Universidade de Aveiro
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Departamento de Química

Helena Beatriz Cinza Santos Leal Ferreira

Estudo de alterações séricas no perfil lipídico de doentes com Lúpus Eritematoso Sistémico

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica Clínica, realizada sob a orientação científica da Drª. Maria do Rosário Gonçalves dos Reis Marques Domingues, Professora Associada com Agregação do Departamento de Química da Universidade de Aveiro, e coorientação da Drª. Ana Margarida Pereira, Técnica Superior de Diagnóstico e Terapêutica, no Serviço de Patologia Clínica do Centro Hospitalar de Baixo Vouga.

O júri

Presidente

Professora Doutora Rita Maria Pinho Ferreira
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Arguente Principal

Professora Doutora Luisa Alejandra Helguero
Professora Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro

Orientador

Professora Doutora Maria do Rosário Gonçalves dos Reis Marques Domingues
Professor Associada com Agregação do Departamento de Química da Universidade de Aveiro

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Palavras-chave Doenças autoimunes; lúpus eritematoso sistémico; lipidómica; espetrometria de massa; perfil lipídico; peroxidação lipídica.

Resumo O lúpus eritematoso sistémico (SLE) é uma das doenças autoimunes mais comuns. O seu desenvolvimento parece estar associado a uma forte predisposição genética, com hiperexpressão constitutiva da atividade dos linfócitos B, por outro lado, também está associado a um aumento do stress oxidativo. A identificação do perfil lipídico típico das doenças autoimunes é um tema que está a emergir, no entanto ainda é pouco explorado. Os pacientes com SLE apresentam alteração do perfil de lipoproteínas, geralmente com diminuição dos níveis de HDL, e aumento dos níveis de VLDL, colesterol total e triglicerídeos. Muito poucos estudos relatam o uso de abordagens lipidómicas no SLE e os resultados aqui reunidos mostram mudanças principalmente no nível de fosfolípidos, com diminuição de algumas espécies de plasmenil, ácidos gordos, com redução de PUFA, e esfingolípidos, com alterações na composição da cadeia acil. Essas alterações podem ser devidas à alteração dos lípidos pela oxidação e aumento dos ROS. Algumas alterações podem estar associadas a alterações na membrana dos linfócitos e à desregulação do sistema imunológico. A análise do perfil lipídico oxidado e não oxidado dos pacientes com SLE revelou a existência de uma considerável variabilidade inter- e intragrupo, o que dificultou a interpretação dos resultados. No entanto, verificou-se que as PE oxidadas atuam como moléculas pró-inflamatórias pois apresentavam menor abundância nos controlos; enquanto que as PS oxidadas estavam diminuídas nos doentes, sendo que estas espécies têm sido associadas a um efeito anti-inflamatório. Os resultados obtidos corroboram o aumento do stress oxidativo no lúpus. Depois de interpretados os resultados, pode-se concluir que o grupo SLE inativo possui um perfil lipídico oxidado distinto devido ao tratamento administrado para o controlo da doença.

Keywords Autoimmune diseases; systemic lupus erythematosus; lipidomics; mass spectrometry; lipidic profile; lipid peroxidation.

Abstract Systemic lupus erythematosus (SLE) is one of the most common autoimmune diseases. Its development seems to be associated with a strong genetic predisposition, with a constitutive hyperexpression of B lymphocytes activity, and it is also associated with an increase in oxidative stress. The identification, using MS techniques, of the lipid profile typical of autoimmune diseases is a topic that is emerging yet far from being completely disclosed. SLE patients have alteration of lipoprotein profile, usually disclosed with lower levels of HDL, and higher levels of VLDL, total cholesterol and triglycerides. Very few studies reported the usage of lipidomic approaches in SLE and results gathered herein showed changes mainly in the level of phospholipids, with decrease of some plasmenyl lipids, fatty acids, with reduction of PUFA, and sphingolipids, with changes in fatty acyl chain composition. These changes can be due to the alteration of lipids by oxidation and increase of ROS. Some alterations can be associated with changes in membrane of lymphocytes and with the deregulation of the immune system. The analysis of the oxidized and non-oxidized lipid profile of SLE patients revealed that there was a considerable inter- and intragroup variability that made difficult the interpretation of the results. Nonetheless, it was verified that oxidized PE act as pro-inflammatory molecules since they showed lower relative abundance in controls; while oxidized PS were decreased in SLE, which have been associated with an anti-inflammatory effect. The results obtained corroborate the increase of oxidative stress in SLE. After careful interpretation of the results, we can conclude that inactive SLE group has a distinct oxidized lipid profile due to the administered treatment for disease management.

Contents

List of Abbreviations

AA	Arachidonic acid
ACN	Acetonitrile
ANA	Antinuclear antibodies
Anti-dsDNA	Anti-double stranded DNA
APC	Antigen-presenting cell
Cer	Ceramides
CL	Cardiolipin
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EPA	Eicosapentaenoic acid
ELISA	Enzyme-linked immunosorbent assay
FA	Fatty acids
FFA	Free fatty acids
GC	Gas chromatography
GlcCer	Glucosylceramides
HDL	High density lipoprotein
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance-liquid chromatography
IIF	Indirect immunofluorescence
LC	Liquid chromatography
LDL	Low density lipoprotein
LPC	Lyso-phosphatidylcholine
LPE	Lyso-phosphatidylethanolamine
LPG	Lyso-phosphatidylglycerol
LPI	Lyso-phosphatidylinositol
LPL	Lyso-phospholipid
LPS	Lyso-phophatidylserine
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
mtDNA	Mitochondrial DNA
NET	Neutrophil extracellular traps

PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PL	Phospholipid
pPC	Plasmenylcholine
pPE	Plasmenylethanolamine
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SLE	Systemic lupus erythematosus
RT	Retention time
SPE	Solid phase extraction
SM	Sphingomyelin
TG	Triglyceride
VLDL	Very low density lipoprotein
XIC	Extracted-ions chromatograms

List of Tables

List of Figures

General Introduction

Part of the text of this chapter was published as follows (Appendices):

H.B. Ferreira, A.M. Pereira, T. Melo, A. Paiva, M.R. Domingues, Lipidomics in autoimmune diseases with main focus on Systemic Lupus Erythematosus, *Journal of Pharmaceutical and Biomedical Analysis* (2019) volume 174, pages 386-395
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1. Autoimmune diseases

Autoimmune diseases are a highly heterogeneous group of disorders that cover a wide range of pathologies. Worldwide, they are known to have an estimated total prevalence of 7.6-9.4%[1]. This type of diseases is extremely debilitating in their acute phases and patients that suffer from autoimmune disorders have their autonomy decreased. These diseases occur when the immune system begins to attack the body itself, recognizing some of the molecules from the own organism, as a foreign and undesirable pathogen[2]. Those "strange" molecules, known as antigens, will induce the natural production of antibodies in plasma[3].

Autoimmune disorders can be classified in two different ways: organ-specific or systemic (Table 1)[4]. In organ-specific autoimmune diseases, the autoimmune response is directed against antigens that are expressed only in a particular organ. Autoantibodies bind to autoantigens in the organ cells and can lead either to their destruction, overstimulation or suppression of the normal cellular function. On the other side, in systemic autoimmune diseases the autoimmune response is directed against autoantigens scattered throughout the organism, which ends into a widespread tissue damage[5].

Table 1. Classification of autoimmune diseases: organ-specific autoimmunity *vs* systemic autoimmunity, and some examples of the most common ones.

Organ-specific autoimmunity	Systemic autoimmunity
Type 1 diabetes	Systemic lupus erythematosus
Autoimmune hepatitis	Sjögren`s syndrome
Hashimoto`s thyroiditis	Rheumatoid arthritis
Goodpasture`s syndrome	Scleroderma
Psoriasis	Systemic Sclerosis
Addison`s disease	Antiphospholipid syndrome
Pernicious anemia	Dermatomyositis
Myasthenia gravis	Vasculitis
Graves` disease	Ankylosing spondylitis
Vitiligo	Polymyositis

Autoimmune disorders are polygenic and each type of autoimmune disease have its distinct clinical phenotype, with specific physiopathology and specific symptoms[6]. Each type of autoimmune disease has a different development and the prognostic is also dissimilar[6]. A characteristic feature of autoimmune diseases is that they have relapse and remission periods[7]. A relapse, or flare, is an acute reactivation of the disease, manifested by the worsening of pre-existing symptoms or the development of new ones. Flare periods occur when the disease activity is enhanced and can manifest themselves in a variety of forms. Usually, treatment during flare episodes requires specific therapy to attenuate symptoms. Meanwhile, remission is a period of inactivity of the disease. There are cases that the disease become permanently inactive, which means, in total remission, however this disease stage is rarely achieved. On the other side, partial remission is more frequent[8]. Relapse periods can occur for no apparent reason. In order to avoid flares, it is vital for the patient to adopt preventive measures. It is advisable to make routine visits to clinicians to reassess the symptoms and the signs, as well as the analysis and examinations that have been carried out. This way the treatment can be readjusted whenever it is necessary. The patient should also have other precautions such as: avoid stress, rest properly and avoid exaggerated physical activity[7]. The administration of the right treatment and adoption of adequate preventive measures are very important so that the patient can eventually achieve remission stage. However, there is a lack of specific diagnostic tools or biomarkers that allow the prediction of the relapse periods. To prevent the appearance of these highly debilitating periods of disease, new studies are needed to understand the mechanism of disease and to find new biomarkers. This should allow tailoring early diagnosis of flare episodes and promote most effective treatment to each patient with a chronic autoimmune disease.

The diagnosis of autoimmune diseases is based on clinical and laboratory data, including serologic tests, the symptoms reported by the patient and the signs observed by the clinician[7]. However, the diagnosis is not always easy to obtain due to insufficient clinical data to ensure a positive diagnosis. Under these circumstances, only the unfolding of the clinical situation will confirm if the autoimmune disease is being responsible for the signs and symptoms of the patient. Moreover, autoimmune diseases have diverse forms of manifesting themselves, which also increases the difficulty of the diagnosis[7,9].

The autoimmune diseases` pathogenesis is not a straightforward process, it is the contribution of several factors (environmental, genetic and hormonal), yet they all work together to disrupt the normal tolerance to the system's own antigens[9]. However, there are certain mechanisms that are common to every autoimmune disease such as: the recognition of the autoantigen as a foreign body (disease initiation), enhanced production of antibodies, amplification of the disease by including multiple pathways of the immune response, chronic inflammation and tissue destruction[6]. Due to the high complexity of autoimmune diseases, this work will only focus on one of the most common autoimmune diseases, systemic lupus erythematosus (SLE).

2. Systemic lupus erythematosus

SLE is a chronic, systemic autoimmune disease and it is characterized by systemic inflammation in multiple organs such as joints, vessels, skin, kidneys and central nervous system[10]. A particular characteristic of SLE is that pathogenic autoantibodies are produced by dysfunctional immunocompetent cells, leading to multiple organ injuries[10,11]. It is most prevalent in women in childbearing age with a very strong 9:1 female to male ratio and the major clinical features of this disease are fatigue and musculoskeletal symptoms[12,13]. Clinical manifestations and the prognostic of the disease are influenced by age of onset and gender[14]. The male gender is often related with higher levels of disease activity, regardless their age or race, at the time of diagnosis. However, during disease course, gender does not seem to dramatically influence the clinical manifestations[15]. The death rate is also different according to the gender, female SLE patients have a lower death rate than men. Patients` age is also important for the cause of death, for patients with ages between 20 and 39 years, the most common cause of death is musculoskeletal and comorbidities that develop as a consequence of SLE, while patients with more than 40 years of age commonly die due to any type of cardiovascular diseases or malignancy[16].

Diagnosis of SLE is based on the criteria established by the American College of Rheumatology and besides the immuno-pathological features, there is also a clinical laboratory profile that suggests that the patient suffers from SLE (Table 2)[8,17]. It comprises mainly full blood cell count (with a decrease of red blood cells, usually associated with anaemia, and/or white blood cells and/or platelets), inflammation parameters (such as increase of C-reactive protein and erythrocyte sedimentation rate) and immunological

changes (as the presence of some antibodies, mainly IgG antinuclear antibodies)[8]. These changes in laboratory parameters are not detected in the same patient at the same time, it is frequent that only a few alterations of these parameters are seen at a particular time of the disease. Also, the laboratory changes can vary from patient to patient[7,8]. In fact, the diagnosis of SLE is somehow difficult in many cases, therefore the interpretation of the laboratory results should be made carefully and only by the attending physician that is aware of the patient`s medical record.

Table 2. Changes in laboratory profile that are most common in SLE disease.

Full blood count	Inflammation parameters	Immunology
↓ Red blood cells (haemolytic anaemia) ↓ White blood cells (lymphopenia) ↓ Platelets (thrombocytopenia)	↑ Erythrocyte sedimentation rate ↑ C-reactive protein	↓ Complement components C3, C4 and CH50 **Presence of**: Antinuclear antibodies Anti-DNA and anti-Sm antibodies Anti-SS-A and anti-SS-B antibodies Anti-RNP antibodies Antiphospholipid antibodies

The development of SLE seems to have a strong genetic predisposition to a dysregulation of the immune system. Hyperexpression of B lymphocyte activity is therefore a consequence of SLE[9,18]. This autoimmune disorder is thus also associated with a polyclonal hypergammaglobulinemia and with elevated titters of antibodies against several auto-antigens, in particular, nuclear antigens. Nonetheless, there are also other factors that contribute to trigger this disease, namely ultraviolet light exposure, cross reactivity with infectious agents, drug hypersensitivities and several stress stimuli[19–21]. Exposure to ultraviolet light, specially UVB, can exacerbate SLE development since it induces apoptosis in keratinocytes and alterations of DNA chemistry and/or location. Sunlight exposition also promotes the production of pro-inflammatory molecules that combined with the cell death mechanisms describe the cutaneous and flare reactions typical of SLE[22]. Infectious agents, such as Epstein-Barr virus (EBV), can also influence the development of SLE and induce flare periods, triggering the disease by molecular

mimicry. It is true that EBV can prompt autoimmune processes, however, SLE patients have an abnormal viral latency period and a dysregulated anti-EBV response[23–25]. All these factors will enable T lymphocytes to recognize the antigen-presenting cell (APC)[26]. T lymphocyte receptor binds to the major histocompatibility complex that is on the surface of the APC. This process is known as antigen presentation and promotes the release of cytokines, inflammation process and B lymphocyte stimulation. B lymphocyte stimulation leads to the hyperproduction of autoantibodies from immunoglobulin G, that are responsible for the tissue damage that is characteristic of SLE[26,27]. These IgG antinuclear antibodies (ANA) that are elevated in SLE target cellular nuclear components. There are several types of ANA and their identification is of utterly importance in SLE diagnosis. In SLE, the most commonly found ANA are anti-native (double stranded) DNA antibodies, although they are not specific of this disease[28].

Genetic factors are one of the major effectors of SLE susceptibility. The development of specific autoantibodies and SLE`s clinical features are genetically encoded, thus the determination of the specific genes that directly contribute to this disease would be a scientific breakthrough that would widely improve the knowledge of this autoimmune disorder. This topic is being intensely investigated (to further clarify this matter please check review article[14]). The development of SLE is also aggravated by epigenetic changes, namely the DNA methylation that is implicated in the pathogenesis of this disorder, although there is not much information regarding hydroxymethylation in this process[14].

Metabolic alterations are also found in patients with SLE, there is evidence that plasma, sera and urine of SLE patients have higher levels of reactive oxygen species (ROS) as well as 8-hydroxy-2`-deoxyguanosine, an oxidative DNA damage biomarker[29,30]. Also, first and second line of defences against mitochondrial ROS, or from other intracellular sources, are found to be decreased in SLE patients[30,31]. First line of cellular antioxidant system (manganese superoxide dismutase and copper/zinc superoxide dismutase), as well as second line of antioxidants enzymes (catalase, glutathione peroxidase 1 and glutathione peroxidase 4), are significantly lower in plasma/serum and in neutrophils/lymphocytes/leukocyte in SLE[30,31]. The disease activity and extent of organ damage are related with glutathione and the ratio of glutathione/oxidized glutathione, which are found to be downregulated in patients with SLE[31,32]. The glucose metabolism is

also altered in lupus, in particular, it was found that leukocytes have lower levels of pyruvate dehydrogenase mRNA and of transcripts of mtDNA-encoded peptides and mitochondrial transcription factor A, that are fundamental for electron-chemical transport, oxidative phosphorylation and mtDNA replication[30]. It was likewise shown that the Krebs cycle in SLE patients is reduced, as well as the levels of the enzymes hexose kinase, glucose phosphate isomerase, phosphofructokinase and glyceraldehyde 3-phosphate dehydrogenase[30,33]. The alteration of DNA and glucose metabolism contribute to an inefficient immune system leading to the development of SLE.

In spite of the difficulties in diagnostics and prediction of relapsing periods, the survival rate of SLE patients has significantly improved over the last decades owing to some progress in early diagnosis. The detection of specific antibodies and the development of more efficient treatment strategies, for both disease and its comorbidities, revealed to be important for the improvement of SLE patients` quality of life. The detection of anti-double stranded DNA (anti-dsDNA) antibodies, ANA and complement activation are used to support either the diagnosis or the evaluation of disease activity of SLE[34]. Disease activity has a direct impact on the complications and associated comorbidities, depending whether it is more or less active. Side effects of immunosuppressant drugs are also a key factor for the development of complications in these patients. In fact, infections are one of the most frequent comorbidities in SLE patients and are responsible for a major contribution to the morbidity and mortality rate of this disorder[14]. People suffering from SLE showed higher probability to have infections of the nervous system and inflammatory bowel diseases. Moreover, they have a substantial higher risk of developing cardiovascular complications (such as coronary heart disease and accelerated atherosclerosis) that may lead to the patient's death. Usually, the peak of occurrence of cardiovascular disease in SLE is observed 7 to 10 years after the diagnosis of the autoimmune disorder[35]. In the next chapter, we will detail the most common diagnostic tools and treatment used nowadays for SLE.

2.1 Diagnosis and Treatment

As reported above, nowadays, SLE diagnostic is based on the assessment of the clinical symptoms and signals evaluated by the physician, and by laboratory tests. The laboratory tests more specific of SLE rely on the detection of specific antibodies, namely the detection of anti-dsDNA antibodies and ANA. Complement components activation are

used to support either the diagnosis or the evaluation of disease activity in SLE[34]. These laboratory tests are made to help clinicians better understand the patients' health status and provide an accurate diagnosis, prognosis and treatment. It is of utmost importance to highlight the fact that inadequate use of such tests can lead to misdiagnosis, inappropriate therapy and needless health-care expenses[36]. The presence of specific autoantibodies is a key parameter for the diagnosis of autoimmune diseases like SLE[37]. Throughout the years, several diagnostic techniques have been developed for the detection of these specific antibodies, always bearing in mind the improvement of the specificity and sensitivity of the analysis. The main techniques used are immunofluorescence, ELISA and recombinant autoantigen technology.

Immunofluorescence

The indirect immunofluorescence (IIF) technique has had beneficial and important implications as a diagnostic method for autoimmune diseases[38]. In IIF, the antigens that have been synthetically produced are recognized by autoantibodies from patients' sera creating specific binding patterns therefore used for diagnostic purposes. "The presence of several antigenic targets on the tissue section results in an excellent overall sensitivity"[2]. Nevertheless, the low specificity of this technique may lead to misinterpretation, which demonstrates the limitations of the IIF for screening purposes. Thus, this technique is being increasingly replaced by enzymatic immunoassays, such as ELISA, that uses antigens defined *à priori*[2]. Nonetheless, IIF is still the most used technique. SLE patients in general have circulating ANA which are primarily detected by IIF. The target for these assays can be either Hep2 cell line or rat liver cryostat sections[4]. However, ANA are not SLE specific, they can also be found in other autoimmune diseases such as Sjögren`s syndrome, rheumatoid arthritis, scleroderma or polymyositis, therefore, a positive test for ANA does not necessarily mean that the patient suffers from SLE. In this way, more diagnostic tests must be performed to confirm the diagnosis.

ELISA

Enzyme-linked immunosorbent assay (ELISA) is a technique that is being more and more accepted in the world of laboratory diagnosis thanks to its high sensitivity and rapid sample throughput[2]. It requires the immobilization of autoantigens on a solid surface so that they are able to interact with autoantibodies through an enzymatic reaction. The quality of the autoantigens used in ELISA will have direct influence not only on the quality

of the screening assays but also on the individual specificity of the technique[2]. Hereby, one major limitation of ELISA`s, with respect to reproducibility of the results and purity, is the autoantigens isolation from natural sources such as human tissues. Anti-dsDNA is a highly specific antinuclear antibody for SLE that is detected using ELISA assays. The quantity of ds-DNA antibodies changes in accordance with disease activity and their measurement has a low sensitivity level, this way, it would be easier to measure changes in blood elements that are targeted by other autoantibodies in order to evaluate disease activity[9].

Recombinant autoantigens

Genetic engineering methods made possible the production of recombinant autoantigens from cloned genes, which allows their applicability in antigen-specific immunotherapy as well as for the analysis of the interaction between antibody and antigen. There are several strategies in order to obtain the complementary DNA encoding for autoantigens[2]. The system that will express the gene of interest, therefore producing the recombinant autoantigen chosen, is dependent on the physico-chemical characteristics of the autoantigen and the purpose for which the recombinant protein is used[39–42]. There are three main prerequisites that this technique should attend to if autoantigens are desired to be used in high quality immunoassays: purity, authenticity and reproducibility[43,44]. Perhaps the most important limitation of the recombinant autoantigen technique is that protein expression in foreign cells can create problems due to instability, insolubility and toxicity of the recombinant product to the host cell[2].

The laboratory techniques described have their own advantages and disadvantages, IIF is being replaced by enzymatic immunoassays, ELISA`s requires autoantigens to have extremely high purity and the technology of recombinant autoantigens is highly demanding. On the other side, recombinant autoantigens are widely used for the diagnosis of autoimmune diseases since it brought major developments onto diagnostic techniques. The same progress is needed to be made with autoantibodies used as controls in diagnostic tests. However, considering that autoimmune diseases are characterized by remission (asymptomatic periods) and relapsing periods (with acute symptoms), that occur with different degrees of severity, more reliable biomarkers are needed to predict, or early diagnose, these periods and to evaluate their degree of severity. They are also important to

avoid disease related damage of affected organs, to decrease associated morbidity and to help evaluating treatment efficiency.

Nowadays the SLE severity and efficacy of treatment is assessed based on the SLE Disease Activity Index (SLEDAI)[8,17]. The SLEDAI evaluates the symptoms of the central nervous and musculoskeletal systems; urinary abnormalities as casts, haematuria, proteinuria and pyuria; mucous membrane involvement; skin changes; serositis such as pleurisy and pericarditis; increased DNA binding; hematologic involvement; low complement levels and fever[8]. SLEDAI is comprised of 24 items according to 9 organ systems, and the index is calculated by assigning a weight to an item that is "present" and summing across all items. Weights are higher for the involvement of an organ system that is more life threatening[17]. Relapsing or flare periods have a SLEDAI score ≥6. A SLEDAI result ≥6 is considered active disease. Remission is considered when the SLEDAI score is less than 6 and that the result corresponds to a 50% decrease when compared to the SLEDAI flare score[17]. It would be fundamental to have clinical diagnostic tests and to identify biomarkers that allow the prediction of these stages, in special the phases of relapsing.

Treatment

SLE treatment is based on a combination of corticosteroids and immunosuppressants, such as methotrexate, cyclophosphamids and cyclosporine. Still, besides their primary function, these drugs have dangerous side effects that should also be monitored, for instance, supressing the ability of the immune system to fight infection and malignancy[6,45]. Hydroxychloroquine is an antimalarial that can also be applied as an SLE therapy and mycophenylate mofetil is being studied for the potential immunosuppressive replacement of cyclophosphamide for the treatment of nephritis[46].

Immunodepletion and immunotherapy are other therapeutic approaches for autoimmune diseases. Immunodepletion uses immunoadsorption columns coupled to various ligands, removing from circulation pathogenic immune complexes[47]. A C1q-protein purified from human blood was used as a ligand for a new immunoadsorption column for treatment of SLE, the results of the study were promising[48]. However, both immunodepletion and immunotherapy are in their early stages and need more research.

Measuring both inflammatory and immunological markers would be beneficial to choose the best course of treatment[9]. Overall, a universal goal of autoimmune diseases`

treatment should be to create therapeutic strategies that induce remission stages without the detrimental side effects.

2.2 The role of lipids in SLE pathogenesis

Autoimmune diseases are strongly influenced by gene mutations and the immune system will be affected as a consequence. SLE pathogenesis is not only associated with genetic dysregulations but it has also been associated with alteration in lipid metabolism including lipid oxidation. In fact, lupus has been strongly associated with oxidative damage in different levels due to the increase of oxidative stress conditions, that can be correlated with mitochondrial disfunction and cell death. Enhanced neutrophil death called NETosis is a characteristic event of lupus pathogenesis. Also, oxidative stress and lipid alteration and oxidation have been related with T lymphocyte dysfunctions, as well as to the systemic inflammation. In all these events, lipids seem to play a fundamental role in their regulation.

2.2.1 Oxidative stress and lipids in SLE

Since autoimmune diseases have an effect on the innate immunity, response neutrophils, as they are the first line of defence of the immune system, will therefore be affected as well[49]. To eliminate pathogens, neutrophils produce superoxide anion, which is a precursor of several types of ROS. It has been reported that neutrophils in SLE are malfunctioning, which is associated with the increase of ROS production that is implicated in the pathogenesis of this disease [49–51]. In this way, uncontrolled or chronic production of ROS by neutrophils may lead to severe oxidative damage in several biomolecules, in particular in lipids of the membranes[52,53]. Also, a very important finding was the fact that ROS production by neutrophils decreased in patients in relapsing phases when compared to patients in remission[49]. This reduction has also been seen in other autoimmune diseases such as multiple sclerosis, Behcet`s disease and Guillain-Barre syndrome[54–56], supporting the importance of neutrophils` dysfunction in autoimmune diseases. A possible explanation for this reduction could be the exhausted status of neutrophils due to overproduction of ROS during active phase of the disease[51]. Another noteworthy aspect was the significant decrease of malondialdehyde levels, a lipid peroxidation product, in neutrophils of patients with lupus[49]. However, within patients, malondialdehyde levels were higher in patients in relapsing than in patients in remission stages, which is indicative of higher levels of

oxidative stress during aggressive periods of this disease[49].

Once SLE is a multifactorial disorder, lipids and the lipid metabolism have been correlated with this autoimmune disease in several ways. The overproduction and increase of ROS and oxidative stress is usually associated with lipid peroxidation and alteration in lipid metabolism[57]. Reaction of ROS with lipids in membranes can lead to the formation of lipid hydroperoxides that can be further degraded into small reactive carbonyl species, such as 4-hydroxynonenal (4-HNE), a toxic aldehyde that can react with proteins and modify their structure and function. The 4-HNE is proven to be significantly elevated in SLE[58]. People with SLE suffer from inflammation, oxidative stress and alteration of energy production pathways, alongside with a prothrombotic state and a disturbance in the lipid profile. Besides malondialdehyde and lipid peroxidation products, elevated levels were also observed for gamma-glutamyl peptides, gamma-glutamyltransferase, leukotriene B4 and 5-hydroxyeicosatetraenoic acid[33]. Lipids` oxidation can also lead to the formation of other products such as F2 isoprostanes, formed by oxidation of arachidonic acid and found in plasma and urine of SLE patients. Isoprostanes are biomarkers of oxidative stress and are usually elevated in acute phases of the disease[57]. Overall, it is well accepted that oxidized lipids have a significant role in the pathogenesis of lupus, however their action is far from being completely elucidated.

2.2.2 NETosis and lipids in SLE

Neutrophils` dysfunction is also associated with their degradation through a specific activation-induced cell death process called NETosis, which is similar to apotosis. The result of this process is the formation of a transient web-like organelle known as neutrophil extracellular trap (NET)[59]. The development of NET has been recognized as a significant mechanism in SLE`s pathogenesis. NETosis is also an important intervenient in self-antigen presentation, which is a characteristic of diseases of the autoimmune system (Figure 1)[60]. NET is responsible for the release of active oxidative enzymes that will induce a proatherogenic mechanism. Those enzymes can modify high density lipoproteins (HDL) to such an extent that they lose their anti-inflammatory and vasoprotective properties and start failing in mediating reverse cholesterol transport[61]. This data is in agreement with the altered lipoprotein profile that is characteristic of SLE` patients that is going to be addressed further ahead. Oxidized lipids, for example oxidized cardiolipin and oxidized phosphatidylethanolamine (PE), have been reported as important players in

apoptosis and ferroptosis[62], it would be interesting to develop more research in order to understand the role of lipids and oxidized lipids in NETosis.

