Universidade de Aveiro Departamento de Química
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Renata Alexandra Boaventura Faria

Perfil lipídico de cérebro e coração de ratinhos com stresse crónico

Lipid profile of brain and heart from mice with chronic stress

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Doutora Maria do Rosário Gonçalves dos Reis Marques Domingues, Professora Auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Cláudia Margarida Gonçalves Cavadas, Professora Auxiliar da Faculdade de Farmácia da Universidade de Coimbra.

Dedico este trabalho aos meus pais, ao Renato, ao Igor e a Eva pelo incansável apoio.

o júri

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palavras-chave

Stresse crónico, cérebro, coração, fosfolípidos, espectrometria de massa, lipidómica

resumo

O stresse crónico é uma resposta natural, adaptativa do organismo a pressões psicológicas e físicas. O stresse crónico tem vindo a ser considerado uma séria preocupação de saúde pública, já que se encontra envolvido com o desenvolvimento de vários distúrbios fisiopatológicos. Algumas das consequências do stresse crónico já descritas incluem intolerância à glucose, inflamação, defeitos cognitivos e especialmente doenças relacionadas com o cérebro, como doenças neurodegenerativas e depressão. As alterações que ocorrem no sistema nervoso, em situações de stresse crónico, relacionam-se com o desenvolvimento de alterações ao nível do cérebro e também com alterações em funções cardíacas. Apesar de várias evidências sugerirem consequências nefastas do stresse crónico, pouco se sabe ainda sobre as suas consequências a nível biomolecular, em particular sobre o seu efeito nos lípidos celulares. Assim, o objectivo deste trabalho foi estudar o perfil fosfolipídico em condições de stresse crónico, no cérebro e no coração de ratinhos. O perfil lipídico foi avaliado usando uma abordagem lipidómica. As diferentes classes de fosfolípidos foram separadas e quantificadas. A avaliação da peroxidação lipídica foi realizada pelo método de FOX II. O extrato lipídico total foi analisado por cromatografia líquida de alta resolução acoplada a espectrometria de massa (HPLC-MS e MS/MS), permitindo a identificação da estrutura dos fosfolípidos, nomeadamente o seu grupo polar e a sua composição em ácidos gordos.

Os resultados revelaram alterações nos níveis cerebrais de fosfolípidos, tendo-se verificado o aumento significativo das fosfatidilcolinas (PC) e uma diminuição significativa dos fosfatidilinositois (PI) e das cardiolipinas (CL). Não foram detetadas, porém, alterações significativas no perfil molecular de cada classe de fosfolípidos, excepto no perfil da classe dos ácidos fosfatídicos (PA). Os nossos resultados comprovam ainda que há a formação de hidroperóxidos da CL no cérebro em situações de stressee crónico. A oxidação da CL é um indicador da apoptose celular que pode ser associado com a morte neuronal. No coração, os nossos resultados revelaram que não existem alterações significativas do perfil fosfolipídico em situações de stresse crónico, havendo apenas alguma variação no conteúdo total das classes.

Assim, concluímos que em condições de stressee crónico os fosfolípidos do cérebro são os mais afectados, havendo mesmo a formação de produtos de oxidação da cardiolipina, sugerindo a ocorrência de importantes alterações a nível mitocondrial.

keywords Chronic stress, brain, heart, phospholipids, mass spectrometry, lipidomic

abstract

Chronic stress is a natural adaptive response to psychological and physical pressures. Chronic stress has been considered a serious public health concern since it is involved in many pathophysiological disturbances. Some of the consequences of chronic stress already described include glucose intolerance, inflammation, cognitive defects and especially diseases related with brain, such as neurodegenerative diseases and depression. The modifications that occur in the nervous system under chronic stress conditions are related with the development of alterations in the brain and also with changes in cardiac functions. Despite of some evidences suggesting adverse effects of chronic stress, little is known about the consequences at the molecular level, especially about its effects in lipids. Thus, the aim of this study was to evaluate the phospholipid profile changes in the brain and in the heart of chronic stressed mice. The lipid profile was evaluated using a lipidomic approach. The different phospholipid classes were separated and quantified. The lipid peroxidation was evaluated by FOX II assay. The total lipid extract was analysed by high performance liquid chromatography mass spectrometry (HPLC-MS and MS/MS), allowing the identification of the detailed structure of phospholipids, namely the polar head and fatty acyl composition.

The results revealed changes in brain levels of phospholipids, with a significant increase in phosphatidylcholine (PC) content and a significant decrease in phosphatidylinositol (PI) and cardiolipin (CL) contents. No significant changes were observed in molecular profile of each phospholipid class, except in phosphatidic acid (PA) class. We were also able to identify CL hydroperoxides after chronic stress, which indicates that the increase in hydroperoxides content verified by FOX II method was due to the oxidation of CL. Interestingly CL oxidation is an early indicator of cell apoptosis that could be associated with neuronal death. In the heart, our results showed no significant changes in PL profile of the molecular species in chronic stress situations, having only some variation in the total content of the classes.

Therefore, we conclude in chronic stress conditions brain phospholipids are the most affected, having the formation of CL oxidation products, suggesting the occurrence of important changes in mitochondria.

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Abbreviations:

α-MSH - melanocytes stimulating hormone
AD - adrenalin
ADP - diphosphate adenosine
ACTH - adrenocorticotrophic hormone
APCI - atmospheric pressure chemical ionization
ATP - triphosphate Adenosine
Cer - ceramid
CL - cardiolipin
CNS - central nervous sytem
CRF - corticotrophin releasing factor
DAG - diacylglycerol
DNA - desoxyribonucleic acid
DSM-IV - Diagnostic and Statistical Manual of Mental Disorders
ESI - electrospray ionization
FA - fatty acids
GC - gas chromatography
GL - glycolipids
GP - glicerophospholipids
GR - glucocorticoid receptor
HPA - hypothalamus-pituitary-adrenal gland axis
HPLC - high performance liquid chromatography
IL - interleukin
MDA - malondialdehyde
MAG - monoacylglycerols
MR - mineralocorticoid receptor
MS - mass spectrometry
MS/MS - tandem mass spectrometry
NA - noradrenalin
PA - phosphatidc acid
PC - phosphatidylcholine
PE - phosphatidylethanolamine
PG - phosphatidylglycerol
PI - phosphatidylinositols
PK - polyketids
PL - phospholipid
POMC - pro-opiomelanocortin
PR - prenol lipids
PS - phosphatidylserine
PUFA - polyunsaturated fatty acid
Q - quadrupole analyzer
ROS - reactive oxygen species
SiO_2 - silica
SL - saccharolipids
SM - sphingomyelin
SNS - sympathetic nervous system
SP - sphingolipids
ST - sterol lipids
TAG - triacylglycerols
TLC - thin layer chromatography
TOF - Time of flight analyzer

I. Introduction

I. Introduction

The exposure of an organism to stress is very common among various tasks of our day and its impact on our quality of life is a high concern. Causes of stress are varied and are not the same for every individual. Thus, the susceptibility to stress and the response to it are variable and dependent on each individual. Sometimes stress can function as a stimulus, encouraging people to achieve certain goals on time and enhance their capacity to carry out their tasks, showing that stress can have a positive effect. However, these positive effects are characteristic of acute stress, while chronic stress, the stress lived on a daily basis, has being considered as a key factor for the development of some pathologies, like depression, neurodegenerative and cardiovascular diseases [1]. In 1979, the United States Public Health Service cited stress as a serious public health concern. Stress-related problems are a main worry and it's estimated that 20-80% of premature deaths are related to diseases of the life style [2]. The relationship between stress, health and disease has strong implications in models of treatment and intervention [3]. The response of the organism to stress has the intention to help the organism to dealing properly with the external and internal changes. Chronic stress causes deep changes in many biochemical and physiological mechanisms and also induces changes in biomolecules, including phospholipids [4].

Phospholipids are important molecules, which have numerous roles in cellular and subcellular membranes. These molecules are essential for maintaining cells integrity and functions. It has been described that changes in phospholipid structure, specially by exposure to oxidative stress, are involved in the onset of inflammatory reactions [5]. Some pathological states developed in chronic stress have been related with an increase in brain's oxidative stress [6]. It causes deep changes in biomolecules, such as lipids, proteins and nucleic acids. Therefore, the toxic effects caused by oxidative stress have been associated with the development of many diseases, including neurodegenerative diseases, diabetes and other diseases related to the aging process [7, 8].

1 Stress: definition and classification

Stress is defined as a generalized and non-specific response to an agent that threatens the homeostasis of the organism [9, 10]. These stress agents are divided in 4 classes: physical agents (like trauma, surgery and/or intensive hot/cold); chemical agents (like lack of oxygen supply and/or acid-base imbalance); physiological agents (pain, haemorrhagic shock and/or heavy physical exercise); and social agents (personal conflicts, problems at work and/or lifestyle changes). Stress is divided in acute stress and chronic stress. Exposure to acute stress allows the organism's adaptation to the changes and affects the brain, thus some responses can be initiated [10, 11]. On the other hand, chronic stress induces maladaptation of the organism, causing the development of disease. So, the duration and the nature of the stressor applied is determinant for the adaptation being protective or damaging [6]. Thus, there are many types of responses to stress agents, being the most studied the stimulus of sympathetic nervous system (SNS) and the stimulus of the hypothalamus-pituitary-adrenal gland (HPA) axis [9]. It is also important to note that the responses from the body to stress can lead to various physiological changes such as increased blood pressure, increased heart rate, mobilization of the body's energy stored, anxiety, mood variations, among many other [10].

1.1 Chronic stress

Chronic stress is a state of continuous threatened homeostasis, in which the organism, to re-establish the equilibrium, triggers a repertory of physiologic and behavioural responses, constituting the adaptive response. The adverse consequences of stress result from the inability of the individual to deal with the stressful stimuli or form maladaptive responses that may restore homeostasis in short-term, but may cause damage at different body systems in long-term. Some studies performed on rats showed that chronic stress induced pathophysiological, biochemical and behavioural disturbances, including diabetes, autoimmune diseases, depression, cognitive defects and male sexual dysfunction [3, 4]. Changes in body weight, in locomotor activity and the corticosterone levels increase are some of the depression phenotypes that appear after the exposure of animal models to chronic stress [4]. These pathological states develop, at least partially,

due to the increase of oxidative stress verified in chronic stress situations, which can lead to neurological disturbances and neurodegenerative diseases [6, 12].

Emotional changes, such as anxiety, depression, anger, apathy and alienation, as consequence of exposure to stress conditions, represent a major financial burden of disease in Europe and the development of strategies for treatment should represent a major socio-economic priority [10]. Moreover chronic stress have different causes, but its effects are confused with other physiological changes and they occur in many organs and systems in the organism [13]. These facts makes the early detection of chronic stress situations extremely important so patients can have a more timely intervention [3].

Many brain related diseases, such as major depression and neurodegenerative diseases, have been associated with chronic stress, due to changes in the brain. Changes caused by chronic stress, like the increase of heart rate and blood pressure, contribute to changes in heart and thus to the development of cardiovascular diseases, a major cause of mortality [1, 14]. However, the molecular changes caused by chronic stress are still to a large extent unknown.

1.2 Effects of chronic stress in the nervous system

One of the main targets of chronic stress and the first to be affected is the nervous system. The stress response is initiated in the brain by the activation of HPA axis and SNS, which are responsible for many physiological changes. However, the duration and nature of the stress agent applied are important factors to determine if the adaptation to stress is protective or, on the other hand, damaging. Chronic stress has many adverse effects in many cellular functions, through the impairment of antioxidant defenses and by promoting the changes associated with oxidative stress, among others [6].

Some studies show that stress and the stress hormones, particularly glucocorticoids and catecholamine's, are related to the induction of changes in molecules linked with structural plasticity [9]. Stress causes loss of synaptic plasticity and density, then promoting changes in the transmission of nervous impulses that can be involved with the development of psychiatric and neurodegenerative diseases [15].

Psychological and physical stress, starting with impulses arising from brain cortical centers and transmitted through the limbic system, results in the release of chemical mediators, including noradrenaline (NA), serotonin and acetylcholine, which activate cells

of paraventricular nucleus of hypothalamus; these cells produce corticotrophin releasing factor (CRF), the coordinator of stress response. CRF passes to the pituitary to produce pro-opiomelanocortin (POMC), a protein that is subsequently cleaved to form the adrenocorticotropic hormone (ACTH), β-endorphin and melanocytes stimulating hormone (α-MSH). CRF together with the CRF derivative from activated neurons of amygdala central nucleus also stimulates *locus coeruleus* to secrete NA in sympathetic nerve endings. The central activation of SNS is also transmitted to the adrenal medulla, where is stimulated the production of adrenalin (AD). ACTH stimulates the adrenal cortex to produce glucocorticoids, which, with catecholamine, AD and NA, are the main stress hormones [9]. SNS is activated in an attempt to ensure the sufficient energy to the body to maintain vital functions, since in stress situations the energy requirements are higher. The results of this activation causes catecholamine plasma content increase that induces an increase in heart rate, dilatation of the bronchi, high blood pressure, metabolic changes for greater release of glucose, and constriction of blood vessels of stomach, kidney, skin and reproductive organs [3, 9]. Normally, this response is an acute response and requires the intervention of parasympathetic nervous system to the organism's adaptation. However, if these stimulus become too repetitive, without a recovery period, it will cause an imbalance between sympathetic and parasympathetic systems, which can cause an incorrect adaptation of the body, enhancing the onset of diseases such as hypertension, diabetes, among others [3]. An increase in this ratio sympathetic/parasympathetic nervous system has been linked with an increase in cardiovascular morbidity and mortality [16].

Stress promotes the activation of HPA axis, resulting, among other things, in glucocorticoids release from adrenal gland through the circulation. When the paraventricular neurons of hypothalamus are stimulated, CRF is released on the pituitary, stimulating the production of α-MSH, β-endorphin and ACTH. In turn, the ACTH stimulates the release of glucocorticoids by the adrenal gland cortex, mainly cortisol in humans and corticosterone in rodents [3].

Studies in animal models show that glucocorticoids have main roles in many peripheral organs and also in the brain, where they bind to intracellular receptors. Two major subtypes of receptors were recognized in brain: the mineralocorticoid receptor (MR), with an elevated affinity for corticosterone and aldosterone, and the glucocorticoid receptor (GR), which has a much lower affinity for the corticosterone and it's the main

regulator, by a mechanism of negative feedback, of the release of corticosterone. Due to the difference in affinity for corticosterone between the two receptors, their degree of occupation is different during the day, depending on the circulating levels of corticosterone. Only after stress exposure, or in a hormone peak in the circadian rhythm the GRs are activated to a considerable extent. Activated glucocorticoid receptors translocate to the nucleus where they regulate the gene transcription, as homodimers, binding directly to the elements of response on DNA, or as monomers, interacting with other transcription factors which bind to DNA. Consequently, changes in the level of activation of the MR and GR receptors will initiate specific changes in cells, particularly on the gene expression pattern. When translated into the proteins, these changes can initiate a slowly but persistent change of cell characteristics [11].

The HPA axis activation affects many functions of the organism. The production of CRF inhibits the gonadal growing and thyroid axis, resulting in a decrease in growth and sexual function. The increase in corticosterone levels enhances metabolic changes, that can result in the increase of endothelial dysfunction, elevated levels of low-density lipoproteins, cholesterol and triacylglycerol, visceral obesity, glucose intolerance and insulin resistance [3].

1.3 Effects of chronic stress in the heart

Cardiovascular diseases (CVD) are the leading cause of death worldwide and were responsible for almost 32% of all deaths in women and 27% in men in 2004. It is expected that in 2030, cardiovascular diseases will cause the deaths of 23,4 millions of people [17].

It is already known that factors like elevated blood levels of cholesterol, hypertension, diabetes mellitus and smoking are risk factors for the development of cardiovascular disorders. Among these, the central nervous system, especially the response to chronic stress seems to be extremely relevant in the pathogenesis of CVD. However, understanding how other factors contribute for this burden is essential to develop new treatment strategies [18].

There are already studies revealing that psychosocial factors, psychiatric and neurologic diseases are positively related with the development of some cardiovascular diseases, like arteriosclerosis and myocardial infarction, although the mechanisms of these interactions are not completely known [1, 19]. Epidemiological data clearly suggests that

depression is in general an independent risk factor for heart diseases [20, 21]. Takotsubo cardiomyopathy (or "stress cardiomyopathy") is probably the most remarkable example of how stress promotes direct heart injuries. Stress cardiomyopathy consists of a transient left ventricular dysfunction triggered by acute emotional or physical stress, whose clinical presentation mimics myocardial infarction [22].

Cardiovascular function is regulated by the autonomic nervous system, which as we know, encompasses two major divisions: sympathetic nervous system and parasympathetic nervous system; the activation ratio sympathetic nervous system/parasympathetic nervous system is fundamental to the pathophysiology of CVD [18]. Chronic activation of the SNS and/or decreased parasympathetic triggering is a remarkable feature of CVD. SNS, by the action of neurotransmitters and neurohormones, contributes to endothelial dysfunction, hypertension and arteriosclerosis; promotes insulin resistance and dyslipidemia; induces left ventricular hypertrophy; and increases the incidence of arrhythmia (Figure 1). In fact, the neurohormonal hypothesis of heart failure states that SNS is persistently activated in patients with heart failure, playing a major role on its progression and establishment [23].

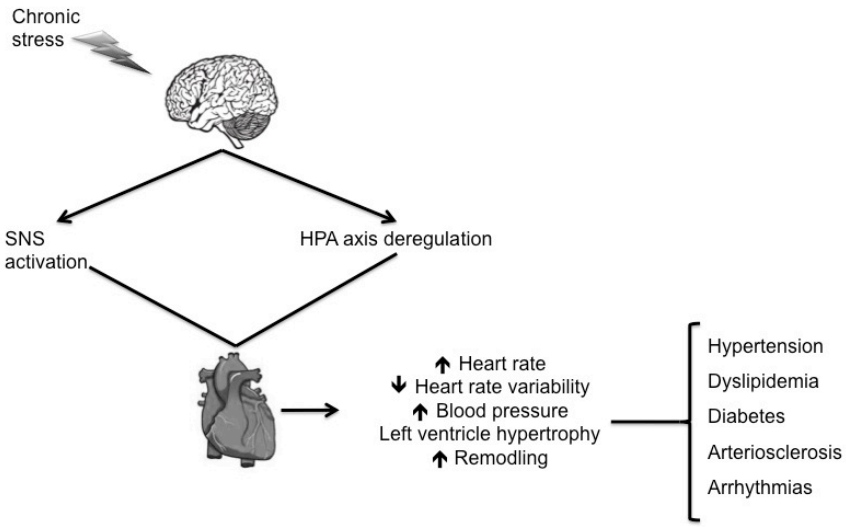

Figure 1. Relationship between the stressed brain and changes in heart that predisposes individuals to the development of different heart disorders (adapted from [18]).

The chronic stress conditions promotes the increase in some cytokines, that together with the stress hormones, catecholamine and glucocorticoids, produce acute phase proteins, like C-reactive protein, which is an important risk factor for myocardial infarction. During chronic stress, cytokines, glucocorticoids and other factors, like the

increase in blood flow and pressure, cause endothelial damage and platelet adhesion. Chronic stress and the acute phase biomarkers promote the progression of the inflammatory response with the activation of macrophages and the consequent formation of free radicals, modification of lipids and the activation of thrombotic events [1].

There are many evidences that SNS activation can alter both the cardiovascular activity and the lipid metabolism, and these changes persist as long as the stress or while SNS is activated [1]. The combination between the SNS activation, inflammation, increased cortisol levels and other not-healthy behaviours can lead to the development of profound changes in heart [19]. Some epidemiologic studies found a correlation between an increase in reactivity of the response to stress, progression of arteriosclerosis, the incidence of ischemic heart disease and mortality [1].

1.4 Chronic stress and the development of disease: Inflammation and Depression

Chronic stress conditions have been associated with many biochemical and physiological changes in the homeostasis of the organism [4]. Chronic stress has been considered an important risk factor for the development of depression [13] and plays a main role in the inflammatory response [9].

1.4.1 Inflammatory response produced by chronic stress

The inflammatory response is a protective mechanism and its relationship with stress response is extremely close. In our days we already know that the nervous system and the immune system are deeply connected, interacting bidirectionally. The central nervous system (CNS), by neuropeptides and neurotransmitters, acts with the immune system, which, in turn, acts back in the brain, inducing changes both in terms of behaviour (response to disease) and in the immune system [9].

