abundance related to the severity of sepsis, being more abundant in septic shock and non-differential in non-infectious SIRS [31]. A confounding factor when analyzing these compounds is the variability of their abundance, sometimes decreased in sepsis, depending on the type and focus of infection (i.e., decrease in lysophosphatidylcholines in community-acquired pneumonia) [32]. This high variability has made its biological interpretation difficult. Interestingly, the compounds of this class identified in our study have a higher mean abundance in sepsis, although with weak univariate statistics but relevance in multivariate differentiation. These compounds are largely associated with lipid peroxidation, whose products may have pro-inflammatory and protective activity against infection [33].

Ceramides play essential roles in cell signaling and contrasting roles within cellular metabolism. Ceramide is involved in cellular responses related to stress, autophagy and apoptosis, whereas S1P, another bioactive lipid of the sphingolipid pathway, stimulates cell survival, proliferation and tissue regeneration [34]. However, it is necessary for further investigation to understand the effect of different lengths of acyl chains on this lipid class. Again, sphingolipids participate in the regulation of the phagosome/lysosome fusion, apoptosis or the inflammatory response [35], facilitating bacterial destruction.

Higher average importance for multivariate model and univariate significance of L-octanoylcarnitine and L-palmitoylcarnitine were found in the sepsis group and just low average importance for gamma-linolenyl carnitine and linoleyl carnitine for the same model. The quaternary ammonium compound carnitine and its acyl esters (acylcarnitines) are essential for the oxidative catabolism of fatty acids and thence for maintaining energy homeostasis in the human body. Downregulation of fatty acid oxidation is evidenced by an increased presence of acylcarnitines in plasma [36]. Their accumulation in the plasma is marked in sepsis non-survivors, indicating a possible mitochondrial dysfunction in energy production. Moreover, it was reported that non-survivor septic patients have mitochondrial dysfunction leading to deficient aerobic catabolism [37] and consequently

elevated plasma concentrations of TCA cycle metabolites. Unused acylcarnitines are reversely transported to the cytoplasm and then into the plasma [38]. Levels of these lipids were found to be lower in SIRS and survivor patients, as reported by other studies [20]. In the present study, we looked for a particular abundance profile for prognostic and diagnostic classifications: L-octanoylcarnitine presented the highest abundance in non-survivor sepsis patients when compared to survivor SIRS patients (lowest abundance), non-survivor SIRS and survivor sepsis patients. Its importance was evaluated by univariate and multivariate prediction methods (Figure 6), with good predictive performance for both diagnoses and prognoses (Figure 5, which identifies it as a possible lipid signature). This compound is the physiologically active form of octanoylcarnitine, an intermediate fatty acid b-oxidation byproduct. In addition to indicating increased lipid oxidation, L-octanoylcarnitine may indicate increased lipid input [39]. A recent study identified low levels of L-octanoylcarnitine as a biomarker of breast cancer (100% positive predictive value) against samples from healthy individuals, in addition to presenting different levels depending on the size of the tumor, as well as high abundance in tumors with high expression of estrogen and progesterone receptors [40]. This may be related to the high metabolic demand of the tumor. Another study on prostate cancer showed a positive relationship between L-octanoylcarnitine levels and the risk of cancer progression in primary and metastatic samples [41]. There is currently no information that relates this acylcarnitine to sepsis, SIRS or the prognosis of these cases. However, a larger, stratified study covering a wider range of compounds (metabolites and proteins) is needed to infer the biological basis of their variable abundance in the cases presented here.

FAHFA 36:4 is a compound that presented a different pattern to those mentioned above. This fatty acid ester of hydroxy fatty acid was found to be more abundant in samples of surviving patients with SIRS when compared to non-survivors with sepsis (less abundant), survivors with sepsis and non-survivors with SIRS. These lipids are endogenous products present in food and mammalian tissues. To date, more than 16 FAHFA families have been determined. Structurally, each family has different fatty acid and hydroxy fatty acid compositions and multiple isomers by the ester bond position. These compounds have anti-inflammatory and anti-diabetic effects [42]. Although it is not known how they perform their biological activity, recent studies link FAHFA to erythroid nuclear factor 2-related factor 2 (Nrf2) [43]. Their presence is related to resolution or regulation of inflammation, including providing protection against potential infection [44]. Therefore, it is not clear whether the low abundance in patients with sepsis and in non-survivors is a depletion or a result of some altered pathway. No studies have been published that relate FAHFA to the progression and outcome of patients with sepsis or SIRS.