Dendritic cells (DC) are considered the professional APCs and their main function is to prime naïve T cells activation[63]. DC have been correlated with SLE pathogenesis, essentially in the induction and progression of this disease[64]. However, their action is not completely clear so far.

In addition to NETosis, apoptosis of SLE lymphocytes, mitochondrial dysfunctions, the enhancement of the inflammation cascade and the oxidative stress conditions that occur in these patients also contribute for the multifactorial aspect of SLE.

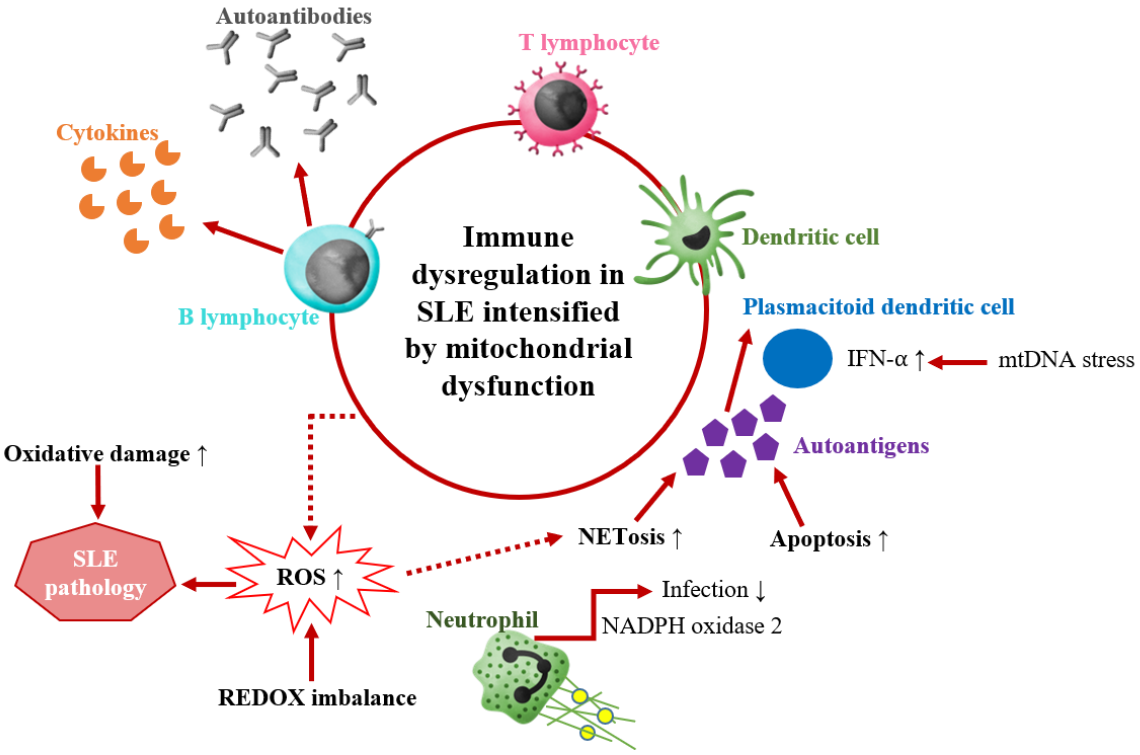

Figure 1. Schematic representation of NETosis` role in SLE pathogenesis. Under sufficient concentrations of ROS, a neutrophil undergoes NETosis to form NET, which are a web-like structure that contains nucleic acid and nucleic protein as well as cell remnants. NET are the source of several autoantigens. Those autoantigens will activate plasmacytoid dendritic cells to release IFN-α and consequently prompt autoimmune destruction. Adapted from H.T. Lee *et al*[11]. Cellular and autoantibodies pictures withdrawn from "BigPicture"[65].

2.2.3 T lymphocyte and mitochondria dysfunction and lipids in SLE

The incorrect functioning of mitochondria in lupus T lymphocytes leads to an elevated mitochondrial transmembrane potential or persistent mitochondrial hyperpolarization[66]. This hyperpolarized state of mitochondria is found to be markedly higher in SLE patients than in healthy controls which may lead to cell death pathways[29,66,67]. Mitochondrial hyperpolarization is linked to an expanded electron transport chain activity and a higher oxygen consumption, which leads to an increased production of ROS and an increase in oxidative stress conditions[68]. Overproduction and inefficient clearance of ROS is thought to underlie abnormal activation and processing of cell-death signals, immune system dysregulation and autoantibody production that are typical of SLE`s pathology[69,70]. Also, the excess of oxidative stress is responsible for alterations in mitochondrial lipids, proteins and DNA, that lead to the malfunctioning of the mitochondria[67]. The existence of severe oxidized damage and oxidative stress in this disease is highlighted as well by the higher levels of 4-hydroxyalkenals in SLE patients, formed during lipid peroxidation enhanced by ROS, confirming altered lipid metabolism in SLE[58].

Chronic inflammation is one key characteristic of SLE and it is exacerbated by oxidative stress and mitochondria dysfunction. Leishangthem *et* al found significantly increased levels of superoxide free radicals in mitochondria of lupus` patients which is suggestive of mitochondria dysfunction[67]. Lipids in mitochondria play an important role in controlling mitochondria functions, they are part of the membrane transport events, therefore the lipid specific regulation of channel transport mechanisms is a decisive aspect related to membrane functions. Malfunctioning of lipid specific mitochondrial membrane transport may have a dangerous effects on cellular health status and raise cell based disease states[71]. T cells dysfunction in SLE have also been correlated with altered lipid metabolism[72]. It was determined that T lymphocytes of SLE patients have an altered glycosphingolipid profile in their membranes, specifically, lactosylceramide, globotriaosylceramide and monosialotetrahexosylganglioside levels were significantly increased when compared with healthy controls[73]. Although glycolipids are not so abundant in cell membranes, they are very important in inflammation/immune response and cell-cell signalling.

3. "Omic" approaches in SLE and in other autoimmune diseases

As mentioned above, disorders of the autoimmune system are the consequence of the interaction between environmental factors, genetic predisposition and a dysregulated immune system. "Omic" studies have helped to better understand autoimmune diseases. Genomics has been developing over the years and an intensive effort is being made to determine the genetic risk *loci* for SLE susceptibility[74]. Although the genetic contribution for SLE susceptibility is important, this disease is not entirely heritable and environmental factors also contribute in some extent. Therefore, an epigenomic analysis may explain part of the missing inheritability [74]. Transcriptomics is another developing approach to better understand autoimmune diseases by studying gene expression levels, using next-generation sequencing of RNA. Transcriptome studies have been crucial to perceive IFN signature in autoimmune diseases since it is not specific from SLE[75–78]. Proteomics brings new opportunities for the diagnosis of autoimmune diseases. Possible applications of proteomics are in antigen-array technology, being the most important the autoantibody profiling of autoimmune diseases to facilitate early diagnosis and treatment[37] (this application has already been reported for multiple sclerosis[79], type 1 diabetes[80] and rheumatoid arthritis[81]); monitoring epitope spreading[79] and tailoring antigen-specific therapy (peptide-based or protein-based tolerizing therapies)[82]. Small molecules or metabolites that are endogenously produced can be measured through metabolomic approaches. This would provide information on the patient`s health, mainly on health status caused by changes in gene expression, diseases, lifestyles and diet[83].

4. Lipidomics in SLE and in other autoimmune diseases

"Omic" studies are very important to understand the overall metabolism of lupus` patients and lipidomic in particular help us to realise how the lipid metabolism is altered in this autoimmune disease. Actually, there is some evidence that there are in fact alterations in lipids and in lipids metabolism in these patients showing the importance that lipid regulation has in SLE pathogenesis.

Always with the goal of improving the diagnostic tools of autoimmune diseases and considering the role of lipids in cellular events, lipidomics is being suggested as a new potential method of diagnosis. It is known that lipids play a very important role in inflammatory processes and act as mediators and biomarkers of many diseases. They are

the precursors of multiple metabolites with powerful bioactivities and are associated with important homeostatic and pathophysiological cellular events[84]. The identification of the lipid profile typical of diseases of the immune system is a topic that is emerging, yet far from being completely disclosed. To face this lack of information, lipidomics arises and hopefully it will be able to provide more knowledge and insight regarding autoimmune disorders.

The identification of the lipid profile at a molecular level in biological systems is nowadays performed using lipidomic approaches which could be used to identify biomarkers and understand the lipidomic signature of autoimmune diseases. The main analytical technique in lipidomics is mass spectrometry (MS) combined with liquid chromatography (LC) or gas chromatography (GC). MS is an extremely sensitive technique and it requires a very small quantity of sample. Lipidomics starts with sample collection followed by lipid extraction. The lipid extract is analysed in a mass spectrometer to identify the individual lipid species, using LC-MS approaches[85]. The acquired data is analysed by using bioinformatic tools and statistical analysis.

In spite of the lack of knowledge regarding the variation of the lipid profile at a molecular level in SLE, there is evidence that lipid metabolism suffers alterations namely in lipoprotein metabolism during the course of SLE disease[58,86–93].

Disturbance of lipoproteins` levels was reported in patients with SLE. Dyslipidemia, an imbalance of lipids in the blood, is known to have a significant influence in atherogenesis[86]. In this way, it was determined a unique dyslipoproteinemia pattern in SLE patients, which is an inappropriate number of lipoproteins in the bloodstream. Dyslipoproteinemia specific of SLE is characterized by low levels of HDL cholesterol and elevated levels of both very low density lipoproteins (VLDL) cholesterol and triglycerides (TG)[87]. Lupus patients also show increased concentrations of oxidized low density lipoproteins (LDL) and an anomalous chylomicron metabolism, which is consistent with the higher levels of 4-HNE[58,88,89]. Levels of oxidized LDL detected by the monoclonal antibody E06 are significantly higher in SLE patients and are associated with cardiovascular disease that develop in SLE[94,95]. SLE`s dyslipoproteinemia has a multifactorial origin and it is yet unclear which factors are definitely involved in the pathophysiology of this disorder[90]. Treatment using some drugs, in particular steroids, contribute to this dyslipoproteinemia specially when they are administrated at a high

dosage. Dyslipidemia can also occur as a consequence of renal failure, one of the most common comorbidities of SLE. Thus, renal involvement and disease activity are some other factors that seem to have the most influence on this characteristic lipoprotein pattern of SLE patients[91–93]. HDL is a cell cholesterol efflux promoter through the reverse cholesterol transport system and prevents LDL oxidation as well, thereby it has an atheroprotective role in the organism. NETosis, as described above, may be a fundamental intervenient in the mechanism implicated in this HDL dysregulation[96]. SLE patients have a much higher risk of developing cardiovascular diseases and this high susceptibility may be partially due to impaired HDL metabolism. In this way, the development of strategies to improve HDL metabolism could have promising effects on the lipid metabolism, ameliorating patients` lipoprotein values and disease management[97,98].

Disease activity is one factor that may have a leading role on the changes of the patients` lipid profile. Differences in the lipid profile between flare and remission need to be considered to better understand lupus lipid metabolism. So far, and to the best of our knowledge, there is only one study that evaluates the changes in lipoprotein values between flare and remission of adult patients with SLE, but no significant differences were observed[90]. The study only detected a tendency of lipoproteins` values during flare to be worse when compared with the same parameters during remission. Patients in flare had higher values of total cholesterol, LDL and TG and lower values of HDL comparing to the same lipoproteins' values in patients in remission. The tendency to higher levels of LD, main targets of ROS, would promote the enhanced ox-LDL levels, with contribution to inflammation and atherogenic risk factors for SLE and relapsing and comorbidities[90].

Lipoprotein dysregulation in lupus patients has been associated with high active phases of the disease. It is also considered a risk factor for the development of cardiovascular diseases, once they have an atherogenic lipoprotein profile[86]. Besides lipoproteins, it is now well known that lipids at molecular level, in the class of lipids found in lipoproteins such as TG, fatty acids (FA), phospholipids (PL) and sphingolipids, can have an important role in the disease and can be used as important biomarkers of the disease.

Also, the study of the variations on the lipid profile at a molecular level using lipidomics has been studied in other autoimmune diseases, for instance rheumatoid arthritis and multiple sclerosis, however little is known about this issue in SLE (Table 3).

Nonetheless, there are already a few studies that provide some evidence considering a possible adaptation of the lipid profile regarding FA, free FA (FFA), PL, lysoPL (LPL), sphingolipids, TG and 4-HNE species profiles in SLE patients.

Table 3. Some autoimmune diseases where lipidomic approaches have been used. Research made on PubMed data base under the terms lipid profile, lipidomic(s), phospholipid(s) and fatty acid (all studies published until 2019 were analysed). All the studies that did not use MS techniques were not taken into consideration.

Autoimmune disease	Lipidomic approach	Class of lipids	Reference
SLE	GC-MS; MDMS-SL	FA; FFA; PL; LPL; Sphingolipids; TG; 4-hydroxyalkenal species	35,58,99–102
Rheumatoid Arthritis	GC-MS; LC-MS/MS	FA; PL	103–110
Multiple Sclerosis	MALDI-TOF/TOF; GC-MS; ESI-MS; LC-MS	PL; FA; Sphingolipids; Very long chain FA	111–116
Systemic Sclerosis	LC-MS/MS	PL; LPL	117
Psoriasis	GC-MS; HPLC-MS	FA; Sphingolipids; PL	118–122
Polymyositis/ Dermatomyositis	GC-MS; LC-MS/MS	FA; TG; PL; LPL; Sphingolipids	123
Celiac disease	HPLC-MS; GC-MS	PL; FA	124–126

Lipidomics revealed a marked increase in TG, individual lyso--phosphatidylethanolamine (LPE) molecular species and total LPE levels, and a significant decrease in some species of PE and phosphatidylcholine (PC), as it is detailed (Table 4)[58,102]. Serum total concentration of TG were increased with markedly differences in its FA composition. It was determined a raise in TG either with 16:1, 20:3, 20:2, 20:1 or 22:6 FA[58]. The most significantly increased LPE species included 20:4 and 22:6 FA[58]. All plasmenylphosphatidylethanolamine (pPE) and plasmenylphosphatidylcholine (pPC) species, which are a subclass of PE and PC respectively, were substantially reduced in SLE patients` serum[58]. The species that suffered the most statistically significant reduction were pPE species of 16:0-20:4 and 18:0-20:4 and pPC species of 18:0-18:0 and 16:0-18:2. The authors attributed the reduction of these species to three major metabolic pathways: decrease of phospholipase A_2 activation, peroxisomal dysfunction and peroxidation-

mediated degradation. Oxidation of pPE is considered the major mechanism responsible for the reduction of pPE levels, which is corroborated by the increase of 4-HNE. It is important to consider that under oxidative stress conditions pPE species have an anti-oxidant role, which also supports the favourable oxidation and decrease of the content of its precursors non-oxidized. Another possible hypothesis proposed by the authors was that the activation of phospholipase A_2 was decreased, however, this hypothesis was discharged because there was not an accumulation of alkenyl LPE. The peroxisomal dysfunction hypothesis was also rejected since it appears to be normal in SLE patients, once plasmanylphosphatidylethanolamine and plasmanylphosphatidylcholine levels were unchanged[58]. pPE may become a biomarker for diagnosis/prognosis of SLE whereas its reduction is strongly associated with disease activity (SLEDAI), oxidative stress (ROS) and proinflammatory cytokines. It has also been reported an increase in plasma levels of PE species (16:0-18:2), (18:0-18:2), (18:1-18:2), (16:0-22:6) and (18:0-22:6), and also an increase of phosphatidylinositol (PI) species with (18:0-18:2)[102]. It was determined a reduction as well in other PE species such as PE (16:0-18:1), (18:1-20:4), (20:0-20:0) and (20:0-20:4), in PI species with (18:1-20:4) and a reduction in lyso-phosphatidylcholine (LPC), namely, LPC 18:2[102].

The total content of sphingomyelin (SM) and ceramide (Cer) species in SLE patients is not different from that found in healthy people. On the other side, it was described that the FA composition of both SM and Cer species was modified in patients with SLE[102]. In SLE patients it was identified a significant increase in SM with N18:0, N18:1 and N22:0 comparing to the healthy control group ("N" stands for the amide linage of the acyl chain). In Cer species, it was determined a significant decrease in Cer with N22:0, N23:0 and N24:0 with a hydroxyl group on the second position of the acyl chain and a significant increase in N24:1[102].

FA composition of plasma and red blood cells can be changed by diet and steroids use, consequently, those alterations also affect circulating lipid profile and thus PL`s composition[35]. The variation of FA in PL have a direct impact on the fluidity of cell membrane of immune cells[127]. Moreover, changes in FA composition in PL of membranes of immune cells can affect the quantity and quality of essential FA, which are released by phospholipases, and to be available for the production of inflammatory mediators that are released by these cells. Essential FA that are used in eicosanoid synthesis derive from PL

pools. For instance, arachidonic acid (AA, 20:4*n*-6) that promotes production of inflammatory molecules, while eicosapentaenoic acid (EPA, 20:5*n*-3) is a precursor of anti-inflammatory mediators[128]. Plasma levels of FA depict recent dietary fat intake while red blood cells levels reflect longer dietary patterns. There are a few reports that suggest a relationship between FFA metabolism and SLE[34,129,130]. FFA can change their levels according with disease activity[131–133].

High levels of saturated FA and low concentrations of PUFA have been detected in plasma of SLE patients, which were correlated with autoimmunity and inflammatory processes[129]. It was also found that oleic acid and AA are decreased in patients with lupus and that higher disease activity are usually associated with a lower level of linoleic acid[34]. Using LC-MS and GC-MS technologies, Wu *et al* found that medium-chain FAs and serum FFA were upregulated, while long-chain FAs (including ω-3 and ω-6 essential FA) were markedly downregulated, which is suggestive of oxidative conditions[134]. Most probably, the decrease of PUFA could be correlated with oxidation of the unsaturated FA due to the increase of ROS products associated with enhanced oxidative stress conditions. However, with GC-MS platforms, Shin and co-workers detected significantly higher levels of palmitoleic and oleic acids (known as anti-inflammatory FA) once they regulate the activation of immune cells (as well as myristic and eicosenoic acids). On the other side, they found markedly decreased concentrations of caproic, caprylic, linoleic, stearic, AA, eicosanoic, behenic, lignoceric and hexacosanoic acids[101]. In general, having in consideration the total content of FFA, saturated FFA levels were reduced and PUFA concentrations were increased[101]. On the contrary, it was also discovered that these patients have lower levels of EPA (which is an ω-3 PUFA) and a reduction of ω-3 index along with a significantly higher ratio of the inflammatory mediator AA to EPA, which is clearly favouring an inflammatory environment[35]. These findings are not in agreement with those reported before presenting the need for more studies in this field. Hereupon, alterations in FA profile in SLE may result from low dietary intake (however it cannot be generalized to every SLE patient due to body mass index differences), lipid peroxidation and/or defects in essential FA desaturation and elongation enzymatic reactions. However, there are controversial findings regarding the changes of FA profiles and its correlation with disease activity which suggests that more studies are needed in this field of lipidomics[101,130]. Some of these MS studies present contradictory results which may be due to the different

methods used for lipid extraction, the type of analysed sample, the number of patients under study or even the chosen lipidomic approach (Table 4).

It seems that lipid metabolism in SLE can be improved with dietary fish oil supplementation which have been shown to have lipid lowering effects. Fish oil has in its composition omega-3 PUFA, specially EPA and DHA. EPA will promote the formation of anti-inflammatory mediators and thus having an anti-atherogenic and anti-inflammatory effect[135]. Therefore, fish oil supplementation is prone to balance immune, atherosclerotic and inflammatory events in patients suffering from lupus[136,137]. It was noticed an increase in EPA and DHA incorporation into cell membranes as well as a decrease in AA after fish oils supplementation, as well as the level of lipoproteins[99]. In fact, TG, VLDL concentrations and the ratio of total to HDL decreased and it was detected a significant elevation of HDL levels in the group of SLE patients that were receiving fish oil supplementation[99]. However, the effects of fish oil supplementation are dependent of the dose administrated. Also, the administration of highly purified EPA alone has also proved to have beneficial effects on the lipid profile of patients with SLE by decreasing the oxidative stress. After treatment, patients showed significantly increased levels of EPA, comparing with the pre-treatment period, as well as markedly decreased concentrations of AA. Thus, the ratio of EPA to AA was also significantly augmented after treatment[100].

Lipid oxidation can lead to alteration of lipid profile by decreasing PUFA, but it can also lead to the formation of toxic small aldehydes. Through multi-dimensional mass spectrometry-based shotgun lipidomics it was found an increase of the levels of 4-hydroxyalkenal species[58]. The elevation of 4-hydroxyalkenals levels alongside with the upregulation of LPE and the increased levels of oxidized LDL mentioned above, are undoubtedly indicative of lipid peroxidation[58,88]. Isoprostanes are biomarkers of lipid peroxidation formed through the non-enzymatic peroxidation of AA. The urinary 8-isoprostane levels were determined to be significantly decreased in SLE patients` after the administration of the EPA treatment[100].

All of these studies clearly demonstrate the role of lipids in inflammatory processes, in immunity, and in the onset and development of autoimmune diseases. Also, lipidomics has potential as a tool to aid both in autoimmune diseases diagnosis and therapeutics by allowing a detailed lipidome profiling of SLE and autoimmune disease patients, which is a first step for the identification of new lipid biomarkers of disease.

Table 4. Main lipid species that showed variation in SLE reported in published lipidomics studies, in PubMed data base, using MS approaches.

Reference	Analytical method	Lipid extraction method	Type of sample	Sample size	Results	
					↓ Reduction	↑ Increase
Aghdassi *et al*[35]	GC-MS	Chloroform/methanol (2:1, v/v)	Red blood cell total lipids (%) Plasma total lipids (%)	33 F	EPA, ω-3 index, total PUFA, total ω-6 Linoleic acid	AA/EPA plasma total trans-FA
Hu *et al*[58]	MDMS-SL	Modified Bligh and Dyer	Serum (nmol/mL serum)	30 F	PE species (16:0-18:1), (16:0-20:4), (18:0-20:4), (18:0-22:4), (18:0-22:5) PC species (18:0-18:0) and (16:0-18:2) TG with 18:2	LPE with 20:4 and 22:6 Total LPE content TG with 16:1, 20:3, 20:2, 20:1, 22:6 4-HNE
Clark *et al*[99]	GC-MS	Modified Bligh and Dyer	Platelet membrane phospholipids	8 F + 4 M	AA	EPA, DHA
Nakamura *et al*[100]	GC-MS	Folch *et al*	Plasma phospholipid fraction (mol%)	5 F + 1 M	Linoleic acid, AA, DHA	EPA/AA, EPA, DPA
Shin *et al*[101]	GC-MS	Paik *et al*	Plasma (FFA%)	41 F	Caproic, caprylic, linoleic, stearic, AA, eicosanoic, behenic, lignoceric and hexacosanoic acids	Myristic, palmitoleic, oleic, and eicosenoic acids
Lu *et al*[102]	MDMS-SL	Modified Bligh and Dyer	Serum (mol%)	30 F	PE species (16:0-18:1), (18:0-20:4), (18:1-20:4), (20:0-20:0), (20:0-20:4) PI species with (18:1-20:4) LPC with 18:2 Ceramides with N22:0, N23:0 and N24:0 with hydroxyl group on the 2nd position of the acyl chain	PE species (16:0-18:2), (18:0-18:2), (18:1-18:2), (16:0-22:6) and (18:0-22:6) PI species with (18:0-18:2) SM with N18:1 and N18:0 Ceramides with N24:1

F-Female; M-Male

5. Phospholipid oxidation formed due to enhanced ROS production

The dyslipoproteinemia pattern characteristic of SLE was determined due to the extensive research of the lipoprotein profile of these patients; the few studies that focused on the molecular lipid profile using MS techniques evidenced that there is in fact alterations in the lipid metabolism regarding adaptations of PL, FA and products of lipid peroxidation, however, to the extent of our knowledge, there are no studies that analysed oxidized PL (ox-PL) in this autoimmune disease. Increase of oxidative stress and ROS production is a hallmark of this disease, therefore it would be expected higher levels of ox-PL in these patients.

Ox-PL are formed due to radical catalysed chemical reactions and are important players in the development of age-related and chronic diseases, inflammation processes, atherosclerotic events and immune responses[138–140]. PL are the main component of cellular membranes therefore their oxidation will induce modifications of the physical properties, such as fluidity and acyl packing, which can lead to apoptotic events once the integrity of the membrane was disrupted[138,141].

PL are formed by two FA, a glycerol backbone, a phosphate group and a polar head group that define the PL class. The unsaturated FA chains present in PL are prone to be oxidized through either radical or nonradical reactions involving enzymatic or nonenzymatic systems. On one side, there are some radical species that are regarded as more reactive and poorly selective such as hydroxyl radicals, •OH, however, on the other side, nonradical species as hydrogen peroxide, H_2O_2, tend to selectively react with PL and are able to diffuse from their point of origin[142]. PL oxidation is influenced not only by the oxidant specie, if it is radical or nonradical, but also by the type of linkage of the FA to the glycerol backbone, ester or ether bonds, and the fatty acyl chain present[140]. The oxidative reactions of PL result in a widespread variety of compounds that are classified according to the nature of the modifications. The products can be classified in either long-chain products, which are products that preserve the PL skeleton; short chain products, formed by cleavage of the unsaturated fatty acyl chains; or adducts, formed by reaction of an oxidation product with molecules containing nucleophilic groups[143].

The identification of ox-PL using MS is rather difficult once they have lower abundances compared with the native PL, therefore their presence is not always detected.

Furthermore, a great variety of oxidation products can be formed during ROS induced oxidation, including long-chain products, with a higher mass than the native PL, and include the products resulting from insertion of oxygen and hypohalous acid molecules; short chain products, with a lower mass than the native PL and result from cleavage of the unsaturated fatty acyl chain; and PL adducts that show products formed by reaction of an oxidation product with molecules containing nucleophilic groups[143], which increases the complexity of the analysis of these oxidized PL in biological samples.

Quantification of ox-PL is also a problematic step since the products of lipid peroxidation are molecules that are typically chemically unstable, mostly the radical species formed in the initial stages of the oxidation process. Besides being chemically unstable, lipid peroxidation products can also be enzymatically metabolized which lowers the concentration of primary ox-PL products and increases that of the more stable end products[139]. Additionally, ox-PL that contain carbonyl groups due to fragmentation can locally react with proteins leading to the formation of adducts, which lowers the concentration of secondary ox-PL products[144]. This way, quantifying ox-PL in biological samples and relate them to the oxidative stress conditions and development of the disease is a laborious task and requires highly advanced lipidomic techniques as well as bioinformatic tools.

6. Objectives

Lipidomics is a scientific field that is in development over the last few years however its applicability in the investigation of autoimmune diseases is still very scarce. MS techniques are a promising tool to better understand autoimmune diseases` lipidome. The aim of this work is to study the lipid profile, with main focus on the oxidized species, of the autoimmune disease SLE, with the intention of trying to identify a molecular lipid profile characteristic of this disease, comparing it with the lipid profile of healthy controls. Lipid profiling of plasma of SLE patients, previously diagnosed, and healthy controls will be evaluated by using HILIC-MS and MS approaches. In addition, we also intend to foster the detection of oxidized PL using the MZmine v2.32 software and to achieve their quantification in plasma samples from human pathologies. Hopefully this work will increase the possibility of identifying lipid biomarkers of SLE and thus contribute to the improvement of diagnostic/prognostic methodologies by using lipidomics approaches. This

approach could give new putative biomarkers for early diagnostics, to predict relapse episodes and evaluate therapy outcomes. Utmost, it could be a promising tool to personalise medicine, allowing the reduction of the morbidity and mortality of SLE.