The hormonal changes that characterize the stress situations can trigger inflammatory reactions. The main stress hormones (catecholamine and glucocorticoids) can induce an acute phase response, which is similar to the response elicited when an organism reacts to an invading microorganism, or sustains trauma and tissue damage. Stress may also activate the primary sensorial neurons, similarly to what happens when a toxin affects the nerves, such as in neurogenic inflammation. Catecholamine and

glucocorticoids effectively initiate a response characterised by the production of cytokines and acute phase markers, as it happens in inflammatory state [9]. Therefore, there is strong evidence showing that stress itself can cause an repeated inflammatory response or chronic episodes can trigger inflammatory diseases. Thus, the hormonal changes that characterise the stress response can induce an inflammatory process [1].

The HPA axis has an important role in inflammatory response regulation, since the secretion of glucocorticoids in stress response decreases the inflammatory activity. Corticosterone has a supressing effect on inflammation, in part, due to the inhibition of pro-inflammatory cytokines production [24, 25]. However, when the corticosterone levels are constantly high, cells may become less sensitive to the inhibitory stimulus of corticosterone, which can lead to the intracellular increase of interleukin-6 (IL-6) and possibly other pro-inflammatory cytokines [24]. It is further noted that, during chronic stress, brain oxidative stress is increased, which also influences the onset of inflammatory reactions [26].

The influence of chronic stress in inflammation becomes more evident with the knowledge that several diseases are related to the increase of pro-inflammatory cytokines, including cardiovascular diseases, osteoporosis, Alzheimer's disease and some cancers [27].

1.4.2 Depression induced by chronic stress and it effects on brain

Combined with chronic stress, depression may trigger modifications in inflammatory response. Moreover, chronic stress is the main factor that predisposes the individuals to the development of depression [13]. Depression has increased worldwide and it can become the main cause for incapacity in the whole world. According to the American Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), dysphoric mood is a core symptom of depression, in conjunction with other symptoms, as cognitive failure, body weight changes, deregulation of HPA axis and sleep patterns [11, 28].

Depression results from a maladaptation to the neuroplastic changes induced by stress, in specific neuronal circuits [13]. Thus, chronic stress acts like a factor that predisposes the appearance of depression. Studies on animal models show that exposure to chronic stress causes behavioural, morphological and hormonal changes, typical of depressive state [27]. Individuals with severe depression have shown inflammation,

manifested by an increase of cytokines, such as tumour necrosis factor (TNF-α), interleukins (IL-1 e IL-6) in peripheral blood and cerebral spinal fluid, as well as increased acute phase proteins, chemokines and adhesion molecules in peripheral blood. The relationship between inflammation and depression is so important that it are currently searched therapies that target the immune system for the treatment of depression [29].

Depression is an extremely incapacitating disease and, in addition to the mortality associated with suicide, makes the patients more susceptible to developing cardiovascular diseases and type 2 diabetes [13]. In addition, recent studies suggest that depression is a independent risk factor for the progression of cardiovascular diseases [30]. The relationship between depression and cardiovascular disease has attracted much attention, since cardiovascular diseases are presented as a major cause of mortality worldwide and depression increases the risk of developing these diseases in two to five times more [14, 19]. The increase in susceptibility to cardiovascular diseases is related to the increase in inflammation and consequently to the increase in pro-inflammatory cytokines [1]. Prospective studies with depressed individuals demonstrated that a major depression history was associated with higher risk of myocardial infarction. However, depression consequences in heart diseases extends to other conditions, including sudden cardiac death and heart failure [18].

All these findings together suggest the importance of interpreting chronic stress as a factor that can trigger many mechanisms, culminating in the development of various diseases, many of them chronic diseases. It is already known that chronic stress causes a very significant increase in brain oxidative stress [6]. Oxidative stress leads to a change in the homeostasis that underlie the development of various pathological situations, such as inflammation, diabetes and arteriosclerosis [26]. The most affected organs in chronic stress conditions are the heart and the brain [18]. These two organs have a high content of lipids and changes related with these biomolecules have already been described in other kind of diseases and/or injuries. However, brain and heart lipid profile changes in chronic stress are not known yet. Taking this into account, it is relevant to investigate the lipid profile in brain and heart after chronic stress situations, more specifically phospholipids, since these are the most abundant lipids in cells and play main roles.

1.5 Lipids and brain diseases

The effect of chronic stress in brain lipid profile was not investigated yet. It is known that an increase in lipid peroxidation occurs [31], but is unknown the oxidised products formed and if there is other kind of changes in lipid profile. However, there are some studies that correlate changes in the lipid profile with the development of brain diseases [32-37].

Several studies described fatty acid composition changes in brain phospholipids in stroke and in neurodegenerative diseases [5, 32, 33, 38]. The phospholipid analysis in *post mortem* human brains shows specific compositional changes in white matter compared with grey matter in patients in the beginning of Alzheimer's disease [26]. Alzheimer's Disease has been associated with altered lipid metabolism and increased lipid peroxidation [39]. In Parkinson's disease, lipid peroxidation markers were also found to be significantly increased in several brain regions [32]. In addition to the studies in brain lipid profile in neurodegenerative diseases, there are also some studies that relate alterations in lipid metabolism with some neurological disorders, namely schizophrenia, bipolar disorders and epilepsy [32, 33].

The biochemical mechanisms underlying the development of depression related to chronic stress are poorly known. The study of physiological processes that occur in brain during depression, based on biochemical mechanisms associated to neuronal membrane modifications, reflect an increase in lipid peroxidation in every brain regions, especially on striatum and hypothalamus [40]. The increase in oxidation destabilizes membranes and causes cell death [41]. The homeostatic impairment in brain regions may explain behavioural changes in individuals with depression [40].

Therefore, it is known that several diseases that affect the brain homeostasis and cause the impairment of lipid metabolism and/or in increase in lipid peroxidation [26]. Thus, it is important to investigate the lipid profile in chronic stress situations.

Taking into account that chronic stress causes the impairment of various cardiac functions, via SNS hyperactivation, it is important to understand how chronic stress acts in heart phospholipid profile.

1.6 Lipids and heart diseases

Several studies correlate changes in phospholipid profile with cardiovascular diseases and cardiomyopathies, although none of them related with chronic stress conditions [42-46]. However, it is known that cardiomyocytes are very sensible to changes in the lipid profile of cell membrane [42]. Changes in lipid profile associated to lipid metabolism changes and/or increase in lipid peroxidation were described in different heart diseases, such as arrhythmias, arteriosclerosis, heart failure and others [5, 26, 42]. Moreover, diabetic cardiomyopathy is characterized by the presence of alterations in lipid composition in the myocardium and with increased lipid peroxidation [44]. Also, many studies demonstrated phospholipid metabolism changes during myocardial ischemia [47-50].

Moreover stress induces myocytolosys that consists in a myofibrillar degeneration in which the cell dies in a hypercontracted state with early myofibrillar damage [51]. The histological changes in stress cardiomyopathy consist in inflammatory cell infiltration, increase in extracellular matrix protein levels and myocytolosys [18].

The effect of chronic stress in cardiomyocytes may lead to changes in phospholipid composition and structure, affecting cell function. Thus it becomes important to study how chronic stress affects heart phospholipids, since these molecules are proved to be important in the development of heart diseases.

2 Lipids

Although in the past, lipids have been considered no more than constituent elements of membranes and storage energy molecules, today it is known that lipids have many other functions, such as cellular signalling mediators, facilitators of cell/cell and cell/protein interactions, hormone precursors (for example, prostaglandins and steroids) and in maintenance of electrochemical gradients [5, 52-54]. These discoveries lead to an increased interest in lipid study and in its definition/classification. Lipids can be defined based on their solubility in organic solvents or by presence of long hydrocarbon chains. Lipids comprise a vast number of chemical molecules, structurally and functionally distinct, resulting from combinations of fatty acids with different structures. Lipids are usually divided in 8 categories, a classification proposed by Fahy and co-workers [55],

which contains distinct subcategories of lipids, so it's easier to classify them according to their structure and functions [53, 56]. These eight categories are fatty acids (FA), glycerophospholipids (GP), glycolipids (GL), sphingolipids (SP), sterol lipids (ST), prenol lipids (PR), saccharolipids (SL) and polyketides (PK) [5, 33, 53, 57].

FAs are defined as the simplest class of lipids and one of the most important since they are basic elements of the majority of lipids. In terms of structure, natural FAs have hydrocarbons chain saturated and unsaturated, and may vary in length (14 to 24 carbon atoms) and number of double bonds (0 to 6 by chain). FAs are precursors of a great variety of bioactive lipid molecules. One of the most common examples of this functionality is arachidonic acid that is a precursor of eicosanoids, which in turn function as signalling molecules, acting on specific receptors and have a vital role in inflammatory processes [56].

GLs include fundamentally monoacylglycerols (MAG), diacylglicerols (DAG) and triacylglycerols (TAG). These molecular species of lipids are variable in the FAs number that is esterified in hydroxyl groups of glycerol. In contrast to MAG and DAG, TAG are very abundant in nature, as main constituents of animal fat. The possibility of binding three different FAs to glycerol to form the TAG molecule makes a great variety of possible molecules. TAGs have an important role in cellular energy storage and, also, as mediators in metabolic processes and diseases, such as diabetes, obesity and arteriosclerosis [56].

STs, essentially constituted by cholesterol and its derivatives, are important constituents of membrane lipids, being signalling regulation and membrane fluidity their main function. Cholesterol is very abundant in mammals and is associated to cardiovascular diseases, due to its appearance in high concentration in these situations [56].

GPs are the most abundant class of lipids, comprising 60% of the total lipids, in the majority of mammalian cells, since they are the main constituents of membranes. This group includes several complex lipids that have extremely important biological functions in cellular signalling [56, 57].

SPs are also a very important class of lipids and they are present in membranes (about 10% of the total lipids). Moreover, they are also very important signalling molecules [56, 58].

2.1 Glycerophospholipids

Glycerophospholipids (commonly named phospholipids) are structurally complex molecules since they are amphipathic, with a polar domain and a nonpolar domain [52]. Phospholipids are the main constituents of cellular membranes, forming the lipid bilayer with the polar head groups on the outside, in contact with aqueous medium, and in the interior stay the long chains of hydrocarbons [52, 57]. This lipid bilayer forms a relatively rigid structure, which gives resistance to cells. The phospholipid bilayer supports proteins, channels and other lipids, allowing exchanges between internal and external environment [26, 54, 59]. Besides components of the lipid membrane, phospholipids have other functions especially as signalling molecules [56, 57]. Metabolites that result from phospholipid metabolism and degradation are extremely important for intracellular signalling and may be involved in processes of cell proliferation and apoptosis [59]. Structurally, phospholipids are constituted by a glycerol backbone, which has three possible binding sites, *sn*-1, *sn*-2 e *sn*-3 (Figure 2). On *sn*-1 and *sn*-2 positions are linked FAs, that can have similar or distinct structural features, saturated, monounsaturated and/or polyunsaturated, promoting a great variety of GPs [26, 57, 59, 60]. It is known, however, that polyunsaturated FAs are preferentially found in *sn*-2 position, while saturated FAs are normally esterified in *sn*-1 position [61, 62]. The FA chains form the non-polar domain of GPs. In *sn*-3 position of the glycerol backbone it's esterified a phosphate molecule, which can bind different polar molecules, such as: choline, ethanolamine, glycerol, inositol and serine (Figure 2). The phosphate molecule linked with one of these molecules or with only a hydrogen linked generates the polar head of GPs, originating each different class of GP [52, 57]. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidic acid (PA) classes are represented in Figure 2.

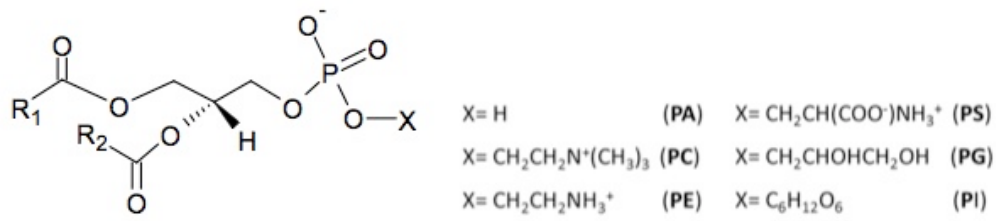

Figure 2. Glycerophospholipids structure. R_1 and R_2 represent the FAs esterified in *sn*-1 e *sn*-2. X represents groups that can bind phosphate molecule, originating classes of glycerophospholipids: (PA) Phosphatidic acid, (PC) phosphatidylcholine, (PE) phosphatidylethanolamine, (PS) phosphatidylserine, (PG) phosphatidylglycerol and (PI) phosphatidylinositol.

The kind of chemical bind that exists in *sn*-1 position also originates three subclasses in each class: phosphatidyl, plasmenyl and plasmanyl subclasses that correspond, respectively, to ester, vinyl ether and alkyl ether bindings between the hydrocarbon chain and glycerol backbone in *sn*-1 position (Figure 3) [56, 59]. In mammals, the ether bindings occur especially in PCs and PEs. The plasmanyl subclasses characteristically exist in PCs, while plasmenyl exists in PEs, with exception in the heart, where predominates plasmenyl PCs. The main signification of subclasses in which the ether bond is presented is related with the production of platelet activation factors and its simultaneous implication with blood coagulation and inflammatory responses. In most cellular membrane lipids, the phosphatidyl subclass of phospholipids is predominant; however in electroactive cellular membrane such as neuronal cells, plasmenyl subclasses are major components of phospholipids [38, 54].

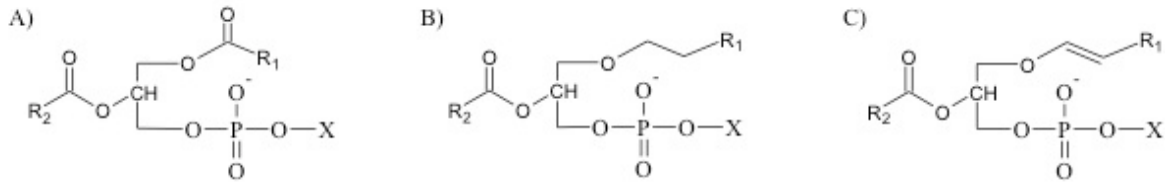

Figure 3. Different classes of phospholipids according to the connection type with the hydrocarbon chain in *sn*-1 position: A) phosphatidyl, B) plasmanyl e C) plasmenyl. R_1 e R_2 represent FAs and X represents the group that binds to the phosphate molecule.

There are also other species named lyso-GP, which have only one of the *sn*-1 or *sn*-2 positions esterified with FA (usually *sn*-1), but they are in the same way considered a GP class [56]. The lyso species act like intermediates in GPs biosynthesis and as second messengers.

Cardiolipin (CL) is also a GP with a different structure from the described previously for other GP compounds (Figure 4). CL (or diphosphatidylglycerol) is a dimeric phospholipid that has two phosphatidic acids and a central glycerol, allowing the existence of four FAs chains in the molecule. The relationship that is created between the three molecules of glycerol generates a single environment for each ester linkage [60, 63]. The fact that two phosphates share the alcohol group is a characteristic with very important implications with respect to physical properties of CL in the lipid membrane, namely in its mobility and conformational flexibility, and also in the assembly of the protein of the complexes of respiratory chain in mitochondria.

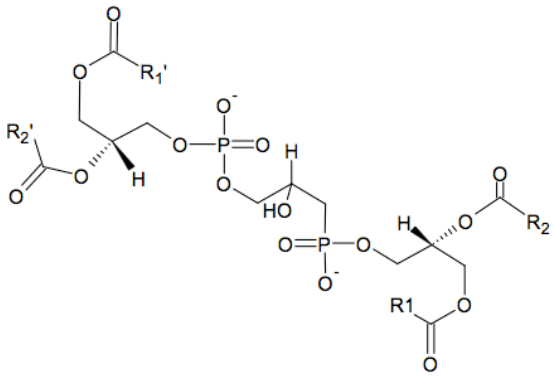

Figure 4. Structure of Cardiolipin. R_1, R_2, R_1' and R_2' represent the fatty acyl chains esterified to glycerol backbone.

2.2 Sphingolipids

SPs are amphipathic lipids that share a common structural feature: a sphingosine backbone as the main chain (Figure 5), which is a long hydrocarbon chain, alkane or alkene (sometimes with more than one double bond), with approximately 14 to 20 carbon atoms, in length, with an amino group in position 2 and hydroxyl substituents in positions 1 and 3 [54, 56, 58]. SPs are classified in different subclasses: ceramide (Cer) (Figure 5),

which have a FA linked to an amide linkage to the sphingosine; sphingomyelin (SM) (Figure 5), which have a FA bound with an amide linkage and a polar head group (phosphocholine) bound to the hydroxyl group in carbon-1 of the sphingosine (these sphingolipid is also frequently considered a phospholipid due to its phosphate group); sphingosilphosphorilcholine, which is like a lyso sphingomyelin that has the phosphocholine group linked with the hydroxyl in position 1, but does not have the FA linked to the amine group; cerebroside and other glycosphingolipids (gangliosides, sulfatides, among others) based on a polar head group linked to the *sn*-1 position of sphingosine [54, 56].

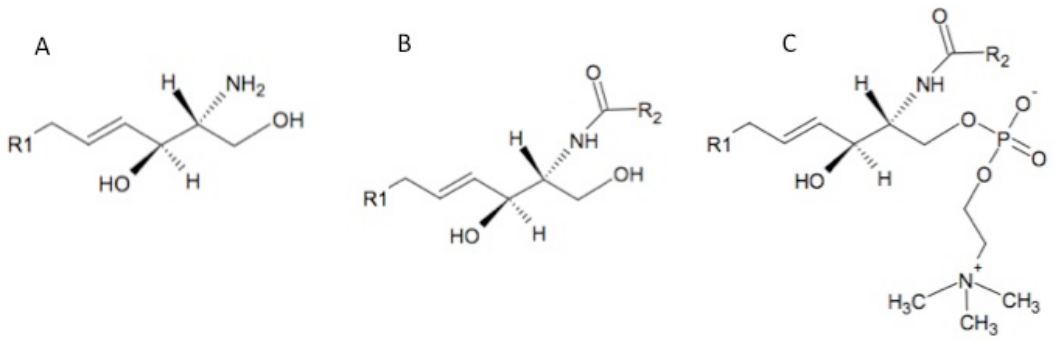

Figure 5. General structure of A) sphingosine, B) ceramide e C) sphingomyelin. R1 represents the hydrocarbon chain of sphingosine and R2 represents the FA linked to the amine group of sphingosine.

The length of the sphingosine hydrocarbon chains is normally well defined into the same species. For example in humans the sphingosine backbone is almost entirely formed by 18 carbon atoms in length, except in cerebral gangliosides, where exist a high quantity of species with 20 carbon atoms, and these are even more frequently found with aging [58] [56]. SPs are recognized by their function as bioactive compounds with big importance in many cellular processes, such as proliferation, differentiation, signalling, inflammation and apoptosis [58].

Since some studies suggest that chronic stress is involved in impairment of cardiac and cognitive/neurological functions [4, 11, 18, 31], in the next section we will summarize the lipid composition of the brain and of the heart.

2.3 Lipids and Brain

Lipids particularly phospholipids (PLs) are fundamental molecules for CNS, both in terms of brain architecture and also by their functions as signalling molecules. These importance becomes extremely evident knowing that CNS has the major quantity of lipids, after adipose tissue, and that they constitute about 50% of dry weight in human brain [62, 64]. Phospholipids represent approximately 25% of the brain dry weight in an adult rat [62]. The nervous system compared with other organs and/or systems in mammals also has the major diversity of lipid classes and lipid molecular species [38]. CNS has a high variety of lipids, including neutral lipids (such as cholesterol and acylglycerols), glycolipids (such as galactosylceramide and gangliosides), sphingolipids (like sphingomyelin) and glycerophospholipids (like phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, among others) [38, 62]. The lipid species with very long hydrocarbon chains (between 20 and 22 carbon atoms) are the most abundant in brain [38]. The distribution and the phospholipid molecules in brain vary depending on the state of development and anatomical, cellular and subcellular localization [62].