In conclusion, this lipidomics study carried out on plasma taken from male patients with sepsis or SIRS assessed relevant lipids for diagnosing. Then, identified lipids from the previous step were assessed as prognostic signatures. Finally, one relevant lipid, L-octanoylcarnitine, was found to be a promising signature for diagnosis and prognosis. Quantification studies of all relevant metabolites highlighted by this study and their physiological and altered levels in human plasma seem to be an interesting matter for further investigation.

4. Materials and Methods

4.1. Study Groups

The study samples came from the Universidade São Francisco (USF) Hospital, Bragança Paulista, São Paulo, Brazil. Male patients admitted to the ICU were evaluated. The project was approved by the Research Ethics Committee of the Universidade São Francisco (CAAE:51356315.5.0000.5514) and was developed at the Intensive Care Unit of Universidade São Francisco Hospital. The following inclusion criteria were adopted for the group of critically ill patients: individuals from 15 to 90 years of age admitted to the intensive care unit, either clinical or surgical, in the period. Female patients and patients receiving special diets were not included in the study to avoid gender-related and diet-related lipidomic profile bias [45]. Following SIRS definition criteria [46], 21 male patients with 2 or more

signs of SIRS and no suspected or confirmed infection were selected for inclusion in the SIRS group. Patients with organ dysfunction and confirmed infection were selected for inclusion in the sepsis group. Clinical data were collected, including severity score (SAPS III and SOFA on the first day of hospitalization). Clinical and demographic data are provided in Table 1. Additionally, a logistic multiple linear regression model was implemented to evaluate the influence of non-lipidomic variables on the classification (diagnostic) variable.

4.2. Sample Collection, Preparation and LC-MS/MS Analysis

Blood samples were collected for daily laboratory monitoring of critically ill patients and aliquots of this material from the first 36 h of hospitalization were used to carry out the analyses of the present study. Labeled ethylenediamine tetraacetic acid (EDTA) blood samples were sent to the Multidisciplinary Research Laboratory of the USF, where the lipidomic analyses were performed. Centrifuged plasma samples were frozen at −80 °C. A mixture of samples from both groups was used as quality control (QC). This pooled sample was divided and extracted along with the remaining samples. $CHCl_3$:MeOH solution (2:1, v/v) was used for extraction with 150 mL of plasma sample. Extracted samples were then vortexed for 30 s and centrifuged at 12,000× RPM for 5 min at 4 °C. The bottom organic layer (450 mL) was collected. Nitrogen-dried samples were stored at −20 °C to await analysis. A solution of isopropanol (IPA)/acetonitrile (ACN)/water (2:1:1, v/v/v) was used to reconstitute samples before analysis.

4.2.1. LC-MS Analysis

Following a method previously published by our group [47], untargeted LC-MS analysis was performed using an ACQUITY UPLC coupled to a XEVO-G2XS QTOF mass spectrometer (Waters, Manchester, UK). Liquid chromatography was performed using an Acquity UPLC CSHC18 column (2.1 × 100 mm, 1.7 mm, Waters). The volume of injection was 1 mL. MS^E mode was used to separately record positive and negative ion modes in the range of 50–2000 *m/z*. The injection order was randomly defined and QC samples were analyzed after every ten injections.

4.2.2. Data Acquisition and Preprocessing

The peak alignment, deconvolution, selection of possible adducts and compound annotation based on MS^E experiments were obtained using Progenesis QI 2.0 software (Nonlinear Dynamics, Newcastle, UK). Search parameters for putative annotation were precursor mass error 5 ppm and fragment tolerance 10 ppm. At this stage, putative identification using LIPID MAPS [48] database and the Human Metabolome Database (HMDB) [49] was defined by fragmentation score, mass accuracy and isotope similarity. Annotation of compounds was classified in accordance with the Metabolomics Standards Initiative (MSI) [50], where ions with some level of match with MS/MS database reached level 2 while compounds putatively identified by exact mass, using the mummichog algorithm, reached level 3. Progenesis QI generated a table of ion intensity by sample and ions. Ions were labeled according to their retention time and mass-to-charge (*m/z*) ratio. Preprocessed data are available as Supplementary Materials files: Spreadsheet_1 Sepsis SIRS negative mode, for negative mode; Spreadsheet_2 Sepsis SIRS positive mode for positive mode.