Experimental Section

7. *Experimental Section*

7.1 Reagents

To perform PL separation from the samples by solid phase extraction, the acetonitrile (ACN) was acquired from Fisher Chemical (lot 1684998) and the formic acid (FA) and ammonium hydroxide were obtained from Fluka Analitical (lot BCBK6176V and lot 63120, respectively). To quantify PL in each sample, dichloromethane was kindly given by Fisher Chemical (lot 1692424), 70% perchloric acid was obtained from Chem-lab NV, Belgium, (product CL00.1612.100), the phosphorous solution was prepared using $NaH2PO_4 \cdot 2H_2O$ from Riedel (lot 04269), molybdate ($NaMoO_4 \cdot H_2O$) was acquired from Panreac, Montplet and Esteban SA, Barcelona, Madrid and the $_L$(+)-ascorbic acid from VWR Chemicals (product 20155294). Internal standards of PL for HILIC-MS analysis were obtained from Avanti® Polar Lipids, Inc (Alabaster, AI, EUA) without any additional purification process. The solvents for LC-MS were ACN, methanol (Fisher Chemical), water and ammonium acetate.

7.2 Plasma samples

In order to study the differences in the lipid profile of SLE disease, plasma samples from SLE patients and healthy controls were collected from Centro Hospitalar Baixo Vouga (CHBV), Hospital de Aveiro. The participating patients were diagnosed with SLE and followed in CHBV prior to sample collection for this study. To allow the participation in this study, all control samples were guaranteed to be healthy, showing no signs of any inflammation parameters. The study protocol was approved by the Ethic Committee of CHBV. It was used a total of 16 SLE samples and 22 plasma samples of healthy controls. Information of SLE patients and controls is shown in Table 5.

Table 5. Demographic and clinical characteristics of the subjects that participated in the study*.

Parameters	Patients (n=16)	Controls (n=22)
Age (years)	41.6 ± 18.5	57.7 ± 18.3
Sex (% women)	100%	45%
Anti-dsDNA antibody presence	9/16	-
Total Cholesterol (mg/dL)	166.9 ± 30.2	165.9 ± 31.1
HDL (mg/dL)	56.0 ± 15.2	65.5 ± 16.2
LDL (mg/dL)	94.8 ± 21.4	86.2 ± 24.1
TG (mg/dL)	112.2 ± 71.3	91.5 ± 22.1

*Values represent the mean ± standard deviation.
CHBV reference values: HDL >45mg/dL; TG <150 mg/dL; LDL <115 mg/dL

7.3 Phospholipid extraction by Hybrid SPE-PL

Solid phase extraction (SPE) is a sample preparation process in which the compounds of interest are separated, from the mixture in which they are included, according with their physical and chemical properties. In this case, SPE was used to separate PL from plasma samples. SPE procedure required three eluents that were prepared prior to PL`s extraction. Eluent 1 consisted of ACN with 1% of FA, that was prepared by adding 250 µL of FA to 25 mL of ACN. Eluent 2 was 25 mL of pure ACN. Eluent 3 was ACN with 5% of ammonium hydroxide, that was prepared by adding 5 mL of 25% ammonium hydroxide to 20 mL of ACN.

After the preparation of the phases, 100 µL of plasma of each sample were mixed with 900 µL of eluent 1 in a Pyrex tube. Each tube was vortexed (Vortex Labinco L46) for 30 seconds and then centrifugated (Mixtasel, Model 540 P Selecta) at 2000 rpm for 5 minutes. The resulting supernatant of each tube was transferred to a Hybrid SPE-PL column (SUPELCO, lot 4497303) that was already placed in a vacuum system *Visiprep SPE Vacuum Manifold* (*Supelco*, Bellefonte, PA). After eluting almost all of the supernatant, the columns were washed with 1 mL of eluent 2 and then with 1 mL of eluent 1. At this point, the collection tubes were replaced by new ones and the PL retained on the Hybrid SPE-PL columns were eluted with two consecutive 1 mL aliquots of eluent 3. The flow-through was collected, dried under nitrogen stream and stored at -20ºC.

7.4 Phosphorus measurement

To determine PL in the lipid extracts, the phosphorus released after acid hydrolysis was quantified using the Bartlett & Lewis method[145]. This method is based on the reaction of molybdate with the released phosphate from the samples, forming phosphomolybdate (yellow). In the presence of ascorbic acid, the phosphomolybdate is reduced to a blue solution. The higher the phosphorus concentration, the bluer the solution becomes. In this way, a colour gradient can be formed, from yellow to blue, depending on the amount of phosphorus that is present.

For the quantification of phosphorus, each extract was first dissolved in 300 µL of dichloromethane. 10 µL of each solution was withdrawn in duplicate for new culture tubes. After drying under nitrogen stream, 125 µL of 70% perchloric acid was added to each tube so that acid hydrolysis could occur. The tubes were placed in the heating block (Stuart SBH200D/3) at 180 ° C for 40 min. During the waiting time, standards containing different phosphorus concentrations were prepared in duplicate using the volumes shown in Table 6.

Table 6. Standards` preparation procedure.

Standard	Phosphorus solution (µL)	70% perchloric acid (µL)	Milli-Q water (µL)	2,5% Molybdate solution (µL)
P0	0	125	825	125
P1	1	125	824	125
P2	2	125	823	125
P3	4	125	821	125
P4	7	125	818	125
P5	10	125	815	125
P6	15	125	810	125
P7	20	125	805	125

The phosphorus solution (100 µg/mL) was previously prepared using $NaH2PO_4 \cdot 2H_2O$ dissolved in Milli-Q water and stored at 4ºC. The 2.5% (m/v) molybdate solution was prepared on time. To prepare 7 ml of this solution, it would be necessary to weigh in an analytical balance 0.175 g of molybdate. After the standards were prepared, they were all vortexed. The standards already have the free phosphate group, so they do not need to undergo acid hydrolysis or go to the heating block.

After the hydrolysis time (40 min) of the samples, the tubes were removed from the block. After cooling the tubes, 825 µL of Milli-Q water and 125 µL of the 2.5%

molybdate solution were added to each tube and vortexed for 1 min. Molybdate should first complex with the sample and do not remain in solution, otherwise ascorbate may bind inappropriately. Thus, good vortexing of the tubes prior to the addition of ascorbic acid is essential.

The 10% (m/v) ascorbic acid solution in Milli-Q water was also prepared on the spot and kept on ice. In the preparation of 7 ml of the solution would be required 0.7 g of $_{L}$(+)-ascorbic acid.

To each sample tube and standard was added 125 µL of the ascorbic acid solution and all tubes were vortexed. All tubes were then placed in a boiling water bath for 10 min, which was then turned into a cold-water bath. After cooling, 200 µL of each tube was pipetted in duplicate into the wells of a 96-well reading plate and absorbances at 797 nm were measured on a microplate reader (Multiskan Go, Thermo Scientific, version 1.00.38).

7.5 Lipid extract analysis by Hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS)

7.5.1 Sample preparation

Lipid extracts obtained from plasma samples were resuspended in dichloromethane in order to have a PL concentration of 1 µg PL/µL. Subsequently, into a vial with a micro-insert, it was added 10 µL of each sample, 4 µL of an internal standards mixture and 86 µL of initial chromatographic phase. The internal standards mixture contained 0.02 µg of PC(14:0/14:0), 0.02 µg of PE(14:0/14:0), 0.012 µg of phosphatidylglycerol (PG, 14:0/14:0), 0.08 µg of PI(16:0/16:0), 0.04 µg of phosphatidylserine (PS, 14:0/14:0), 0.08 µg of phosphatidic acid (PA, 14:0/14:0), 0.02 µg of LPC(19:0), 0.02 µg of SM(d18:1/17:0) and 0.08 µg of cardiolipin (CL, 14:0/14:0/14:0/14:0). The initial chromatographic phase consisted of two mobile phases in a proportion of 10% of eluent A (50% of acetonitrile, 25% of methanol, 25% of water and 10 mM of ammonium acetate) and 90% of eluent B (60% of acetonitrile, 40% of methanol and 10 mM of ammonium acetate).

7.5.2 Data acquisition

PL were separated according to the polarity of the head of the substituent group through HILIC using an Ascentis Si HPLC Pore column (100 mm x 1 mm; 3 µm, Sigma-Aldrich) inserted in a high performance-liquid chromatography (HPLC) system (Ultimate 3000 Dionex, Thermo Fisher Scientific, Bremen, Germany) with an autosampler coupled

online to a Q-Exactive™ hybrid quadrupole Orbitrap™ mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).

5 μL of each sample mixture were placed in the autosampler and each individual sample was injected into the HPLC column, at a flow rate of 50 μL/min with a temperature of 35ºC. Elution started with 10 % of mobile phase A, which was held isocratically for 2 minutes, followed by a linear increase to 90% of mobile phase A within 13 minutes and maintained for 2 minutes. After that, conditions returned to the initial settings in 13 minutes (3 min to decrease to 10% of phase A and a re-equilibration period of 10 min prior next injection). PL analysis was obtained by a Q-Exactive™ orbitrap mass spectrometer with a heated electrospray ionization source that operated simultaneously in both positive (electrospray voltage of 3.0 kV) and negative mode (electrospray voltage of -2.7 kV). The sheath gas flow was 15 U, auxiliary gas was 5 U, the capillary temperature was 250ºC with maximum spray current of 100, the S-lenses RF was 50 U and the probe`s temperature was 130ºC. Mass spectra acquisition method was data-dependent, full scans were acquired in a m/z values range of 400-1600, with a 70.000 high resolution, automatic gain control target of $1x10^6$ and maximum injection time of 100 ms. The top 10 most abundant precursor ions were selected to be fragmented in the collision cell HCD. Normalized collision energy™ ranged between 20, 25 and 30 eV, and MS/MS spectra obtained were the ones combining the information obtained with the three collision energies. The tandem mass spectra (MS/MS spectra) were obtained with a 17.500 resolution, automatic gain control target of $1x10^5$, an isolation window of 1 *m/z* and scan range of 200-2000 *m/z*. Maximum accumulated ions were established at 100 ms for MS spectra and 50 ms for MS/MS spectra. The cycles consisted in one full scan mass spectrum, and ten data-dependent MS/MS scans were repeated continuously throughout the experiments, with the dynamic exclusion of 60 seconds and intensity threshold of $1x10^4$.

In order to detect a characteristic lipid pattern of SLE, the acquired spectrum of each sample was analysed using the data acquisition software Xcalibur v3.3 (Thermo Fisher Scientific, USA). PL identification was performed comparing the experimental measurements with the exact lipid mass. To correctly identify a PL specie, the error must be less than 5 ppm. To confirm exactly which PL was identified, MS/MS spectra interpretation was required. After identification, quantification of molecular species was performed through integration of chromatographic peaks. Mass spectra were processed and

integrated using the software MZmine v2.32. During the processing of raw data acquired in full MS mode, all peaks with raw intensity lower than $1x10^4$ were excluded.

Relative quantification was performed by exporting peak area values into a computer spreadsheet. To normalize the data, peak areas of the extracted-ion chromatograms (XIC) of each lipid molecular species were divided by the sum of total XIC areas of the identified PL species.

7.6 Statistical analysis

Statistical analysis of PL, total cholesterol, HDL, LDL and TG concentrations was carried out using PRISM® GraphPad Software, Inc. (La Jolla, CA, USA). The Mann-Whitney U test was performed, and statistical differences were represented with the following symbols of significance level *, significantly different $p<0.05$. To perform multivariate analysis of data matrices (normalized peak areas of lipid species in the different samples) it was used Metaboanalyst[146]. The data was log-transformed and Pareto scaled before Principal Component Analysis (PCA) and Partial-least Squares Discriminant Analysis (PLS-DA). The results were visualized in scores scatter plots to see the discriminant ability of the method. Univariate data analysis was also performed using Metaboanalyst to produce Heatmaps showing the top 20 variables contributing for sample discrimination.

Results and Discussion

8. Results & Discussion

8.1 Plasma samples characterization

In order to evaluate the changes of the lipid profile in serum of SLE patients, 16 disease samples and 22 healthy control samples were collected from CHBV, as summarized in Table 5. All patients and controls were adults. Only 9 of the 16 patients diagnosed with SLE had the antibody anti-dsDNA, which is in agreement with the literature since this antibody is not SLE specific (~70% of SLE patients have anti-dsDNA[6]), hence the need for new diagnostic methods to overcome the difficulty of the diagnosis[28].

By the analysis of Table 5, it can be seen that SLE affects mainly women, especially in childbearing age, in accordance with the information reported in the literature[7]. The lipoprotein profile of SLE patients was not statistically different from the lipoprotein profile of the healthy controls (Figure 2). Nonetheless, lipoprotein profile of SLE showed a tendency to be altered and all patients have lower levels of HDL and higher levels of TG and LDL than controls, suggestive of a dyslipoproteinemia pattern in SLE plasma samples, also in accordance with previous reports for this disease[87]. The determined values were under CHBV reference values for these parameters, although SLE patients had a tendency to have their lipoprotein values near or even out of the maximum limit of the references. Lipid lowering drugs (corticosteroids) for SLE treatment could be responsible for the non-significant statistical differences.

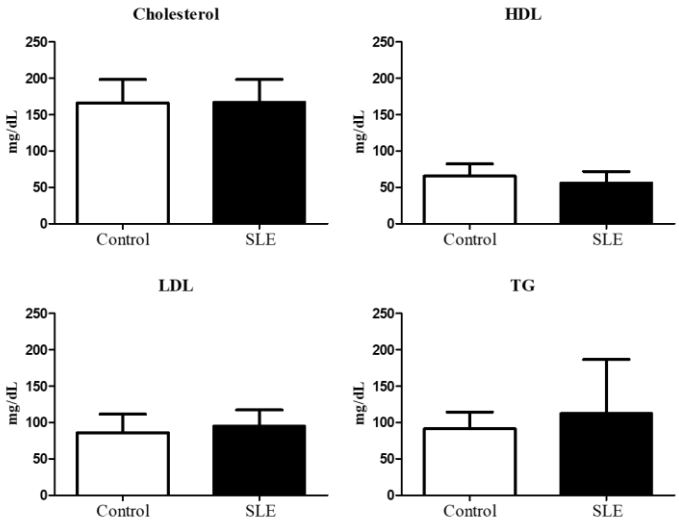

Figure 2. Plasma lipid profile: levels of cholesterol, HDL, LDL and TG of SLE patients and healthy controls. Mann-Whitney U test did not reveal differences statistically significant.

8.1.1 Lipid extraction and phospholipid quantification

Total PL extracts were obtained by SPE and the total amount in PL was quantified by using the method of Bartlett & Lewis[145]. For this, standards with known phosphorus concentrations were prepared in order to build a calibration curve that allowed the determination of the phosphorus amount and, consequently, of PLs in each sample. The quantity of phosphorus (μg) determined was multiplied by 25 to have the total mass in PL. This conversion factor results from the division of the average molecular mass of a PL (770) by the molecular mass of phosphorus (31), this way, 770/31 = 25. The amount of PL from each extract obtained from the plasma samples had an average value of 66.8 ± 26.1 μg for SLE patients and 47.8 ± 24.8 μg for healthy controls (Figure 3). The significantly higher amount of PL in SLE samples, may be explained by the dyslipoproteinemia characteristic of these patients and thus a higher content in lipoproteins` main transporter of lipids in plasma.

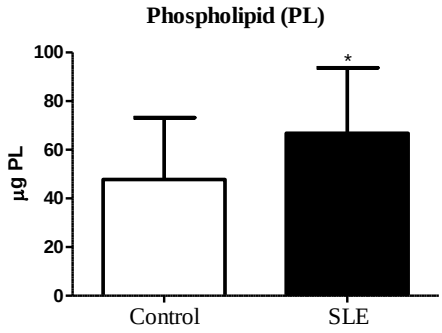

Figure 3. PL quantification of SLE and Control samples. The results were compared with the Mann-Whitney U test: *p<0.05.

8.1.2 Analysis of the lipid extracts by high resolution HILIC-MS and MS/MS.

The PL extracts obtained were analysed by high resolution HILIC-MS and MS/MS in a Q-Exactive™ orbitrap mass spectrometer, as described in chapter 7.5. Analysis were made in both positive and negative ion modes. The interpretation of the LC-MS data allowed the identification and quantification of PL species. The identification of each PL specie was made based on the retention time (RT), once each PL class typically displays a characteristic RT range, *m/z* value and exact mass of the ions identified in the MS spectra, along with characteristic fragment ions or neutral losses for each class found in MS/MS spectra. The identification of the fatty acyl chain composition was obtained also through interpretation of MS/MS spectra of each PL species. In negative ion mode, it is possible to detect and identify carboxylate anions, $RCOO^-$ ions, which enables the identification of the fatty acids present in a specific PL specie. Oxidized PL are expected to be formed in SLE, since this condition is associated with exacerbation of ROS production. Thus oxidized PL, specially oxidized PC and PE that are the most abundant PL in plasma, were also detected using LC-MS and MS/MS approaches as described for the non oxidized species. The identification was based on the comparison with the exact mass through a homemade data base that displays the calculated mass for the long chain oxidation products, including hydroxyl (PL+O) and hydroperoxyl derivatives (PL+2O) of each PL class (Figure 4-B/C). These oxidized classes are the ones that are expected to be formed *in vivo* in higher abundance considering previous studies[147,148]. We also considered the short chain oxidation products, mostly reported such as the ones with short end chain with C9, with terminal aldehyde and carboxilic group derived from oxidative cleavage of linoleic acid esterified to

PL(Figure 4-D/E); and with C5, derived from oxidative cleavage of arachidonic acid esterified to PL (Figure 4-F/G). The detailed explanation on how the identification of the most abundant species from each PL class was made is described in full detail along the following text.

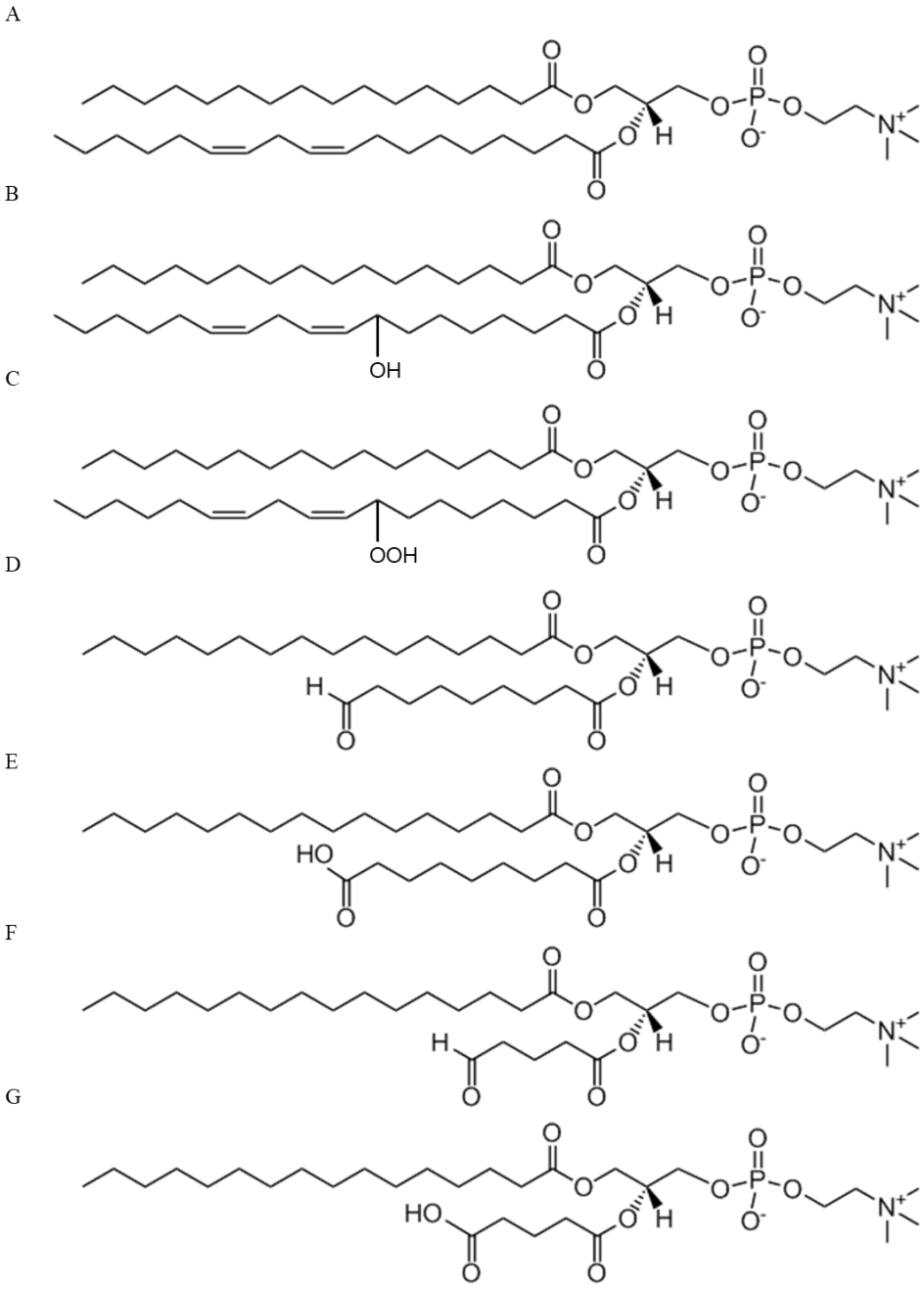

Figure 4. Structural features of a PL class in its native form and oxidized derivatives. **A:** PL in native form [PC(34:2)]. **B:** PL hydroxyl derivative [PC(34:2)+O]. **C**: PL hydroperoxyl derivative [PC(34:2)+2O]. **D:** PL short chain oxidation product with C9 with terminal aldehyde [PC(16:0/C9aldehyde)]. **E:** PL short chain oxidation product with C9 with terminal carboxylic [PC(16:0/C9carboxylic)]. **F:** PL short chain oxidation product with C5 with terminal aldehyde [PC(16:0/C5aldehyde)]. **G:** PL short chain oxidation product with C5 with terminal carboxylic [PC(16:0/C5carboxylic)].

PC and LPC can be identified by LC-MS in positive ion mode by the ions $[M+H]^+$ and in negative ion mode by the ions $[M+CH_3COO]^-$. The structural features can be inferred by MS/MS in both ion modes. In the MS/MS of the $[M+H]^+$ ions it is possible to see the product ion at *m/z* 184, which corresponds to the phosphocholine polar head group. In the MS/MS spectrum of the $[M+CH_3COO]^-$ ion, which corresponds to a mass shift of plus 58 Da, it is possible to see the product ion at *m/z* 168, corresponding to the demethylated phosphocholine polar head group. The presence of these product ions, retention time at which these species were identified and comparison to exact mass measurements allows to confirm the presence of PC and LPC molecular species. The identification of the fatty acyl chains is confirmed by the identification of the $RCOO^-$ ions in the MS/MS spectrum of the $[M+CH_3COO]^-$ ions. In Figure 5 it is shown, as an example, the MS and MS/MS spectra of PC(34:1), correspondent to PC(16:0/18:1), and its hydroxyl derivative correspondent to $[PC(34:1)+O+H]^+$.

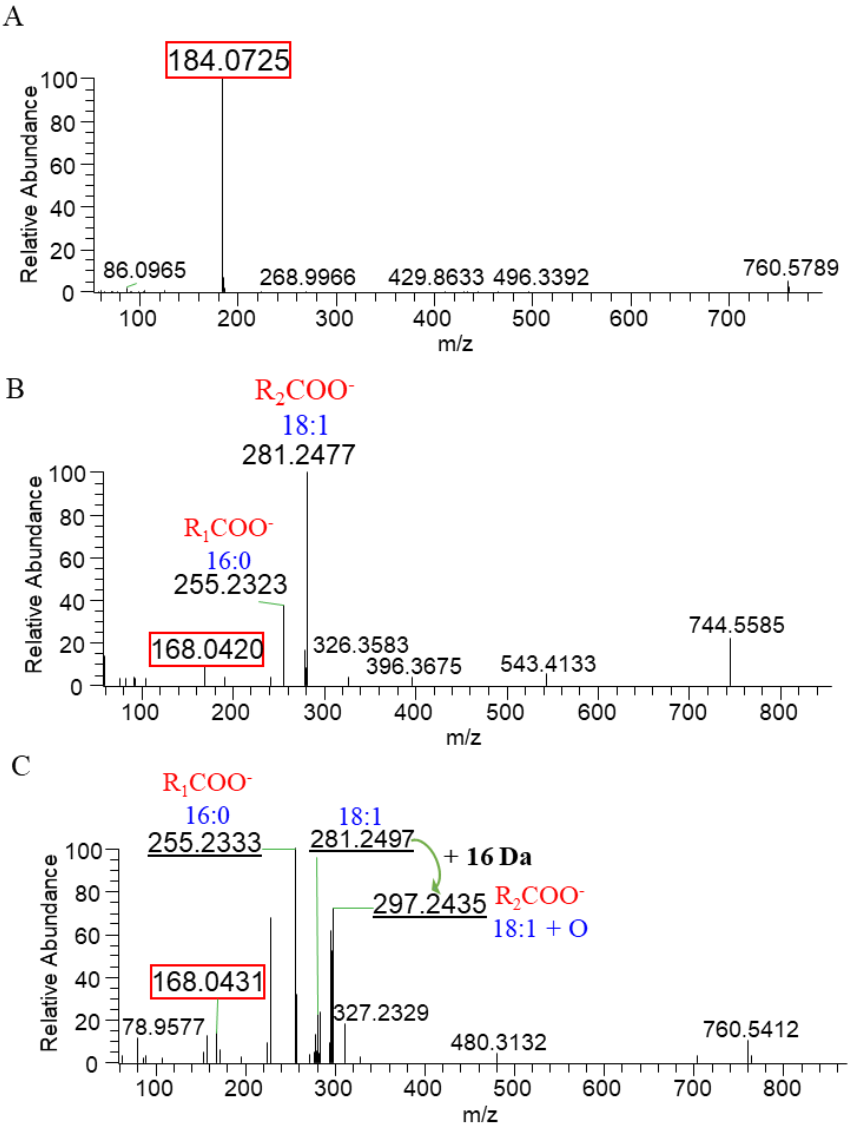

Figure 5. PC identification. **A:** MS/MS spectrum of the $[M+H]^+$ ion of PC(34:1) (*m/z* 760.5812). **B:** MS/MS spectrum of the $[M+CH_3COO]^-$ ion of PC(34:1) (*m/z* 818.6440). **C:** MS/MS spectrum of the $[M+CH_3COO]^-$ ion of PC(34:1)+O (*m/z* 834.4994). Fragment ions characteristic of PC and LPC classes are highlighted in a red box.

- **Positive mode:** $[M+H]^+$ ions
  - ✓ MS/MS of the precursor at *m/z* 760.5812 [PC(34:1)] in full scan (A).
  - ✓ MS/MS spectrum showed the abundant product ion at *m/z* 184.0725, which corresponds to the phosphocholine polar head group (A).
- **Negative mode:** $[M+CH_3COO]^-$ ions, mass shift of plus 58 Da
  - ✓ MS/MS of the precursor at *m/z* 818.6440 [PC(34:1)] in full scan (B).
  - ✓ MS/MS of the precursor at *m/z* 834.4994 [PC(34:1)+O] in full scan (C).

- ✓ MS/MS spectra showed product ions at *m/z* 168.0420 (B) and m/z 168.0431 (C), which corresponds to the demethylated phosphocholine polar head group.

- **Fatty acyl chain confirmed in the negative mode by carboxylate anions:**
  - ✓ (B) It is possible to see the product ion at *m/z* 255.2323, which corresponds to the palmitic acid (16:0) (R_1COO^-)
  - ✓ (B) It is possible to see the product ion at *m/z* 281.2477, which corresponds to the oleic acid (18:1) (R_2COO^-)
  - ✓ (C) It is possible to see the product ion at *m/z* 255.2333, which corresponds to the palmitic acid (16:0) (R_1COO^-)
  - ✓ (C) It is possible to see the product ion at *m/z* 297.2435, which corresponds to the hydroxyl derivative of oleic acid (18:1+O) (R_2COO^-)
- **Identification based on MS data:**
  - ✓ (B) PC(34:1); [PC(16:0/18:1)]
  - ✓ (C) PC(34:1+O); [PC(16:0/18:1+O)]

PE and LPE can be identified by LC-MS in positive ion mode by the ions $[M+H]^+$ and in negative ion mode by the ions $[M-H]^-$. The structural features can be inferred by MS/MS in both ion modes. In the MS/MS of the $[M+H]^+$ ions it is possible to see the product ion formed by typical neutral loss of 141 Da, which corresponds to the loss of the phosphoethanolamine group. In the MS/MS spectrum of the $[M-H]^-$ ions, it is possible to see the product ion at *m/z* 140, corresponding to the deprotonated phosphoethanolamine group. The neutral loss, the presence of the product ion, retention time at which these species were identified and comparison to exact mass measurements allows to confirm the presence of PE and LPE molecular species. The identification of the fatty acyl chains is confirmed by the identification of the $RCOO^-$ ions in the MS/MS spectrum of the $[M-H]^-$ ions. In Figure 6 it is shown, as an example, the MS and MS/MS spectra of PE(36:4), correspondent to PE(16:0/20:4) or PE(18:2/18:2), and its hydroxyl derivative correspondent to $[PE(36:4)+O+H]^+$.