Neuronal lipids have many specific functions in nervous system. Signalling function of lipids in the nervous system is apparently much more complicated than simply as secondary messengers [38]. The lipid membrane homeostasis in neurons is of extreme importance for preventing the loss of synaptic plasticity, cellular death and neurodegeneration [39].

Main fatty acids esterified to glycerol in brain phospholipids [62] are presented in Table 1. Fatty acids in brain are mainly polyunsaturated fatty acids that are very susceptible to oxidation. Oxidation of FA and phospholipids (PLs) or other lipids lead to loss of their function, affecting cellular homeostasis. Besides oxidized lipids can also be pro-inflammatory signalling molecules. Oxidized PL can be degraded by phospholipases realising the oxidized FA, affecting PL composition and membrane properties [32].

Table 1. Fatty acyl chains mostly found in brain phospholipids, nomenclature and the possibility of suffering oxidation [62].

Number of carbons: Number of double bounds	FA	Prefix	Saturation State	Oxidizable?
12:0	Dodecanoic	Lauroyl	Saturated	No
14:0	Tetradecanoic	Myiristoyl	Saturated	No
16:0	Hexadecanoic	Palmitoyl	Saturated	No
18:0	Octadecanoic	Stearoyl	Saturated	No
18:1	9-Octadecanoic	Oleoyl	Monounsaturated	No
18:2	9,12-Octadecadienoic	Linoleoyl	Polyunsaturated	Yes
20:4	5,8,11,14-Eicosatetraenoic	Arachidonoyl	Polyunsaturated	Yes
22:5	7,10,13,16,19-Docosapentaenoic	Docosapentaenoyl	Polyunsaturated	Yes
22:6	4, 7,10,13,16,19-Docosahexenoic	Docosahexaenoyl	Polyunsaturated	Yes

Taking into account that the dynamic functional state of neuronal membrane is dependent on its composition, even modest changes in fatty acyls composition of PLs, such as gain/loss of arachidonic acid, can lead to membrane dysfunction. Lately arachidonic acid has caused much interest since it was suggested that this is a very important FA for the functioning and development of the brain [32]. The understanding of the signal transduction mediated by arachidonic acid and its metabolism in brain may lead to some clues for a variety of neurological disorders and neurodegenerative diseases. Many drugs, namely mood stabilizers, have as target the turnover and enzymatic pathways of arachidonic acid [65, 66].

The fact that brain is an organ extremely rich in unsaturated lipids and the importance of their homeostasis demonstrated here reveals the importance of the study of changes related to lipids in diseases that affect the brain.

2.3.1 Oxidative stress and lipid peroxidation in brain

As mentioned previously, chronic stress and oxidative stress are particularly related, especially in brain changes. Oxidation is a natural occurring process, however when the homeostasis of an organism is altered, begins a process called oxidative stress [67].

Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the ability of a biological system to detoxify the organism of these radicals, an imbalance between the systems pro-oxidant/anti-oxidant occurs [68]. The majority of ROS are produced in low quantities, to be used in oxidation/reduction reactions of cellular signalling and are important as a way to prevent the aging and in combat to invasive microorganisms. The loss of antioxidant defences, or the increase in ROS production enhances progressive cellular damage and the decline in physiological function. The exaggerated production of these radicals causes changes in all cellular components, from proteins, nucleic acids and lipids, culminating in cell death [34]. The phospholipid oxidation, usually called lipid peroxidation has been associated with the changes in structure of these biomolecules and consequently impairment of the cellular function.

Oxidative stress causes many changes in a variety of phospholipids found in living beings, resulting in a variety of oxidative products. These products can have distinct biological activities, that depend not only on the location, but also on the nature of that changes [69]. Oxidized phospholipids in biological membranes induce changes in physical properties like fluidity, which can produce a big impact in membrane integrity, causing apoptosis [7]. Lipid peroxidation has been indicated as a factor responsible for triggering many diseases such as arteriosclerosis, diabetes and neurodegenerative diseases [70].

Brain is an extremely vulnerable organ to oxidative stress due to the high content of PUFAs, in phospholipids, which are extremely susceptible to lipid peroxidation (especially arachidonic acid (20:4) and docosahexanoic acid (22:6)). On the other hand, the brain consumes elevated oxygen quantities for energy production, has elevated quantities of transition metals that can act as pro-oxidants, and it has little levels of antioxidant defences, compared with other organs [8, 34]. Overall, oxidation is probable to occur. It is known that oxidative stress contributes to the neurons degeneration in CNS, both in the aging process and in neurodegenerative diseases [8]. Chronic stress produces a deregulation in HPA axis, which modifies the antioxidant defences, increasing the vulnerability of different brain regions to lipid peroxidation [6, 31]. The increase in catabolism in stress situations associated with increasing energetic needs of the organism also leads to the increase in ROS production [71].

Some studies show that exists an increase in lipid peroxidation in brain after stress exposure [8]. However, these studies evaluate the increase in malondialdehyde (MDA), a

secondary product of lipid peroxidation, but little specific. The achievement of a more specific study of the oxidative changes in phospholipids in chronic stress situations is extremely relevant, since the susceptibility to phospholipid oxidative modifications is entirely dependent on structure. These oxidised phospholipids have a high variety of biological effects both directly, reacting with proteins and nucleic acids, or indirectly, through receptors mediated pathways [72]. Also, oxidized PL may be degradated, which could lead to changes in PL content or PL profile.

2.4 Lipids and Heart

Lipids in general and phospholipids in particular are extremely important molecules for the heart, since changes associated to these biomolecules cause the impairment of cardiac function, promoting the development of cardiomyopathy [5]. The heart has a great variety of lipids, like cholesterol, triacylglicerides and phospholipids [73]. Phospholipids have many functions in cardiomyocytes, both in terms of structure and in signaling events. The importance of phospholipids in the heart is even more evident knowing that cardiomyocytes are highly sensible to lipid changes, becoming extremely important the maintenance of the homeostasis of these biomolecules in the heart [42].

The most abundant classes of phospholipids in the heart, as well as in the other organs, are the phosphatidylcholines and phosphatidylethanolamines. However, the heart is a muscular organ, so it has a high content in mitochondria, which consequently makes it very rich in cardiolipin. Cardiolipin has a main importance in heart, where it is the third most abundant class of phospholipids. Changes in metabolism and oxidation products of cardiolipin have been implicated with the development of many heart diseases [5, 74].

In the heart the saturated fatty acyl chains are very abundant (C16:0 and 18:0), but this organ has also many PUFAs (especially, C18:2 and C20:4), which makes it also susceptible to oxidation. However, the heart is more resistant to oxidative stress than brain, since it has more antioxidant defences [75].

So far no studies investigated heart phospholipids in chronic stress conditions, but as we know these conditions propitiate the impairment of many cardiac functions, becoming important the study of the possible changes caused by chronic stress in heart phospholipid profile.

Mass spectrometry has recently been used as the main analytical technique for analysis of lipid/phospholipid oxidation products and also in profiling cell or tissue phospholipids, an approach that often involves preparative chromatography techniques. These methodologies are generically known as Lipidomics.

3 Lipidomics

Lipidomics have been target of a great development in the last decade and can be defined as the systematic study and in a large-scale of structure, function and interactions of lipids with other lipids, proteins and other molecules present in biological samples (for example, biological fluids, tissues and cells). It includes also the study of lipid changes that occur during pathophysiological disturbances, in response to different stimuli. Analysis of lipids is achieved through the integration of many techniques, like MS and chromatography [56, 76]. Lipidomics is considered a branch of metabolomics, since lipids are biological metabolites. The role of lipids in the formation of cellular membranes makes them both ligands and substrates for proteins, suggesting that advances in lipidomics can have great implications in genomics, proteomics and metabolomics [33].

Lipidomics usually involve several steps: extraction, separation and analysis. Extraction of lipids from tissues or cells is achieved by methods that exploit the high solubility of lipids in organic solvents. Most common extraction methods for phospholipid extraction are the Bligh and Dyer and Folch methods. Phospholipid separation is usually achieved by chromatographic methods, allowing the separation and identification of different phospholipid classes. Phospholipid content of each class can be measured by phosphorous assay that measures the inorganic phosphate present in the sample. In addition, analysis of the lipid profile is accomplished by mass spectrometry (Figure 6). This methodology is also applied in the analysis of lipid peroxidation products that are generated in vivo conditions, named as oxidative lipidomics [33].

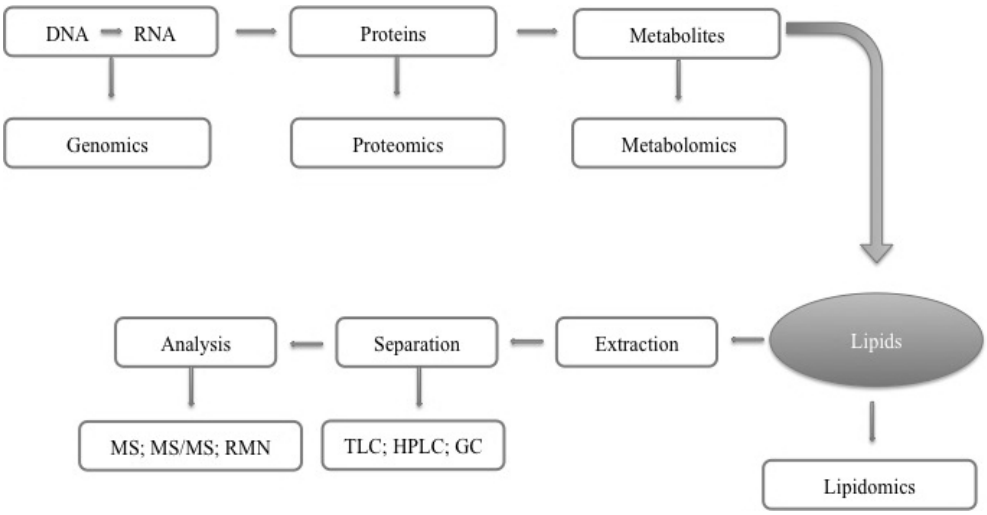

Figure 6. Lipidomics: strategy for lipids extraction and analysis.

3.1 Methods for phospholipid extraction

Lipid extraction takes into account the fact that lipids are soluble in organic solvents. Using different combinations of organic solvents it is possible to enrich the extracts in the lipids of interest. Nonpolar solvents can be used when we want to extract neutral lipids, like esters of fatty acids and acylglycerols. On the other hand, when we want an extract rich in polar lipids, such as phospholipids and glycolipids, it's imperative to use more polar solvents (for example, methanol and chloroform) [57]. There are two methods widely used in phospholipid extraction: the Folch method [77] and the Blight and Dyer method [78], which use the same combination of solvents (chloroform/methanol), but in different proportions, in which phospholipids are obtained in the hydrophobic and less dense phase. Methanol has the ability to break bounds between protein-lipid associations and inactivates lipases. In turn, chloroform dissolves lipids. Due to the unsaturated bonds present in the majority of phospholipids they are easy to oxidase, so the samples should be always maintained on ice to avoid this problem [59]. This extraction should be very careful, since it's a critical step and it will influence the posterior analysis and the final results.

3.2 Phospholipid Separation Methods

Since phospholipid extracts obtained from biological samples include different phospholipid classes, it is necessary their separation. This separation is normally done by chromatographic methods. The most common method is thin-layer chromatography (TLC), however, more recently, high performance liquid chromatography (HPLC) has become popular.

3.2.1 Principles of TLC in the separation of phospholipid classes

TLC is one of the techniques more versatile and efficient for the separation of lipid complex mixtures. This technique can be used for separation of compounds from a complex mixture, but also, to quantify, identify and purify these compounds. TLC is used routinely in lipid analysis, since it does not require sophisticated instrumentation and allows a fast, simple and economic separation of the main classes of lipids and/or phospholipids [76].

In TLC is possible the separation of complex mixtures in a stationary phase, because there is a difference of polarity between the components and the stationary and mobile phase of the chromatography. This chromatographic technique uses a mobile phase that is composed by a liquid (eluent) and a stationary solid phase [79, 80].

In separation of phospholipid classes it is used, normally, glass plates covered with silica particles (SiO_2) distributed evenly, originating the stationary phase of chromatography, an extremely polar surface. Thus, the polar portion of phospholipids interacts strongly with the silica plate. The components can bind in two ways to the plate: by hydrogen bonds and dipole-dipole interactions. There are many modifications that can be done in the silica plate to improve the separation of phospholipid classes, such as the coating of the plate with boric acid, that promotes the increase in resolution [80]. The mobile phase of TLC is a system of solvents that vary in their polarity. The use of appropriate solvent mixtures allows the separation of the different classes of lipids [57]. The migration of phospholipids in silica plate depends on the composition of each PL and on the eluent and affinity of phospholipids with mobile phase. Therefore more non-polar components have great affinity with the mobile phase (generally solvent mixture non-polar) and they will be eluting ahead of the more polar compounds of the sample, which

have a great affinity to the stationary phase [79]. PLs with different polar head groups have different polarity, migrating differently along the TLC plate, allowing their separation Figure 7.

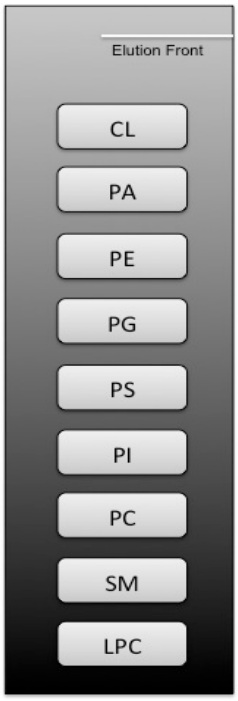

Figure 7. Scheme of the normal elution order of the main phospholipids classes in a TLC plate using the eluent $CHCl_3$: MeOH: H2O: trietilamine. PLs with different polar head groups have different polarity, migrating differently along the TLC plate, allowing their separation.

After a chromatographic run it is necessary the use of detection reagents to visualize the separation results. Generally is used a dye that allows the visualization of the spots. However, choosing a dye is not easy when we want to separate lipids for analysis by mass spectrometry, since the coloration cannot cause molecular changes in the analyte. A dye extremely useful is primuline, that binds by non-covalent bindings to nonpolar FAs of lipids, visible with UV, so it does not affect the subsequent analysis [81]. The separated classes can be extracted from the TLC spots, using appropriated solvents, and used for quantification by phosphorous assay or analysis by mass spectrometry.

3.2.2 Principles of high performance liquid chromatography (HPLC) coupled with mass spectrometry in the analysis of phospholipid classes

The high performance liquid chromatography (HPLC) is a separation technique in which the components of a sample are separated by distribution between the mobile phase (a flowing liquid) and a stationary phase (sorbents packed inside a column) [38, 76, 82]. HPLC has good reproducibility and high resolution [76, 79].

The principles of HPLC are similar to TLC. However, it has a great advantage over TLC, because it decreases the exposure of the sample to atmospheric oxygen, decreasing the risk of auto-oxidation of phospholipids [80]. HPLC is an automated technique and is measured in terms of capacity, efficiency, selectivity and resolution. The capacity and resolution of the column are variables and dependent on the column manufacturer, whereas the handler can control efficiency and resolution, to some extent. To obtain the best separation, the efficiency of the chromatographic system must be optimized in order to minimize band dragging. The column must have the capability to retain the solutes and it should have the appropriate selectivity to resolve the analytes of interest [83].

A typical HPLC system consists of a pump, an injector, a column, a detector, and a data-handling device (Figure 8).

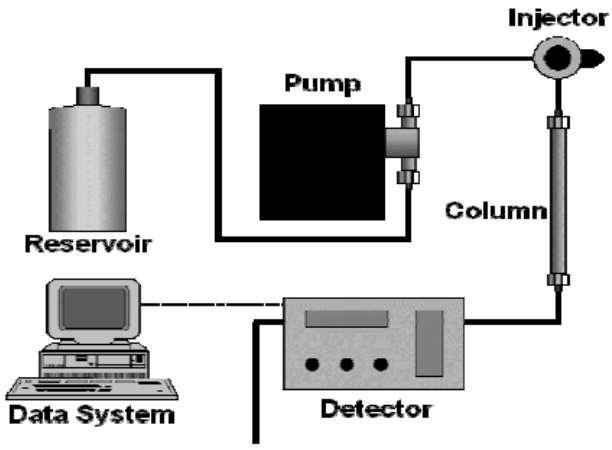

Figure 8. Schematic representation of the main HPLC constituents.

Although direct infusion mass spectrometry offers some advantages for analysis of phospholipids, HPLC can be coupled with MS, allowing the separation of the different phospholipid classes and simultaneous analysis in molecular components [52, 84]. Separation of phospholipid classes can be achieved by normal phase HPLC-MS, with

increased sensitivity for minor components, comparatively to the direct analysis of samples [85]. It is possible to quantify the phospholipid species, but it is important in this case to use internal standards, which have a similar response in MS conditions, in comparison with the analytes [52, 84]. The elution of the phospholipid species depends on the chemical properties, the head group, chemistry, acyl chain length and degree of unsaturation [86].

3.3 Mass Spectrometry (MS)

Mass spectrometry is an analytical technique with high sensibility, selectivity, specificity and very fast, that can be used with many purposes, such as the identification of unknown compounds and the evaluation of the oxidation extension of some molecules. This technique is based on the detection of ions after their separation according to their mass (m) and charge (z) ratio, *m/z*. The data obtained is presented in a mass spectrum, in which in the x-axis is located the ratio mass/ion charge (*m/z*) and in the y-axis is presented the relative abundance (like a relative intensity) of the ion in the mixture, after normalization in relation to the most abundant ion [59].

The mass spectrometer is constituted by 4 principle components: a system to introduce the sample into the source of ionization; the ionization source; the mass analyzer; and the detector (Figure 9). For a MS analysis first the sample is introduced in the ionization source by an input system. The way that the sample is introduced can vary, since it can be introduced directly or through other coupled devices to the spectrometer, namely HPLC and gas chromatography (GC). Then, the sample molecules are ionized, in the ionization source producing an ion beam in gaseous phase. The ionization sources that exist are many, but nowadays electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the most popular. After the sample ionization, the resulting ions are selected and separated according to their *m/z* ratio, in the analyzer. The analyzers can vary, differing in its characteristics such as sensibility and resolution. Finally, in the detector, ions are collected and characterized by the production of a signal, which intensity is related to the number of detected ions. The detector is connected to a computer where all the information received is integrated and transformed in the mass spectra. Thus, and taking into account that the ionization sources and the analyzers can vary, is possible the construction of a great variety of mass spectrometers [57, 59]. Mass spectrometers are instruments very complex that have a very important characteristic that

is a high vacuum present on the instrument (10^{-5} to 10^{-7} Torr), which increases the free space of ions by the spectrometer, without occurring reaction or changes in ions. Thus, the majority of reactions are unimoleculars, which can reveal structural information about the compound being analysed [57, 59].

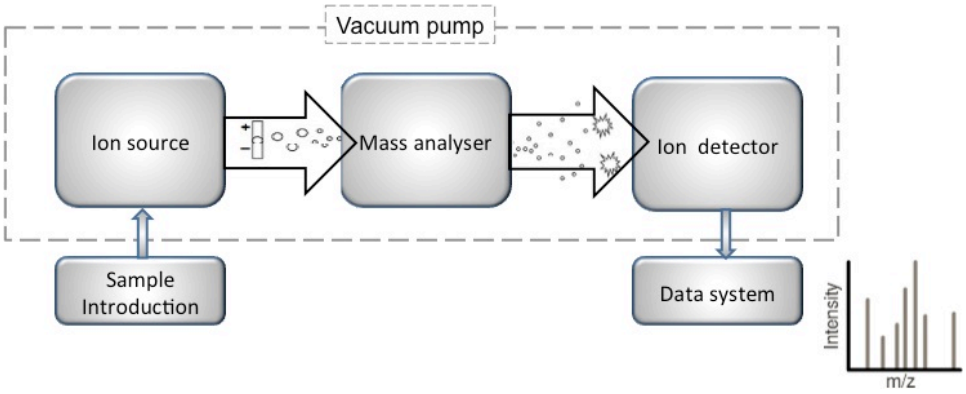

Figure 9. Main mass spectrometer components. For MS analysis, samples can be injected directly or by a system copulated to the mass spectrometer. Then they are ionized, pass to the mass analyser and then to the ion detector. The final result is the generation of a mass spectrum.