4.3. Statistical Analysis

MetaboAnalystR 3.0 [51], statTarget2 [52] and Bioconductor package manager using R programming language [53], were used to perform statistical analyses. Quality control based signal correction was performed using random forest implementation (QC-RFSC) [54]. According to the "80% rule" [55], peaks present in more than 80% of the samples of each group were kept for further analysis. The K-nearest neighbor algorithm was used to impute the remaining missing values. Further data filtering removed variables with low variance based on the interquartile range (IQR) [56]. Then, the corrected data were log-transformed and normalized using the Pareto scale [57].

4.3.1. Exploratory Analysis

For univariate descriptive analyses, a volcano plot was used to represent features with FDR-adjusted p-values < 0.05 using t-test and 2-fold intensity between groups for each m/z. Principal component analysis (PCA) was used to distinguish sample cluster distribution in the first two principal components. A heatmap and unsupervised hierarchical clustering of 50 features with the lowest adjusted p-value < 0.05 depicts differential peaks.

4.3.2. Analysis of Biomarkers for Diagnosis

The biomarker analysis module implemented in the MetaboanalystR package was used on the MS peak intensities table for all the samples for detecting relevant features for diagnostic classification. The random forest method, a classification ensemble algorithm, was used for classification and feature selection models. To construct ROC curves, balanced sub-sampling and Monte Carlo cross-validation (MCCV) with two thirds (2/3) of the samples for training were used to evaluate feature importance. The test subgroup (1/3 of samples) was used to build a classification model for top n (1 to 100) important features. The performance and confidence interval of each model were calculated, repeating the procedure multiple times. The RF model produces a reduced list of features ranked by value of importance. All the features obtained here were then used in the annotation stage.

4.3.3. Putative Identification of Lipids and Metabolomics Pathway Analysis

In addition to the putative identification using Progenesis QI described above, the mummichog V2 algorithm [58] was used for MS peaks, without prior annotation. This method identifies lipids based on mass-to-charge ratios (m/z), p-values, fold change, retention time and mixed analytical mode (positive and negative ions), which were used to interrogate the KEGG library. Molecular weight tolerance at 5 ppm and a customized adduct list were used. Only lipidic matched compounds with registered LipidMaps entries were kept. A final manually curated list of identified lipids was obtained using Progenesis QI putative identification and the mummichog-identified lipid list. Using the identified compound list, metabolomics pathway analysis (MetPA) was used to identify biological pathway impact associated with the differences between study groups.

4.3.4. Performance Evaluation of Diagnostic Biomarkers Used for Prognostic Prediction

To assess whether the lipids identified as diagnostic biomarkers could also be predictive for prognostic classification, these lipids were used to build a random forest predictive model for the prognosis. The most relevant lipid was further individually evaluated as a diagnostic and prognostic individual biomarker. For a more stringent evaluation as a possible biomarker, the predictive model tested a subgroup of unlabeled samples. A random forest model was then trained with the labeled subgroup of samples for a single compound, thus alleviating the training bias for which it was initially selected in the diagnostic classification. ANOVA two-way was used for final clustering and visualization of lipid relevant to both diagnosis and prognosis categories.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-1989/10/9/359/s1, Figure S1: PCA with quality controls, Figure S2: Negative mode ROC for diagnosis, Figure S3: Positive mode ROC for diagnosis, Figure S4: ROC for prognosis, Table S1: Ranked lipids for prognosis, Table S2: Pathway impact analysis, Table S3: Logistic linear model for all baseline characteristics, Spreadsheet_1: Sepsis_SIRS negative mode, Spreadsheet_2: Sepsis_SIRS positive mode.