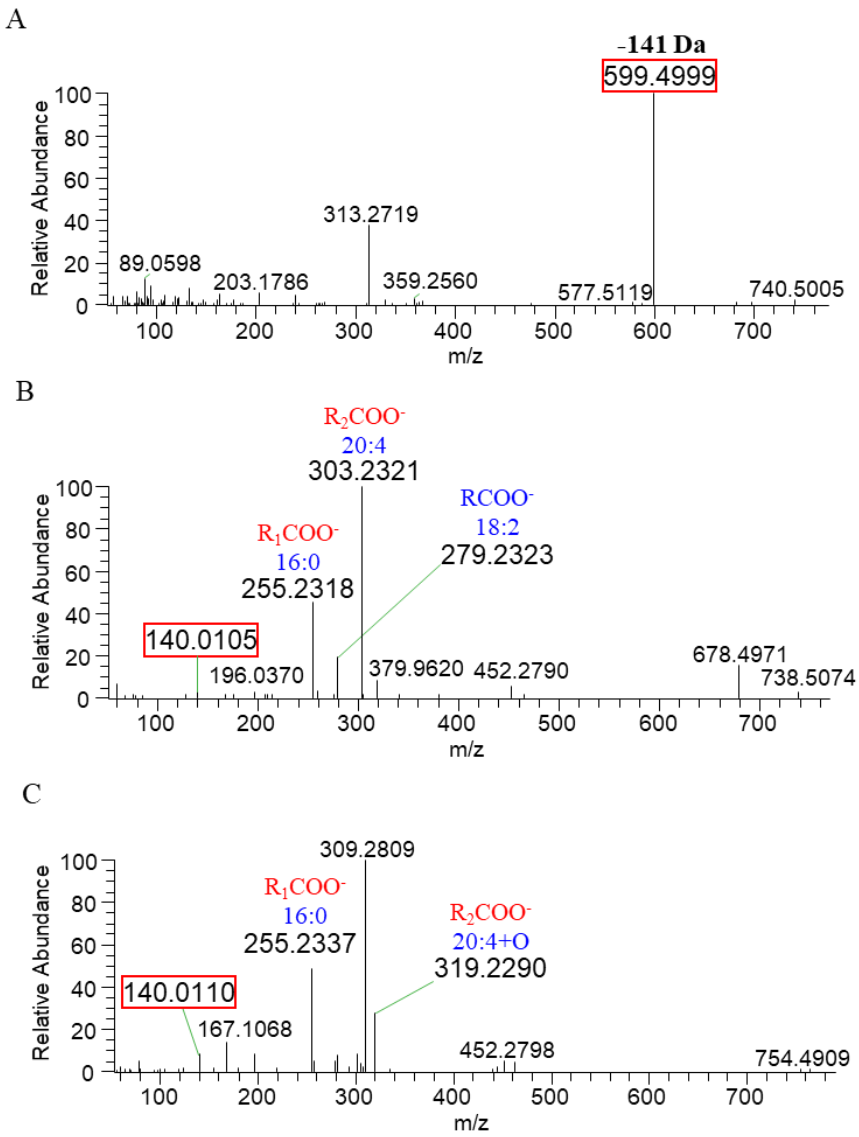

Figure 6. PE identification. **A:** MS/M spectrum of the $[M+H]^+$ ion of PE(36:4) (*m/z* 740.5197). **B:** MS/MS spectrum of the $[M-H]^-$ ion of PE(36:4) (*m/z* 738.5050). **C:** MS/MS spectrum of the $[M-H]^-$ ion of PE(36:4)+O (*m/z* 754.4909). Fragment ions characteristic of PE and LPE classes are highlighted in a red box.

- **Positive mode:** $[M+H]^+$ ions
  - ✓ MS/MS of the precursor at *m/z* 740.5197 [PE(36:4)] in full scan (A).
  - ✓ MS/MS spectrum showed product ion at *m/z* 599.4999, formed due to neutral loss of 141.0198 Da, which corresponds to the loss of the phosphoethanolamine polar head group (A).
- **Negative mode:** $[M-H]^-$ ions
  - ✓ MS/MS of the precursor at *m/z* 738.5050 [PE(36:4)] in full scan (B)

- ✓ MS/MS of the precursor at *m/z* 754.4909 [PE(36:4)+O] in full scan (C)
- ✓ MS/MS spectra showed product ions at *m/z 140.0105* (B) and *m/z* 140.0110 (C), which corresponds to the phosphoethanolamine polar head group.

➢ **Fatty acyl chain confirmed in the negative mode by carboxylate anions:**

- ✓ (B) It is possible to see the product ion at *m/z* 255.2318, which corresponds to the palmitic acid (16:0) (R_1COO^-)
- ✓ (B) It is possible to see the product ion at *m/z* 303.2321, which corresponds to the arachidonic acid (20:4) (R_2COO^-)
- ✓ (B) It is possible to see the product ion at *m/z* 279.2323, which corresponds to the linoleic acid (18:2) ($RCOO^-$)
- ✓ (C) It is possible to see the product ion at *m/*z 255.2337, which corresponds to the palmitic acid (16:0) (R_1COO^-)
- ✓ (C) It is possible to see the product ion at *m/z* 319.2290, which corresponds to the hydroxyl derivative of arachidonic acid (20:4+O) (R_2COO^-)

➢ **Identification based on MS data:**

- ✓ (B) PE(36:4); [PE(16:0/20:4)/PE(18:2/18:2)]
- ✓ (C) PE(36:4)+O; [PE(16:0/20:4+O)]

PG and LPG can be identified by LC-MS in the negative ion mode by the ions $[M-H]^-$. The structural features can be inferred by MS/MS also in the negative ion mode. In the MS/MS spectrum of the $[M-H]^-$ ions, it is possible to see the product ion at *m/z* 171, which corresponds to the phosphoglycerol group. The presence of this product ion, retention time at which these species were identified and comparison to exact mass measurements allows to confirm the presence of PG and LPG molecular species. The identification of the fatty acyl chains is confirmed by the identification of the $RCOO^-$ ions in the MS/MS spectrum of the $[M-H]^-$ ions. In Figure 7 it is shown, as an example, the MS/MS spectra of LPG(14:0). Oxidized PG and LPG species were identified only by comparison to exact mass measurements.

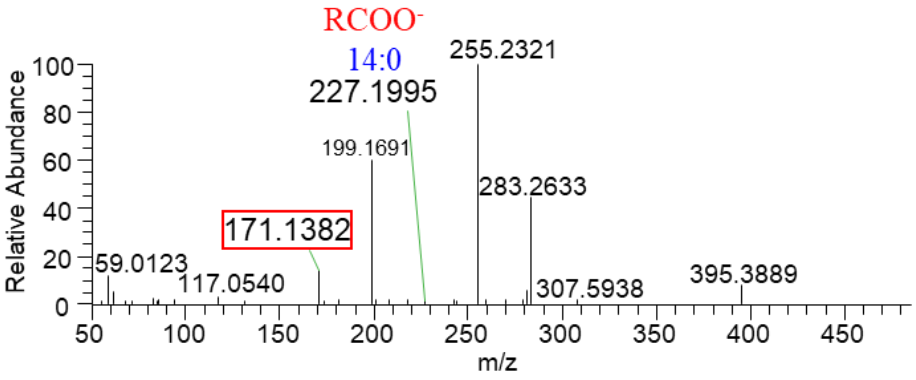

Figure 7. LPG identification. MS/MS spectrum of the $[M-H]^-$ ion of LPG(14:0) (*m/z* 455.2405). Fragment ion characteristic of PG and LPG classes is highlighted in a red box.

- **Negative mode:** $[M-H]^-$ ions
  - ✓ MS/MS of the precursor at *m/z* 455.2405 [LPG(14:0)] in full scan
  - ✓ MS/MS spectrum showed product ion at *m/z* 171.1382, which corresponds to the phosphoglycerol group.
- **Fatty acyl chain confirmed in the negative mode by carboxylate anions:**
  - ✓ It is possible to see the product ion at *m/z* 227.1995, which corresponds to the myristic acid (14:0) ($RCOO^-$)
- **Identification based on MS data:**
  - ✓ LPG(14:0)

PI and LPI can be identified by LC-MS in the negative ion mode by the ions $[M-H]^-$. The structural features can be inferred by MS/MS also in the negative ion mode. In the MS/MS spectrum of the $[M-H]^-$ ions, it is possible to see the product ion at *m/z* 241, which corresponds to the phosphatidylinositol polar head group. The presence of this product ion, retention time at which these species were identified and comparison to exact mass measurements allows to confirm the presence of PI and LPI molecular species. The identification of the fatty acyl chains is confirmed by the identification of the $RCOO^-$ ions in the MS/MS spectrum of the $[M-H]^-$ ions. In Figure 8 it is shown, as an example, the MS/MS spectra of LPI(18:0). Oxidized PI and LPI species were identified only by comparison to exact mass measurements.

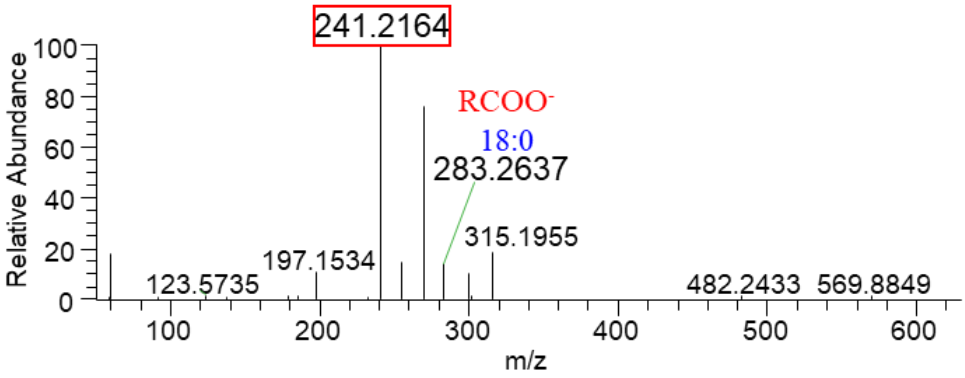

Figure 8. LPI identification. MS/MS spectrum of the $[M-H]^-$ ion of LPI(18:0) (*m/z* 599.3204). Fragment ion characteristic of PI and LPI classes is highlighted in a red box.

Negative mode: $[M-H]^-$ ions

- ✓ MS/MS of the precursor at *m/z* 599.3204 [LPI(18:0)] in full scan
- ✓ MS/MS spectrum showed product ion at *m/z* 241.2164, which corresponds to the phosphatidylinositol polar head group.

➢ **Fatty acyl chain confirmed in the negative mode by carboxylate anions:**

- ✓ It is possible to see the product ion at *m/z* 283.2637, which corresponds to the stearic acid (18:0) ($RCOO^-$)

➢ **Identification based on MS data:**

- ✓ LPI(18:0)

PS and LPS can be identified by LC-MS in the negative ion mode by the ions $[M-H]^-$. The structural features can be inferred by MS/MS also in the negative ion mode. In the MS/MS spectrum of the $[M-H]^-$ ions, it is possible to see the product ion formed by typical neutral loss of 87 Da, which corresponds to the loss of the serine from the polar head group. The presence of the product ion, retention time at which these species were identified and comparison to exact mass measurements allows to confirm the presence of PS and LPS molecular species. The identification of the fatty acyl chains is confirmed by the identification of the $RCOO^-$ ions in the MS/MS spectrum of the $[M-H]^-$ ions. The identification of PS and LPS species, either in the native form or oxidized, was made based only on the exact mass measurements. In Figure 9 it is shown the MS/MS spectra of PS(36:1), correspondent to PS(18:0/18:1), as an example, since this PS molecular specie was not identified in this work.

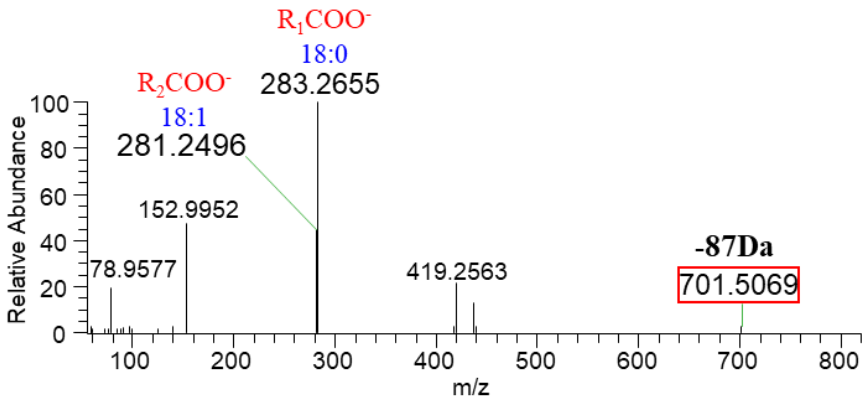

Figure 9. PS identification. MS/MS spectrum of the $[M-H]^-$ ion of PS(36:1) (*m/z* 788.5442). Fragment ion characteristic of PS and LPS classes is highlighted in a red box.

- **Negative mode:** $[M-H]^-$ ions
  - ✓ MS/MS of the precursor at *m/z* 788.5442 [PS(36:1)] in full scan.
  - ✓ MS/MS spectrum showed product ion at *m/z* 701.5069, formed due to neutral loss of 87 Da, which corresponds to the serine from polar head group.
- **Fatty acyl chain confirmed in the negative mode by carboxylate anions:**
  - ✓ It is possible to see the product ion at *m/z* 283.2655, which corresponds to the stearic acid (18:0) (R_1COO^-)
  - ✓ It is possible to see the product ion at *m/z* 281.2496, which corresponds to the oleic acid (18:1) (R_2COO^-)
- **Identification based on MS data:**
  - ✓ PS(36:1); [PS(18:0/18:1)].

SM can be identified by LC-MS in positive ion mode by the ions $[M+H]^+$ and in negative ion mode by the ions $[M+CH_3COO]^-$. The structural features can be inferred by MS/MS in both ion modes. In the MS/MS of the $[M+H]^+$ ions it is possible to see the product ion at *m/z* 184, which corresponds to the phosphocholine polar head group. In the MS/MS spectrum of the $[M+CH_3COO]^-$ ions, which corresponds to a mass shift of plus 58 Da, it is possible to see the product ion at *m/z* 168, corresponding to the demethylated phosphocholine polar head group, and the product ion formed by typical neutral loss of 74 Da, which corresponds to the loss of methyl ethanoate (CH_3COOCH_3). The presence of

these product ions, retention time at which these species were identified and comparison to exact mass measurements allows to confirm the presence of SM molecular species. The identification of SM species, either in the native form or oxidized, was made based only on the exact mass measurements, the fatty acyl chains could not be identified. In Figure 10 it is shown, as an example, the MS and MS/MS spectra of $[SM(d42:3)+O+H]^+$ which is the hydroxyl derivative of SM(d42:3). MS/MS spectra of any other SM species were not found.

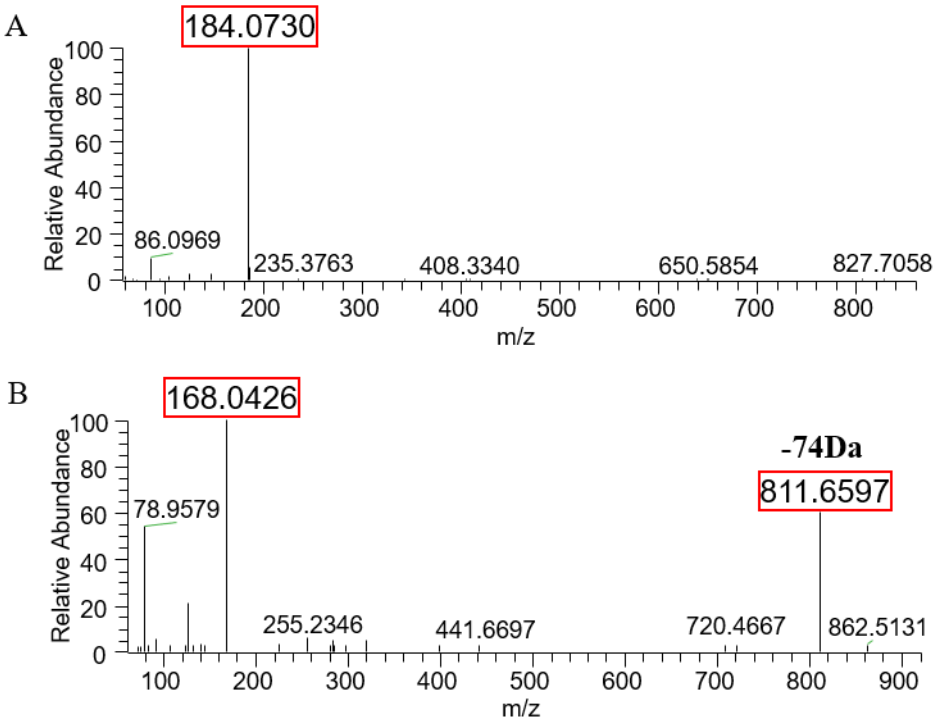

Figure 10. SM identification. **A:** MS/MS spectrum of the $[M+H]^+$ ion of SM(d42:3)+O (*m/z* 827.5322). **B:** MS/MS spectrum of the $[M+CH_3COO]^-$ ions of SM(d42:3)+O (*m/z* 885.5399). Fragment ions characteristic for the SM class are highlighted in a red box.

- **Positive mode:** $[M+H]^+$ ions
  - ✓ MS/MS of the precursor at *m/z* 827.5322 [SM(d42:3)+O] in full scan (A).
  - ✓ MS/MS spectrum showed product ion at *m/z* 184.0730, which corresponds to the phosphocholine polar head group (A).
- **Negative mode:** $[M+CH_3COO]^-$ ions, mass shift of plus 58 Da
  - ✓ MS/MS of the precursor at *m/z* 885.5399 [SM(d42:3)+O] in full scan (B).

- ✓ MS/MS spectrum showed product ions at *m/z* 811.6597, formed due to neutral loss of 74 Da, which corresponds to the loss of CH_3COOCH_3 (B).
- ✓ MS/MS spectrum showed product ions at *m/z* 168.0426 which corresponds to the demethylated phosphocholine polar head group (B).

- **Fatty acyl chain:** could not be identified
- **Identification based on MS data:**
  - ✓ SM(d42:3)+O

8.2 Study of the ox-PL profile of SLE patients

Considering that oxidative stress is a phenomena that is enhanced in SLE and that PL in plasma are an easy target of ROS, we firstly looked for ox-PL in plasma of SLE and control samples.

It was identified 45 different ox-PL species belonging to 7 different classes: PC (with both diacyl and alkyl-acyl species), PE (with both diacyl and alkyl-acyl species), LPC, PG, PI, PS and SM (Table 7 and Table 8). PC, LPC, PE and SM species were identified in the positive mode while PI, PG and PS species were identified in negative mode. PC, LPC and PE species were identified through the exact mass and MS/MS spectra analysis while PG, PI, PS and SM species were identified only through the exact mass.

Table 7. ox-PL molecular species identified in SLE samples by HILIC-MS.

Lipid specie (C:N)	Theoretical *m/z*	Observed *m/z*	Error (ppm)	Fatty acyl chains (C:N)	Formula
PC identified as $[M+H]^+$					
PC(34:2)+2O	790.5598	790.5566	**4.0478**	16:0/18:2+2O	C42H81NO10P
PC(34:2)+O	774.5649	774.5617	**4.1314**	16:0/18:2+O	C42H81NO9P
PC(36:3)+2O	816.5755	816.5721	**4.1637**	16:0/20:3+2O and 18:0/18:3+2O and 18:1/18:2+2O	C44H83NO10P
PC(36:4)+2O	814.5598	814.5568	**3.6830**	16:0/20:4+2O and 16:2+2O/20:2	C44H81NO10P
PC(36:4)+O	798.5649	798.5668	**-2.3793**	16:0/20:4+O and 16:1/20:3+O and 18:1/18:3+O and 18:2/18:2+O	C44H81NO9P
PC(36:5)+2O	812.5442	812.5403	**4.7997**	16:2/20:3+2O	C44H79NO10P
PC(40:4)+O	854.6275	854.6253	**2.5742**	*	C48H89NO9P
PC(40:6)+O	850.5962	850.5925	**4.3499**	*	C48H85NO9P

LPC identified as $[M+H]^+$					
LPC(16:1)+O	510.3196	510.3174	**4.3110**	16:1+O	C24H49NO8P
LPC(18:1)+O	538.3509	538.3491	**3.3435**	18:1+O	C26H53NO8P
LPC(18:2)+2O	552.3301	552.3281	**3.6210**	18:2+2O	C26H51NO9P
LPC(18:2)+O	536.3352	536.3330	**4.1019**	18:2+O	C26H51NO8P
LPC(18:3)+2O	550.3145	550.3121	**4.3611**	18:3+2O	C26H49NO9P
LPC(20:1)+2O	582.3771	582.3744	**4.6362**	*	C28H57NO9P
LPC(20:1)+O	566.3822	566.3837	**-2.6484**	*	C28H57NO8P
LPC(20:4)+O	560.3352	560.3324	**4.9970**	20:4+O	C28H51NO8P
LPC(22:1)+O	594.4135	594.4132	**0.5047**	*	C30H61NO8P
LPC(22:2)+2O	608.3927	608.3901	**4.2736**	*	C30H59NO9P
LPC(22:2)+O	592.3978	592.3994	**-2.7009**	*	C30H59NO8P
LPC(22:6)+O	584.3352	584.3325	**4.6206**	*	C30H51NO8P
LPC(24:1)+2O	638.4397	638.4371	**4.0724**	*	C32H65NO9P
PE identified as $[M+H]^+$					
PE(16:0/C9carboxylic)	624.3877	624.3869	**1.2813**	*	C30H59NO10P
PE(34:1)+O	734.5336	734.5345	**-1.2253**	*	C39H77NO9P
PE(34:2)+2O	748.5129	748.5147	**-2.4048**	16:2+2O/18:0	C39H75NO10P
PE(36:4)+2O	772.5129	772.5104	**3.2362**	16:0/20:4+2O	C41H75NO10P
PE(36:4)+O	756.5179	756.5159	**2.6437**	16:0/20:4+O and 18:1/18:3+O	C41H75NO9P
PE(36:5)+2O	770.4972	770.4953	**2.4659**	*	C41H73O10NP
PE(38:5)+2O	798.5285	798.5251	**4.2578**	*	C43H77NO10P
PE(40:7)+O	806.5336	806.5310	**3.2237**	*	C45H77NO9P
PG identified as $[M-H]^-$					
PG(16:0/C5aldehyde)	581.3091	581.3114	**-3.9566**	*	C27H50O11P
PG(18:0/C5aldehyde)	609.3404	609.3401	**0.4923**	*	C29H54O11P
PG(18:0/C9aldehyde)	665.4030	665.4040	**-1.5028**	*	C33H62O11P
PI identified as $[M-H]^-$					
PI(18:0/C9aldehyde)	717.4190	717.4208	**-2.5090**	*	C33H66O14P
PI(18:0/C9carboxylic)	733.4139	733.4142	**-0.4090**	*	C33H66O15P
PS identified as $[M-H]^-$					
PS(16:0/C9aldehyde)	650.3669	650.3661	**1.2301**	*	C31H57NO11P
PS(18:0/C9aldehyde)	678.3982	678.3957	**3.6852**	*	C33H61NO11P
SM identified as $[M+H]^+$					
SM(d40:3)+O	799.6329	799.6295	**4.2520**	-	C45H88N2O7P

C – carbons; N – number of double bonds; *identified based on exact mass measurements, no FA acyl-chain fragments observed

Table 8. ox-PL molecular species identified in control samples by HILIC-MS.

Lipid specie (C:N)	Theoretical *m/z*	Observed *m/z*	Error (ppm)	Fatty acyl chains (C:N)	Formula
PC identified as $[M+H]^+$					
PC(32:1)+O	748.5492	748.5458	**4.5421**	*	C40H79NO9P
PC(34:2)+2O	790.5598	790.5592	**0.7590**	16:0/18:2+2O	C42H81NO10P
PC(34:2)+O	774.5649	774.5631	**2.3239**	16:0/18:2+O	C42H81NO9P
PC(36:2)+2O	818.5911	818.5903	**0.9773**	*	C44H85NO10P
PC(36:2)+O	802.5962	802.5927	**4.3608**	16:0/20:2+O and 18:0/18:2+O	C44H85NO9P
PC(36:3)+2O	816.5755	816.5761	**-0.7348**	16:0/20:3+2O and 18:0/18:3+2O and 18:1/18:2+2O	C44H83NO10P
PC(36:4)+2O	814.5598	814.5568	**3.6830**	16:0/20:4+2O and 16:2+2O/20:2	C44H81NO10P
PC(36:4)+O	798.5649	798.5629	**2.5045**	16:0/20:4+O and 18:1/18:3+O and 18:2/18:2+O	C44H81NO9P
PC(36:5)+2O	812.5442	812.5412	**3.6921**	16:2/20:3+2O	C44H79NO10P
PC(40:4)+O	854.6275	854.6279	**-0.4680**	*	C48H89NO9P
PC(40:6)+O	850.5962	850.5926	**4.2323**	*	C48H85NO9P
LPC identified as $[M+H]^+$					
LPC(16:1)+O	510.3196	510.3176	**3.9191**	16:1+O	C24H49NO8P
LPC(18:1)+O	538.3509	538.3497	**2.2290**	18:1+O	C26H53NO8P
LPC(18:2)+2O	552.3301	552.3286	**2.7158**	18:2+2O	C26H51NO9P
LPC(18:2)+O	536.3352	536.3335	**3.1697**	18:2+O	C26H51NO8P
LPC(18:3)+2O	550.3145	550.3135	**1.8171**	18:3+2O	C26H49NO9P
LPC(20:1)+2O	582.3771	582.3766	**0.8586**	*	C28H57NO9P
LPC(20:1)+O	566.3822	566.3800	**3.8843**	*	C28H57NO8P
LPC(20:4)+O	560.3352	560.3329	**4.1047**	20:4+O	C28H51NO8P
LPC(20:5)+2O	574.3145	574.3137	**1.3930**	*	C28H49NO9P
LPC(20:5)+O	558.3196	558.3186	**1.7911**	*	C28H49NO8P
LPC(22:6)+O	584.3352	584.3333	**3.2516**	*	C30H51NO8P
LPC(24:1)+2O	638.4397	638.4392	**0.7832**	*	C32H65NO9P
LPC(24:1)+O	622.4448	622.4446	**0.3213**	*	C32H65NO8P
PE identified as $[M+H]^+$					
PE(18:0/C9carboxylic)	652.4190	652.4179	**1.6860**	*	C32H63NO10P
PE(34:2)+2O	748.5129	748.5140	**-1.4696**	16:2+2O/18:0	C39H75NO10P
PE(36:4)+O	756.5179	756.5176	**0.3966**	*	C41H75NO9P
PE(38:4)+O	784.5492	784.5483	**1.1472**	*	C43H79NO9P
PE(40:7)+O	806.5336	806.5342	**-0.7439**	*	C45H77NO9P
PS identified as $[M-H]^-$					
PS(18:0/C9aldehyde)	678.3982	678.3971	**1.6215**	18:0	C33H61NO11P

PS(18:0/C9carboxylic)	694.3931	694.3923	**1.1521**	*	C33H61NO12P

C – carbons; N – number of double bonds; *identified based on exact mass measurements, no FA acyl-chain fragments observed

Due to its lower abundance, the identification of the fatty acyl chains was not possible for every ox-PL species. In such cases, its identification was made by the exact mass measurements. It was expected the identification of more ox-PL species in SLE samples due to the higher oxidative stress conditions that develop during this disease, however, the number of ox-PL determined was similar between categories. The total number of oxidized species identified in SLE samples were 37 while in control samples were 31.

Table 9 summarizes which ox-PL species are present in which condition to better understand what is the variability of the oxidized species between groups.

Table 9. Comparison of the ox-PL species identified in each condition. ✖ - not identified; ✓- identified.