MS has been widely used in lipidomic analysis since the combination of selectivity, specificity, sensibility and speed makes this technique extraordinarily useful for lipid and phospholipid analysis [57]. With the appearance of "soft ionization techniques" like MALDI, ESI and atmospheric pressure chemical ionization (APCI) for MS, possibly coupled to HPLC, made possible the fast and sensible analysis of the majority of lipids achievable in one analysis [33]. Many complementary analytical approaches are currently used in lipidomics.

3.3.1 Ionization Methods

The first ionization method developed was the electron impact method (EI), which was only applied to volatile and thermostable compounds. In order to overcome this limitation, it has emerged the ionization by bombardment with fast ions or atoms (FAB) that allowed the analysis by MS of non-volatile and thermally unstable samples. More recently ESI and MALDI were developed to allow the analysis of non-volatile and thermolable molecules, such as biomolecules.

FAB-MS was used in the 90's for the analysis of natural phospholipids. Since that moment it was possible to analyse directly intact phospholipid structures and preserve the information inherited to their chemical structure. However, this ionization technique had some problems associated, like the complications of the mass spectra with the appearance of ions due to the matrix and the decomposition of molecular ions during the ionization. The emergence of ESI eliminated these problems and also improved the sensibility in detection, since it is able to preserve the lipid molecular structure after ionization. ESI is nowadays the most used method in the phospholipid analysis [52, 59, 85].

ESI is an ionization technique used for the mass spectrometry analysis of polar compounds that was initially developed by Fenn and colleagues. ESI is performed by applying a strong electric field under atmospheric pressure to a liquid passing through a capillary tube with a slow flow. The field induces charge accumulation at the liquid surface located at the end of the capillary to spray the mobile phase into highly charged droplets. These droplets then pass through a heated inert gas for desolvation prior to mass spectrum analysis of individual ionic species [54, 80, 87].

Depending on the chemical properties of the molecules both negative and positive ions can be formed. The major advantages of ESI-MS are high accuracy, sensitivity, reproducibility, applicability to complex phospholipid solutions without prior derivatization, and as "soft ionization technique", molecules are not broken apart, instead they remain intact. ESI-MS of lipids represents one of the most sensitive and direct methods to assess changes in cellular lipidome directly from lipid extracts of biological samples [54, 59, 88].

The ions formed by ESI are then transported to the analyzer. ESI sources can be combined with distinct types of analyzers such as quadrupole (Q), ion trap, time- of-flight (TOF), cyclotron resonance of Fourier / Fourier transform ion cyclotron (FT-ICR) and orbitrap [59].

3.3.2 Analyzers

After being ionized, ions formed are separated by the analyzer, according to their *m/z* ratio. Every analyzer differs in its precision (the error in the exact mass determination compared with the theoretical value), in resolution (the value of mass m divided by the mass difference between two ion profiles with a small mass difference); dynamic range

and capability to perform tandem mass spectrometry. There are three main types of analyzers that are commonly used for phospholipids analysis by mass spectrometry: quadrupole (Q), ion trap and time of flight (TOF). These analyzers can be used individually in mass spectrometers, or can be combined into complex instruments, the most commonly used Q-TOF, Q-Trap and triple quadrupoles [59].

Quadrupole - The quadrupole is composed by four parallel rolls to which is applied a current that affects the trajectory of the ions traveling through the central route between the 4 rolls. For certain voltages, only ions of a specific *m*/*z* ratio can pass through the quadrupole filter, while others are carried away as uncharged molecules. By varying the electrical signals from one quadrupole, it is possible to vary the range of the *m*/*z* transmitted. The Qs have many advantages such as being relatively low-cost, easy to use and capable of providing accurate results [57, 59].

Time of Flight - A time of flight analyzer (TOF) uses the differences on time that accelerated ions leads to reach the detector based on their mass differences. The ions are accelerated by pulsed electric field and the accelerated particles pass through a flight tube of varying length. The essential principle of flight time analyzer is based on all ions accelerated to the same energy, i.e. with equal energy and will travel at speeds inversely proportional to the square roots of their masses. So the lighter ions with high-speed reach the detector earlier than heavier ions with low speed [59].

Linear Ion Trap - During this study we used a linear ion trap mass spectrometer. Linear ion trap is a multipole ion-dimensional, where the ions are confined to radial dimension by a quadrupole field and to an axial dimension by means of an electric field in the trap extremity [57].

Linear ion trap can be combined with other mass analyzers. It can be used to isolate ions of selected mass to charge ratios, to perform tandem mass spectrometry experiments, and to study the molecule chemistry [59].

3.3.3 Tandem Mass Spectrometry (MS/MS)

In analysis of a phospholipid sample by MS, one of the advantages of the soft ionization methods is the absence of fragmentations, which allows a rigorous determination of the molecular mass of the components of a mixture. However, in these conditions is obtained little information about the molecular structure of phospholipids.

For this effect it is necessary to proceed to the ion fragmentation inducing the dissociation of the ions formed in the source. This technique is called tandem mass spectrometry or MS/MS [59].

MS/MS involves many steps: i) selection and isolation of the ions of interest; ii) fragmentation in a collision cell by interaction with a gas; iii) the product ions are separated by its *m/z* and are characterized by a second mass analyzer (Figure 10). The base structure of a tandem mass spectrometer includes two analyzers, separated by a collision chamber. This technique is named MS/MS, however the number of steps in a ion trap mass spectrometer can increase in order to perform MS^n (in which n represents the number of ion generations to be analysed) [89].

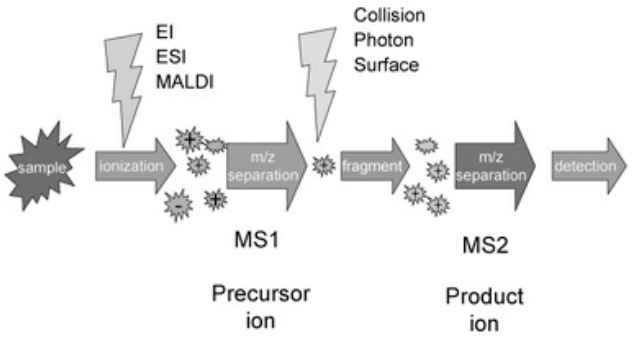

Figure 10. Schematic representation of the MS/MS analysis. The ion of interest is selected and then, by fragmentation, generate several product ions that are subsequently analysed.

Using MS/MS technology we can draw several conclusions regarding the structure of the molecules and the analysis of the MS/MS spectrum gives us both the most frequent breaks and the more favourable. [85]. MS/MS has been used for the study and characterization of many molecules, including phospholipids.

3.3.4 Phospholipids and Mass Spectrometry

MS has a key role in phospholipid analysis, because it allows the detection and determination of these biomolecules in different complex mixtures. Information about the molecular weight of each lipid species can be determined from a first analysis of the MS spectrum. In a second approach, a certain ion with a specific *m/z* value, corresponding to a single phospholipid, can be further analysed by MS/MS. MS/MS gives detailed

information that is necessary for the lipid structural characterization and has the selectivity necessary for determination of the lipid species present in complex mixtures [90]. The analysis by ESI identify both positively or negatively charged ions, since each phospholipid class has the capacity to form positive or negative ions, depending on the structural features of the polar head group [59]. PCs, PSs, PEs and SMs can be analysed in the positive mode, forming ions $[M + H]^+$ or $[M + X]^+$ (X = Na, Li, K), while PGs, PIs, PAs, PSs, PEs, CLs and Cer's are analysed in the negative mode, forming ions $[M - H]^-$ [89].

PCs are characterised structurally by the presence of quaternary nitrogen, with a positive charge, forming an ion $[MH]^+$. The MS/MS spectrum of the ion $[MH]^+$ shows an abundant ion at *m/z* 184, which corresponds to the polar head of PC $[H_2PO_4(CH_2)_2N(CH_3)_3]^+$. Other product ions with low relative abundance can be formed corresponding to the loss of the fatty acyl chains placed at the *sn*-1 (R_1COOH and $R_1=C=O$) and *sn*-2 (R_2COOH and $R_2=C=O$). FAs may be identified by the formation of the ion $[M+H-R_2=C=O]^+$ with higher abundance than the formation of the ion $[M+H-R_1=C=O]^+$, which allows us to distinguish the position of certain FA in the glycerol backbone. In positive mode PCs can also ionize as $[MNa]^+$, forming product ions due to the loss -59 Da $(NCH3)_3$, -183 Da $HPO_4(CH_2)_2N(CH_3)_3$, -205 Da $NaHPO_4(CH_2)_2N(CH_3)_3$, and to the loss of the FAs presented in the positions *sn*-1 and *sn*-2 of the glycerol backbone [59, 89]. In Figure 11 are represented the MS/MS spectra of PC (16:0/18:1) both as $[MH]^+$ at *m/z* 760.6 and $[MNa]^+$ at *m/z* 782.6.

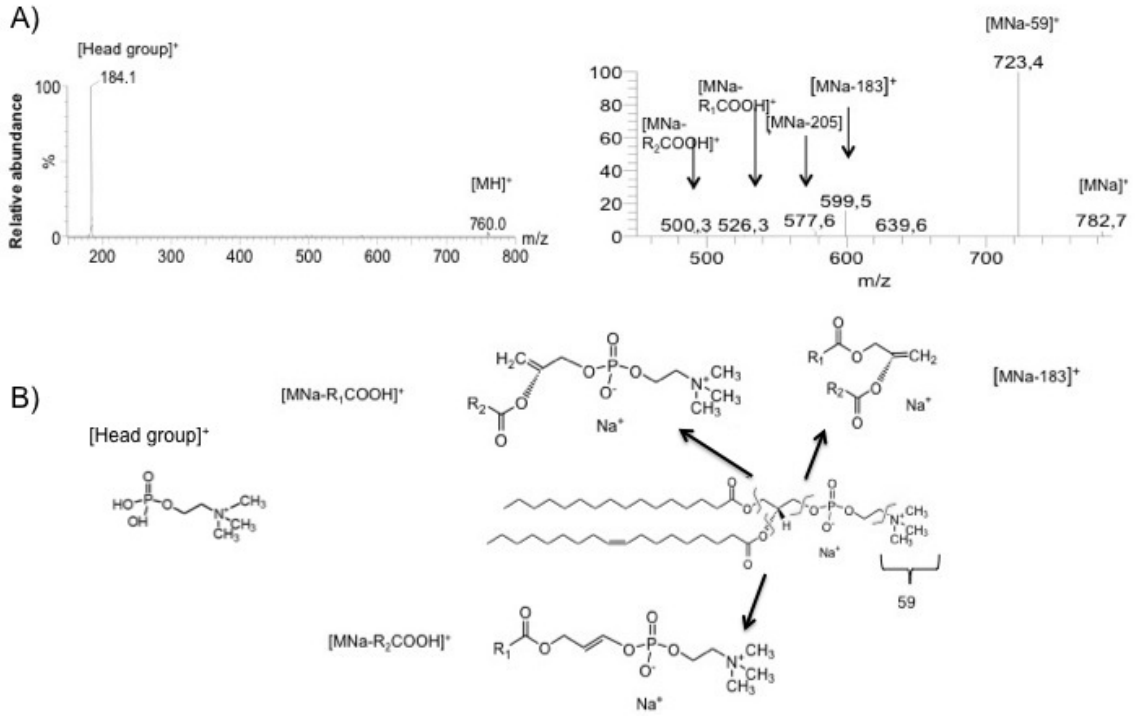

Figure 11. A) MS/MS spectra of the $[MH]^+$ ion at *m/z* 760.6 and the $[MNa]^+$ adduct at *m/z* 782.6, of the PC (16:0/18:1), B) showing the fragmentation patterns of the MS/MS fragments of the $[MNa]^+$ of this phospholipid class and the loss of the polar head group at *m/z* 184 (relative to the MS/MS spectrum $[MH]^+$).

SMs and PCs have in common the polar head group constituted by choline, which means that they ionize in similar ways. SMs also form $[MH]^+$ and $[MNa]^+$ ions. The fragmentation of the $[MH]^+$ ion also produces an abundant ion at *m/z* 184, which corresponds to the polar head of SM $[H_2PO_4(CH_2)_2N(CH_3)_3]^+$. $[MNa]^+$ ions form products due to the loss -59 Da $(NCH3)_3$, -183 Da $HPO_4(CH_2)_2N(CH_3)_3$, -205 Da $NaHPO_4(CH_2)_2N(CH_3)_3$, and to the loss of the FA bound by an amide linkage. However, is very easy to distinguish the spectra of PCs and SMs, since the first when protonated shows at pairs *m/z* values, whereas the second have odd *m/z* values, since they have one more nitrogen atom [91, 92]. In Figure 12 is presented the MS/MS spectra of SM $[MH]^+$ and $[MNa]^+$ ions at *m/z* 703.5 and 725.5, respectively, of the SM (d18:1/16:0).

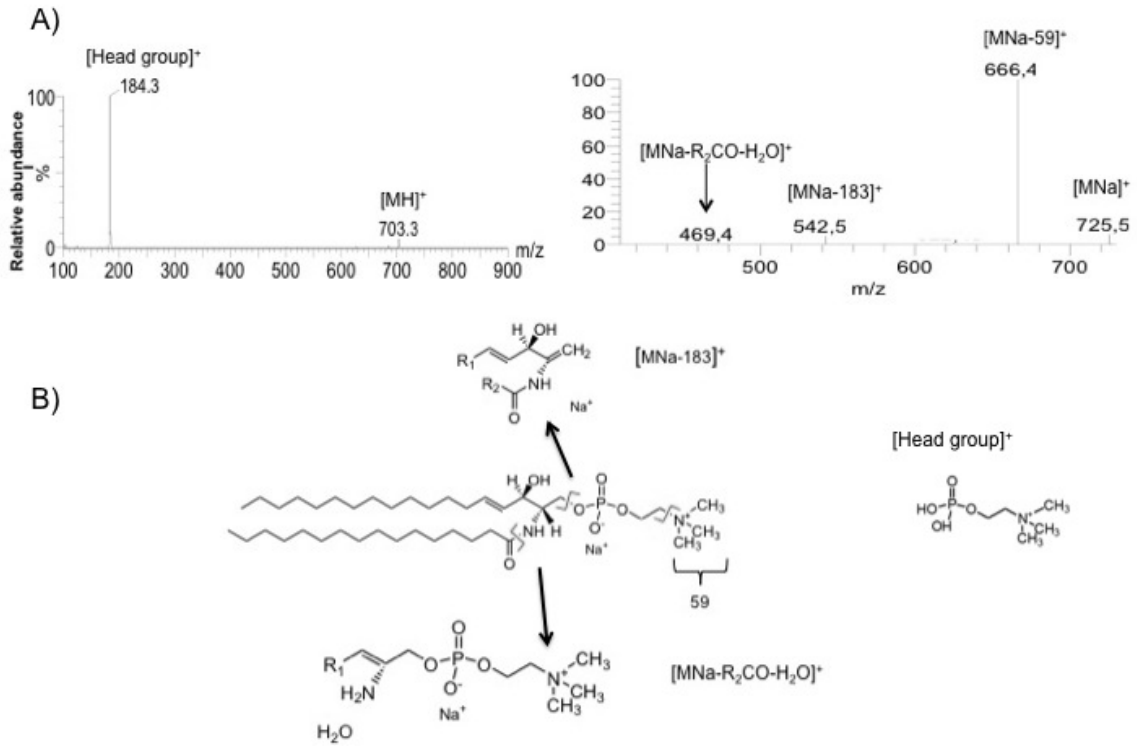

Figure 12. A) MS/MS spectra of SM $[MH]^+$ and $[MNa]^+$ ions at *m/z* 703.5 and 725.5, respectively, of SM (d18:1/16:0). B) The fragmentation products characteristic of this class, showing the loss of the polar head at *m/z* 184 in MS/MS spectrum of $[MH]^+$ ion and the other breaks relative to the formation of $[MNa]^+$ ion.

PEs can form $[MH]^+$ and $[MNa]^+$ ions in the positive mode and $[M-H]^-$ ions in the negative mode. MS/MS spectra of the $[MH]^+$ and $[MNa]^+$ ions shows an ion due to the loss of 141 Da, corresponding to the loss of the polar head ($HPO_4(CH_2)_2NH_3$). The fragmentation of the $[M-H]^-$ ions leads to abundant carboxylate anions ($RCOO^-$), allowing the identification of the FAs in *sn*-1 (R_1COO^-) and in *sn*-2 (R_2COO^-) positions. The relative abundance of the ion R_2COO^- is normally higher than the relative abundance of the ion R_1COO^- [69]. In Figure 13 it is represented an example of the MS/MS spectrum of PE $[M-H]^-$ ion at *m/z* 790.5, corresponding to PE (18:0/20:4).

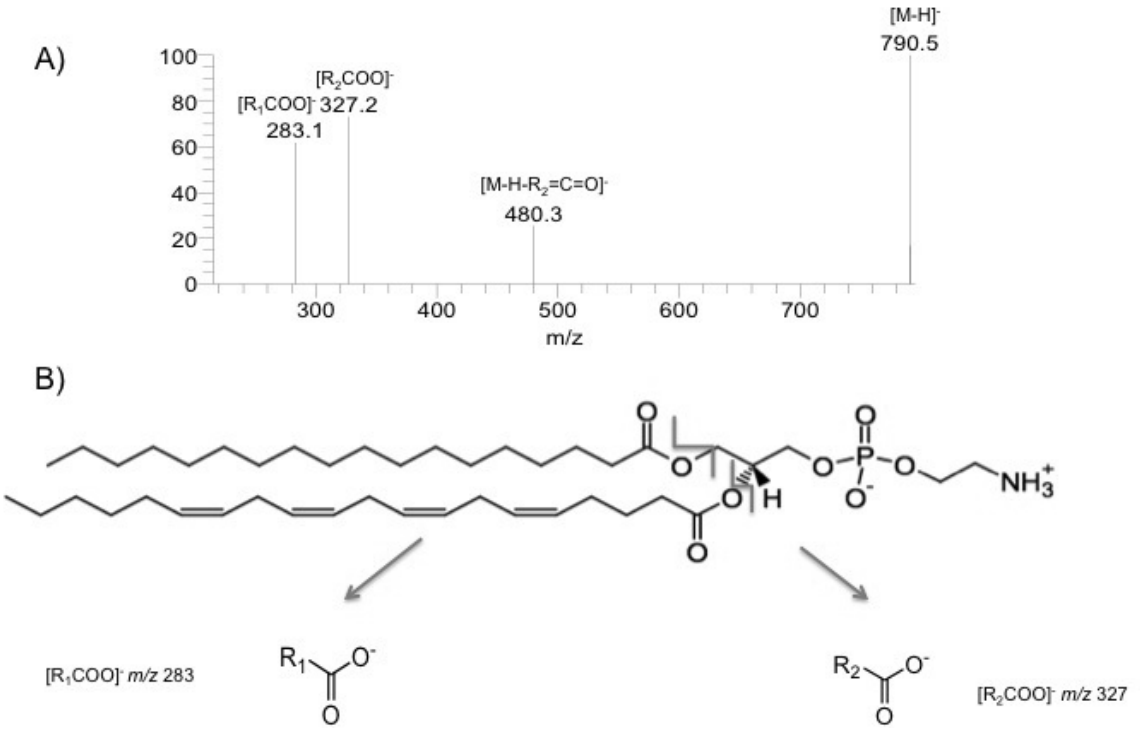

Figure 13. A) MS/MS spectrum of the PE $[M-H]^-$ ion at *m/z* 790.5 (PE C18:1/20:4). B) Fragmentation patterns of PE class in the negative mode.

PSs ionize preferentially in the negative mode, forming $[M-H]^-$ ions [69]. The fragmentation of $[M-H]^-$ ions shows the loss of the serine group (-87 Da) and carboxylate ions R_1COO^- (more abundant) and R_2COO^- [69, 93], as showed in Figure 14 that represents the MS/MS spectrum for PS $[M-H]^-$ ion at *m/z* 760.4, relative to PS (16:0/18:1).