Author Contributions: Conceptualization, P.d.O.C. and F.A.L.M.; methodology, G.C.M., S.S.-V. and A.M.A.P.F., M.C.F.M., G.K.D.d.S. and A.M.P.; validation, G.C.M., S.S.-V. and A.M.A.P.F.; formal analysis, S.S.-V. and G.C.M.; investigation, S.S.-V., A.M.A.P.F. and G.C.M.; resources, P.d.O.C., G.C.M. and A.M.P.; data curation, S.S.-V.; writing—original draft preparation, S.S.-V. and G.C.M.; writing—review and editing, P.d.O.C., A.M.P. and F.A.L.M.; visualization, G.C.M. and S.S.-V.; supervision, P.d.O.C.; project administration, P.d.O.C.; funding acquisition, P.d.O.C. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

SIRS	systemic inflammatory response syndrome
SOFA	sequential organ failure assessment
qSOFA	quick SOFA
APACHE evaluation	acute physiology and chronic health
PCT	procalcitonin
SD	standard deviation
BMI	body mass index
CRP	C-reactive protein
SAPS	simplified acute physiology score
COPD	chronic obstructive pulmonary disease
AP	arterial pressure
AKI	acute kidney injury
mmHg	millimeters of mercury
mg	milligram
dL	deciliter
INR	international normalized ratio
mm3	cubic millimeter
PaO2	partial pressure of oxygen
FiO2	fraction of inspired oxygen
ICU	intensive care unit
UTI	urinary tract infection
USF	Universidade São Francisco
QC	quality control
PCA	principal component analysis
AUC	area under curve
ROC	receiver operating characteristic
RF	random forest
MSI	Metabolomics Standards Initiative
QC-RFSC correction	quality control random forest based signal
IQR	interquartile range
MCCV	Monte Carlo cross-validation
MS	mass spectrometry
HMDB	Human Metabolome Database
PC	phosphatidylcholine
PG	phosphatidylglycerol
ANOVA	analysis of variance
UPLC	ultra performance liquid chromatography
ACN	MS data-independent acquisition
MS^E	n acetonitrile
EDTA	ethylenediamine tetraacetic acid
QTOF	quadrupole time-of-flight mass spectrometry
FAHFA	fatty acid esters of hydroxy fatty acids
TCA	tricarboxylic acid cycle
GSL	glycosphigolipids
LC-MS	liquid chromatography–mass spectrometry

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Supplementary Materials: The following are available online at http://www.mdpi.com/2218-1989/10/9/359/s1

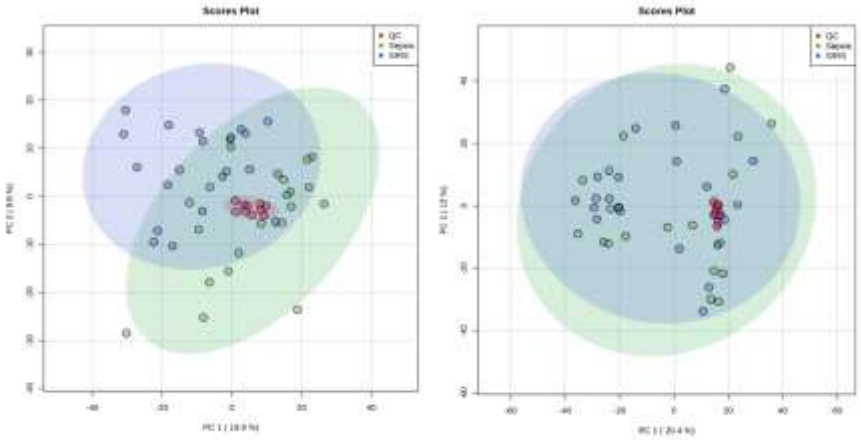

Figure S1: PCA with quality controls. Spreadsheet_1: Sepsis_SIRS negative mode, Spreadsheet_2: Sepsis_SIRS positive mode.