Ox-PL species	SLE	Control
PC(34:2)+2O	✓	✓
PC(34:2)+O	✓	✓
PC(36:2)+2O	✖	✓
PC(36:2)+O	✖	✓
PC(36:3)+2O	✓	✓
PC(36:4)+2O	✓	✓
PC(36:4)+O	✓	✓
PC(36:5)+2O	✓	✓
PC(40:4)+O	✓	✓
PC(40:6)+O	✓	✓
LPC(16:1)+O	✓	✓
LPC(18:1)+O	✓	✓
LPC(18:2)+2O	✓	✓
LPC(18:2)+O	✓	✓
LPC(18:3)+2O	✓	✓
LPC(20:1)+2O	✓	✓
LPC(20:1)+O	✓	✓

LPC(20:4)+O	✓	✓
LPC(20:5)+2O	✗	✓
LPC(20:5)+O	✗	✓
LPC(22:1)+O	✓	✗
LPC(22:2)+2O	✓	✗
LPC(22:2)+O	✓	✗
LPC(22:6)+O	✓	✓
LPC(24:1)+2O	✓	✓
LPC(24:1)+O	✗	✓
PE(16:0/C9carboxylic)	✓	✗
PE(18:0/C9carboxylic)	✗	✓
PE(34:1)+O	✓	✗
PE(34:2)+2O	✓	✓
PE(36:4)+2O	✓	✗
PE(36:4)+O	✓	✓
PE(36:5)+2O	✓	✗
PE(38:4)+O	✗	✓
PE(38:5)+2O	✓	✗
PE(40:7)+O	✓	✓
PG(16:0/C5aldehyde)	✓	✗
PG(18:0/C5aldehyde)	✓	✗
PG(18:0/C9aldehyde)	✓	✗
PI(18:0/C9aldehyde)	✓	✗
PI(18:0/C9carboxylic)	✓	✗
PS(16:0/C9aldehyde)	✓	✗
PS(18:0/C9aldehyde)	✓	✓
PS(18:0/C9carboxylic)	✗	✓
SM(d40:3)+O	✓	✗

On one side, there are several ox-PL species that were identified in both conditions. Those lipid species were quantified by integration of the chromatographic peaks and it was performed statistical analysis to see if there were significant differences between the two

conditions. On the other side, there are also some ox-PL species that are only present in one group. In the SLE category it was not identified LPC(20:5)+O, which is an ox-PL with an omega-3 FA, and PS(18:0/C9carboxylic), while in the control group the quantity of ox-PL that were not identified when compared with SLE was considerably higher. LPC(22:1)+O; LPC(22:2)+2O; LPC(22:2)+O; PE(16:0/C9carboxylic); PE(34:1)+O PE(36:4)+2O; PE(36:5)+2O; PE(38:5)+2O; PS(16:0/C9aldehyde) and all ox-PG and ox-PI species were identified solely in SLE and not identified in control samples. Those species could be formed due to the enhanced ROS production and elevated oxidation levels in SLE disease. They could also be contributors to pro-inflammatory signalling in SLE. Further studies are needed to understand these findings and to evaluate if these could be used as biomarkers of this disease or relapsing episodes.

Multivariate analysis showed, by PCA scores scatter plots (Figure 11), that all SLE and control samples tended to cluster away from each other (both in positive and negative modes), revealing to be a good model for discriminating these two conditions.

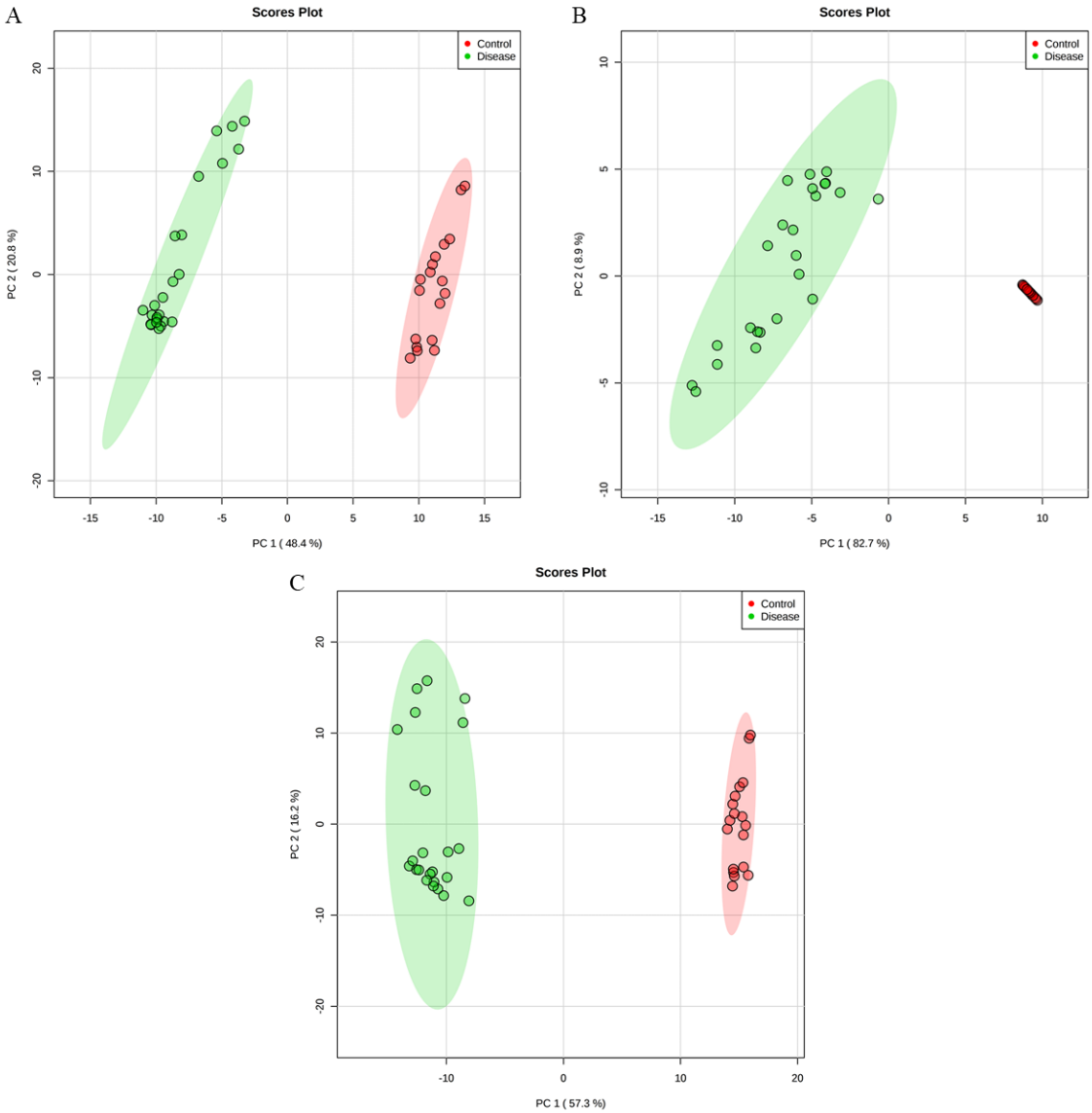

Figure 11. Multivariate analysis of HILIC-MS oxidized data collected from SLE and control samples. **A:** PCA scores scatter plot of ox-PL in positive mode. **B:** PCA scores scatter plot of ox-PL in negative mode. **C:** PCA scores scatter plot of ox-PL in both positive and negative modes.

Univariate analysis shows the top 20 variables that reflect the most important oxidized lipid species that differentiate disease from healthy controls and are represented in individual heatmaps (for both positive and negative modes), shown in Figure 12.

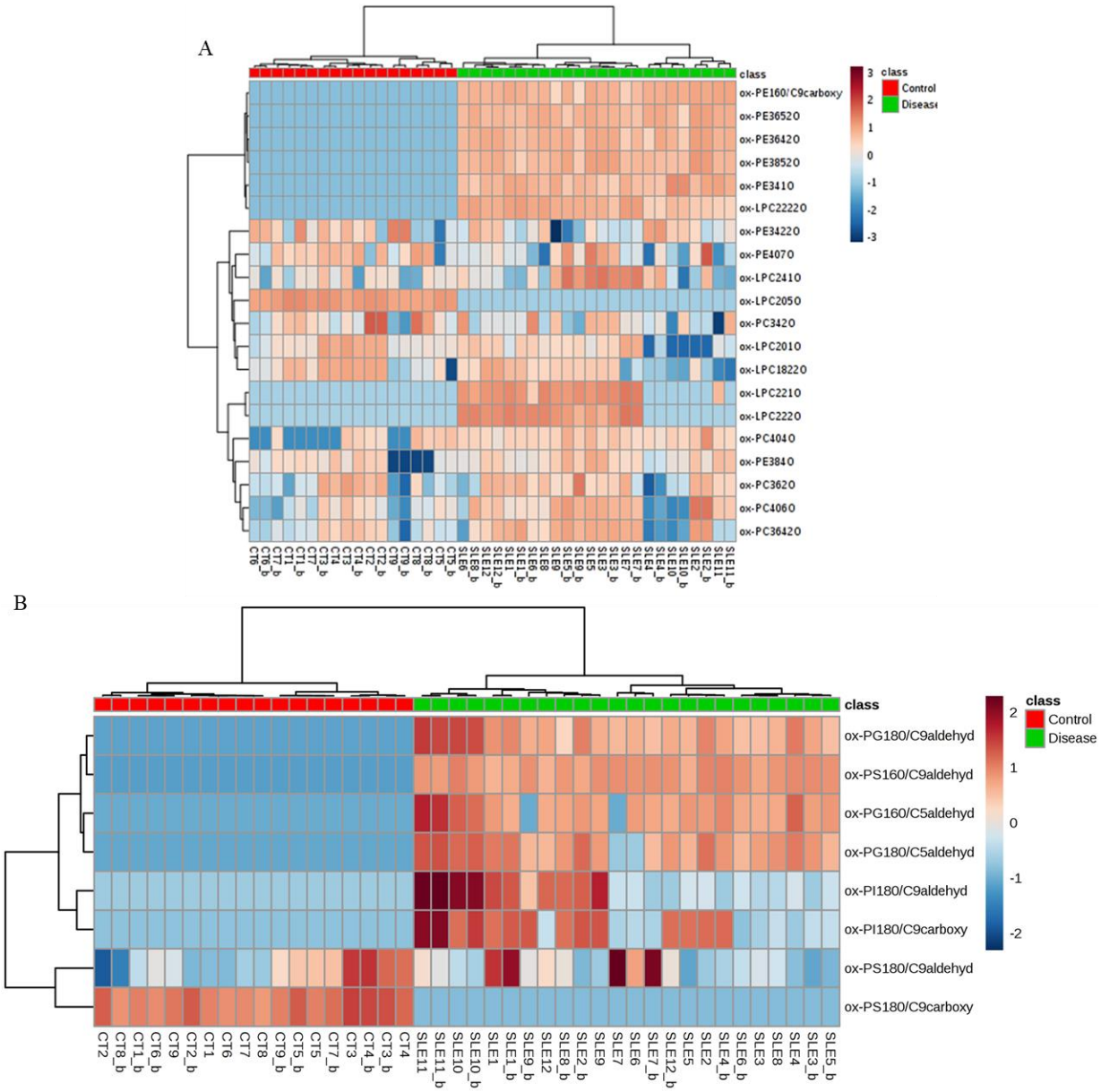

Figure 12. Univariate and clustering analysis considering the top 20 most discriminatory ox-PL species **A:** Heatmap of ox-PL in positive mode. **B:** Heatmap of ox-PL in negative mode.

The univariate and clustering analysis considering the top 20 most discriminatory ox-PL species, illustrated in Figure 12, showed that in the positive mode (Figure 12-A), oxidized LPC is an important class to discriminate disease from controls contributing with seven species. LPC(22:2)+2O; LPC(22:1)+O and LPC(22:2)+O are significantly increased in SLE while LPC(20:5)+O has the opposite behaviour, being considerably higher in the control group. LPC(24:1)+O shows a tendency to be increased in SLE samples, meanwhile

LPC(20:1)+O and LPC(18:2)+2O showed to be higher in controls. However, it is important to notice that these species have intragroup variability. The species that most contribute to differentiate SLE from control samples are hydroperoxyl derivatives (PL+2O) of PE species, which are significantly upregulated in the disease category. Oxidized PE species [PE(16:0/C9carboxylic), PE(36:5)+2O, PE(36:4)+2O, PE(38:5)+2O and PE(34:1)+O] are markedly increased in disease samples, which reflects the conditions of oxidative damage in SLE patients. Colombo *et al* found that oxidized PE have a pro-inflammatory effect in inflammatory diseases characterized by oxidative stress[149]. Since SLE is characterized by constant inflammation, it was expected that the species that promote inflammatory responses (oxidized PE) were upregulated, which is in agreement with the results obtained in our study. On the other hand, that same study also found that oxidized PS have an anti-inflammatory effect in peripheral blood immune cells[149]. In our work, the univariate analysis in the negative mode (Figure 12-B) revealed that PS(18:0/C9aldehyde) and PS(18:0/C9carboxylic) are significantly decreased in SLE samples. This means that patients have less of these anti-inflammatory species to regulate inflammation. However, PS(16:0/C9aldehyde) is markedly increased in the disease category, revealing the necessity of more studies with a higher quantity of samples to confirm the contradictory results. In the negative mode it was also revealed a significant increase of oxidized PI [PI(18:0/C9aldehyde) and PI(18:0/C9carboxylic)] and oxidized PG [PG(18:0/C9aldehyde); PG(16:0/C5aldehyde) and PG(18:0/C5aldehyde)] species which have not been yet reported in the literature.

As there is a considerable variability in the disease and control groups, we refined our analysis by clustering the samples into 3 different categories: healthy controls, active SLE and inactive SLE. For that, it was analysed the raw data of a previous study with SLE patients[150], searching for ox-PL, something that the previous work did not look at. There were 10 SLE samples (active, n=5; inactive, n=5) and 5 healthy control samples that were collected from Centro Hospitalar e Universitário de Coimbra. There was no other information regarding those samples. Lipid extraction and phosphorus measurements followed the same protocols described in chapters 7.3 and 7.4. respectively. PL were separated according to the polarity of the head of the substituent group through HILIC using an *Ascentis Si HPLC Pore* (15 cm x 1.0 mm, 3 μm*; Sigma-Aldrick*) inserted in a

HPLC system *HPLC Thermo Accela,* with an autosampler coupled online to the *Q-Exactive Orbitrap*™ mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).

The detailed explanation on how the identification of the most abundant species from each PL class was made is the same as described in full detail in chapter 8.1.2. It was identified 40 ox-PL species belonging to 5 different classes: PC (with both diacyl and alkyl-acyl species), PE (with both diacyl and alkyl-acyl species), PI, PS and SM (Table 10). PC, PE and SM species were identified in the positive mode while PI and PS species were identified in negative mode. PC and PE species were identified through the exact mass and MS/MS spectra analysis while PI, PS and SM species were identified only through the exact mass.

Table 10. Oxidized PL molecular species identified in the clustered samples: active SLE; inactive SLE; healthy controls.

Lipid specie (C:N)	Theoretical *m/z*	Observed *m/z*	Error (ppm)	Fatty acyl chains (C:N)	Formula
PC identified as $[M+H]^+$					
★PC(16:0/C5aldehyde)	594.3771	594.3754	**2.8601**	*	C29H57NO9P
★PC(16:0/C9carboxylic)	666.4346	666.4323	**3.4512**	*	C33H65NO10P
★PC(18:0/C5carboxylic)	638.4033	638.4018	**2.3496**	*	C31H61NO10P
★PC(18:0/C9aldehyde)	678.4710	678.4739	**-4.2743**	*	C35H69NO9P
★PC(32:1)+O	748.5492	748.5457	**4.6757**	16:0/16:1+O	C40H79NO9P
★PC(34:1)+O	776.5805	776.5788	**2.1891**	16:0/18:1+O	C42H83NO9P
PC(34:2)+2O	790.5598	790.5582	**2.0239**	*	C42H81NO10P
PC(34:2)+O	774.5649	774.5634	**1.9366**	16:0/18:2+O	C42H81NO9P
PC(36:2)+O	802.5962	802.5938	**2.9903**	18:0/18:2+O	C44H85NO9P
PC(36:3)+2O	816.5755	816.5735	**2.4493**	16:0/20:3+2O and 18:0/18:3+2O	C44H83NO10P
PC(36:4)+2O	814.5598	814.5583	**1.8415**	18:1/18:3+2O	C44H81NO10P
PC(36:4)+O	798.5649	798.5633	**2.0036**	16:0/20:4+O	C44H81NO9P
PC(36:5)+2O	812.5442	812.5432	**1.2307**	16:0/20:5+2O and 16:2+2O/20:3 and 18:2/18:3+2O	C44H79NO10P
★PC(36:6)+2O	810.5285	810.5269	**1.9740**	16:2/20:4+2O	C44H77NO10P
★PC(36:6)+O	794.5336	794.5313	**2.8948**	*	C44H77NO9P
PC(40:4)+O	854.6275	854.6257	**2.1062**	18:0/22:4+O	C48H89NO9P
★PC(40:6)+2O	866.5911	866.5903	**0.9232**	*	C48H85NO10P
PC(40:6)+O	850.5962	850.5941	**2.4689**	18:0/22:6+O	C48H85NO9P
PE identified as $[M+H]^+$					

★PE(18:0/C5aldehyde)	580.3614	580.3610	**0.6892**	*	C28H55NO9P
★PE(34:1)+2O	750.5285	750.5286	**-0.1332**	*	C39H77NO10P
PE(34:2)+2O	748.5129	748.5138	**-1.2024**	16:2+2O/18:0	C39H75NO10P
★PE(36:2)+2O	776.5442	776.5451	**-1.1590**	*	C41H79O10NP
PE(36:4)+2O	772.5129	772.5138	**-1.1650**	16:0/20:4+2O and 16:1/20:3+2O and 16:2/20:2+2O and 18:1+2O/18:3 and 18:2/18:2+2O	C41H75NO10P
PE(36:4)+O	756.5179	756.5180	**-0.1322**	16:0/20:4+O and 18:1/18:3+O	C41H75NO9P
PE(36:5)+2O	770.4972	770.4974	**-0.2596**	16:1+2O/20:4 and 16:2+2O/20:3 and 18:2/18:3+2O	C41H73O10NP
★PE(38:3)+2O	802.5598	802.5586	**1.4952**	*	C43H81NO10P
★PE(38:4)+2O	800.5442	800.5441	**0.1249**	16:2/22:2+2O and 18:0/20:4+2O	C43H79NO10P
PE(38:4)+O	784.5492	784.5472	**2.5492**	18:0/20:4+O and 18:1/20:3+O	C43H79NO9P
PE(38:5)+2O	798.5285	798.5262	**2.8803**	*	C43H77NO10P
★PE(38:5)+O	782.5336	782.5329	**0.8945**	*	C43H77NO9P
★PE(40:4)+2O	828.5755	828.5755	**0.0000**	*	C45H83NO10P
★PE(40:5)+2O	826.5598	826.5561	**4.4764**	20:1+2O/20:4	C45H81O10NP
★PE(40:8)+2O	820.5129	820.5148	**-2.3156**	*	C45H75O10NP
★PE(40:8)+O	804.5179	804.5171	**0.9944**	18:2/22:6+O	C45H75O9NP
★PE(42:10)+O	828.5179	828.5184	**-0.6035**	*	C47H75O9NP
PI identified as $[M-H]^-$					
★PI(34:2)+2O	865.5078	865.5036	**4.8526**	*	C43H78O15P
PS identified as $[M-H]^-$					
★PS(40:6)+O	850.5234	850.5222	**1.4109**	*	C46H77NO11P
SM identified as $[M+H]^+$					
SM(d40:3)+O	799.6329	799.6322	**0.8754**	-	C45H88N2O7P
★SM(d42:1)+O	831.6955	831.6932	**2.7654**	-	C47H96N2O7P
★SM(d42:3)+O	827.6642	827.6617	**3.0205**	-	C47H92N2O7P

C – carbons; N – number of double bonds; *identified based on exact mass measurements, no FA acyl-chain fragments observed

Ox-PL species marked with ★ were not identified in the previous set of samples (disease/controls). The remaining species are common to both sets of samples. The species of the LPC and PG classes were not identified in this set of samples (active SLE/inactive SLE/controls).

A multivariate analysis was applied to understand the behaviour of the ox-PL species present in the clustered samples. Since we could only identify two ox-PL species in the negative mode, the statistical analysis of both modes was performed at the same time, unstead of being performed seperatly as in the previous set of samples. PCA scores scatter plot did not show a good discriminant ability, therefore, to maximize separation between pre-defined classes, it was performed PLS-DA (Figure 13-A) which showed a mild discriminant ability where the three groups cluster away from each other however with some dispersion of values. Univariate analysis shows the top 20 variables that reflect the most important oxidized lipid species that differentiate the three categories and is represented in a heatmap (for both positive and negative modes), shown in Figure 13-B.

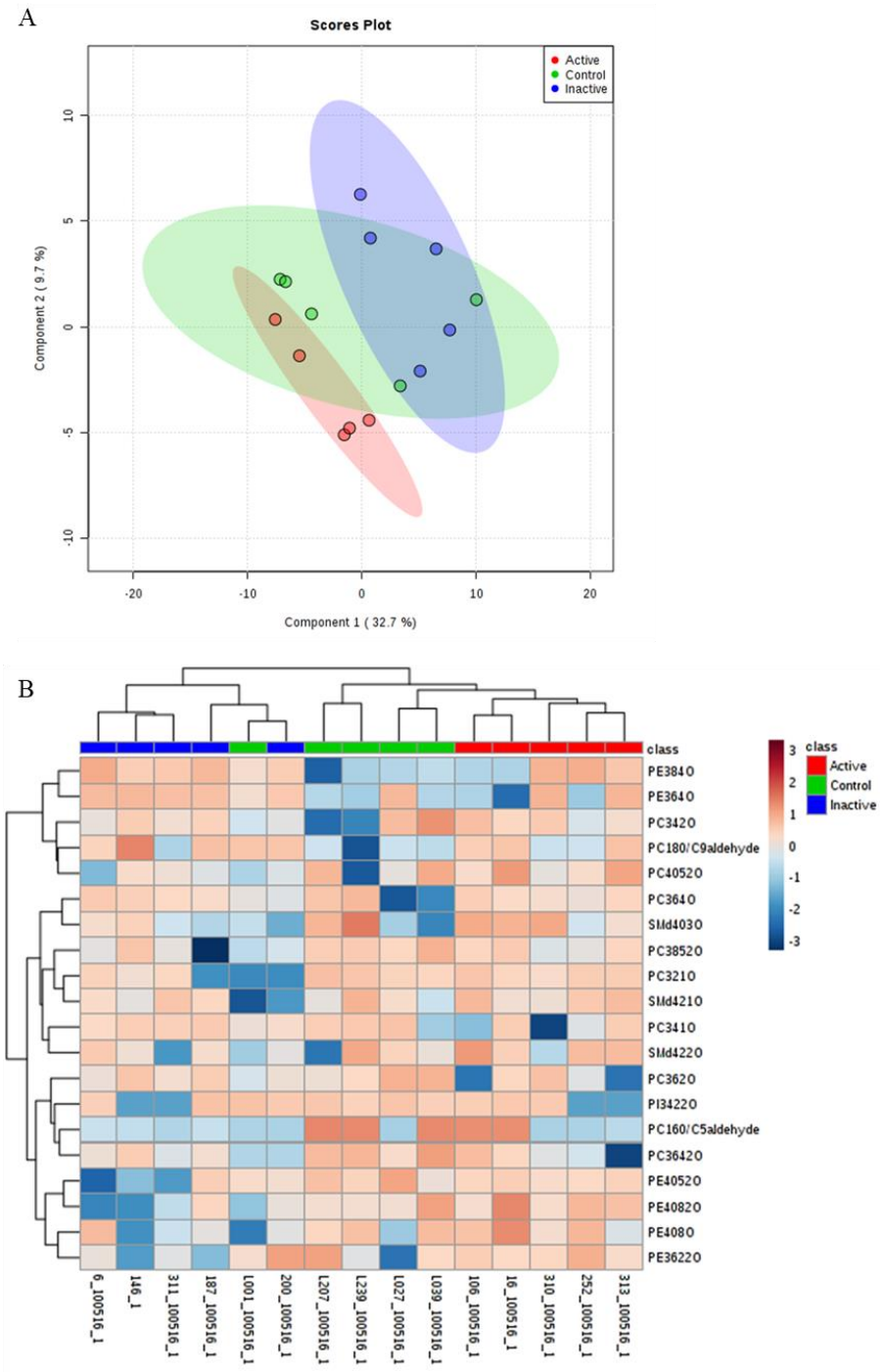

Figure 13. A: Multivariate analysis of HILIC-MS oxidized data. PLS-DA scores scatter plot of ox-PL in both positive and negative modes. **B:** Univariate and clustering analysis considering the top 20 most discriminatory ox-PL species from the samples clustered in active SLE (red), inactive SLE (blue) and healthy controls (green). Heatmap of both modes to discriminate the three study groups.

The univariate and clustering analysis considering the top 20 most discriminatory ox-PL species, illustrated in Figure 13-B, revealed that the three sample categories show a tendency to cluster away from each other, mainly active SLE and inactive SLE. PC is the most abundant class of PL present in cell membranes and in plasma, therefore it would be expected to identify higher quantities of PC species rather than any other class of PL[151]. Oxidized PC is the most discriminatory class contributing with ten different species with the addition of one/two O atoms and short chain products, PC(18:0/C9aldehyde); PC(34:2)+O; PC(40:5)+2O; PC(36:4)+O; PC(38:5)+2O; PC(32:1)+O; PC(34:1)+O; PC(36:2)+O; PC(16:0/C5aldehyde) and PC(36:4)+2O. PE is the second most abundant class of PL and in this case is the second most discriminatory class to differentiate the three groups with six oxidized species (hydroxy and hydroperoxyl derivatives), PE(38:4)+O; PE(36:4)+O; PE(40:5)+2O; PE(40:8)+2O; PE(40:8)+O and PE(36:2)+2O. Oxidized PE species are present in all groups (controls, active and inactive SLE) with significant increase in active SLE, revealing its pro-inflammatory activity in accordance with the literature[149]. These results are also in accordance with data obtained from the previous data set reported above. In inactive SLE, most oxidized PE species showed lower abundance such as the case of [PE(40:5)+2O; PE(40:8)+2O; PE(40:8)+O and PE(36:2)+2O]. This could be due to lower inflammatory response in the inactive form of SLE disease, therefore associated with lower concentration of pro-inflammatory molecules. This effect is also most probably due to the therapeutics that are used in SLE. Antimalarial drugs are stabilizers of the immune system, also reducing inflammation. Corticosteroids are used as a powerful anti-inflammatory drug, however, they also promote energy production, functioning as a lipid lowering drug as well. Immunosuppressants are used to weaken the immune system, thus lowering the autoimmune inflammatory response. This way, a possible cause for this reduction of oxidized PE species in inactive SLE group may be the treatment administrated to SLE patients that controls inflammatory processes (anti-inflammatory, antimalarial, corticosteroids and immunosuppressant drugs) and regulate lipid metabolism (corticosteroids that act as lipid-lowering treatment for dyslipoproteinemia). Oxidized SM species identified are present in all three groups however with different concentrations. The control group shows significant and inconsistent variations between samples, which could be due to the number of analysed samples (n=5). Oxidized SM relative abundances in inactive SLE are markedly lower than

in active SLE. PI(34:2)+2O is present in control samples and in the active and inactive SLE categories undergo intragroup variability. There are no previous reports in the literature regarding these ox-PL species, therefore, more studies are needed to unveil the behaviour of these biomolecules.

8.3 Study of the lipid profile of SLE patients

Since ox-PL are degraded by phospholipase A_2 to form LPL and as lipid metabolism is regulated in response to inflammation, we evaluated the change in the non-oxidized lipid profile. Once the main focus of this work is the study of the oxidized lipid profile, the analysis of the PL in their native form was performed only on the two initial groups under study (SLE disease/controls).

It was identified 120 different PL species belonging to 10 different classes: PC (with both diacyl and alkyl-acyl species), PE (with both diacyl and alkyl-acyl species), LPC, LPE, PG, LPG, PI, LPI, PS and SM (Table 11 and Table 12). PC, LPC, PE, LPE and SM species were identified in the positive mode while PI, LPI, PG, LPG and PS species were identified in negative mode. PC, LPC, PE and LPE species were identified through the exact mass and MS/MS spectra analysis while PG, LPG, PI, LPI, PS and SM species were identified only through the exact mass.