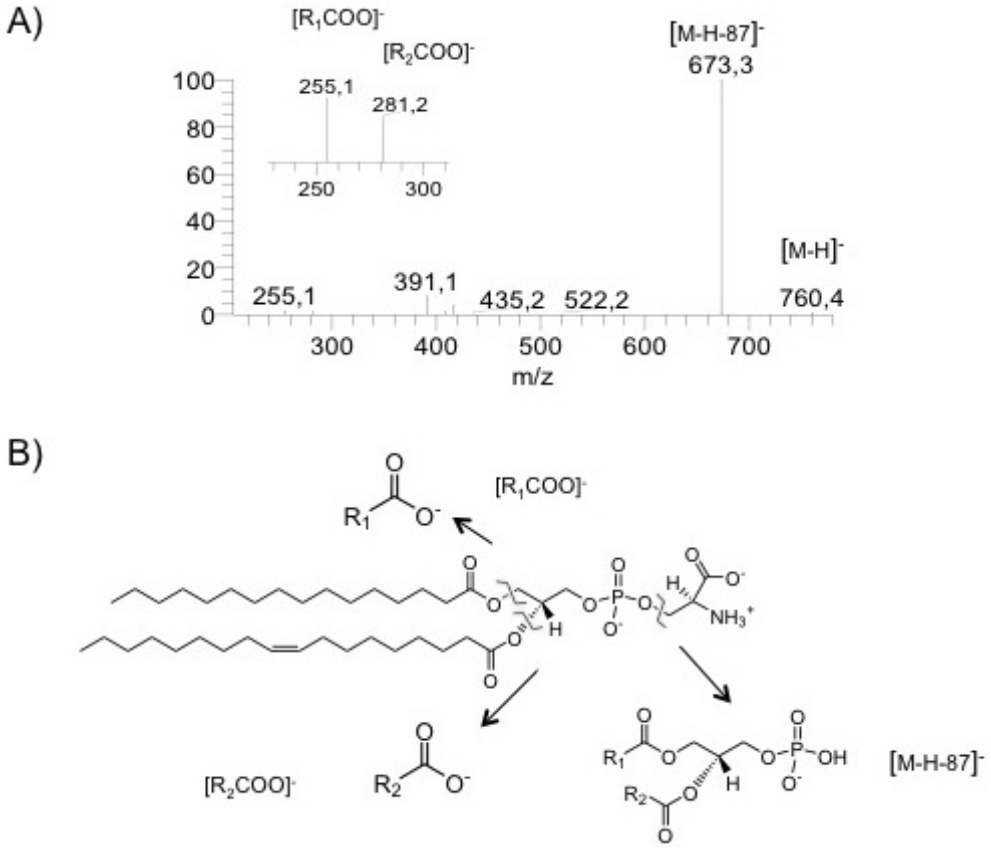

Figure 14. A) MS/MS spectrum of the PS $[M-H]^-$ ion at *m/z* 760.4 (PS C16:0/18:1) and B) the typical fragmentation pathways of this class.

PAs class ionize preferentially in the negative mode with formation of $[M-H]^-$ ions. In MS/MS spectrum it is possible to see $RCOO^-$ ions. The carboxylate anion in *sn*-1 (R_1COO^-) is typically more abundant than the carboxylate ion in *sn*-2 (R_2COO^-). It's also possible to visualize ions that correspond to the neutral losses of FAs in *sn*-1 and *sn*-2 like ketenes ($[M-H-R=C=O]^-$) or carboxyl acids ($[M-H-RCOOH]^-$) [69]. In this cases the loss of FA in *sn*-2 position, as acid or as ketene, is more favourable that the loss in *sn*-1 [89]. In Figure 15 is represented the MS/MS spectrum of PA $[M-H]^-$ ion at *m/z* 675.3, relative to PA (16:0/18:0).

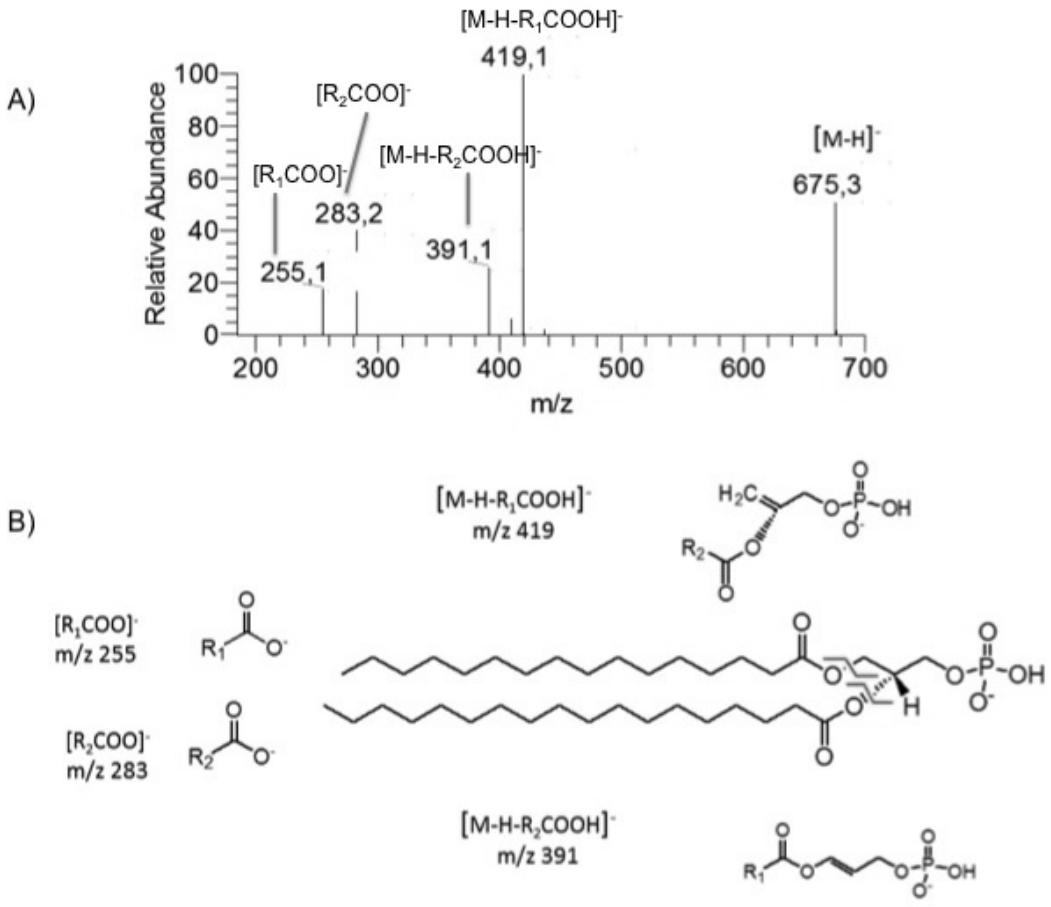

Figure 15. A) MS/MS spectrum of the PA $[M-H]^-$ ion at *m/z* 675.3. B) Fragmentation patterns of PA (16:0/18:0).

PIs ionize also preferentially in the negative mode with formation of $[M-H]^-$ in MS spectra. The MS/MS spectrum of the $[M-H]^-$ ion shows a characteristic product ion at *m/z* 241, corresponding to the phosphoinositol. FAs chains are preferentially loss in the form of ($[M-H-R_xCO_2H]$). The relative intensity of the carboxylate anions, R_1COO^- and R_2COO^-, is very close, however R_1COO^- is a little more abundant than R_2COO^- [94]. Still, it is also possible to see the product ions corresponding to the loss of fatty acyl species combined with loss of inositol (-162 Da) $[M-H-162-R_1COOH]^-$ e $[M-H-162-R_2COOH]^-$. In Figure 16 it is represented MS/MS spectrum PI $[M-H]^-$ ion at *m/z* 885.5 as an example and the main fragmentation pathways observed for PI (18:0/20:4).

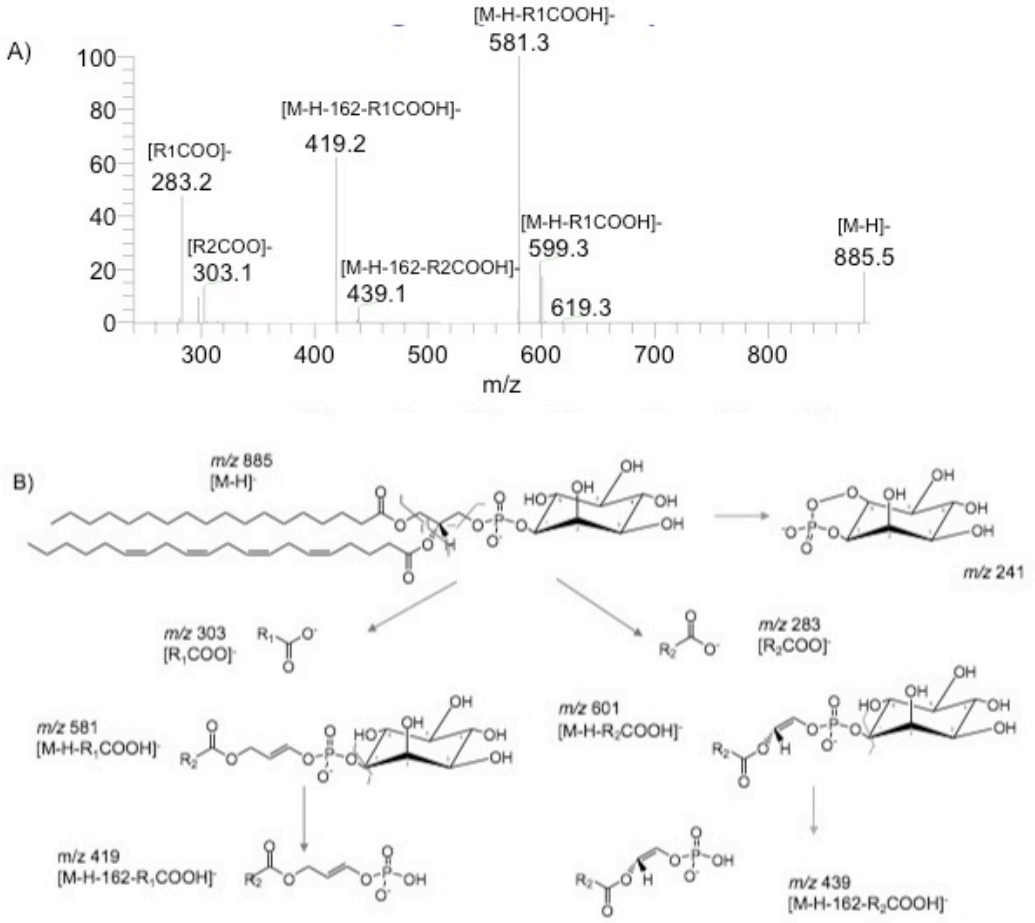

Figure 16. A) MS/MS spectrum of the $[M-H]^-$ ion at *m/z* 885.5, relative to PI (18:0/20:4). B) The fragmentation patterns of this phospholipid class.

PGs form preferentially $[M-H]^-$ ions. MS/MS spectrum shows characteristic fragments with *m/z* 153 (glycerophosphate) and with *m/z* 171, due to neutral loss of the glycerol polar head group. The MS/MS spectra of PG species can also show loss of fatty acyl chains at *sn*-2 position as ketene ($[M-H-R_2=C=O]^-$), acid ($[M-H-R_2CO_2H]^-$) or carboxylate anion (R_2COO^-) are the most favourable; loss of *sn*-1 fatty acyl is more favourable as acid (R_1CO_2H) than as ketene ($R_1=C=O$) [89, 94]. In Figure 17 is represented the MS/MS spectrum of the $[M-H]^-$ ion at *m/z* 773.4 of PG (18:1/18:1) and the corresponding fragmentation patterns.

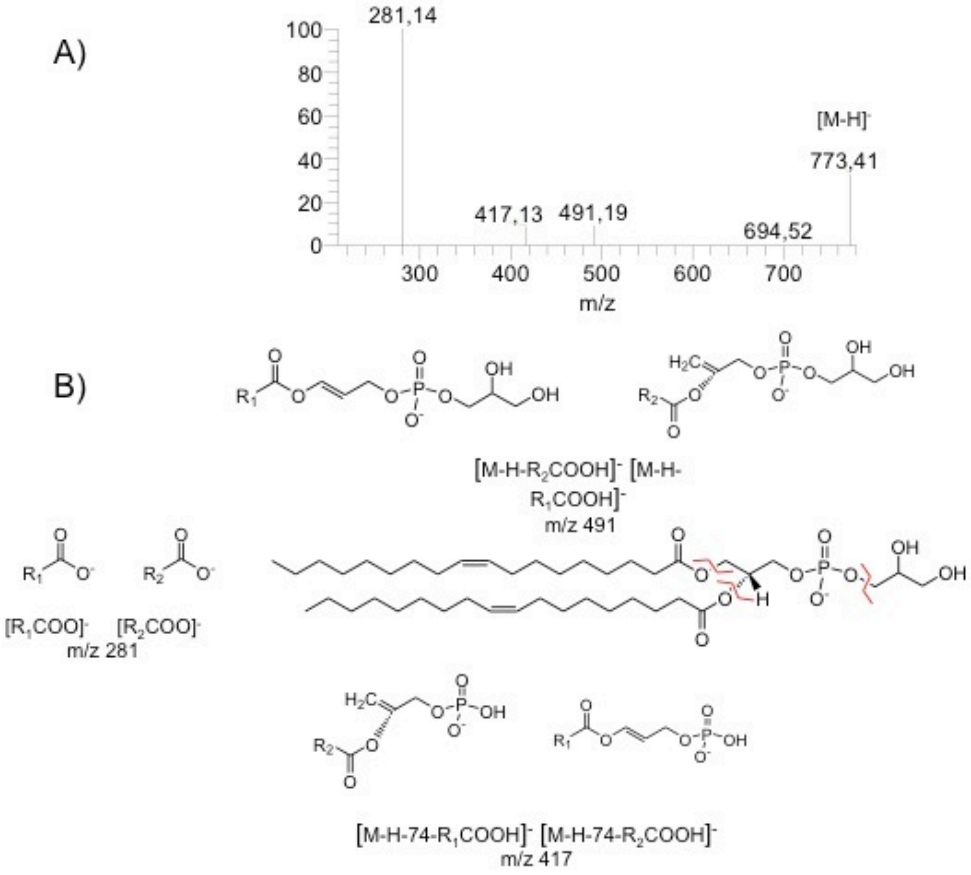

Figure 17. A) MS/MS spectrum of PG $[M-H]^-$ at *m/z* 773.4 (PG C18:1/18:1). B) Fragmentation patterns of PGs.

CL also ionizes preferentially in the negative mode, forming ions of the type $[M-H]^-$ or $[M-2H]^{2-}$ [69]. CL fragmentation shows product ions corresponding ($[M-H-R_xCO_2H]^-$; phosphatidic acid ($[PA-H]^-$); PA with a glycerol molecule ($[PA+56-H]^-$), with a gycerolphosphate ($[PA+136-H]^-$) and with the loss of a fatty acyl chain ($[PA-H-R_2'COOH]^-$) and the four acyl chains $[R_xCOO]^-$. In Figure 18 is represented the characteristic MS/MS spectrum $[M-H]^-$ ion at *m/z* 1447.7 and $[M-2H]^{2-}$ ion at *m/z* 723.6 of CL $(C18:2)_4$.

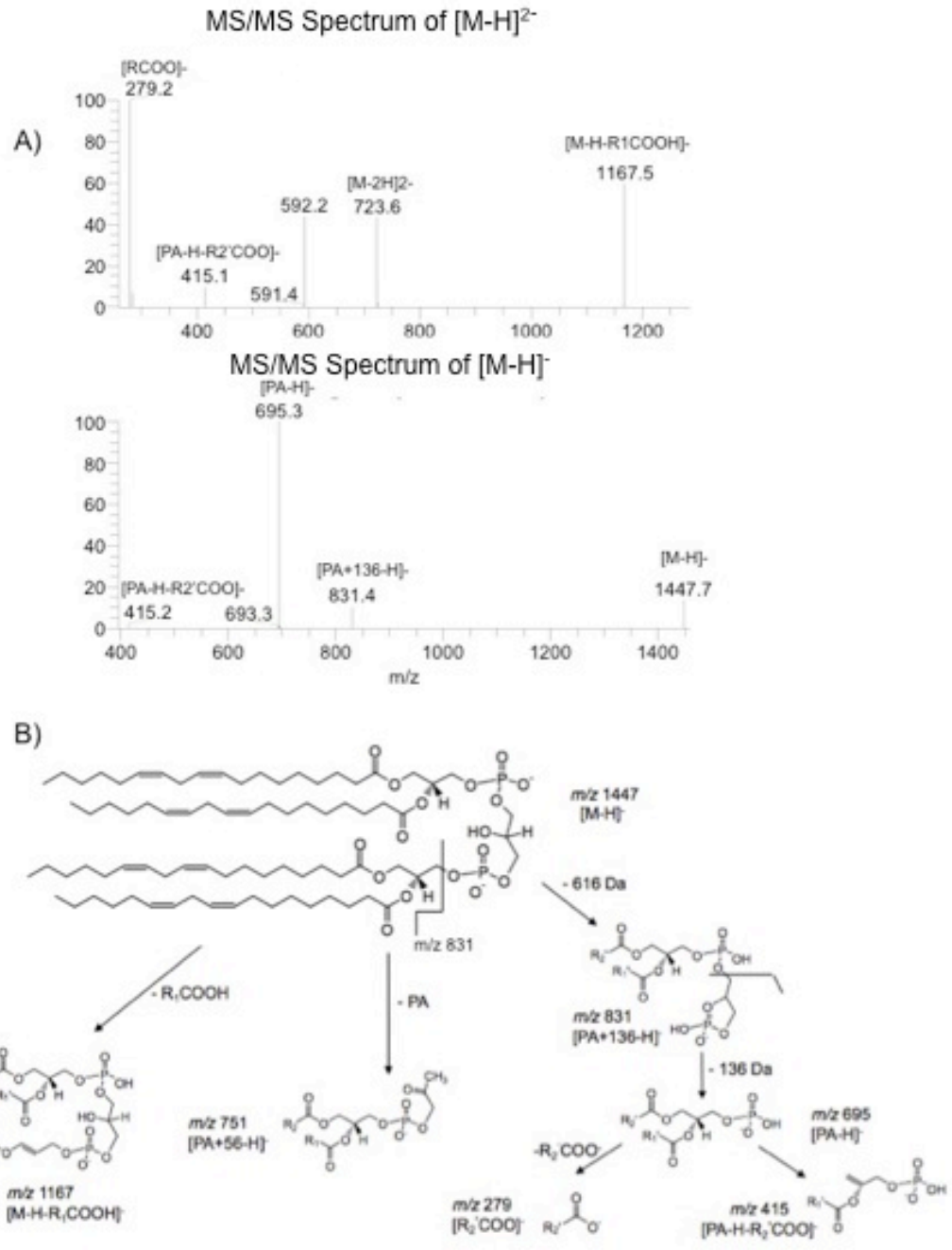

Figure 18. A) MS/MS spectra of $[M-H]^-$ ion at *m/z* 1447.7 and $[M-2H]^{2-}$ ion at m/z 723.6 of CL $(C18:2)_4$. B) Fragmentation patterns of these phospholipid species.

MS has been used to identify phospholipids both in their native form as oxidized species, providing structural data about phospholipids and their oxidative products. The products observed in MS/MS of oxidized phospholipids allows the identification of changes in FAs and specific characteristics, like the presence of new functional groups in

the molecule and its localization in the hydrocarbon chain [61]. The different oxidation products may be responsible for distinct biological effects thus increasing the relevance of identifying each oxidation product in order to understand their specific biological significance and effects [95].

Several studies reporting the analysis of brain and heart lipids using mass spectrometry were carried out in the recent years, especially the study of lipid changes in diseases that affect these organs. Many researchers used lipidomic approaches for the study of phospholipids and their oxidation products in brain and heart injuries, like in traumatic brain injury [35, 36, 61]; in altered lipid metabolism in brain injury and disorders [32-34]; in lipids oxidation in the development of depression [40]; in the study of the effects of diabetes in heart lipids [44, 45]; in the study of cardiolipin loss and oxidation in cardiovascular diseases [43], among others.