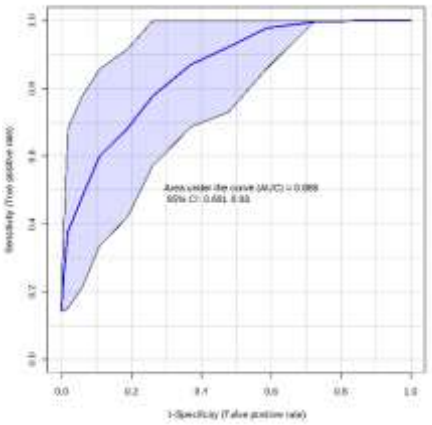

Figure S2: Negative mode ROC for diagnosis

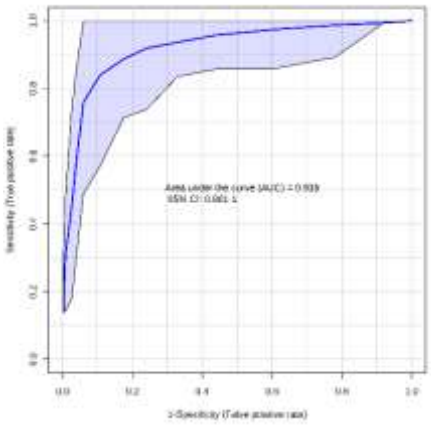

Figure S3: Positive mode ROC for diagnosis

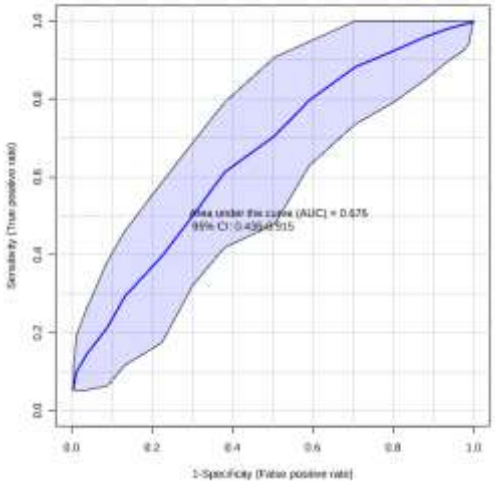

Figure S4: ROC for prognosis

Table S1: Ranked lipids for prognosis

	Rank Freq.	Importance	died	survived
L-Octanoylcarnitine	1	3.513626717	High	Low
2-Methoxyestrone 3-glucuronide	1	1.718238274	High	Low
17-Hydroxyprogesterone	1	1.233574707	Low	High
FAHFA 364	1	1.155257279	High	Low
6-Hydroxyhexanoic acid	1	0.963067176	Low	High
Mevalonic acid	1	0.595508632	High	Low
N-palmitoyl serine	1	0.516400324	High	Low
PGE2 1,15-lactone	1	0.291425156	High	Low
13-L-Hydroperoxylinoleic acid	1	0.234356698	Low	High
Leukotriene F4	1	0.159415145	High	Low
Arachidonic acid	1	0.076876218	High	Low
PS431	1	-0.11000578	Low	High
Dehydroepiandrosterone sulfate	1	-0.11269207	Low	High
AS 1-5	1	-0.12625387	High	Low
Docosahexaenoic acid	1	-0.15022169	High	Low
Glycocholic acid	1	-0.25270521	High	Low
2-Amino-4-oxopentanoic acid	1	-0.26198412	Low	High
LysoPCO-180	1	-0.28352821	High	Low
Linoleyl carnitine	1	-0.30963904	Low	High
Gamma-linolenyl carnitine	1	-0.31811153	High	Low
PGO-320	1	-0.41293186	Low	High
PS406	1	-0.42322095	High	Low
Tetrahydropersin	1	-0.47996744	Low	High
Cerd161/180	1	-0.48994484	High	Low
PS160/160	1	-0.58137871	High	Low
13S-hydroxyoctadecadienoic acid	1	-0.60429275	Low	High
PSO-350	1	-0.64148266	High	Low
L-Palmitoylcarnitine	1	-0.64503568	Low	High
Cerd361	1	-0.68997562	High	Low
S-aminomethyldihydrolipoamide	1	-0.76169645	Low	High
PC 447	1	-0.96686047	Low	High
Prostaglandin E2	1	-1.05037926	High	Low
7-2,4,6-trihydroxy-2,5,5,8a-tetramethyl-decahydronaphthalen-1-ylmethoxy-2H-chromen-2-one	1	-1.13911619	High	Low