Table 11. Native PL molecular species identified in SLE samples by HILIC-MS.

Lipid specie (C:N)	Theoretical *m/z*	Observed *m/z*	Error (ppm)	Fatty acyl chains (C:N)	Formula
PC identified as $[M+H]^+$					
PC(28:0)	678.5074	678.5046	**4.1267**	14:0/14:0	C36H73NO8P
PC(30:0)	706.5387	706.5358	**4.1045**	14:0/16:0	C38H77NO8P
PC(30:1)	704.5230	704.5199	**4.4001**	*	C38H75NO8P
PC(32:1)	732.5543	732.5510	**4.5048**	14:0/ 18:1 and 16:0/16:1	C40H79NO8P
PC(32:2)	730.5387	730.5362	**3.4221**	14:0/18:2	C40H77NO8P
PC(34:2)	758.5700	758.5671	**3.8230**	16:0/18:2	C42H81NO8P
PC(34:3)	756.5543	756.5513	**3.9653**	14:0/20:3 and 16:0/18:3 and 16:1/18:2	C42H79NO8P
PC(36:2)	786.6013	786.5977	**4.5767**	16:0/20:2 and 18:0/18:2 and 18:1/18:1	C44H85NO8P
PC(36:3)	784.5856	784.5817	**4.9708**	16:0/20:3 and 18:1/18:2	C44H83NO8P
PC(36:4)	782.5700	782.5665	**4.4724**	16:0/20:4 and 16:1/20:3 and 18:2/18:2	C44H81NO8P

PC(38:4)	810.6013	810.5976	**4.5645**	16:0/22:4 and 18:0/20:4 and 18:1/20:3 and 18:2/20:2	C46H85NO8P
PC(38:6)	806.5700	806.5665	**4.3394**	16:0/22:6 and 18:2/20:4	C46H81NO8P
PC(40:4)	838.6326	838.6312	**1.6694**	18:0/22:4	C48H89NO8P
LPC identified as $[M+H]^+$					
LPC(16:0)	496.3403	496.3382	**4.2310**	16:0	C24H51NO7P
LPC(16:1)	494.3247	494.3226	**4.2482**	16:1	C24H49NO7P
LPC(18:0)	524.3716	524.3695	**4.0048**	18:0	C26H55NO7P
LPC(18:1)	522.3560	522.3540	**3.8288**	18:1	C26H53NO7P
LPC(18:2)	520.3403	520.3384	**3.6515**	18:2	C26H51NO7P
LPC(20:0)	552.4029	552.4004	**4.5257**	20:0	C28H59NO7P
LPC(20:1)	550.3873	550.3848	**4.5423**	20:1	C28H57NO7P
LPC(20:2)	548.3716	548.3691	**4.5590**	20:2	C28H55NO7P
LPC(20:4)	544.3403	544.3388	**2.7556**	20:4	C28H51NO7P
LPC(22:1)	578.4186	578.4172	**2.4204**	*	C30H61NO7P
LPC(22:2)	576.4029	576.4029	**0.0000**	*	C30H59NO7P
LPC(22:4)	572.3716	572.3689	**4.7172**	22:4	C30H55NO7P
LPC(22:5)	570.3560	570.3539	**3.6819**	*	C30H53NO7P
LPC(22:6)	568.3403	568.3379	**4.2228**	22:6	C30H51NO7P
LPC(24:1)	606.4487	606.4477	**1.6489**	*	C32H65NO7P
PE identified as $[M+H]^+$					
PE(28:2)	632.4291	632.4304	**-2.0556**	*	C33H63NO8P
PE(32:1)	690.5074	690.5043	**4.4895**	16:0/16:1	C37H73NO8P
PE(34:2)	716.5230	716.5197	**4.6056**	16:0/18:2	C39H75NO8P
PE(34:3)	714.5074	714.5048	**3.6389**	16:0/18:3 and 16:1/18:2	C39H73O8NP
PE(34:4)	712.4917	712.4926	**-1.2632**	*	C39H71NO8P
PE(36:4)	740.5230	740.5197	**4.4563**	16:0/20:4 and 18:2/18:2	C41H75NO8P
PE(36:5)	738.5074	738.5063	**1.4895**	*	C41H73O8NP
PE(38:4)	768.5543	768.5514	**3.7733**	18:0/20:4	C43H79O8NP
PE(38:6)	764.5230	764.5198	**4.1856**	16:0/22:6 and 18:1/20:5 and 18:2/20:4	C43H75O8NP
PE(38:7)	762.5074	762.5071	**0.3934**	*	C43H73NO8P
PE(40:6)	792.5543	792.5509	**4.2899**	18:0/22:6	C45H79O8NP
PE(40:7)	790.5387	790.5348	**4.9333**	18:1/22:6	C45H77NO8P
PE(42:10)	812.5230	812.5243	**-1.6000**	*	C47H75O8NP
PE(48:12)	892.5856	892.5816	**4.4814**	*	C53H83O8NP
LPE identified as $[M+H]^+$					
LPE(14:1)	424.2464	424.2477	**-3.0643**	*	C19H39NO7P
LPE(16:0)	454.2934	454.2913	**4.6226**	16:0	C21H45NO7P
LPE(18:0)	482.3247	482.3228	**3.9393**	18:0	C23H49NO7P
LPE(18:1)	480.3090	480.3071	**3.9558**	*	C23H47NO7P

LPE(18:2)	478.2934	478.2913	**4.3906**	18:2	C23H45NO7P
LPE(20:5)	500.2777	500.2766	**2.1988**	20:5	C25H43NO7P
LPE(22:6)	526.2934	526.2910	**4.5602**	22:6	C27H45NO7P
PG identified as $[M-H]^-$					
PG(32:2)	717.4707	717.4714	**-0.9756**	*	C38H70O10P
PG(34:2)	745.5020	745.5018	**0.2683**	*	C40H74O10P
PG(40:7)	819.5176	819.5207	**-3.7827**	*	C46H76O10P
LPG identified as $[M-H]^-$					
LPG(12:0)	427.2097	427.2094	**0.7022**	*	C18H36O9P
LPG(14:0)	455.2410	455.2433	**-5.0523**	14:0	C20H40O9P
LPG(20:4)	531.2723	531.2738	**-2.8234**	*	C26H44O9P
LPG(24:1)	593.3818	593.3846	**-4.7187**	*	C30H58O9P
LPG(24:2)	591.3662	591.3691	**-4.9039**	*	C30H56O9P
LPG(24:3)	589.3505	589.3513	**-1.3574**	*	C30H54O9P
PI identified as $[M-H]^-$					
PI(32:1)	807.5024	807.5024	**0.0000**	*	C41H76O13P
PI(44:5)	967.6276	967.6271	**0.5167**	*	C53H92O13P
LPI identified as $[M-H]^-$					
LPI(18:0)	599.3196	599.3204	**-1.3348**	18:0	C27H52O12P
LPI(20:1)	625.3353	625.3368	**-2.3987**	*	C29H54O12P
LPI(24:1)	681.3979	681.3985	**-0.8805**	*	C33H62O12P
LPI(24:2)	679.3822	679.3834	**-1.7663**	*	C33H60O12P
PS identified as $[M-H]^-$					
PS(36:0)	790.5598	790.5586	**1.5179**	*	C42H81NO10P
PS(36:1)	788.5442	788.5430	**1.5218**	*	C42H79NO10P
PS(38:1)	816.5755	816.5747	**0.9797**	*	C44H83NO10P
PS(38:2)	814.5598	814.5598	**0.0000**	*	C44H81NO10P
PS(40:1)	844.6068	844.6047	**2.4864**	*	C46H87NO10P
PS(40:2)	842.5911	842.5884	**3.2044**	*	C46H85NO10P
SM identified as $[M+H]^+$					
SM(d30:1)	647.5128	647.5103	**3.8609**	-	C35H72N2O6P
SM(d32:1)	675.5441	675.5413	**4.1448**	-	C37H76N2O6P
SM(d32:2)	673.5285	673.5257	**4.1572**	-	C37H74N2O6P
SM(d34:1)	703.5754	703.5722	**4.5482**	-	C39H80N2O6P
SM(d34:2)	701.5598	701.5563	**4.9889**	-	C39H78N2O6P
SM(d36:1)	731.6067	731.6033	**4.6473**	-	C41H84N2O6P
SM(d36:2)	729.5911	729.5876	**4.7972**	-	C41H82N2O6P
SM(d38:2)	757.6224	757.6192	**4.2237**	-	C43H86N2O6P
SM(d38:3)	755.6067	755.6031	**4.7644**	-	C43H84N2O6P
SM(d40:2)	785.6537	785.6498	**4.9640**	-	C45H90N2O6P
SM(d42:3)	811.6693	811.6657	**4.4353**	-	C47H92N2O6P

C – carbons; N – number of double bonds; *identified based on exact mass measurements, no FA acyl-chain fragments observed

Table 12. Native PL molecular species identified in control samples by HILIC-MS.

Lipid specie (C:N)	Theoretical *m/z*	Observed *m/z*	Error (ppm)	Fatty acyl chains (C:N)	Formula
PC identified as $[M+H]^+$					
PC(28:0)	678.5074	678.5058	**2.3581**	14:0/14:0	C36H73NO8P
PC(30:0)	706.5387	706.5362	**3.5384**	14:0/16:0	C38H77NO8P
PC(30:1)	704.5230	704.5225	**0.7097**	*	C38H75NO8P
PC(32:0)	734.5700	734.5665	**4.7647**	16:0/16:0	C40H81NO8P
PC(32:1)	732.5543	732.5516	**3.6857**	14:0/ 18:1 and 16:0/16:1	C40H79NO8P
PC(32:2)	730.5387	730.5372	**2.0533**	14:0/18:2	C40H77NO8P
PC(34:1)	760.5856	760.5820	**4.7332**	16:0/18:1	C42H83NO8P
PC(34:2)	758.5700	758.5675	**3.2957**	16:0/18:2	C42H81NO8P
PC(34:3)	756.5543	756.5521	**2.9079**	14:0/20:3 and 16:0/18:3 and 16:1/18:2	C42H79NO8P
PC(34:4)	754.5387	754.5358	**3.8434**	*	C42H77NO8P
PC(36:2)	786.6013	786.5983	**3.8139**	16:0/20:2 and 18:0/18:2 and 18:1/18:1	C44H85NO8P
PC(36:3)	784.5856	784.5822	**4.3335**	16:0/20:3 and 18:1/18:2	C44H83NO8P
PC(36:4)	782.5700	782.5680	**2.5557**	16:0/20:4 and 16:1/20:3 and 18:2/18:2	C44H81NO8P
PC(36:5)	780.5543	780.5508	**4.4840**	16:0/20:5 and 16:1/20:4	C44H79NO8P
PC(38:3)	812.6169	812.6162	**0.8614**	16:0/22:3 and 18:0/20:3 and 18:1/20:2 and 18:2/20:1	C46H87NO8P
PC(38:4)	810.6013	810.5980	**4.0711**	16:0/22:4 and 18:0/20:4 and 18:1/20:3 and 18:2/20:2	C46H85NO8P
PC(38:5)	808.5856	808.5839	**2.1024**	16:0/22:5	C46H83NO8P
PC(38:6)	806.5700	806.5680	**2.4796**	16:0/22:6 and 18:2/20:4	C46H81NO8P
PC(38:8)	802.5387	802.5376	**1.3707**	*	C46H77NO8P
PC(40:4)	838.6326	838.6285	**4.8889**	*	C48H89NO8P
PC(40:5)	836.6169	836.6158	**1.3148**	*	C48H87NO8P
PC(40:6)	834.6013	834.5980	**3.9540**	18:0/22:6	C48H85NO8P
PC(40:7)	832.5856	832.5844	**1.4413**	18:1/22:6 and 20:3/20:4	C48H83NO8P
PC(40:8)	830.5699	830.5675	**2.8896**	18:2/22:6	C48H81NO8P
PC(42:6)	862.6326	862.6301	**2.8981**	*	C50H89NO8P
PC(42:7)	860.6169	860.6160	**1.0458**	*	C50H87NO8P
PC(44:12)	878.5700	878.5658	**4.7805**	*	C52H81NO8P
LPC identified as $[M+H]^+$					
LPC(14:0)	468.3090	468.3075	**3.2030**	14:0	C22H47NO7P
LPC(16:0)	496.3403	496.3387	**3.2236**	16:0	C24H51NO7P

LPC(16:1)	494.3247	494.3229	**3.6413**	16:1	C24H49NO7P
LPC(18:0)	524.3716	524.3700	**3.0513**	18:0	C26H55NO7P
LPC(18:1)	522.3560	522.3546	**2.6802**	18:1	C26H53NO7P
LPC(18:2)	520.3403	520.3389	**2.6905**	18:2	C26H51NO7P
LPC(18:3)	518.3247	518.3236	**2.1222**	18:3	C26H49NO7P
LPC(18:4)	516.3090	516.3076	**2.7116**	*	C26H47NO7P
LPC(20:0)	552.4029	552.4009	**3.6205**	20:0	C28H59NO7P
LPC(20:1)	550.3873	550.3854	**3.4521**	20:1	C28H57NO7P
LPC(20:2)	548.3716	548.3697	**3.4648**	20:2	C28H55NO7P
LPC(20:3)	546.3560	546.3544	**2.9285**	*	C28H53NO7P
LPC(20:4)	544.3403	544.3380	**4.2253**	20:4	C28H51NO7P
LPC(20:5)	542.3247	542.3237	**1.8439**	20:5	C28H49NO7P
LPC(22:4)	572.3716	572.3695	**3.6689**	22:4	C30H55NO7P
LPC(22:5)	570.3560	570.3546	**2.4546**	*	C30H53NO7P
LPC(22:6)	568.3403	568.3385	**3.1671**	22:6	C30H51NO7P
LPC(24:1)	606.4487	606.4479	**1.3192**	*	C32H65NO7P
PE identified as $[M+H]^+$					
PE(30:3)	658.4448	658.4436	**1.8225**	*	C35H65NO8P
PE(34:2)	716.5230	716.5206	**3.3495**	16:0/18:2	C39H75NO8P
PE(34:3)	714.5074	714.5055	**2.6592**	*	C39H73O8NP
PE(34:4)	712.4917	712.4939	**-3.0878**	*	C39H71NO8P
PE(36:4)	740.5230	740.5213	**2.2957**	*	C41H75NO8P
PE(36:5)	738.5074	738.5061	**1.7603**	*	C41H73O8NP
PE(38:4)	768.5543	768.5523	**2.6023**	18:0/20:4	C43H79O8NP
PE(38:6)	764.5230	764.5207	**3.0084**	16:0/22:6 and 18:1/20:5 and 18:2/20:4	C43H75O8NP
PE(38:7)	762.5074	762.5067	**0.9180**	*	C43H73NO8P
PE(40:10)	784.4917	784.4882	**4.4615**	*	C45H71NO8P
PE(40:6)	792.5543	792.5520	**2.9020**	18:0/22:6	C45H79O8NP
PE(40:7)	790.5387	790.5359	**3.5419**	18:1/22:6	C45H77NO8P
PE(40:9)	786.5074	786.5066	**1.0172**	*	C45H73O8NP
PE(42:9)	814.5387	814.5370	**2.0871**	*	C47H77O8NP
PE(42:10)	812.5230	812.5250	**-2.4615**	*	C47H75O8NP
PE(48:12)	892.5856	892.5824	**3.5851**	*	C53H83O8NP
LPE identified as $[M+H]^+$					
LPE(14:1)	424.2464	424.2472	**-1.8857**	*	C19H39NO7P
LPE(16:0)	454.2934	454.2921	**2.8616**	16:0	C21H45NO7P
LPE(18:0)	482.3247	482.3233	**2.9026**	18:0	C23H49NO7P
LPE(18:1)	480.3090	480.3076	**2.9148**	*	C23H47NO7P
LPE(18:2)	478.2934	478.2917	**3.5543**	18:2	C23H45NO7P
LPE(18:3)	476.2777	476.2767	**2.0996**	18:3	C23H43NO7P

LPE(20:3)	504.3090	504.3077	**2.5778**	20:3	C25H47NO7P
LPE(20:4)	502.2934	502.2914	**3.9817**	20:4	C25H45NO7P
LPE(20:5)	500.2777	500.2767	**1.9989**	20:5	C25H43NO7P
LPE(22:4)	530.3247	530.3231	**3.0170**	22:4	C27H49NO7P
LPE(22:5)	528.3090	528.3067	**4.3535**	*	C27H47NO7P
LPE(22:6)	526.2934	526.2913	**3.9902**	22:6	C27H45NO7P
PG identified as $[M-H]^-$					
PG(32:1)	719.4863	719.4855	**1.1119**	*	C38H72O10P
PG(32:2)	717.4707	717.4718	**-1.5332**	*	C38H70O10P
PG(34:2)	745.5020	745.5020	**0.0000**	*	C40H74O10P
PG(34:3)	743.4863	743.4854	**1.2105**	*	C40H72O10P
LPG identified as $[M-H]^-$					
LPG(12:0)	427.2097	427.2096	**0.2341**	*	C18H36O9P
LPG(14:0)	455.2410	455.2405	**1.0983**	14:0	C20H40O9P
LPG(18:4)	503.2410	503.2410	**0.0000**	*	C24H40O9P
LPG(20:4)	531.2723	531.2720	**0.5647**	20:4	C26H44O9P
PI identified as $[M-H]^-$					
PI(30:2)	777.4554	777.4525	**3.7301**	*	C39H70O13P
PI(34:2)	833.5180	833.5145	**4.1991**	*	C43H78O13P
PI(40:1)	919.6276	919.6275	**0.1087**	*	C49H92O13P
LPI identified as $[M-H]^-$					
LPI(24:4)	675.3509	675.3501	**1.1846**	*	C33H56O12P
PS identified as $[M-H]^-$					
PS(36:0)	790.5598	790.5597	**0.1265**	*	C42H81NO10P
PS(36:1)	788.5442	788.5438	**0.5073**	*	C42H79NO10P
PS(38:1)	816.5755	816.5748	**0.8572**	*	C44H83NO10P
PS(38:2)	814.5598	814.5598	**0.0000**	*	C44H81NO10P
PS(40:1)	844.6068	844.6064	**0.4736**	*	C46H87NO10P
PS(40:2)	842.5911	842.5904	**0.8308**	*	C46H85NO10P
SM identified as $[M+H]^+$					
SM(d30:1)	647.5128	647.5108	**3.0887**	-	C35H72N2O6P
SM(d32:1)	675.5441	675.5420	**3.1086**	-	C37H76N2O6P
SM(d32:2)	673.5285	673.5266	**2.8210**	-	C37H74N2O6P
SM(d34:1)	703.5754	703.5729	**3.5533**	-	C39H80N2O6P
SM(d34:2)	701.5598	701.5567	**4.4187**	-	C39H78N2O6P
SM(d36:1)	731.6067	731.6041	**3.5538**	-	C41H84N2O6P
SM(d36:2)	729.5911	729.5885	**3.5636**	-	C41H82N2O6P
SM(d36:3)	727.5754	727.5719	**4.8105**	-	C41H80N2O6P
SM(d38:1)	759.6380	759.6344	**4.7391**	-	C43H88N2O6P
SM(d38:2)	757.6224	757.6199	**3.2998**	-	C43H86N2O6P
SM(d38:3)	755.6067	755.6037	**3.9703**	-	C43H84N2O6P

SM(d40:1)	787.6693	787.6685	**1.0157**	-	C45H92N2O6P
SM(d40:2)	785.6537	785.6506	**3.9458**	-	C45H90N2O6P
SM(d40:3)	783.6380	783.6341	**4.9768**	-	C45H88N2O6P
SM(d42:2)	813.6850	813.6816	**4.1785**	-	C47H94N2O6P
SM(d42:3)	811.6693	811.6664	**3.5729**	-	C47H92N2O6P

C – carbons; N – number of double bonds; *identified based on exact mass measurements, no FA acyl-chain fragments observed

It is evident that in the negative mode the majority of the PL species were identified based only on the exact mass measurements. On the contrary, in the positive mode there was better ionization, which allowed the identification of each individual FA of the majority of the PL species identified in this mode.

Table 13 summarizes which native PL species are present in which group to acknowledge which species have different behaviours between categories.

Table 13. Comparison of the PL species identified in each category. ✘ - not identified; ✓- identified.

PL species	SLE	Control
PC(30:0)	✓	✓
PC(30:1)	✓	✓
PC(32:0)	✓	✓
PC(32:1)	✓	✓
PC(32:2)	✓	✓
PC(34:1)	✘	✓
PC(34:2)	✓	✓
PC(34:3)	✓	✓
PC(34:4)	✘	✓
PC(36:2)	✓	✓
PC(36:3)	✓	✓
PC(36:4)	✓	✓
PC(36:5)	✘	✓
PC(38:3)	✘	✓
PC(38:4)	✓	✓
PC(38:5)	✘	✓

PC(38:6)	✓	✓
PC(38:8)	✓	✓
PC(40:4)	✓	✓
PC(40:5)	✗	✓
PC(40:6)	✗	✓
PC(40:7)	✗	✓
PC(40:8)	✗	✓
PC(42:6)	✗	✓
PC(42:7)	✗	✓
PC(44:12)	✗	✓
LPC(14:0)	✗	✓
LPC(16:0)	✓	✓
LPC(16:1)	✓	✓
LPC(18:0)	✓	✓
LPC(18:1)	✓	✓
LPC(18:2)	✓	✓
LPC(18:3)	✗	✓
LPC(18:4)	✗	✓
LPC(20:0)	✓	✓
LPC(20:1)	✓	✓
LPC(20:2)	✓	✓
LPC(20:3)	✗	✓
LPC(20:4)	✓	✓
LPC(20:5)	✗	✓
LPC(22:1)	✓	✗
LPC(22:2)	✓	✗
LPC(22:4)	✓	✓
LPC(22:5)	✓	✓
LPC(22:6)	✓	✓
LPC(24:1)	✓	✓
PE(28:2)	✓	✗
PE(30:3)	✗	✓

PE(32:1)	✓	✗
PE(34:2)	✓	✓
PE(34:3)	✓	✓
PE(34:4)	✓	✓
PE(36:4)	✓	✓
PE(36:5)	✓	✓
PE(38:4)	✓	✓
PE(38:6)	✓	✓
PE(38:7)	✓	✓
PE(40:6)	✓	✓
PE(40:7)	✓	✓
PE(40:9)	✗	✓
PE(40:10)	✗	✓
PE(42:9)	✗	✓
PE(42:10)	✓	✓
PE(48:12)	✓	✓
LPE(14:1)	✓	✓
LPE(16:0)	✓	✓
LPE(18:0)	✓	✓
LPE(18:1)	✓	✓
LPE(18:2)	✓	✓
LPE(18:3)	✗	✓
LPE(20:3)	✗	✓
LPE(20:4)	✗	✓
LPE(20:5)	✓	✓
LPE(22:4)	✗	✓
LPE(22:5)	✗	✓
LPE(22:6)	✓	✓
PG(32:1)	✗	✓
PG(32:2)	✓	✓
PG(34:2)	✓	✓
PG(34:3)	✗	✓

PG(40:7)	✓	✗
LPG(12:0)	✓	✓
LPG(14:0)	✓	✓
LPG(18:4)	✗	✓
LPG(20:4)	✓	✓
LPG(24:1)	✓	✗
LPG(24:2)	✓	✗
LPG(24:3)	✓	✗
PI(30:2)	✗	✓
PI(32:1)	✓	✗
PI(34:2)	✗	✓
PI(40:1)	✗	✓
PI(44:5)	✓	✗
LPI(18:0)	✓	✗
LPI(20:1)	✓	✗
LPI(24:1)	✓	✗
LPI(24:2)	✓	✗
LPI(24:4)	✗	✓
PS(36:0)	✓	✓
PS(36:1)	✓	✓
PS(38:1)	✓	✓
PS(38:2)	✓	✓
PS(40:1)	✓	✓
PS(40:2)	✓	✓
SM(d30:1)	✓	✓
SM(d32:1)	✓	✓
SM(d32:2)	✓	✓
SM(d34:1)	✓	✓
SM(d34:2)	✓	✓
SM(d36:1)	✓	✓
SM(d36:2)	✓	✓
SM(d36:3)	✗	✓

SM(d38:1)	✗	✓
SM(d38:2)	✓	✓
SM(d38:3)	✓	✓
SM(d40:1)	✗	✓
SM(d40:2)	✓	✓
SM(d40:3)	✗	✓
SM(d42:2)	✗	✓
SM(d42:3)	✓	✓

On one side, there are several native PL species that were identified in both conditions. Those lipid species were quantified by integration of the chromatographic peaks and it was performed statistical analysis to see if there were significant differences between the two conditions. On the other side, there are also some PL species that are only present in one group. There are several PC and PE species [PC(38:3); PC(38:5); PC(40:4); PC(40:5); PC(40:6); PC(40:7); PC(40:8); PC(42:6); PC(42:7); PC(44:12); PE(40:9); PE(40:10) and PE(42:9)] that were not identified in the disease category. PE and PC species in SLE samples have fatty acids with smaller carbon chains and less unsaturated, which is probably due to the oxidation processes that may occur in SLE. It is also seen that LPC(18:3); LPC(18:4); LPC(20:3) and LPC(20:5) were only identified in the control group. These LPC species contain omega-3 PUFA and are reported to be anti-atherogenic once they modulate the inflammatory response induced by LPC with saturated or monounsaturated FA[152]. This way, this finding is in agreement with the atherogenic lipoprotein profile characteristic of the SLE disease. LPI [LPI(18:0); LPI(20:1); LPI(24:1) and LPI(24:2)] and LPG [LPG(24:1); LPG(24:2) and LPG(24:3)] species were only identified in SLE category, which could be explained by the increased oxidative conditions of this disease. As there are no reports regarding these PL classes in SLE, further studies are needed to understand these findings and evaluate if these could be used as biomarkers of the disease or relapsing episodes.

In order to determine which PL classes differ within conditions, contributing this way to the greater significance of the results, it was performed a multivariate statistical analysis. PCA scores scatter plots revealed good discriminant ability where SLE and

control samples tended to cluster away from each other, both in positive and negative modes (Figure 14).

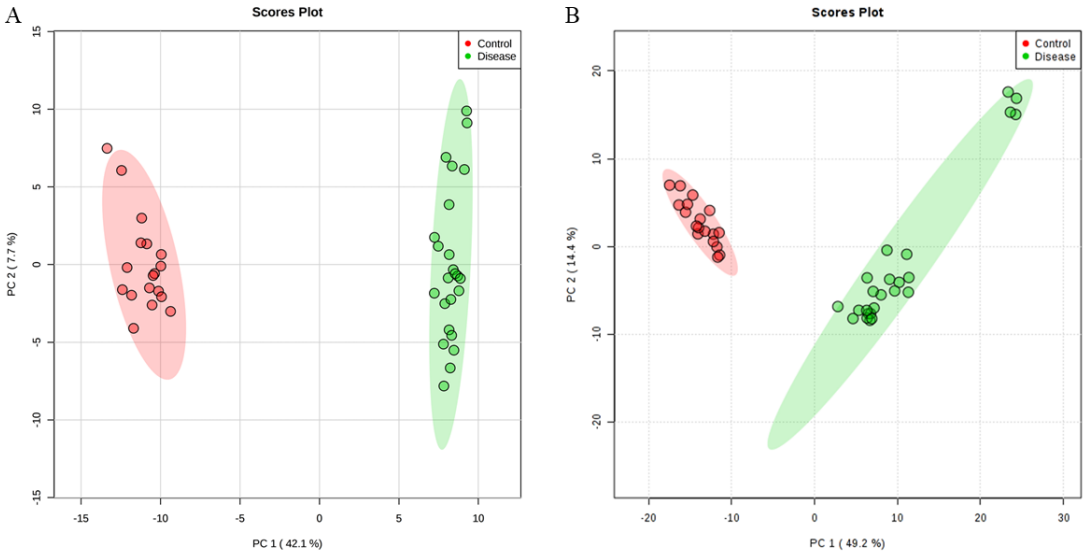

Figure 14. Multivariate analysis of HILIC-MS data collected from SLE and control samples. **A:** PCA scores scatter plot of PL species in positive mode. **B:** PCA scores scatter plot of PL species in negative mode.

Univariate analysis showed the top 20 variables that reflect the most important lipid species that differentiate disease from healthy controls and are represented in heatmaps (for both positive and negative modes), shown in Figure 15.