Glycerophosphocholines (PC) are the most studied class of phospholipids by mass spectrometry and oxidized products have been identified by MS in biological fluids and tissues [69]. Khaselev and Murphy (1999) reported the results of the oxidation by Fenton reaction of PE from bovine brain [96]. Gugiu *et al.* (2006) reported PE truncated oxidation products by liquid chromatography-tandem mass spectrometry (LC-MS/MS) from rat retina [97]. Several studies identified phosphatidylserine hydroperoxy and hydroxy species using ESI-MS and ESI-MS/MS in negative mode. Bayir and collaborators identified the presence of PS hydroperoxides derivatives in traumatic brain injury after controlled cortical impact [36]. Tyurin *et al.* reported the formation of hydroperoxy and hydroxyl PS derivatives during apoptosis induced in neurons by staurosporine [61] and in cells and tissues after pro-apoptotic and pro-inflammatory stimuli [41]. Kagan and collaborators studied cardiolipin oxidation induced by different conditions in several biological samples by ESI-MS and MS/MS [36, 41, 98]. Maciel *et al.* identified new short chain products o cardiolipin oxidation in nephrotoxic drug-induced disturbances in rat kidney tissue [99]. Melo *et al.* identified sphingolipids oxidation products in *in vitro* analysis by ESI-MS and ESI-MS/MS [100].

However, until this moment no study was performed to evaluate lipid changes that may occur in chronic stress conditions, in the brain and in the heart. Still, it is important to notice the importance of these two organs in chronic stress conditions, the main organs involved in the biochemical changes that occur in these conditions.

4 Aims

Chronic stress has been suggested has a risk factor for the development of several neurological and cardiac diseases. These diseases cause changes in biochemical and physiological mechanisms and are associated with alterations in various biomolecules, including phospholipids. However, as mentioned previously, lipid research in chronic stress conditions is scarce. Chronic stress conditions in brain have been correlated both with increased ROS production and also with deregulation of memory and cognitive function. One of the aims of this work is to investigate the changes in phospholipid brain profile under chronic stress conditions, evaluating the phospholipid oxidation, by finding the oxidation phospholipid products that may be forming in this organ. The phospholipid profile and the oxidation products are identify and characterized by mass spectrometry.

It was already reported that a stressed brain causes impairment of many cardiac functions, providing the onset and development of cardiovascular diseases. However, it is still unclear the changes that this impairment could cause at the biomolecular level, namely in phospholipids. Therefore another aim of this study was to evaluate the phospholipid profile changes in heart of mice with chronic stress, in order to understand the changes caused by chronic stress and its relation with the ones caused in brain.

Thus, the general aim of this study was to evaluate the changes in lipidome that occur due to chronic stress in the brain and in the heart, using a lipidomic approach involving HPLC-MS analysis, in order to better understand the side effects of chronic stress and which organ is more affected.

II. Materials and Methods

II. Materials and Methods

1 Chemicals

The phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidic acid (PA), sphingomyelin (SM), phosphatidylglycerol (PG), lysophosphatdylcholine (LPC), lysophosphatidylinositol (LPI), phosphatidylethanolamine (LPE), lysophosphatidic acid (LPA) and cardiolipin (CL) standards were purchased from Sigma-Aldrich (Madrid, Spain), triethylamine (Acros Organics), chloroform (HPLC grades), methanol (HPLC grades), ethanol (Panreac), primuline (Sigma) were used without further purification. TLC silica gel 60 plates with concentration zone (2.5x20cm) were purchased from Merck (Darmstadt, Germany).

2 Biological Samples

As biological samples we used brains and hearts from mice, specie B57BL/6, male with 8/9 weeks old in the beginning of the protocol. These mice were divided in two groups: the control group, which were not exposed to the stress protocol, and the stress group, which was exposed to the protocol of chronic unpredictable stress, for 21 days. The organs used in this protocol were provided by CNC - Centre for Neuroscience and Cell Biology of Coimbra.

3 Experimental Procedure for Chronic Unpredictable Stress

The mice were exposed to a chronic unpredictable stress protocol, as described by Lu and colleagues [101], which consists of applying some stressors to mice, daily during 21 days. These stressors are applied once a day, in the following order: exposure to the box with wet shavings; pairing with another stressed mice; cold bath (15ºC, 20 min); enclosure (2h); foot shock (0.7mA, 3s, given intermittently during a total time of 5min); exposure to the apparatus of foot shock without the shocks; light off in the light phase of the cycle and box without shavings; inclined box 45º; deprivation of water and food; access to the empty bottle; exposure to the box with wet shavings; pairing with another stressed mice; cold bath (15ºC, 20 min); enclosure (3h); foot shock (0.7mA, 3s, given intermittently during a total time of 5min); exposure to the apparatus of foot shock without the shocks; deprivation of water and food; access to the empty bottle; enclosure (4h); light off in the light phase of the cycle and box without shavings; inclined box 45º.

The evaluation of the stress was made by many tests: comparison of weight gain; behavioural tests, 24h after the last stressor, the mice were exposed to the forced swimming test, the open-field test and the labyrinth y test; the measure of the amount of corticosterone; and measure of the weight of the adrenal gland.

4 Phospholipids Extraction

All the brain and heart samples were homogenised with 1 mL potassium phosphate buffer (50mM, 7,0 pH). Phospholipids were extracted from the brain and from the heart of mice by the Blight and Dyer method [78]. Briefly, 3.75mL of $CHCl_3$: MeOH (1:2 v/v) were added to 1mL of sample (homogenised tissue), well mixed in a vortex and incubated in ice for 30min. Then, 1.25 mL of $CHCl_3$ and 1.25 mL of H_2O were added and mixed well. After, the mixture was centrifuged at 1000g, 5 min, at room temperature (centrifuge Mixtasel Centrifuge (Selecta)), obtaining two phases: the aqueous phase on top and the organic phase below, from where lipids were collected. The extracts were dry in stream of nitrogen and stored at -20ºC, for subsequent analysis.

5 Separation of phospholipids classes by thin-layer chromatography (TLC)

Separation of PL classes by TLC from the total lipid extract was performed using plates of silica gel 60 with concentration zone 2,5x20 cm. Before the separation, the plates were washed in a solution of $CHCl_3$: MeOH (1:1 v/v) and treated with a solution of boric acid in ethanol (2.3% w/v). The plates with spots containing about 30 µg of sample were developed in a mixture of solvents $CHCl_3$: MeOH: H_2O: trietilamine (30:35:7:35, v/v/v/v). To reveal the phospholipid spots, the TLC plates were sprinkle with a primuline solution (50 µg/100 mL acetone: water, 80/20, v/v), and visualized with a UVlamp (λ=254nm). After the identification of the phospholipid spots, by comparison with phospholipid standards, the spots were scraped off from the plates and quantified using the phosphorous assay [102].

6 Quantification of phospholipids using phosphorous assay

In order to determine the content of phospholipids of each extract and compare with the phospholipid content of each class separated by TLC, a phosphorous quantification method was realized in accordance with the protocol of Bartlett and Lewis (1970) [102]. In brief, 650 µL of percloric acid (70% m/v) were added to the samples, which were incubated 45 min at 180 ºC in a heating bloc (Stuart, U.K.). To all samples we

added 3.3 mL of H_2O, 0.5 mL of ammonium molibdate (2.5 g ammonium molibdate/100 mL of H_2O) and 0.5 mL of ascorbic acid (10 g ascorbic acid / 100 mL of H_2O), and vortexed after the addition of each solution, following by the incubation in a bath at 100ºC, 5 min. Additionally we prepared standards with 0.1 to 2 μg of phosphate, which suffer the same treatment of the samples. Finally, we measured the absorbance of standards and samples at 800 nm, in a microplate reader (Multiscan 90, ThermoScientific). The percentage of each phospholipid class was calculated, relating the amount of phosphorous in each spot to the total amount of phosphorous in the sample, thus giving the relative abundance of each phospholipid class.

7 Quantification of Lipid hydroperoxides using Ferric-xylenol orange (FOX) assay

The ferric-xylenol orange (FOX) assay was first described by Gupta [103] and has been used to measure the amount of hydroperoxides in samples, which provides information about the oxidative stress. The method used was FOX II, because this is an adaptation of the initial method, which allows the measurement of lipid hydroperoxides. In brief, 50 μL of sample before and after reduction (50 μL of sample was incubated for 20 min at 48 ºC with triphenylphosphine, 1 mg/mL in methanol) was added to 950 μL of the following solution (FOX reagent), and incubated at room temperature in the dark 30 min, before measurement at 560 nm: 100 μM xylenol orange, 250 μM Fe^{2+}, 25 mM H_2SO_4, and 4 mM BHT in 90% (v/v) methanol. Additionally standards of 0.01 to 0.4 mM of hydrogen peroxide were prepared, and were submitted to the same samples treatment.

8 Protein Quantification

For protein quantification we used the DC-kit: we added 20 μL of the reagent S to 1mL of reagent A (an alkaline cooper tartrate solution), to do reagent A'. Standards of BSA 0.2 mg/mL to 1.5 mg/mL were also prepared. Then, 5 μL of each standard were mixed and each sample with 25 μL of reagent A' and 200 μL of reagent B (a dilute Folin Reagent), and incubated at room temperature 15min. The absorbance at 750 nm was read using a microplate reader (Multiscan 90, ThermoScientific).

9 HPLC Instrumentation

In order to identify the molecular species and their changes in chronic stress phospholipid classes were separated by LC-MS, using a HPLC system (Waters Alliance

2690) coupled to an electrospray (ESI) linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA). The mobile phase A consisted of 10% water and 55% acetonitrile with 35% (v/v) of methanol. The mobile phase B consisted of acetonitrile 60%, methanol 40% with 10mM ammonium acetate. 15 µL of total lipid extract were diluted in the mobile phase A and reaction mixture was introduced into a Ascentis Si HPLC Pore column (15 cm×1.0 mm, 3 µm) (Sigma-Aldrich). The solvent gradient was programmed as follows: gradient started with 0% of A and linear increased to 100% of A during 20 min, and held isocratically for 35 min, returning, to the initial conditions in 5 min. The flow rate through the column was 16 µL/ min obtained using a pre-column split (Acurate, LC Packings, USA) [104]. LC-MS was performed with an internal standard to confirm and quantify the ions variations observed in the spectrum according to the Lipid Maps methods [85]. The PL standards used were PC (14:0/14:0), PS (14:0/14:0), PI (16:0/16:0), PE (14:0/14:0), PA (14:0/14:0) and CL (14:0/14:0/14:0/14:0).

10 Electrospray mass spectrometry conditions

The phospholipid analysis was carried out in positive and negative mode on electrospray (ESI) linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA). ESI conditions on linear ion trap mass spectrometer were as follows: electrospray voltage was 4.7 kV in negative mode and 5 kV in positive mode; capillary temperature was 275ºC and the sheath gas flow was 25 units. An isolation width of 0.5 Da was used with a 30 ms activation time for MS/MS experiments. Full scan mass spectrum and MS/MS spectrum were acquired with a 50 ms and 200 ms maximum ionization time, respectively. Normalized collision energy^{TM} (CE) was varied between 17 and 20 (arbitrary units) for MS/MS. Data acquisition and treatment of results was carried out with an Xcalibur data system (V2.0).

11 Statistical Analysis

Results were presented as means ± standard error (SD). Differences among experimental groups were determined by students t-test. P-values <0.05 were considered to indicate statically significance.

III. Results and Discussion

III. Results and Discussion

This work focused on the identification of changes in phospholipid profile of the brain and of the heart of mice after exposure to chronic stress conditions.

Using brain and heart of chronic stressed mice we were able to evaluate the changes in lipidome and understand the extension of the lesions in the organism induced by chronic stress. These two organs are key organs related to the main symptoms associated with chronic stress.

This study could give new insights on the knowledge of how chronic stress may be involved in the development of diseases.

In this chapter, we will show the results obtained in the characterization of the lipid profile of the brain and of the heart of control and chronic stressed mice (CTL and Stress, respectively) by mass spectrometry with electrospray ionization, using a lipidomic approach. Three complementary strategies were used: a) separation of phospholipids by TLC and quantification of the relative amount of each PL class content; b) quantification of hydroperoxides using FOX II assay, in order to evaluate possible lipid peroxidation; c) analysis of the total phospholipid extract by HPLC-MS and MS/MS. Results and discussion will be divided in two sections: analysis of brain phospholipid profile of chronic stressed mice and analysis of heart phospholipid profile of chronic stressed mice.

1 Analysis of brain phospholipid profile of chronic stressed mice

In order to evaluate possible changes in brain phospholipid profile between chronic stress and controls, total lipid extracts were obtained from the total brain of mice. To know the amount of phospholipids and proteins per µg of tissue we weighted each organ, quantified the total lipid and protein content in each extract and the obtained results are presented in Table 2.

Table 2. Quantification of phospholipids (PLs) and proteins from brain per µg of tissue in controls and chronic stressed mice and relation between µg of PLs per µg of proteins.

	µg of PLs per µg of Tissue	µg of Proteins per µg of Tissue	µg of PLs per µg of Proteins
Control	1.22E-02 ± 2.50E-03	9.21E-02 ± 6.19E-03	1.32E-01 ± 2.34E-02
Stress	1.56E-02 ± 3.17E-03	9.03E-02 ± 6.86E-03	1.73E-01 ± 2.19E-02

Results represent mean± SD of three brains analysed (n=3) in each group.

The analysis of data presented in the Table 2 indicates that stress is associated with a tendency to an increase of the amount of PLs per µg of tissue and a tendency to a decrease in protein content per µg of tissue. Although, these changes are not statistically significant, this increase in PLs/proteins ratio may be related to some metabolic changes in brain under chronic stress conditions [6]. However, this data needs to be explored.

1.1 Changes in relative abundance of different classes of phospholipids in the brain

In this work we evaluated the differences between the relative content of each class of phospholipids. For accomplish this, total lipid extracts were fractionated by TLC and each class was identified with pure standards applied in the same TLC plate. These approach allowed the separation of seven classes of phospholipids: sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidic acid (PA) and cardiolipin (CL) (Figure 19).

TLC is a very valuable technique for the separation of PL classes, being extremely fast and simple [79].

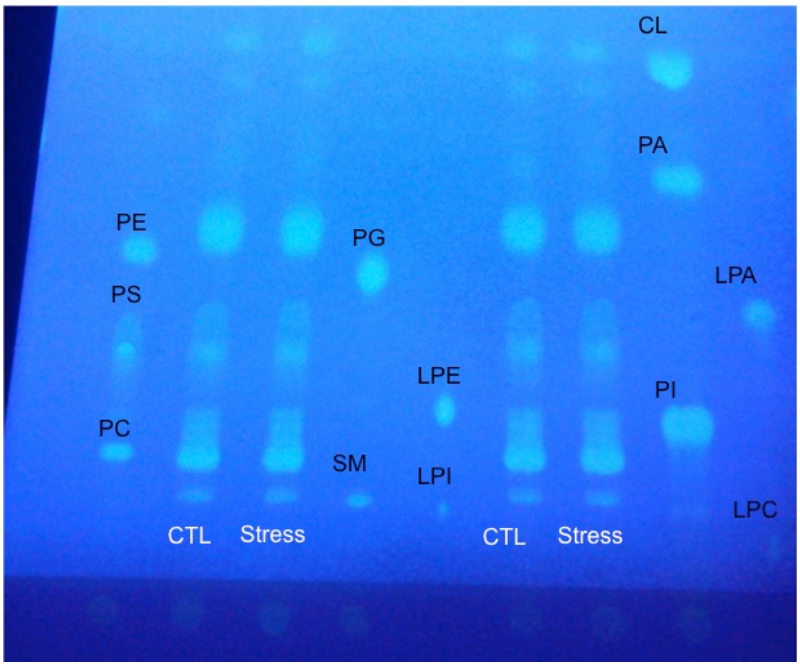

Figure 19. Thin-layer chromatography of total lipid extract obtained from mice brain control (CTL) and with chronic stress (Stress). Phospholipid standards were also applied: (PC) - Phosphatidylcholine; (PS) - Phosphatidylserine; (PE) - Phosphatidylethanolamine; (SM) - Sphingomyelin; (PG) - Phosphatidylglycerol; (LPI) - Lysophosphatdylinositol; (LPE) - Lysophosphatidylethanolamine; (PI) - Phosphatidylinositol; (PA) - Phosphatidic Acid; (CL) - Cardiolipin; (LPC) - Lysophosphatidylcholine; (LPA) - Lysophosphatidic acid.

After the separation of each PL class, we quantified the PL content of each TLC spot, by phosphorous assay described by Bartlett and Lewis (1970), thereby obtaining a relative amount of each PL class in the total lipid extract (Figure 20). This analysis was carried out in triplicate for each sample and three different samples were analysed for each experimental group.

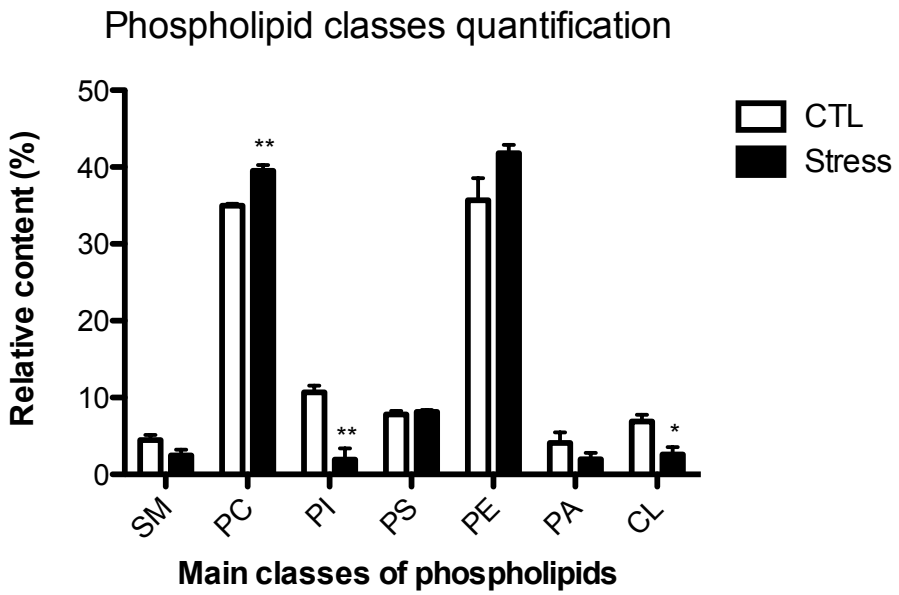

Figure 20. Relative content of phospholipid classes in controls (CTL) and chronic stress situations (Stress) in total lipid extract obtained from mice brain. The phospholipid classes were separated by thin-layer chromatography and the phosphorous content of each spot was calculated taking in account the amount of phosphorous in the total lipid extract. Phospholipid classes were separated and quantified: (SM) - Sphingomyelin; (PC) - Phosphatidylcholine; (PI) - Phosphatidylinositol; (PS) - Phosphatidylserine; (PE) - Phosphatidylethanolamine; (PA) - Phosphatidic Acid; (CL) - Cardiolipin. * $p < 0.05$ versus control, ** $p < 0.01$ versus control, n=3 independent experiments.

Several changes occur in phospholipid profile of chronic stress group. The exposure to chronic stress caused an increase in most abundant PL classes in brain (PC and PE). PS class did not show variation. In addition it was possible to see a decrease in the relative content of PI, SM, PA and CL. In the control group the relative abundance of classes follows the order PC>PE>PI>PS>CL>SM>PA. However, this order is changed in chronic stress group, becoming PE>PC>PS>CL>SM>PI>PA (Figure 20). Both in the control group (CTL) as in the chronic stress group (Stress), phospholipid classes more abundant in brain were phosphatidylcholines (CTL=34%; Stress=40%) and phosphatidylethanolamines (CTL=33%; Stress=41%), corroborating the information available in literature, where is stated that these are the main classes of phospholipids in mice brain and they together constitute approximately 70 % of total brain phospholipids [105]. Although PCs and PEs increased in chronic stress, only the increase verified in PCs is statistically significant. The decrease of PI and CL were also statistically significant.

In Figure 21 are presented the main pathways synthesis of phospholipids, which form a complex network of metabolic pathways, whereby it becomes difficult to identify only with these data the causes of the variations in PL classes, but we can try to take some clues.