Table S2: Pathway impact analysis

	Total	Expected	Hits	Raw p	-log(p)	Holm adjust	FDR	Impact
Linoleic acid metabolism	5	0.051613	1	0.050622	2.983	1	1	0
Biosynthesis of unsaturated fatty acids	36	0.37161	2	0.051323	2.97	1	1	0
Arachidonic acid metabolism	36	0.37161	2	0.051323	2.97	1	1	0.3135
Terpenoid backbone biosynthesis	18	0.18581	1	0.17123	1.765	1	1	0.11429
Ether lipid metabolism	20	0.20645	1	0.18845	1.669	1	1	0.14458
Steroid hormone biosynthesis	85	0.87742	2	0.21753	1.525	1	1	0.09615
Glycerophospholipid metabolism	36	0.37161	1	0.31467	1.156	1	1	0.04701
Fatty acid degradation	39	0.40258	1	0.33618	1.09	1	1	0
Primary bile acid biosynthesis	46	0.47484	1	0.38394	0.957	1	1	0.00805

Table S3: Logistic linear model for all baseline characteristics

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	0.252387	12.59109	0.020045	0.984008
Age	0.044553	0.060754	0.733345	0.463348
BMI	0.095452	0.379431	0.251567	0.801376
SAPSIII	-0.03001	0.104549	-0.28705	0.774074
Risk of death	-0.02466	0.10518	-0.23446	0.814627
SOFA	-0.32056	0.481632	-0.66557	0.505689
Systemic hypertension	-37.2832	26621.91	-0.0014	0.998883
Diabetes mellitus	11.26478	29532.89	0.000381	0.999696
Coronary insufficiency	-40.8265	49717.1	-0.00082	0.999345
COPD	-40.2845	19296.76	-0.00209	0.998334
Neoplasm	-65.1783	38574.23	-0.00169	0.998652
N Organ dysfunction	-23.9739	59078.71	-0.00041	0.999676
AP	26.33647	59078.71	0.000446	0.999644
Lactate	23.68586	59078.71	0.000401	0.99968
AKI	65.78835	83621.61	0.000787	0.999372
Total bilirubin	-15.4047	57284.91	-0.00027	0.999785
INR	-13.9761	62602.3	-0.00022	0.999822
Platelets	41.83436	58239.31	0.000718	0.999427
PaO2/FiO2	41.27298	62623.81	0.000659	0.999474

4. CONCLUSÃO

Conforme exposto nos capítulos anteriores, os autores concluem que:

- ✓ Os pacientes sépticos e com choque séptico possuem importantes alterações no metabolismo lipídico, em especial nos fosfolipídios, esfingolipídios e no metabolismo dos ácidos graxos;
- ✓ Foi possível identificar moléculas lipídicas diferenciais de resposta a um insulto infeccioso em pacientes com sepse tanto em comparação a pacientes saudáveis quanto em pacientes com SIRS;
- ✓ A redução de importantes mediadores anti-inflamatórios, os ácidos graxos poliinsaturados da família *n*-3 e os ésteres de ácidos graxos ramificados de ácidos graxos hidroxilados (FAHFA 36:4) foram evidenciados em pacientes com diagnóstico de sepse;
- ✓ Os resultados apontam para uma elevação nos níveis de derivados de carnitina, moléculas relacionadas a distúrbios da oxidação mitocondrial dos ácidos graxos, em especial a L-octanoilcarnitina identificada como candidato a biomarcador que poderá corroborar no diagnóstico e prognóstico da sepse.

Os autores sugerem a realização de mais estudos prospectivos, respaldados em modelos de estimativa de tamanho amostral e testes de poder estatístico, desenhados para investigar o comportamento destas moléculas frente a uma infecção e de correlacionar com desfechos clínicos importantes. Ainda, a descoberta de um único biomarcador ideal, com alta sensibilidade e especificidade, de fácil acessibilidade e baixo custo envolve padrões rigorosos de validação intra e inter laboratorial afim de estabelecer sua utilidade clínica.

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