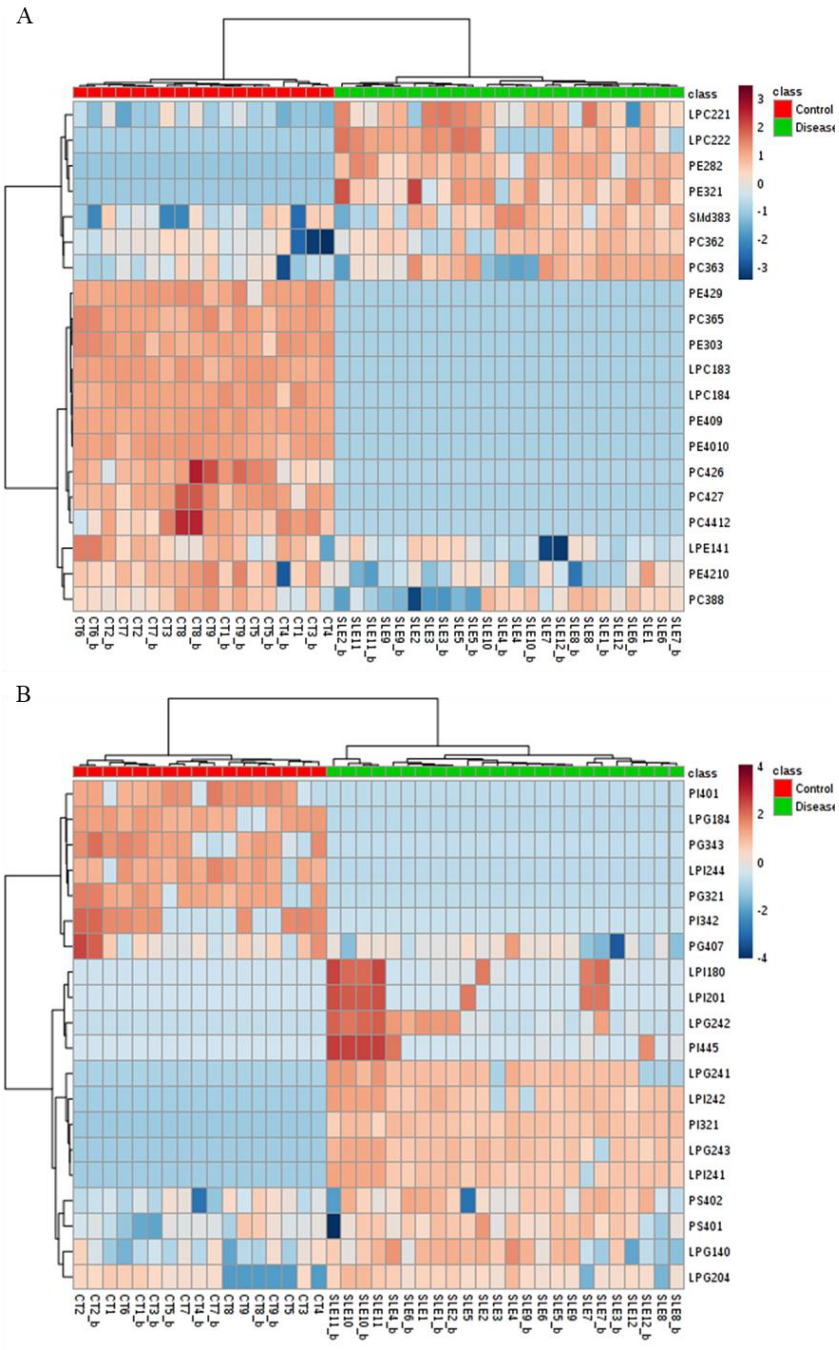

Figure 15. Univariate and clustering analysis considering the top 20 most discriminatory ox-PL species. **A:** Heatmap of PL in positive mode. **B:** Heatmap of PL in negative mode.

The univariate and clustering analysis considering the top 20 most discriminatory PL species, illustrated in Figure 15, showed that in the positive mode (Figure 15-A) PE and PC are the PL classes that most contribute to differentiate SLE from control samples. PE species PE(42:9), PE(30:3), PE(40:9), PE(40:10) and PE(42:10), are significantly lower in disease samples, which can be due to oxidative degradation, while PE(32:1) and PE(28:2)

are considerably higher in this condition. It is also seen that PC(36:5), PC(42:6), PC(42:7), PC(44:12) and PC(38:8) are markedly downregulated in SLE samples, which can also be explained by oxidative degradation. On the other side, PC(36:2) and PC(36:3) significantly increase their relative content in SLE patients. PE and PC species in control samples have longer carbon chains and have more unsaturations, which are prone to oxidation that is favoured in SLE samples. LPC(18:3) and LPC(18:4) have considerably higher relative content in the control group, on the contrary, LPC(22:1) and LPC(22:2) have significantly higher relative content in the disease group. LPC with PUFA are considered anti-inflammatory[152], and thus their reduction in SLE can probably be associated with a higher inflammatory state in SLE. An interesting difference between groups is the behaviour of SM and LPE species. SM(d38:3) is relatively elevated in SLE samples while LPE(14:1) has the opposite behaviour, being with higher relative abundance in healthy controls. In the negative mode (Figure 15-B) there is a considerable inter- and intragroup variability that can be explained by the lower abundance of these species. LPG class is the most discriminatory in this mode, contributing with six species. LPG(18:4) is significantly increased in control samples, on the other side, LPG(24:2), LPG(24:1) and LPG(24:3) have the contrary behaviour. LPG(14:0) and LPG(20:4) show a tendency to be elevated in the SLE group, however they present intra- and intergroup variability. LPI is the second most discriminatory class, LPI(24:4) is considerably raised in controls while LPI(18:0), LPI(20:1), LPI(24:2) and LPI(24:1) have higher relative abundances in SLE samples. PI(34:2) and PI(40:1), are significantly increased in control samples meanwhile PI(32:1) and PI(44:5) are markedly raised in SLE samples. The disease group has more LPL species than the control group, that can be formed by degradation of PL by phospholipase A_2[153], which is indicative of the elevated lipid oxidation processes that develop in SLE. PG class have intragroup variability, nonetheless, PG(32:1) and PG(34:3) increase significantly in the control group. PG(40:7) shows a tendency to have higher abundances in the control group however it displays a deep both intra- and intergroup heterogenous behaviour. PS(40:2) and PS(40:1) show an opposite tendency, being augmented in disease.

Likewise the oxidized species, the lipidic signature of both disease and control groups revealed deep intra- and intergroup variability and dispersion of values which made difficult the interpretation of the results. Nonetheless we were able to determine some PL species, mainly oxidized, that contribute to differentiate conditions.

Conclusions, Final remarks & Future work

9. *Conclusions, Final remarks & Future work*

SLE is a chronic, systemic autoimmune disease characterized by a constant inflammatory state and elevated levels of oxidative stress, correlated with mitochondrial dysfunctions and cell death processes like NETosis. Lipidomics help us realise how the lipid metabolism is altered in this autoimmune disease. There are evidences that there are in fact alterations in lipids and in lipids metabolism in these patients showing the importance that lipid regulation has in SLE pathogenesis. Following the objectives of this work, we analysed the oxidized and non-oxidized lipid profile of SLE patients, in order to identify a characteristic lipidic signature of SLE. Data collection regarding the laboratory parameters of SLE patients revealed the non-specificity of anti-dsDNA antibodies in this disease unveiling the difficulty of SLE diagnosis. The discovery of a new biomarker for this disease would be a suitable breakthrough.

The results gathered in this study showed that SLE patients and controls had a similar number of identified ox-PL species, something that we did not foreseen, but there is some variation in their relative content. Due to the enhanced ROS production and oxidative conditions characteristic of SLE, we would expect to identify more ox-PL species in SLE samples. However, although several oxidized PC were identified in both conditions, there are others that were only observed in SLE or in controls. Comparing the two conditions, disease (SLE) vs control, it was visible a good separation between groups. Oxidized LPC and oxidized PE were the most discriminatory species in the positive mode. There were several oxidized PE species that were not found in control samples which is suggestive of the increased inflammatory conditions in SLE. In the negative mode, oxidized PS species were also important to discriminate categories once they have an anti-inflammatory effect and were not detected in SLE samples, possibly due to a lower content, lower than the threshold selected for the detection of peaks in LC-MS. Our findings are in agreement with those reported before for inflammatory diseases with excessive oxidative stress conditions, like SLE[149].

Since there was a considerable variability of ox-PL species identified in the two groups, it was performed an analysis with three clustered sample groups, healthy controls, active SLE and inactive SLE. Multivariate analysis allowed to discriminate both groups. Univariate analysis showed that oxidized PC contributes more for the discrimination followed by oxidized PE. Oxidized PE are significantly increased in active SLE,

evidencing its role as a pro-inflammatory biomolecule. Active SLE is characterised by relapsing periods where inflammation is a key factor for the exacerbation of pre-existing symptoms or the development of new ones. The lower content of oxidized PE in inactive SLE, where inflammation is expected to be lower than in active SLE, indicates that in SLE, oxidized PE should have a role as signalling and regulatory lipids in inflammatory activation. SLE patients in an inactive state should have a lower inflammatory as well as oxidative status (except when in flare periods - active SLE) that justifies the lower oxidized PE content than in active SLE.

The non-oxidized lipidic signature of SLE and controls was also evaluated. There was a good discrimination between disease and control categories. Univariate analysis of the positive mode showed that PE species were the most discriminatory followed by PC and LPC classes. We verified that PE and PC species with longer carbon chains and more unsaturations showed higher relative content in controls, and thus a lower relative content in SLE. SLE samples have those species significantly reduced or even absent due to the higher levels of lipid oxidation processes induced by ROS, associated with NETosis and inflammation. Oxidized PL are known to be important players in cell death, as it is case of oxidized cardiolipin in apoptosis and oxidized PE in ferroptosis, two different types of controlled cell deaths[62]. LPC species with omega-3 PUFA were found to be decreased in SLE which contributes to the atherogenic lipoprotein profile of this disease. This type of LPC are considered anti-inflammatory and anti-atherogenic and their lower abundance in SLE may also be considered a characteristic of this disease. Univariate analysis of the negative mode revealed deep inter- and intragroup heterogeneity in the behaviour of several PL species due to their lower abundance. The most discriminatory species in this mode belonged to LPG and LPI classes. We also found that LPL species are significantly elevated in the disease group, reassuring the raised peroxidation processes in SLE. Similarly to what happened with the oxidized lipid profile, it was verified that the native lipid species also have high variability among categories.

Oxidized PE and oxidized PS showed variation in SLE, with opposite trend, with a higher content for oxidized PE and lower for oxidized PS in SLE. Oxidized PE is considered a pro-inflammatory molecule while oxidized PS seems to be associated with an anti-inflammatory role, according with the literature[149], which seems to justify their role in SLE. Nonetheless, more studies are needed to confirm the results. Inactive SLE group has

a distinct oxidized lipid profile due to a lower inflammation state conceivably due to the treatment for disease management. Immunosuppressant, anti-inflammatory and lipid-lowering drugs may be the cause for this lipidic signature of inactive SLE patients.

Overall, the results gathered in the present work showed that the software MZmine v2.32 is a suitable tool to detect and quantify oxidized PL and that more studies are needed to unveil the role of PL in general, PL in SLE, and in both forms of disease, active and inactive. The changes in lipid profiling could give new clues for the discovery of new biomarkers of the disease and could also be valuable for early diagnosis, prediction of relapsing periods or for new therapeutic strategies. A valuable addition for future works would be to increase the number of samples to decrease the significant intragroup variability. Clustering of the samples proved to be informative regarding the oxidized lipidic signature, therefore, the analysis of a higher number of clustered samples (active SLE, inactive SLE and controls) would be beneficial for future works. Also, since SLE has a strong 9:1 female to male ratio, it would be interesting to analyse and compare the lipid profile between genders.

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10. References

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Appendices

Title: Lipidomics in autoimmune diseases with main focus on Systemic Lupus Erythematosus

Author: Helena Beatriz Ferreira Ana Margarida Pereira Tânia Melo Artur Paiva M. Rosário Domingues

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Highlights:

Systemic Lupus Erythematosus is an inflammatory chronic disease that lacks tools for prognostics;

Lipids and oxidized lipids have important role in inflammation and disease progression in SLE;

Lipidomics as tool to identify new lipid biomarkers of SLE relapsing episodes;

Lipidomics to identify lipid biomarkers to ensure better patient care and personalized medicine;

Lipidomics in autoimmune diseases with main focus on Systemic Lupus Erythematosus

Helena Beatriz Ferreira[a], Ana Margarida Pereira[b], Tânia Melo[a,c], Artur Paiva[d,e,f], M. Rosário Domingues[a,c*]

[a] Mass Spectrometry Center & QOPNA, Chemistry Department of University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[b] Clinical pathology department, Centro Hospitalar do Baixo Vouga, Av. Artur Ravara 3810-501 Aveiro, Portugal

[c] Departament of Chemistry & CESAM & ECOMARE, University of Aveiro, Campus Universitário de Santiago 3810-193 Aveiro, Portugal

[d] Unidade de Gestão Operacional em Citometria, Centro Hospitalar e Universitário de Coimbra (CHUC, Portugal).

[e] Coimbra Institute for Clinical and Biomedical Research (iCBR), Faculty of Medicine, University of Coimbra, (Coimbra, Portugal).

ortugal).

[f] Instituto Politécnico de Coimbra, ESTESC-Coimbra Health School, Ciências Biomédicas Laboratoriais (Portugal).

*Corresponding author. Rosário Domingues

Tel.:+351 234 370 698

E-mail address: mrd@ua.pt

Abstract

Autoimmune diseases (AID) are a heterogeneous group of disorders that have in common a chronic inflammation and dysregulation of the immune system. Systemic lupus erythematosus (SLE) is one of the most frequent systemic autoimmune diseases characterized by autoimmune phenomena in multiple organs. The tests used for evolution and prognosis assessment are either non-specific or non-sensitive, impairing an adequate therapeutics. To face this drawback, lipidomics is being used to provide more knowledge and insights regarding autoimmune disorders. Through lipidomic approaches using MS, it is possible to identify and quantify the level of lipid molecular species in the biological system and this could be useful to identify biomarkers and to better understand the pathophysiology of autoimmune diseases. There are some evidence that lipids and oxidized lipids can play a key role in AID pathogenesis. Although this field has been scarcely explored, there are some studies that reported variations on the lipid profile at a molecular level using lipidomic approaches based on MS in SLE. The results gathered herein showed changes mainly in the level of phospholipids, with decrease of some plasmenyl lipids, fatty acids, with reduction of PUFA, and sphingolipids, with changes in fatty acyl chain composition. These changes may be the result of lipids` modifications due to oxidation and increase of ROS. Some alterations can be associated with changes in membrane of lymphocytes and with the deregulation of the immune system. Thus, exploring the knowledge from modern lipidomic approaches in the study of the role of lipids and oxidized lipids, in oxidative stress and in inflammatory diseases, could contribute for the identification of new lipid biomarkers. Lipid biomarkers are promising tools to prognosis and treatment monitoring, tailored for the best therapeutic response and highest safety to ensure better patient care and to be used for personalized medicine.

Keywords Autoimmune diseases; systemic lupus erythematosus; lipidomics; mass spectrometry; biomarkers; lipid peroxidation.

Abbreviations: AA, arachidonic acid; ANA, antinuclear antibodies; APCs, antigen-presenting cells; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; FFA, free fatty acids; LPC, lyso-phosphatidylcholine; LPE, lyso-phosphatidylethanolamine; LPL, lyso-phospholipid; mtDNA, mitochondrial DNA; NET, neutrophil extracellular traps; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; pPC, plasmenylphosphatidylcholine; pPE, plasmenylphosphatidylethanolamine; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SLE, systemic lupus erythematosus; SM, sphingomyelin; TG, triglyceride;.

Contents

1. Autoimmune diseases

Autoimmune diseases are a highly heterogeneous group of disorders that cover a wide range of pathologies. Worldwide, they are known to have an estimated total prevalence of 7.6-9.4% [1]. This type of diseases is extremely debilitating in their acute phases and patients have their autonomy decreased. These diseases occur when the immune system begins to attack the body itself, recognizing some of the molecules from the own organism, as a foreign and undesirable pathogen [2]. The increased production of those antigens will induce the production of antibodies present in plasma [3]. The autoimmune diseases` pathogenesis is not a straightforward process, and it has the contribution of several factors (environmental, genetic and hormonal), yet they all work together to disrupt the normal tolerance to the system's own antigens [4]. However, there are certain mechanisms that are common to every autoimmune disease such as: the recognition of the autoantigen as a foreign body (disease initiation), enhanced production of antibodies, amplification of the disease by including multiple pathways of the immune response, chronic inflammation and tissue destruction [5].

The diagnosis of autoimmune diseases is based on clinical and laboratory data, including serologic tests, the symptoms reported by the patient and the signs observed by the clinician [6]. However, the diagnosis is not always easy to obtain due to insufficient clinical data and specific markers to ensure a positive diagnosis. Under these circumstances, only the unfolding of the clinical situation will confirm if the autoimmune disease is being responsible for the signs and symptoms of the patient. Moreover, autoimmune diseases have diverse forms of manifesting themselves, which also increases the difficulty of the diagnosis [4,6].

Autoimmune disorders can be classified in two different ways: organ-specific or

systemic (Table 1) [7]. In organ-specific autoimmune diseases, the autoimmune response is directed against antigens that are expressed only in a particular organ. Autoantibodies bind to autoantigens in the organ cells and can lead either to their destruction, overstimulation or suppression of the normal cellular function. On the other side, in systemic autoimmune diseases the autoimmune response is directed against autoantigens scattered throughout the organism, which ends into a widespread tissue damage [8].

Table 1. Classification of autoimmune diseases: organ-specific autoimmunity *vs* systemic autoimmunity, and some examples of the most common ones.

Organ-specific autoimmunity	**Systemic autoimmunity**
Type 1 diabetes	Systemic lupus erythematosus
Autoimmune hepatitis	Sjögren`s syndrome
Hashimoto`s thyroiditis	Rheumatoid arthritis
Goodpasture`s syndrome	Scleroderma
Psoriasis	Systemic Sclerosis
Addison`s disease	Antiphospholipid syndrome
Pernicious anemia	Dermatomyositis
Myasthenia gravis	Vasculitis
Graves` disease	Ankylosing spondylitis
Vitiligo	Polymyositis

Autoimmune disorders are polygenic and each type of autoimmune disease have its distinct clinical phenotype, with specific physiopathology and specific symptoms [5]. Each type of autoimmune disease has a different development and their prognostics are also dissimilar [5]. A characteristic feature of autoimmune diseases is that they have relapse

and remission periods [6]. A relapse, or flare, is an acute reactivation of the disease, manifested by the worsening of pre-existing symptoms or the development of new ones. Flare periods occur when the disease activity is enhanced and can manifest themselves in a variety of forms. Usually, treatment during flare episodes, requires specific therapy to attenuate symptoms. Meanwhile, remission is a period of inactivity of the disease. There are cases that the disease becomes permanently inactive, which means, in total remission, however this disease stage is rarely achieved. On the other side, partial remission is more frequent [9]. Relapse periods can occur for no apparent reason. In order to avoid flares, it is vital for the patient to adopt preventive measures or prediction tools. It is advisable to make routine visits to clinicians to reassess the symptoms and the signs, as well as the analysis and examinations that have been carried out. This way the treatment can be readjusted whenever it is necessary. The patient should also have other precautions such as: avoid stress, rest properly and avoid exaggerated physical activity [6]. However, there is a lack of specific diagnostic tools or biomarkers that allow the prediction of the relapse periods. To prevent the appearance of these highly debilitating periods of disease, new studies are needed to understand the mechanism of disease and to find new biomarkers. This should allow tailoring early diagnosis of flare episodes and promote most effective treatment to each patient with a chronic autoimmune disease.

Due to the high complexity of autoimmune diseases, this paper will only focus on one very common autoimmune disease, systemic lupus erythematosus (SLE) [4].

2. Systemic lupus erythematosus

SLE is a chronic, systemic autoimmune disease and it is characterized by systemic inflammation in multiple organs such as joints, vessels, skin, kidneys and central nervous

system [10]. A particular characteristic of SLE is that pathogenic autoantibodies are produced by dysfunctional immunocompetent cells, leading to multiple organ injuries [10,11]. It is most prevalent in women in childbearing age with a very strong 9:1 female to male ratio and the major clinical features of this disease are fatigue and musculoskeletal symptoms [12,13]. Clinical manifestations and the prognostic of the disease are influenced by age of onset and gender [14]. The male gender is often related with higher levels of disease activity, regardless their age or race, at the time of diagnosis. However, during disease course, gender does not seem to dramatically influence the clinical manifestations [15]. The death rate is also different according to the gender; female SLE patients have a lower death rate than men. Patients` age is also important for the cause of death; for patients with ages between 20 and 39 years, the most common cause of death is musculoskeletal and comorbidities that develop as a consequence of SLE, while patients with more than 40 years of age commonly die due to any type of cardiovascular diseases or malignancy, caused by the normal ageing process in combination with the added inflammation due to SLE [16].

Diagnosis of SLE is based on the criteria established by the American College of Rheumatology and besides the immuno-pathological features, there is also a clinical laboratory profile that suggests that the patient suffers from SLE (Table 2) [9,17]. It comprises mainly full blood cell count (with a decrease of red blood cells, usually associated with anaemia, and/or white blood cells and/or platelets), inflammation parameters (such as increase of C-reactive protein and erythrocyte sedimentation rate) and immunological changes (as the presence of some antibodies, mainly IgG antinuclear antibodies) [9]. The changes in the biochemical parameters are found in other autoimmune diseases, only the immunological parameters are specific for SLE. These changes in

laboratory parameters are not detected in the same patient at the same time, it is frequent that only a few alterations of these parameters are seen at a particular time of the disease. Also, the lab test results can vary from patient to patient [6,9]. In fact, the diagnosis of SLE is somehow difficult in many cases, therefore the interpretation of the laboratory results should be made carefully and only by the attending physician that is aware of the patient`s medical record.

Table 2. Principal alterations on laboratory parameters observed in SLE disease.

Blood count	Inflammation parameters	Immunology
		↓ Complement components C3, C4 and CH50
↓ Red blood cells (haemolytic anaemia)	↑ Erythrocyte sedimentation rate	**Presence of**:
↓ White blood cells (lymphopenia)	↑ C-reactive protein	Antinuclear antibodies
↓ Platelets (thrombocytopenia)		Anti-DNA and anti-Sm antibodies
		Anti-SS-A and anti-SS-B antibodies
		Anti-RNP antibodies
		Antiphospholipid antibodies

The development of SLE seems to have a strong genetic predisposition to a dysregulation of the immune system. Hyperexpression of B lymphocyte activity is therefore a consequence of SLE [4,18]. This autoimmune disorder is thus also associated with a polyclonal hypergammaglobulinemia and with elevated titters of antibodies against several auto-antigens, in particular, nuclear antigens. Nonetheless, there are also other factors that contribute to trigger this disease, namely ultraviolet light exposure, cross reactivity with infectious agents, drug hypersensitivities and several stress stimuli [19–21].

Exposure to ultraviolet light, specially UVB, can contribute to the pathogenesis and exacerbation of SLE, since it induces apoptosis in keratinocytes and alterations in DNA. Sunlight exposition also promotes the production of pro-inflammatory molecules that combined with the cell death mechanisms describe the cutaneous and flare reactions typical of SLE [22]. Infectious agents, such as Epstein-Barr virus, can also influence the development of SLE and induce flare periods, triggering the disease by molecular mimicry. It is true that EBV can prompt autoimmune processes, however, SLE patients have an abnormal viral latency period and a dysregulated anti-EBV response[23–25]. All these factors will enable T lymphocytes to recognize peptides presented by antigen-presenting cells (APCs) [26]. This process is known as antigen presentation and promotes the release of cytokines, inflammation process and B lymphocyte stimulation. However, T lymphocyte malfunction could be the result of defective APCs. In fact there are some studies that reflect the dysfunction of APCs in autoimmune diseases [27–29]. B lymphocyte stimulation leads to the hyperproduction of IgG autoantibodies that are responsible for the tissue damage, which is a characteristic of SLE [18,26,30]. These IgG antinuclear antibodies (ANA) that are elevated in SLE target cellular nuclear components. There are several types of ANA and their identification is of utterly importance in SLE diagnosis. In SLE, the most commonly found ANA are anti-native (double stranded) DNA antibodies, although they are not specific of this disease [31].

Genetic factors are one of the major effectors of SLE susceptibility. The development of specific autoantibodies and SLE`s clinical features are genetically encoded, thus the determination of the specific genes that directly contribute to this disease would be a scientific breakthrough that would widely improve the knowledge of this autoimmune disorder. This topic is being intensely investigated and will not be discussed

in this paper (to further clarify this matter please check review article [14]). The development of SLE is also aggravated by epigenetic changes, namely the DNA methylation that is implicated in the pathogenesis of this disorder, although there is not much information regarding hydroxymethylation in this process [14].

Metabolic alterations are also found in patients with SLE; there is evidence that plasma, sera and urine of SLE patients have higher levels of reactive oxygen species (ROS) as well as 8-hydroxy-2`-deoxyguanosine, an oxidative DNA damage biomarker [32,33]. Also, first and second line of defences against mitochondrial ROS, or from other intracellular sources, are found to be decreased in SLE patients [33,34]. First line of cellular antioxidant system (manganese superoxide dismutase and copper/zinc superoxide dismutase), as well as second line of antioxidant enzymes (catalase, glutathione peroxidase 1 and glutathione peroxidase 4), are significantly lower in plasma/serum and in neutrophils/lymphocytes/leukocyte in SLE [33,34]. The disease activity and extent of organ damage are related with glutathione and the ratio of glutathione/oxidized glutathione, which are found to be downregulated in patients with SLE [34,35]. The glucose metabolism is also altered in lupus, in particular, it was found that leukocytes have lower levels of pyruvate dehydrogenase mRNA and of transcripts of mtDNA-encoded peptides and mitochondrial transcription factor A, which are fundamental for electron-chemical transport, oxidative phosphorylation and mtDNA replication [33]. It was likewise shown that the Krebs cycle in SLE patients is reduced, as well as the levels of the enzymes hexose kinase, glucose phosphate isomerase, phosphofructokinase and glyceraldehyde 3-phosphate dehydrogenase [33,36]. The alteration of DNA and glucose metabolism contributes to an inefficient immune system leading to the development of SLE.

In spite of the difficulties in diagnostics and prediction of relapsing periods, the survival rate of SLE patients has significantly improved over the last decades owing to some progress in early diagnosis. The detection of specific antibodies and the development of more efficient treatment strategies for both disease and its comorbidities revealed to be important for the improvement of SLE patients` quality of life. The detection of anti-double stranded DNA (anti-dsDNA) antibodies, ANA and complement activation are used to support either the diagnosis or the evaluation of disease activity of SLE [37]. Disease activity has a direct impact on the complications and associated comorbidities, depending whether it is more or less active. Side effects of immunosuppressant drugs are also a key factor for the development of complications in these patients. In fact, infections are one of the most frequent comorbidities in SLE patients and are responsible for a major contribution to the morbidity and mortality rate of this disorder [14]. People suffering from SLE showed higher probability to have infections of the nervous system and inflammatory bowel disease. Moreover, they have a substantial higher risk of developing cardiovascular complications (such as coronary heart disease and accelerated atherosclerosis) that may lead to the patient's death. Usually, the peak of occurrence of cardiovascular disease in SLE is observed 7 to 10 years after the diagnosis of the autoimmune disorder [38].

2.1 Diagnosis

As reported above, nowadays, SLE diagnostic is based on the assessment of the clinical symptoms and signals evaluated by the physician, and by laboratory tests. The laboratory tests more specific of SLE relies on the detection of specific antibodies, namely the detection of anti-dsDNA , ANA, anti-Sm and anti-RNP antibodies [7,39] The antibodies anti-Sm are detectable in SLE patients comprised in a percentage of 5 and 30% while anti-RNP are detectable in 25-47% of SLE patients. These antibodies are detected by counter

279 immunoelectrophoresis (CIE), immunoblot and ELISA, and are quite important in clinics.
280 Expression of anti-Sm is always associated with anti-RNP in patients with SLE. Although
281 its mechanism remains uncertain, they seem to have some modulatory effect on monocytes
282 [39,40].