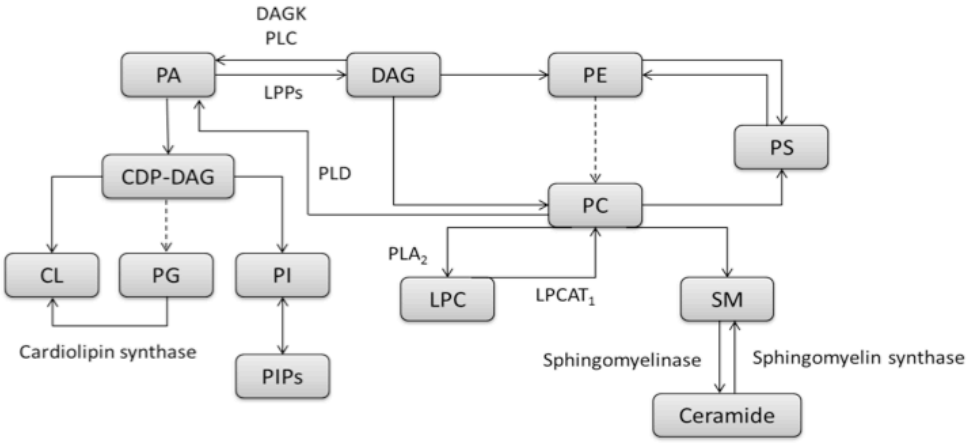

Figure 21. Main pathways of biosynthesis of phospholipids. The phosphatidic acid (PA) is the basis for the formation of all other phospholipids. The addition of an inositol group originates phosphatidylinositol (PI). This class can be phosphorylated in some inositol sites forming PIPs (phosphatidylinositol phosphorylated). PA can also generate PG (phosphatidylglycerol) and CL (cardiolipin), and they can be converted in each other. PA and diacylglycerol (DAG) are precursores of phosphatidylethanolamine (PE) and phosphatidylcholine. Both are involved in synthesis of phosphatidylserine (PS). Besides that, PE can generate PC and PS can generate PE. The hydrolysis of a fatty-acid chain of PC, by PLA_2 (phospholipase A_2), produces lysophosphatydilcholine (LPC). Finally, sphyngomielin (SM) that can be genarated by PC, when suffers the action of sphyngomielinase releases ceramide (Cer).

PCs and PEs are the most abundant phospholipids in cellular membranes and they play a main role in membrane structure. The increase verified in these two classes may be related with a structural change in neuronal membranes.

Sphingomyelin class showed a decrease, although not statistically significant, in its relative abundance, which, in addition to the possibility of being synthetizing PCs and PEs, may be explained by the decomposition of SM by the sphingomyelinase, producing ceramide (Cer) [33]. The accumulation of ceramide can lead to many biological consequences, as the increase of cytokines, formation of reactive oxygen species (ROS), interruption of the mitochondrial respiratory chain, apoptosis, among others [54].

Another class of phospholipids, which undergoes change, is phosphatidylinositol. This class showed statistically significant decrease with chronic stress, which may be due

to the increase of phospholipases action or due to a decrease of the biosynthesis of this phospholipid class. Some studies correlate the decrease of PIs to injuries or diseases associated with brain [64, 106], suggesting the existence of profound changes in metabolism of this biomolecules. PIs are very important in signalling events. These molecules can be phosphorylated with formation of molecules named phosphoinositides phosphorylated (PIPs), which vary in its functions depending on the position of phosphorylation in the inositol head group. These molecules are extremely important since they are responsible for triggering signalling cascades, playing a main role in cell metabolism and apoptotic cellular events [107].

As shown in Figure 21 PAs are the molecular basis for the formation of all other phospholipids. The decrease of this class after chronic stress may be related with the increase in biosynthesis of PCs and PEs. However, PAs are also involved in cell proliferation, differentiation and in signalling events, suggesting that this decrease can be related to the activation of these kind of events.

Cardiolipin also suffers a statistically significant decrease in chronic stress situations. CL is a very important and well-studied phospholipid; it is almost exclusive located on mitochondrial inner membrane and is involved in the assembly of the electron transport chain complexes responsible for the oxidative phosphorylation. The decrease in CL has been associated with mitochondrial dysfunction that was related with several pathophysiological conditions, like aging and neurodegenerative diseases. CL due to its structure (four fatty-acid chains that can be polyunsaturated) is very susceptible to oxidation. Oxidation of CL may lead to its release in the cytoplasm, propitiating the decrease in CL content. Moreover, the deregulation of CL in chronic stress situations may be due to an increase in oxidative stress. CL is closely related to the phenomena of apoptosis, thus the decrease in CL and therefore its deregulation may be related to neuronal loss observed in chronic stress [63].

1.2 Increased lipid hydroperoxides in mice brain after exposure to chronic stress

Chronic stress is correlated with the increase in oxidative stress in brain [31]. Some authors have studied the increase of oxidative stress, in chronic stress, in many biomolecules, inclusive lipids [8, 31]. However, these studies were carried out measuring the maloniladheyde (MDA), a decomposition product of lipid hydroperoxides. This assay

is very nonspecific for the evaluation of lipid peroxidation. On the other hand, taking into account the mechanism of lipid peroxidation (Figure 22), we know that lipid hydroperoxides are the primary products of lipid oxidation and have been used in several studies to evaluate the oxidation status of cells or tissues [61, 62].

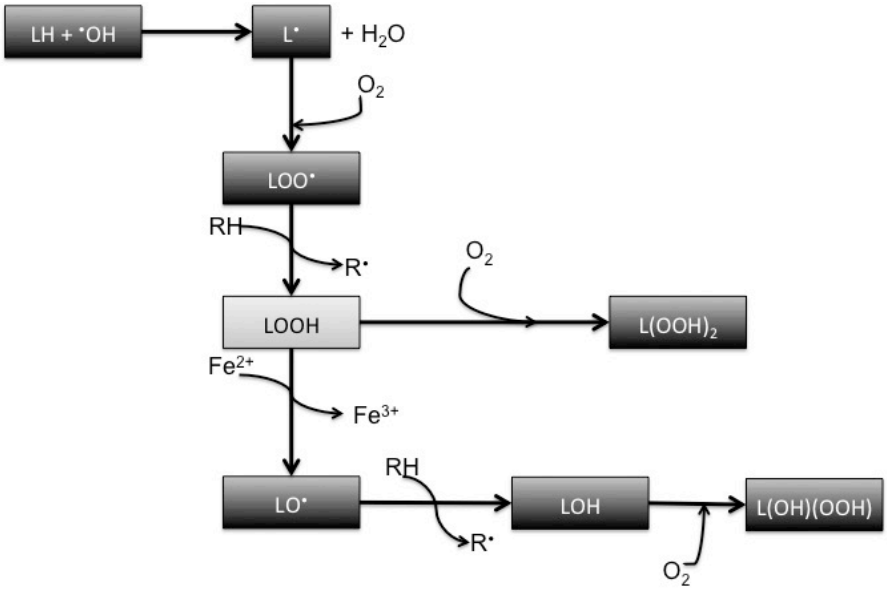

Figure 22. General lipid peroxidation mechanism. Oxidation is initiated by the attack of a hydroxyl radical (•OH), capable of abstracting a hydrogen atom from a reactive methyl group of a polyunsaturated lipid (LH), forming a lipid alkyl radical (L•). Then propagation occurs with the addition of an oxygen molecule, forming a lipid peroxyl radical (LOO•), which can abstract a hydrogen atom from adjacent polyunsaturated fatty acid in membrane to produce a lipid hydroperoxide (LOOH) and a second lipid radical. The resulting peroxide may be cleaved, by reduced metals, such as Fe^{2+}, producing alkoxyl (LO•) or epoxyperoxyl (OLOO•) radicals, and both stimulate the chain reaction of lipid peroxidation by abstracting additional hydrogen atoms.

Therefore we used FOX II assay (ferrous oxidation xylenol orange assay) to measure lipid hydroperoxides in total lipid extract of brain [108]. This method is based on the oxidation of Fe^{2+} to Fe^{3+} by the hydroperoxides, at low pH, which forms a complex with xylenol orange dye that is then measured spectrophotometrically at 560 nm [109]. Results obtained with FOX II assay in the total lipid extract, allowed the observation of a statistically significant increase of hydroperoxides in the chronic stress group (Figure 23). These results were obtained measuring hydroperoxides in three samples of control group and three of chronic stress group. This increase was found to be statistically significant.

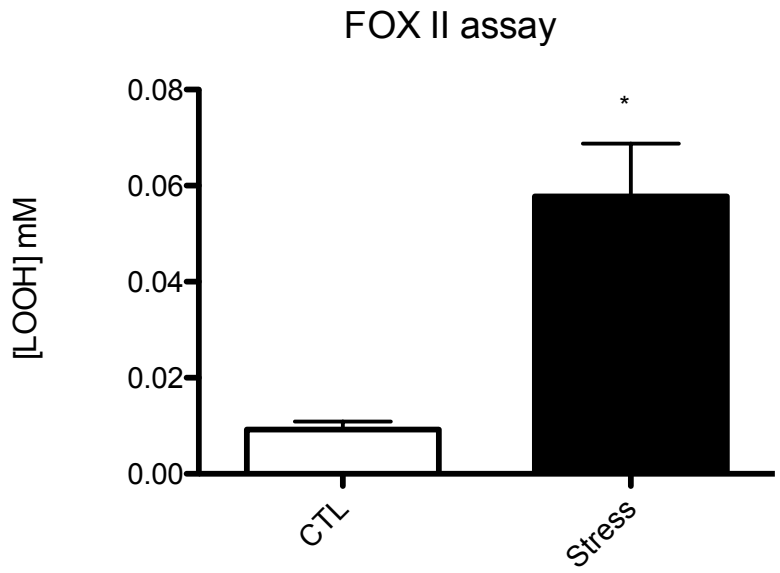

Figure 23. Concentration of lipid hydroperoxides in samples of control group (CTL) and chronic stress group (Stress), evaluated by FOX II assay. * $p < 0.05$ versus control; n=3 independent experiments.

Thus, there is a strong evidence that chronic stress causes an increase in lipid peroxidation. As we know lipid peroxidation is a reaction that causes many changes in the homeostasis of the organism, since it is involved in cellular damage and may explain the neuronal loss verified in stress situations. This phenomenon is also involved in tissue damage, aging and neurodegenerative diseases [31]. Our results showed that there is an increase in oxidative stress in chronic stress conditions, which may be responsible for the neurological side effects of chronic stress.

It is currently known that chronic stress is particularly involved with the development of depression and other neurological disorders. Depression has increased extremely in the last years, and that fact can be involved with the increase in stress situations, which we are exposed in our days. The study of physiological processes that occur in brain during depression, based on the modification of the neuronal membranes, showed an increase in lipid peroxidation in brain [40]. This increase in oxidation destabilizes membranes and causes the cell death. The homeostatic imbalance that is verified in brain regulating regions can explain the behavioural changes of depressive individuals [40].

1.3 Analysis by HPLC-MS of brain phospholipid classes

HPLC-MS is a technique that allows the separation of phospholipid classes and at the same time allows the analysis by MS and MS/MS of each class. The molecular species of each class were identified in order to evaluate if the changes in relative content of PL occur at the molecular level and to evaluate which PL class is most affected by oxidative stress. PC, SM and LPC classes were analysed in the positive mode, while PE, PA, PS, PI e CL were analysed in the negative mode. The chromatogram showing the elution of each phospholipid class is shown in Figure 24.

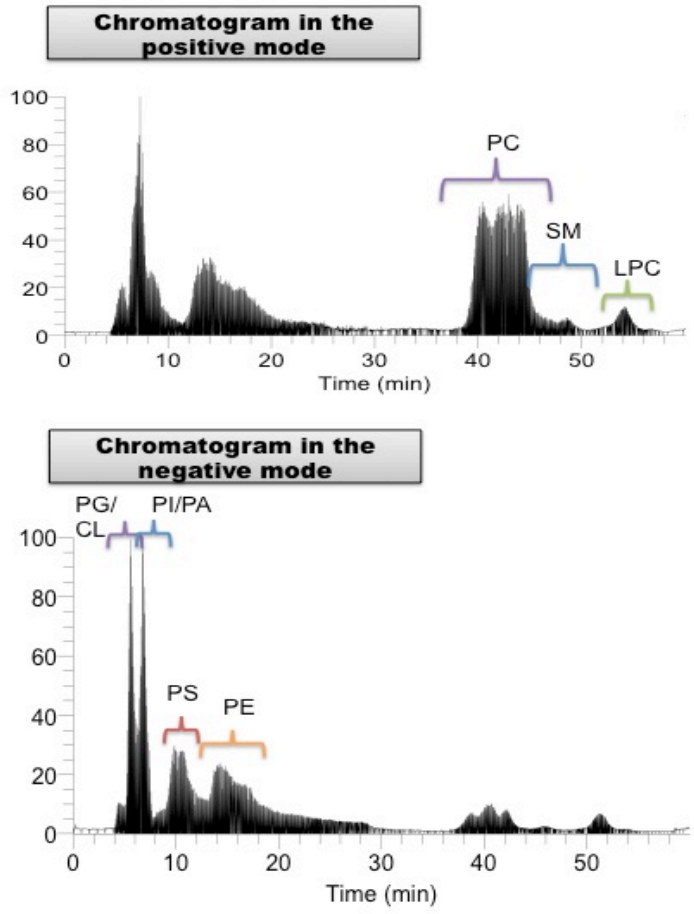

Figure 24. Chromatograms in positive and negative mode, showing where each class elutes, in the mode where they were analysed.

1.3.1 Analysis of PC profile

PCs are the most abundant phospholipids found in cell membranes, where they play an important structural role. PCs ionize preferentially in the positive mode, forming $[MH]^+$ ions and the MS/MS analysis of PCs shows a characteristic ion fragment at *m/z* 184. The most abundant PC species identified are presented in Table 3 and the MS/MS of each was performed to confirm the fatty acyl composition of the ions. We were able to identify two groups of molecular species: diacyl-PC and alkenyl-PC.

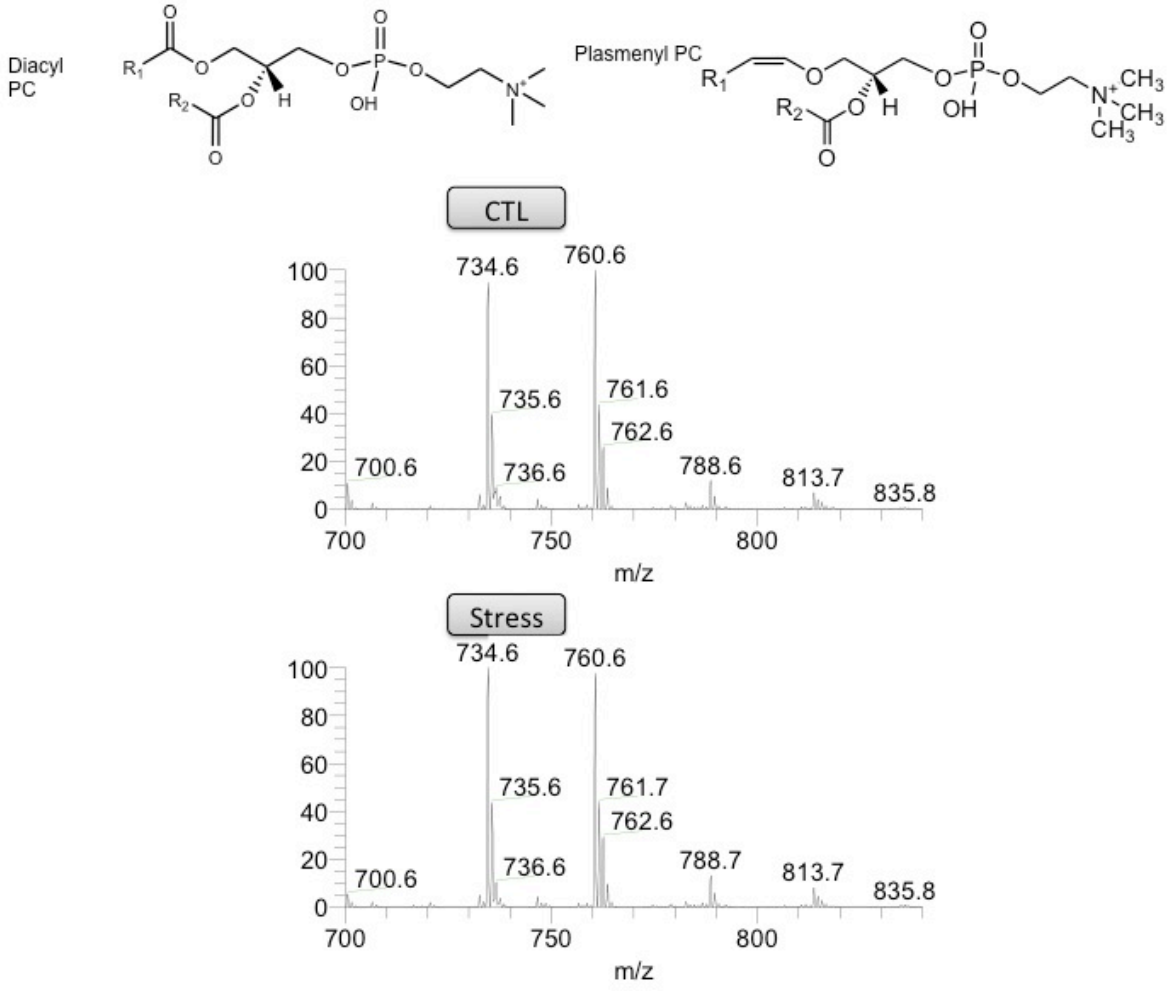

Figure 25. General structure of diacyl and plasmenyl PC class; HPLC-MS spectra of PC class in the positive mode with formation of $[MH]^+$ ions, in control (CTL) and in chronic stress situations (Stress). Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: *m/z* for each ion.

Table 3. Identification of $[MH]^+$ ions observed in the MS spectra in PC; p - an *sn-1* vinyl ether (alkenyl- or plasmalogen) linkage. Phospholipids are designated as follows: diacyl 34:1 PC, where 34 indicates the summed number of carbon atoms at both the *sn-1* and *sn-2* positions and 1 designates the summed number of double bonds at both positions.

	Diacyl Species			Alkenyl Species		
Class	*m/z* $[MH]^+$	C:N	Fatty Acyl Chains	*m/z* $[MH]^+$	C:N	Fatty Acyl Chains
PC	732.6	32:1	16:0/16:1	716.6	32:1p	16:0p/16:1
	734.6	32:0	16:0/16:0	718.6	32:0p	16:0p/16:0
	756.7	34:3	16:1/18:2	736.6	34:5p	16:2p/18:3
	758.7	34:2	16:0/18:2	738.6	34:4p	16:2p/18:2
	760.6	34:1	16:0/18:1	744.6	34:3p	16:1p/18:2
	762.6	34:0	16:0/18:0	746.7	34:0p	16:0p/18:0
	780.7	36:5	16:1/20:4	764.7	36:5p	16:1p/20:4
	782.7	36:4	16:0/20:4	772.7	36:1p	18:0p/18:1
	784.7	36:3	18:1/18:2	774.7	36:0p	18:0p/18:0
	786.7	36:2	18:0/18:2			
	788.7	36:1	18:0/18:1			
	790.7	36:0	18:0/18:0			
	806.6	38:6	16:0/22:6			
	808.7	38:5	18:1/20:4			
	810.7	38:4	18:0/20:4			
	812.7	38:3	18:1/20:2			
	814.7	38:2	18:0/20:2			
	816.7	38:1	18:0/20:1			
	818.6	38:0	18:0/20:0			
	834.7	40:6	18:0/22:6			

Although PC class relative content increases with chronic stress the PC molecular composition does not change, since MS spectrum is similar for both situations (control and stress - Figure 25). The increase in relative abundance of PC class may be explained by the decrease in SM class, which can be a precursor of PCs. This increase can also mean that some changes occur in neuronal membranes that are necessary to be explored.

1.3.2 Analysis of SM profile

SMs are a very important class of phospholipids, since they have important structural functions and at the same time, when in specific domains such as membrane "rafts", they can play an important role in signalling pathways [100]. As PC, SM species ionize in positive mode as $[MH]^+$ forming a typical choline phosphate fragment ion at *m/z* 184. They can be easily differentiated since PC appear at pair *m/z* values, whereas protonated molecules of SM exhibit odd *m/z* values due to the presence of an additional nitrogen atom in SM. The major SM molecules identified are resumed in Table 4.