283 Complement components activation are used to support either the diagnosis or the
284 evaluation of disease activity in SLE [37]. These laboratory tests are made to help
285 clinicians to better understand the patients' health status and to provide the patient an
286 accurate diagnosis, prognosis and treatment. It is of utmost importance to highlight the fact
287 that inadequate use of such tests can lead to misdiagnosis, inappropriate therapy and
288 needless health-care expenses [41]. The presence of specific autoantibodies is a key
289 parameter for the diagnosis of autoimmune diseases like SLE [42]. Throughout the years,
290 several diagnostic techniques have been developed for the detection of these specific
291 antibodies, always bearing in mind the improvement of the specificity and sensitivity of
292 the analysis. The main techniques used are immunofluorescence (IIF), enzyme-linked
293 immunosorbent assay (ELISA) and recombinant autoantigen technology.
294
295 These laboratory techniques have their own advantages and disadvantages; IIF is being
296 replaced by enzymatic immunoassays, ELISA`s requires autoantigens to have extremely
297 high purity and the technology of recombinant autoantigens is highly demanding. On the
298 other side, recombinant autoantigens are widely used for the diagnosis of autoimmune
299 diseases since it brought major developments onto diagnostic techniques. The same
300 progress is needed to be made with autoantibodies used as controls in diagnostic tests.
301 However, considering that autoimmune diseases are characterized by remission
302 (asymptomatic periods) and relapsing periods (with acute symptoms) that occur with

different degrees of severity, more reliable biomarkers are needed to predict, or early diagnose, these periods and to evaluate their degree of severity. They are also important to avoid disease related damage of affected organs, to decrease associated morbidity and to help evaluating treatment efficiency.

2.2 The role of lipids in SLE pathogenesis

Autoimmune diseases are strongly influenced by gene mutations and the immune system will be affected as a consequence. SLE pathogenesis is not only associated with genetic dysregulations but it has also been associated with alteration in lipid metabolism including lipid oxidation. In fact, lupus has been strongly associated with oxidative damage in different levels due to the increase of oxidative stress conditions, which can be correlated with mitochondrial dysfunction and cell death. Enhanced neutrophil death called NETosis is a characteristic event of lupus pathogenesis . Also, oxidative stress and lipid alteration and oxidation have been related with T lymphocyte dysfunctions, as well as to the systemic inflammation. In all of these events, lipids seem to play a fundamental role in their regulation.

2.2.1 Oxidative stress and lipids in SLE

Since autoimmune diseases have an effect on the innate immunity, response neutrophils, as they are the first line of defence of the immune system, will therefore be affected as well [43]. To eliminate pathogens, neutrophils produce superoxide anion, which is a precursor of several types of ROS. It has been reported that neutrophils in SLE are malfunctioning, which is associated with the increase of ROS production that is implicated in the pathogenesis of this disease [43–45]. In this way, uncontrolled or chronic production of ROS by neutrophils may lead to severe oxidative damage in several

biomolecules, in particular in lipids of the membranes [46,47]. Also, a very important finding was the fact that ROS production by neutrophils decreased in patients in relapsing phases when compared to patients in remission [43]. This reduction has also been seen in other autoimmune diseases such as multiple sclerosis, Behcet`s disease and Guillain-Barre syndrome [48–50], supporting the importance of neutrophils` dysfunction in autoimmune diseases. A possible explanation for this reduction could be the exhausted status of neutrophils due to overproduction of ROS during active phase of the disease [45]. However, Elloumi *et al* suggested that in neutrophils, after the oxidative burst and the production of ROS, there is a second stage with lower levels of ROS production in these immune cells, inferred by the observed decrease of malondialdehyde levels, a lipid peroxidation product, in neutrophils of patients with lupus [43]. They also observed that, within patients, malondialdehyde levels were higher in patients in relapsing than in patients in remission stages, which is indicative of higher levels of oxidative stress during aggressive periods of this disease. [43].

SLE is a multifactorial disorder, lipids and lipid metabolism have been correlated with this autoimmune disease in several ways. The overproduction and increase of ROS and oxidative stress is usually associated with lipid peroxidation and alteration in lipid metabolism [51]. Reaction of ROS with lipids in membranes can lead to the formation of lipid hydroperoxides that can be further degraded into small reactive carbonyl species, such as 4-hydroxynonenal (4-HNE), a toxic aldehyde that can react with proteins and modify their structure and function. The 4-HNE is proven to be significantly elevated in SLE [52]. People with SLE suffer from inflammation, oxidative stress and alteration of energy production pathways, alongside with a prothrombotic state and a disturbance in the lipid profile. Besides malondialdehyde and lipid peroxidation products, elevated levels

were also observed for gamma-glutamyl peptides, gamma-glutamyltransferase, leukotriene B4 and 5-hydroxyeicosatetraenoic acid [36]. Lipids` oxidation can also lead to the formation of other products such as F2 isoprostanes, formed by oxidation of arachidonic acid, and found in plasma and urine of SLE patients [53]. Isoprostanes are biomarkers of oxidative stress and are usually elevated in acute phases of the disease [51]. Overall, it is well accepted that oxidized lipids have a significant role in the pathogenesis of lupus, however their action is far from being completely elucidated.

2.2.2 NETosis and lipids in SLE

Neutrophils` dysfunction is also associated with their degradation through a specific activation-induced cell death process called NETosis, which is similar to apoptosis. The result of this process is the formation of a transient web-like organelle known as neutrophil extracellular trap (NET) [54]. The development of NET has been recognized as a significant mechanism in SLE`s pathogenesis, that is associated with NETosis, characteristic of this AID (Figure 1) [55]. NET is responsible for the release of active oxidative enzymes that will induce a proatherogenic mechanism. Those enzymes can modify HDL to such an extent that they lose their anti-inflammatory and vasoprotective properties and start failing in mediating reverse cholesterol transport [56]. This data is in agreement with the altered lipoprotein profile that is characteristic of SLE` patients and is going to be addressed further ahead.. Oxidized lipids, for example oxidized cardiolipin and oxidized phosphatidylethanolamine (PE), have been reported as important players in apoptosis and ferroptosis, it would be interesting to develop more research in order to understand the role of lipids and oxidized lipids in NETosis.

In addition to NETosis, apoptosis of SLE lymphocytes, mitochondrial dysfunctions, the enhancement of the inflammation cascade and the oxidative stress conditions that occur

in these patients also contribute for the multifactorial aspect of SLE. Dendritic cells (DC) are considered the professional APCs and their main function is to prime naïve T cells activation [57]. DC have been correlated with SLE pathogenesis, essentially in the induction and progression of this disease [58]. However, their action is not completely clear so far.

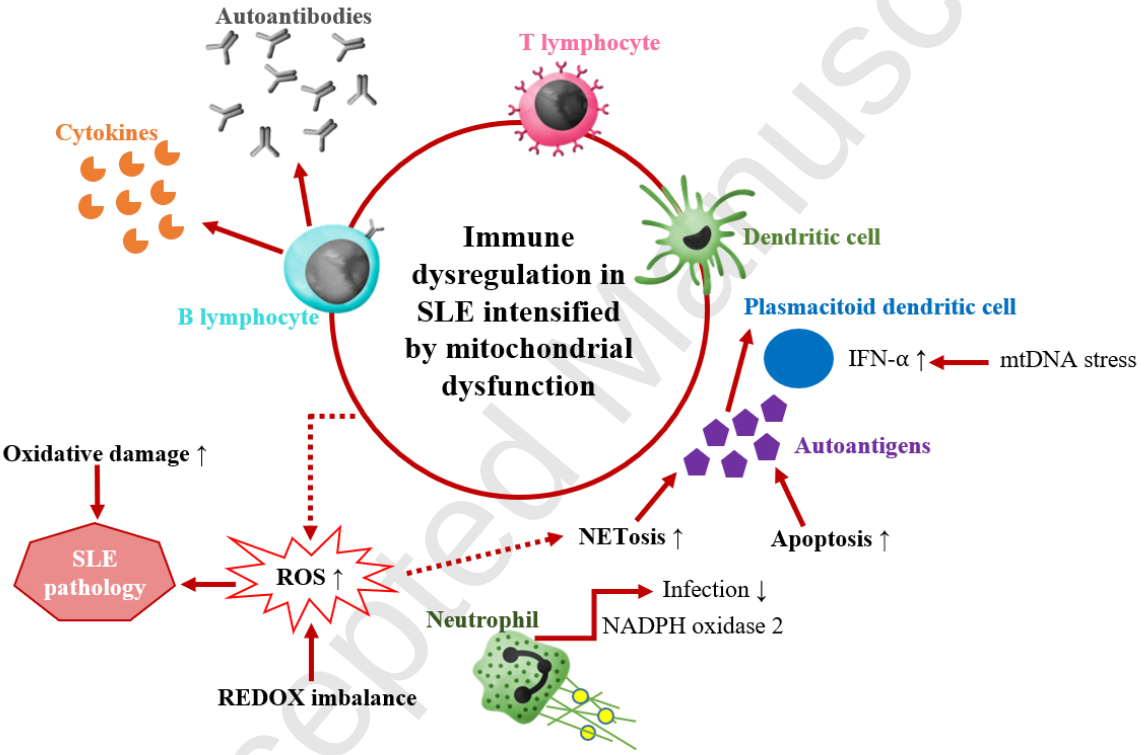

Figure 1. Schematic representation of NETosis` role in SLE pathogenesis. Under sufficient concentrations of ROS, a neutrophil undergoes NETosis to form NET, which is a web-like structure that contains nucleic acid and nucleic protein as well as cell remnants. NET is the source of several autoantigens. Those autoantigens will activate plasmacytoid dendritic cells to release IFN-α and consequently prompt autoimmune destruction. Adapted from H.T. Lee *et al* [11]. Cellular and autoantibodies pictures withdrawn from "BigPicture" [59].

2.2.3 T lymphocytes, mitochondrial dysfunction and lipids in SLE

The incorrect functioning of mitochondria in lupus T lymphocytes leads to an elevated mitochondrial transmembrane potential or persistent mitochondrial hyperpolarization [60]. This hyperpolarized state of mitochondria is found to be markedly higher in SLE patients than in healthy controls which may lead to cell death pathways [32,60,61].

Chronic inflammation is one key characteristic of SLE and it is exacerbated by oxidative stress and mitochondria dysfunction. Leishangthem *et* al found significantly increased levels of superoxide anion free radicals in mitochondria of lupus` patients which is suggestive of mitochondria dysfunction [61]. Lipids in mitochondria play an important role in controlling mitochondria functions; they are part of the membrane transport events; therefore, the lipid specific regulation of channel transport mechanisms is a decisive aspect related to membrane functions. Malfunctioning of lipid specific mitochondrial membrane transport may have a dangerous effects on cellular health status and raise cell based disease states [62]. T cell dysfunction in SLE have also been correlated with altered lipid metabolism [63]. It was determined that T lymphocytes of SLE patients have an altered glycosphingolipid profile in their membranes, specifically, lactosylceramide, globotriaosylceramide and monosialotetrahexosylganglioside levels were significantly increased when compared with healthy controls [64]. Although glycosphingolipids are not so abundant in cell membranes, they are very important in inflammation/immune response and cell-cell signalling.

There are some evidences that alterations in lipid profile and in lipid metabolism in these patients have an important role in SLE pathogenesis. Therefore, it is of utmost importance to improve the screening methodologies to evaluate the alterations in lipid

profile of SLE and other AID patients by using robust and accurate approaches. Omics platforms and lipidomics, in particular, are suitable tools to contribute to understand the role of dysregulation in lipid metabolism in SLE and also in other AID. Besides, lipidomics is also important to comprehend how these alterations are reflected in the complexity of the biologic processes and for fingerprinting lipid profile alterations for new potential biomarkers.

3. Lipidomics in SLE and in other autoimmune diseases

The identification of the lipid profile at a molecular level in biological systems is nowadays performed using lipidomic approaches which could be used to identify biomarkers and to understand the contribution of lipids in disease pathogenesis, useful for new therapeutic strategies. The main analytical technique in lipidomics is MS combined with LC or GC. MS is an extremely sensitive technique and it requires a very small quantity of sample. Lipidomics starts with sample collection followed by lipid extraction The lipid extract is analysed in a mass spectrometer to identify the individual lipid species, usually using a LC-MS approaches [65]. The acquired data is analysed by using bioinformatics tools and statistical analysis.

In spite of the lack of knowledge regarding the variation of the lipid profile at a molecular level in SLE, there is evidence that lipid metabolism suffers alterations namely in lipoprotein metabolism during the course of SLE disease [52,66–73].

435

436 Disturbance of lipoproteins levels was reported in patients with SLE. Dyslipidemia,
437 an imbalance of lipids in the blood, is known to have a significant influence in
438 atherogenesis [66]. Dyslipoproteinemia deregulation was observed in SLE, namely by low
439 levels of HDL cholesterol and elevated levels of both VLDL cholesterol and triglycerides
440 (TG) [67]. Lupus patients also show increased concentrations of oxidized LDL and an
441 anomalous chylomicron metabolism, which is consistent with the higher levels of 4-HNE
442 [52,68,69]. Levels of oxidized LDL detected by the monoclonal antibody E06 are
443 significantly higher in SLE patients and are associated with cardiovascular disease that
444 develop in SLE [74,75]. SLE`s dyslipoproteinemia has a multifactorial origin and it is yet
445 unclear which factors are definitely involved in the pathophysiology of this disorder [70].
446 Treatment using some drugs, in particular steroids, contribute to this dyslipoproteinemia
447 especially when they are administrated at a high dosage. Dislypidemia can also occur as a
448 consequence of renal failure, one of the most common comorbidities of SLE. Thus, renal
449 involvement and disease activity are some other factors that seem to have the most
450 influence on this characteristic lipoprotein pattern of SLE patients [71–73]. HDL is a cell
451 cholesterol efflux promoter through the reverse cholesterol transport system and prevents
452 LDL oxidation as well, thereby it has an atheroprotective role in the organism. NETosis, as
453 described above, may be a fundamental intervenient in the mechanism implicated in this
454 HDL dysregulation [76]. SLE patients have a much higher risk of developing
455 cardiovascular diseases and this high susceptibility may be partially due to impaired HDL
456 metabolism. In this way, the development of strategies to improve HDL metabolism could
457 have promising effects on the lipid metabolism, ameliorating patients` lipoprotein values
458 and disease management [77,78].

Disease activity is one factor that may have a leading role on the changes of the patients` lipid profile. Differences in the lipid profile between flare and remission need to be considered to better understand lupus lipid metabolism. So far, and to the best of our knowledge, there is only one study that evaluates the changes in lipoprotein values between flare and remission of adult patients with SLE, but no significant differences were observed [70]. The study only detected a tendency of lipoproteins` values during flare to be worse when compared with the same parameters during remission. Patients in flare had higher values of total cholesterol, LDL and TG and lower values of HDL comparing to the same lipoproteins' values in patients in remission[70]. The tendency to higher levels of LDL, main targets of ROS, would promote the enhance ox-LDL levels, with contribution to inflammation and atherogenesis risk factors for SLE relapsing and comorbidities.Lipoprotein dysregulation in SLE patients has been associated with high active phases of the disease. It is also considered a risk factor for the development of cardiovascular diseases, once they have an atherogenic lipoprotein profile [66]. Lipidomics is a high throughput method that allows to evaluate the variation of lipids at a molecular level and has been used for the study of several chronic diseases. It is now well known that changes in lipids at molecular level can occur as a consequence of metabolism adaptation in a disease environment therefore can be used as important biomarkers of the disease. Lipidomics at molecular level is important to disease pathology explanation, identification of changes in lipid metabolism, discovery of new biomarkers and definition of new treatment strategies [79]. The study of the variations on the lipid profile at a molecular level using lipidomics has been applied in some autoimmune diseases, for instance rheumatoid arthritis and multiple sclerosis, however little is known about this issue in SLE (Table 3). Nonetheless, there are already a few studies that provide some evidence

considering a possible adaptation of the lipid profile regarding FA, free FA (FFA), PL, lysoPL (LPL), sphingolipids, TG and 4-HNE species profiles in SLE patients.

Table 3. Lipidomics studies on AID. Research made on PubMed data base under the terms lipid profile, lipidomic(s), phospholipid(s) and fatty acid (all studies published until 2019 were analysed). All the studies that did not use MS techniques were not taken into consideration.

Autoimmune disease	Lipidomic approach	Class of lipids	Reference
Systemic Lupus Erythematosus	GC-MS; MDMS-SL	FA; FFA; PL; LPL; Sphingolipids; TG; 4-hydroxyalkenal species	[38,52,80–83]
Rheumatoid Arthritis	GC-MS; LC-MS/MS	FA; PL	[84–91]
Multiple Sclerosis	MALDI-TOF/TOF; GC-MS; ESI-MS; LC-MS	PL; FA; Sphingolipids; Very long chain FA	[92–97]
Systemic Sclerosis	LC-MS/MS	PL; LPL	[98]
Psoriasis	GC-MS; HPLC-MS	FA; Sphingolipids; PL	[99–103]
Polymyositis/ Dermatomyositis	GC-MS; LC-MS/MS	FA; TG; PL; LPL; Sphingolipids	[104]
Celiac disease	HPLC-MS; GC-MS	PL; FA	[105–107]

Lipidomics revealed a marked increase in TG, individual lyso--phosphatidylethanolamine (LPE) molecular species and total LPE levels, and a significant decrease in some species of PE and phosphatidylcholine (PC), as it is detailed (Table 4)[52,83]. Serum total concentration of TG were increased, with markedly differences in its FA composition. It was determined a raise in TG either with 16:1, 20:3, 20:2, 20:1 or

22:6 FA [52]. The most significantly increased LPE species included 20:4 and 22:6 FA [52]. All plasmenylphosphatidylethanolamine (pPE) and plasmenylphosphatidylcholine (pPC) species, which are a subclass of PE and PC respectively, were substantially reduced in SLE patients` serum [52]. The species that suffered the most statistically significant reduction were pPE species of 16:0-20:4 and 18:0-20:4 and pPC species of 18:0-18:0 and 16:0-18:2. The authors attributed the reduction of these species to three major metabolic pathways: decrease of phospholipase A_2 activation, peroxisomal dysfunction and peroxidation-mediated degradation. Oxidation of pPE is considered the major mechanism responsible for the reduction of pPE levels, which is corroborated by the increase of 4-HNE. It is important to consider that under oxidative stress conditions pPE species have an anti-oxidant role, which also supports the favourable oxidation and decrease of the content of its non-oxidized precursors. Another possible hypothesis proposed by the authors was that the activation of phospholipase A_2 was decreased, however, this hypothesis was discharged because there was not an accumulation of alkenyl LPE. The peroxisomal dysfunction hypothesis was also rejected since it appears to be normal in SLE patients, once plasmanylphosphatidylethanolamine and plasmanylphosphatidylcholine levels were unchanged [52]. pPE may become a biomarker for diagnosis/prognosis of SLE whereas its reduction is strongly associated with disease activity (SLEDAI), oxidative stress (ROS) and pro-inflammatory cytokines. It has also been reported an increase in plasma levels of PE species (16:0-18:2), (18:0-18:2), (18:1-18:2), (16:0-22:6) and (18:0-22:6), and also an increase of phosphatidylinositol (PI) species with (18:0-18:2) [83]. It was determined a reduction as well in other PE species such as PE (16:0-18:1), (18:1-20:4), (20:0-20:0) and (20:0-20:4), in PI species with (18:1-20:4) and a reduction in lyso-phosphatidylcholine (LPC), namely, LPC 18:2 [83].

The total content of sphingomyelin (SM) and ceramide species in SLE patients is not different from that found in healthy people. On the other side, it was described that the FA composition of both SM and ceramide species was modified in patients with SLE[83]. In SLE patients it was identified a significant increase in SM with N18:0, N18:1 and N22:0 comparing to the healthy control group ("N" stands for the amide linage of the acyl chain). In ceramide species, it was determined a significant decrease in ceramides with N22:0, N23:0 and N24:0 with a hydroxyl group on the second position of the acyl chain and a significant increase in N24:1[83].

FA composition of plasma and red blood cells can be changed by diet and steroids use, consequently, those alterations also affect circulating lipid profile and thus PL`s composition [38]. The variation of FA in PL have a direct impact on the fluidity of cell membrane of immune cells [108]. Moreover, changes in FA composition in PL of membranes of immune cells can affect the quantity and quality of essential FA, which are released by phospholipases and to be available for the production of inflammatory mediators that are released by these cells. Essential FA that are used in eicosanoid synthesis derive from PL pools. For instance, arachidonic acid (AA, 20:4*n*-6) that promotes production of inflammatory molecules, while eicosapentaenoic acid (EPA, 20:5*n*-3) is a precursor of anti-inflammatory mediators [109]. Plasma levels of FA depict recent dietary fat intake while red blood cells levels reflect longer dietary patterns. There are a few reports that suggest a relationship between FFA metabolism and SLE [37,110,111]. FFA can change their levels according with disease activity [112–114].

High levels of saturated FA and low concentrations of PUFA have been detected in plasma of SLE patients, which were correlated with autoimmunity and inflammatory processes [110]. It was also found that oleic acid and AA are decreased in patients with

lupus and that higher disease activity are usually associated with a lower level of linoleic acid [37]. Using LC-MS and GC-MS technologies, Wu *et al* found that medium-chain FAs and serum FFA were upregulated, while long-chain FAs (including ω-3 and ω-6 essential FA) were markedly downregulated, which is suggestive of oxidative conditions [115]. Most probably, the decrease of PUFA could be correlated with oxidation of the unsaturated FA due to the increase of ROS products associated with enhanced oxidative stress conditions. However, with GC-MS platforms, Shin and co-workers detected significantly higher levels of palmitoleic and oleic acids (known as anti-inflammatory FA) once they regulate the activation of immune cells (as well as myristic and eicosenoic acids). On the other side, they found markedly decreased concentrations of caproic, caprylic, linoleic, stearic, AA, eicosanoic, behenic, lignoceric and hexacosanoic acids [82]. In general, having in consideration the total content of FFA, saturated FFA levels were reduced and PUFA concentrations were increased [82]. On the contrary, it was also discovered that these patients have lower levels of EPA (which is an ω-3 PUFA) and a reduction of ω-3 index along with a significantly higher ratio of the inflammatory mediator AA to EPA, which is clearly favouring an inflammatory environment [38]. These findings are not in agreement with those reported before presenting the need for more studies in this field. Hereupon, alterations in FA profile in SLE may result from low dietary intake (however it cannot be generalized to every SLE patient due to body mass index differences), lipid peroxidation and/or defects in essential FA desaturation and elongation enzymatic reactions. However, there are controversial findings regarding the changes of FA profiles and its correlation with disease activity, which suggests that more studies are needed in this field of lipidomics [82,111]. Some of these MS studies present contradictory results which

may be due to the different methods used for lipid extraction, the type of analysed sample, the number of patients under study or even the chosen lipidomic approach (Table 4).

It seems that lipid metabolism in SLE can be improved with dietary fish oil supplementation, which have been shown to have lipid lowering effects. Fish oil has in its composition omega-3 PUFAs, specially EPA and DHA. EPA will promote the formation of anti-inflammatory mediators and thus having an anti-atherogenic and anti-inflammatory effect [116]. Therefore, fish oil supplementation is prone to balance immune, atherosclerotic and inflammatory events in patients suffering from lupus [117,118]. It was noticed an increase in EPA and DHA incorporation into cell membranes as well as a decrease in AA after fish oils supplementation, as well as the level of lipoproteins [80]. In fact, TG, VLDL concentrations and the ratio of total to HDL decreased, and it was detected a significant elevation of HDL levels in the group of SLE patients that were receiving fish oil supplementation [80]. However, the effects of fish oil supplementation are dependent of the dose administrated. Also, the administration of highly purified EPA alone has proved to have beneficial effects on the lipid profile of patients with SLE by decreasing the oxidative stress. After treatment, patients showed significantly increased levels of EPA, comparing with the pre-treatment period, as well as markedly decreased concentrations of AA [81].

Through multi-dimensional mass spectrometry-based shotgun lipidomics it was found an increase of the levels of 4-hydroxyalkenal species [52]. The elevation of 4-hydroxyalkenals levels alongside with the upregulation of LPE and the increased levels of oxidized LDL mention above, are undoubtedly indicative of lipid peroxidation [52,68]. Isoprostanes are biomarkers of lipid peroxidation formed through the non-enzymatic

peroxidation of AA. The urinary 8-isoprostane levels were determined to be significantly decreased in SLE patients` after the administration of the EPA treatment [81].

All of these studies clearly demonstrate the role of lipids in inflammatory processes, in immunity, and in the onset and development of AID. Also, lipidomics has potential as a tool to aid both in AID diagnosis and therapeutics by allowing a detailed lipidome profiling of SLE and AID patients, which is a first step for the identification of new lipid biomarkers of disease.

Table 4. Main lipid species that showed variation in SLE reported in published lipidomics studies, in PubMed data base, using MS approaches.

4. Concluding remarks and future perspectives

Lipids have a plethora of important biological functions and play key roles in many intra and intercellular signalling pathways that are related with the maintenance of cell and tissue homeostasis. Alterations in lipid profile and individual lipid molecular species are important players of several pathologies, including SLE and other AID. There are evidences that there are alterations in lipids and in lipids metabolism in SLE patients, showing the importance that lipid regulation has in SLE pathogenesis. SLE is usually associated with dyslipidemia. Some studies also reported alterations of some molecular lipid species, from particular classes of lipids, such as variation of plasmalogens and FA. Oxidized PL were also reported in few studies. Alteration in lipids seems to be a common issue in SLE, that has been scarcely addressed and are far from being completely elucidated. Therefore, it is of outmost importance to improve the screening methodologies

to evaluate the alterations in lipid profile of SLE, also useful in other AID. Lipidomics is nowadays the best methodological approach to understand the modulation of lipid metabolism in SLE, and advance knowledge on SLE`s pathology. Lipidomics is a promising tool to better understand autoimmune diseases` lipidome, enabling the identification of potential disease biomarkers. Thus, it would contribute to a more precise and early diagnosis, evaluation of disease progression, to predict relapse episodes, and evaluate therapy outcomes. Utmost, lipidomics approaches could be a promising tool to personalise medicine, allowing the reduction of the morbidity and mortality of SLE and other AID.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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945 **List of Tables**

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956 **List of Figures**

964
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970 *Table 4.* ***Main lipid species that showed variation in SLE reported in published lipidomics***
971 ***studies, in PubMed data base, using MS approaches.***

Reference	Analytical method	Lipid extraction method	Type of sample	Sample size	Results	
					↓ Reduction	↑ Increase
Aghdassi *et al* [38]	GC-MS	Chloroform/methanol (2:1, v/v)	Red blood cell total lipids (%) Plasma total lipids (%)	33 F	EPA, ω-3 index, total PUFA, total ω-6 Linoleic acid	AA/EPA plasma total trans-FA
Hu *et al* [52]	MDMS-SL	Modified Bligh and Dyer	Serum (nmol/mL serum)	30 F	PE species (16:0-18:1), (16:0-20:4), (18:0-20:4), (18:0-22:4), (18:0-22:5) PC species (18:0-18:0) and (16:0-18:2) TG with 18:2	LPE with 20:4 and 22:6 Total LPE content TG with 16:1, 20:3, 20:2, 20:1, 22:6 4-HNE
Clark *et al* [80]	GC-MS	Modified Bligh and Dyer	Platelet membrane phospholipids	8 F + 4 M	AA	EPA, DHA
Nakamura *et al* [81]	GC-MS	Folch *et al*	Plasma phospholipid fraction (mol%)	5 F + 1 M	Linoleic acid, AA, DHA	EPA/AA, EPA, DPA
Shin *et al* [82]	GC-MS	Paik *et al*	Plasma (FFA%)	41 F	Caproic, caprylic, linoleic, stearic, AA, eicosanoic, behenic, lignoceric and hexacosanoic acids	Myristic, palmitoleic, oleic, and eicosenoic acids
Lu *et al* [83]	MDMS-SL	Modified Bligh and Dyer	Serum (mol%)	30 F	PE species (16:0-18:1), (18:0-20:4), (18:1-20:4), (20:0-20:0), (20:0-20:4) PI species with (18:1-20:4) LPC with 18:2 Ceramides with N22:0, N23:0 and N24:0 with hydroxyl group on the 2nd position of the acyl chain	PE species (16:0-18:2), (18:0-18:2), (18:1-18:2), (16:0-22:6) and (18:0-22:6) PI species with (18:0-18:2) SM with N18:1 and N18:0 Ceramides with N24:1

F- Female; M-Male