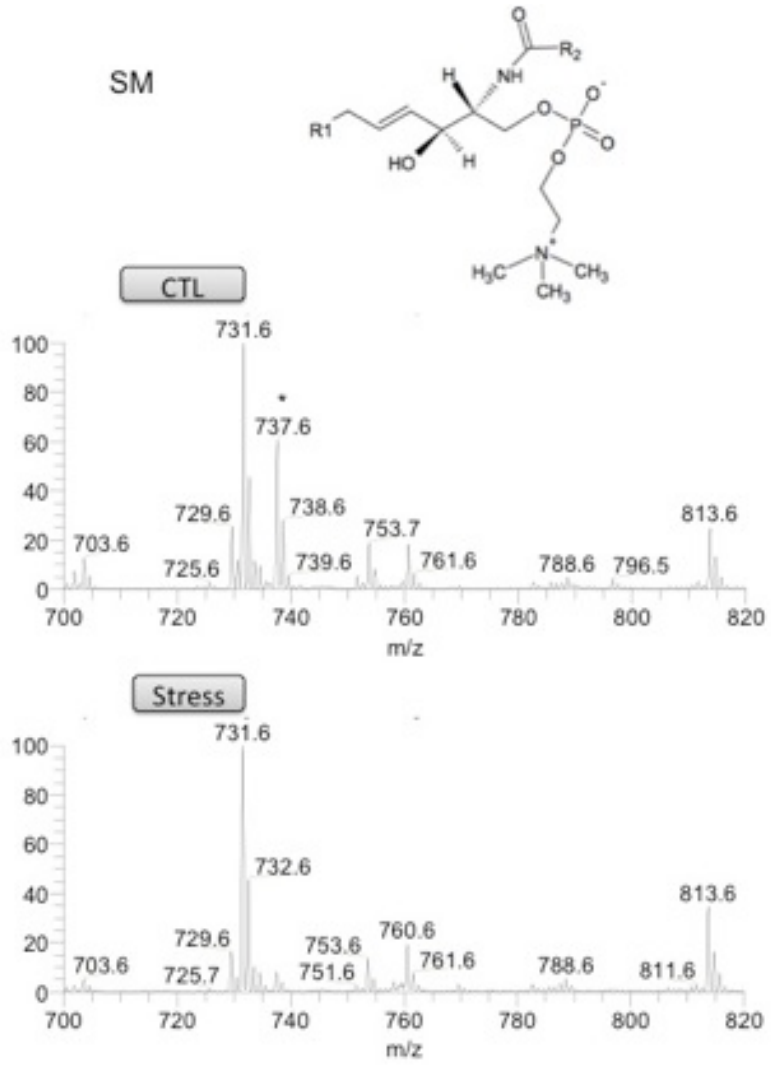

Figure 26. General structure of SM class; HPLC-MS spectra of SM class in the positive mode with formation of $[MH]^+$ ions, in control (CTL) and in chronic stress situations (Stress). Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: m/z for each ion. The ions marked are those that suffer changes in chronic stress situations, namely they have a decrease. * 737.6 peak correspondent to the eluent.

Table 4. Identification of $[MH]^+$ ions observed in the MS spectra in SM. Phospholipids are designated as follows: d18:1/16:0 SM, where d18:1 indicates the sphingosine chain and 16:0 indicates the fatty acyl residue.

Class	*m/z* $[MH]^+$	C:N	Fatty Acyl Chains
SM	701.7	34:2	d18:1/16:1
	703.6	34:1	d18:1/16:0
	705.7	34:0	d18:0/16:0
	725.7	36:4	d18:1/18:3
	729.6	36:2	d18:1/18:1
	731.6	36:1	d18:1/18:0
	733.6	36:0	d18:0/18:0
	751.7	38:5	d18:1/20:4
	753.7	38:4	d18:0/20:4
	755.7	38:3	d18:1/20:2
	757.6	38:2	d18:1/20:1
	759.6	38:1	d18:1/20:0
	761.6	38:0	d18:0/20:0
	783.7	40:3	d18:1/22:2
	785.7	40:2	d18:1/22:1
	787.6	40:1	d18:1/22:0
	789.6	40:0	d18:0/22:0
	805.7	42:6	d18:0/24:6
	807.6	42:5	d18:1/24:4
	809.7	42:4	d18:1/24:3
	811.7	42:3	d18:1/24:2
	813.7	42:2	d18:1/24:1
	815.6	42:1	d18:1/24:0
	817.7	42:0	d18:0/20:0
	833.8	44:6	d18:0/26:6
	835.7	44:5	d18:1/26:4

By the analysis of SM spectra (Figure 26) we see that SM profile is not altered in chronic stress conditions. However, in the total SM content we showed that this class suffers a decrease. Deregulation of sphingolipid metabolism leads to the onset and progression of various diseases such as neurodegenerative diseases, cardiovascular diseases, chronic inflammation and/or cancer. Sphingomyelin is an important constituent in nervous system and is one of the precursors of Cer and sphingosine found in membranes, which are important signalling molecules [92].

1.3.3 Analysis of LPC profile

Due to its very low abundance, the LPC class was not detected in TLC, however we were able to identify some ions of this class in HPLC-MS (Table 5). LPCs were analysed in the positive ion mode, with the formation of ions $[MH]^+$. Also in this class (as we can see in Figure 27) the differences between the two spectra are not significant.

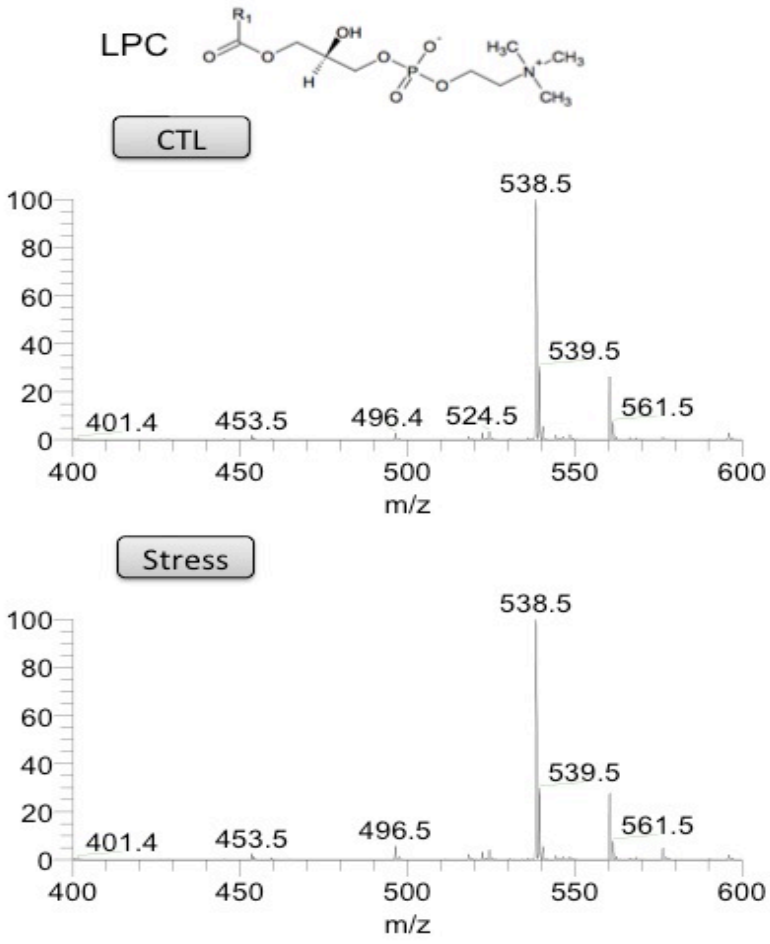

Figure 27. General structure of LPC class; HPLC-MS of LPC class in the positive mode with formation of $[MH]^+$ ions, in control (CTL) and in chronic stress situations (Stress). Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: *m/z* for each ion.

Table 5. Identification of $[MH]^+$ ions observed in the MS spectra in LPC; p - an *sn-1* vinyl ether (alkenyl- or plasmalogen) linkage. Lysophosphatidylcholines are designated as follows: diacyl 18:3 PC, where 18 indicates the number of carbon atoms at both the *sn-1* position and 3 designates the number of double bonds at this position.

	Acyl Species		Akenyl Species	
Class	*m/z* $[MH]^+$	C:N	*m/z* $[M+H]^+$	C:N
LPC	496.4	16:0	476.5	16:2p
	518.5	18:3	478.7	16:1p
	520.5	18:2	538.5	20:4p
	522.4	18:1	540.5	20:3p
	524.5	18:0	542.5	20:2p
	550.6	20:1	544.4	20:1p
	568.4	22:6	546.5	20:0p
	570.4	22:5		
	572.6	22:4		
	576.4	22:2		
	578.5	22:1		
	580.5	22:0		
	596.1	24:6		
	598.5	24:5		
	600.5	24:4		
	604.8	24:2		
	606.5	24:1		

1.3.4 Analysis of PE profile

PE is one of the most abundant classes of PLs in cells. PEs are especially localized in the inner leaflet of the plasma membrane and are important for membrane fluidity. PEs may also affect the Ca^{2+} transport process [110]. The molecular species of PE class were analysed in the negative mode by the formation of $[M-H]^-$ ions. PE species identified included two groups of molecular species: diacyl-PE and alkenyl-PE (Table 6 and Figure 28).

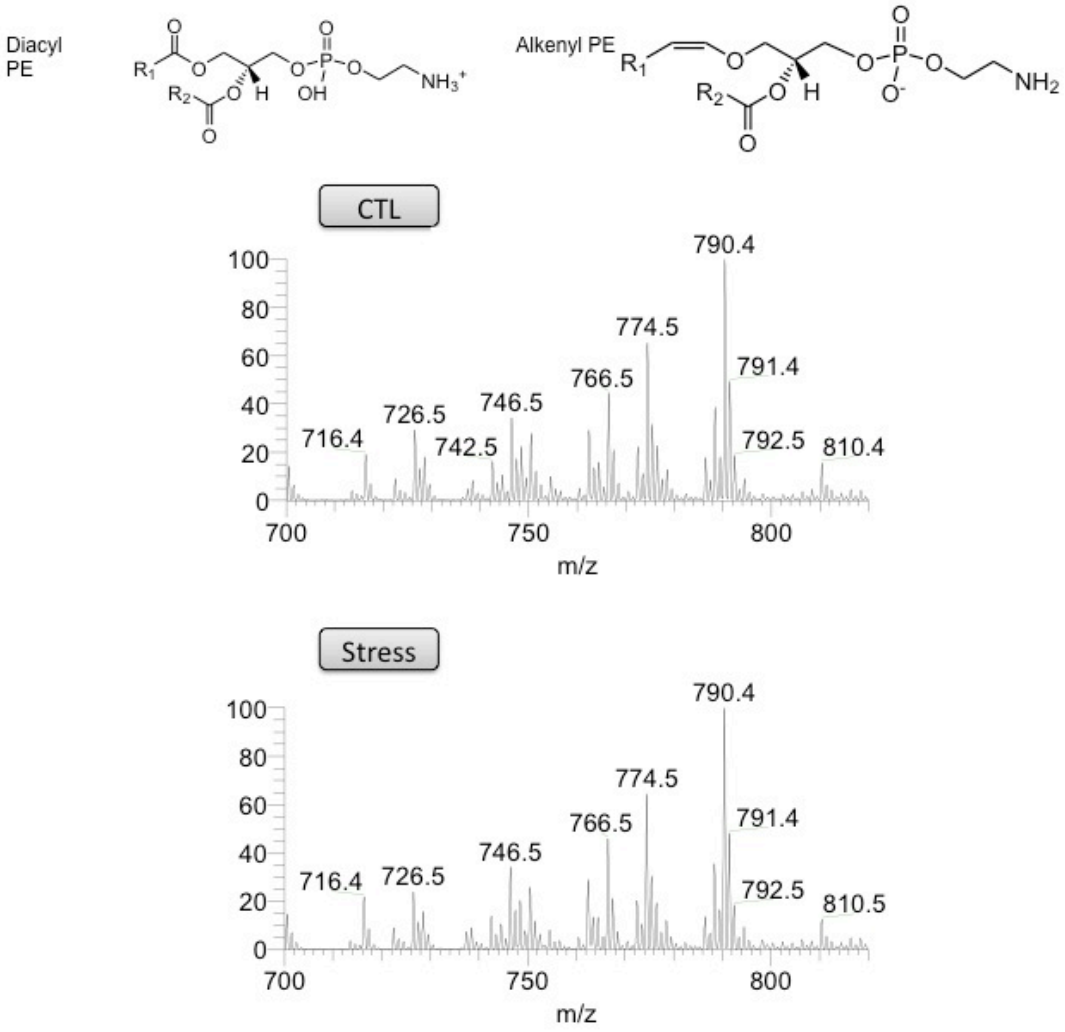

Figure 28. General structure of diacyl PE and alkenyl PE; HPLC-MS spectra of PE class in the negative mode with formation of $[M-H]^-$ ions, in control (CTL) and in chronic stress situations (Stress). Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: *m/z* for each ion.

Table 6. Identification of $[M-H]^-$ ions observed in the MS spectra of PE; p - an *sn-1* vinyl ether (alkenyl- or plasmalogen) linkage. Phospholipids are designated as follows: diacyl 40:6 PE, where 40 indicates the summed number of carbon atoms at both the *sn-1* and *sn-2* positions and 6 designates the summed number of double bonds at both positions.

	Diacyl Species			Alkenyl Species		
Class	*m/z* $[M-H]^-$	C:N	Fatty Acyl Chains	*m/z* $[M-H]^-$	C:N	Fatty Acyl Chains
PE	714.5	34:2	16:0/18:2	700.5	34:1p	16:0p/18:1
	716.4	34:1	16:0/18:1	702.5	34:0p	18:0/16:0
	718.4	34:0	16:0/18:0	722.5	36:4p	16:0p/20:4
	738.4	36:4	16:0/20:4	724.5	36:3p	18:1p/18:2
	740.4	36:3	18:1/18:2	726.5	36:2p	18:0p/18:2
	742.5	36:2	18:1/18:1	728.5	36:1p	18:0p/18:1
	744.5	36:1	18:0/18:1	730.5	36:0p	18:0p/18:0
	746.5	36:0	18:0/18:0	748.5	38:5p	18:1p/20:4
	762.4	38:6	16:0/22:6	750.5	38:4p	18:0p/20:4
	764.5	38:5	18:1/20:4	752.5	38:3p	18:1p/20:2
	766.5	38:4	18:0/20:4	754.5	38:2p	18:1p/20:1
	768.5	38:3	18:1/20:2	756.6	38:1p	18:0p/20:1
	770.4	38:2	18:0/20:2	758.5	38:0p	18:0p/20:0
	772.5	38:1	18:0/20:1	774.5	40:6p	18:0p/22:6
	790.5	40:6	18:0/22:6	776.5	40:5p	18:1p/22:4
	792.4	40:5	18:1/22:4	778.5	40:4p	18:0p/22:4
	794.4	40:4	18:0/22:4	780.5	40:3p	18:1p/22:2
	796.5	40:3	18:1/22:2	782.4	40:2p	18:0p/22:2
	798.4	40:2	18:0/22:2	784.4	40:1p	18:1p/22:0
	800.5	40:1	18:0/22:1	786.5	40:0p	18:0p/22:0
	802.4	40:0	18:0/22:0			

All these ions identified in MS were analysed by MS/MS, which allowed the identification of the two fatty acyl/alkenyl residues of both diacyl- and alkenyl-PE species and a proposal for their localization in glycerol backbone (Table 6).

Although analysis of phospholipid content revealed an increase in the relative content of PE class, no significant differences were found in molecular profile of PE species. The increase in the relative content of PEs can be involved with changes in membrane fluidity in chronic stress situations.

1.3.5 Analysis of PS profile

PS is an acidic natural occurring phospholipid, which is found on the cellular membrane of a great variety of organisms [111]. It is an essential component of biological membranes and has important regulatory functions in cells, being most abundant in the inner leaflet of the plasma membrane [64, 112-114]. PS is more abundant in brain, than in other tissues, representing about 15% of total phospholipid pool in human brain [112]. PS has been extensively studied, due to its effects in brain, such as the improvement in the NA^+/K^+-ATPase activity and its effects in memory recovery [115]. PS has also been used as a nutritional supplement, since it has an important role in neuronal modulation and excitability, signal transduction and neurotransmitter activity. PS is maintained in the inner leaflet membrane due to the aminophospholipid tranlocase activity, a member of the fllipase family enzymes [116]. During apoptosis or cellular damage, an increase in intracellular levels of Ca^{2+} triggers two events: the inhibition of aminophospholipid translocase and stimulation of scramblases, enzymes that stimulate the transbilayer randomization of PS, thus causing the PS switch to the external leaflet, where it is recognized by the macrophages. The result of these reactions is the elimination of apoptotic neuronal cells, during the cerebral development and neural inflammation. In addition, Ca^{2+} also stimulates the cytosolic enzymes that catalyse the synthesis of this phospholipid [64, 93, 117]. Apoptosis is accompanied by the generation of ROS mainly by mitochondria, which causes the oxidation of different classes of phospholipids, including PS oxidation [114].

PS molecular species were also analysed in the negative mode, showing $[M-H]^-$ ions. In PS mass spectra we only were able to identify diacyl-species. PS species identified are presented in Table 7.

Differences between the mass spectra of control and stress situations were not significant (Figure 29), neither the differences in quantification of the PS class, as reported before.

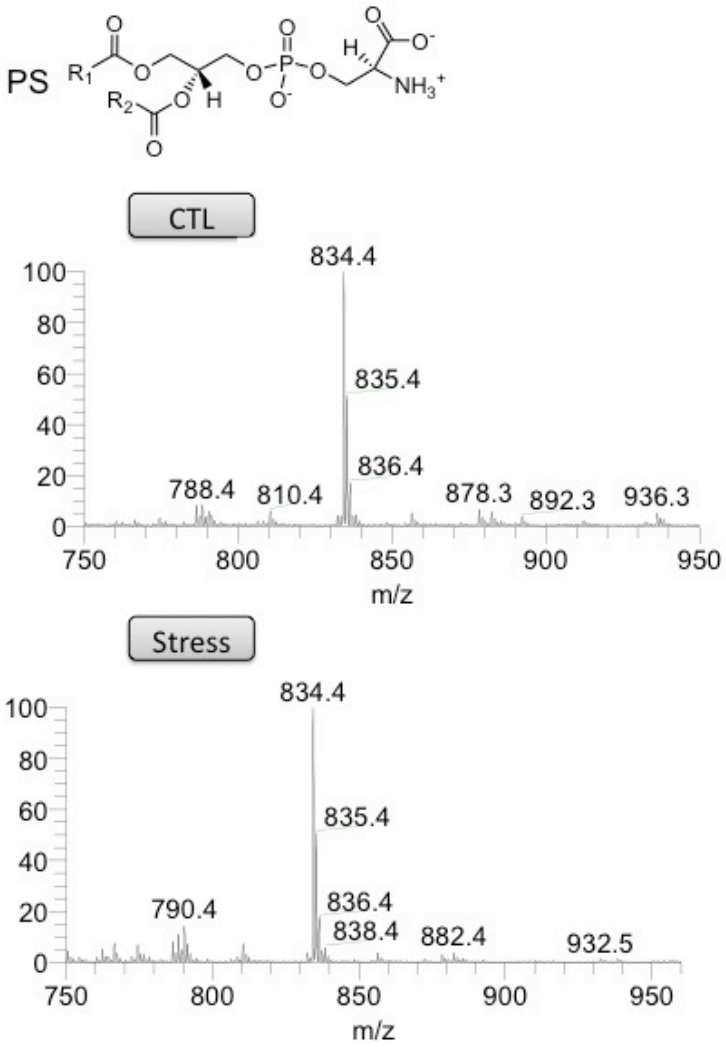

Figure 29. General structure of PS class; HPLC-MS spectra of PS class in the negative mode with formation of $[M-H]^-$ ions, in control (CTL) and in chronic stress situations (Stress). Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: *m/z* for each ion.

Table 7. Identification of $[M-H]^-$ ions observed in the MS spectra of PS. Phospholipids are designated as follows: diacyl 40:6 PS, where 40 indicates the summed number of carbon atoms at both the *sn-1* and *sn-2* positions and 6 designates the summed number of double bonds at both positions.

Class	*m/z* $[M-H]^-$	C:N	Fatty Acyl Chains
PS	782.3	36:4	16:0/20:4
	784.4	36:3	18:1/18:2
	786.4	36:2	18:1/18:1
	788.4	36:1	18:0/18:1
	790.4	36:0	18:0/18:0
	802.4	38:8	16:2/22:6
	804.3	38:7	16:1/22:6
	806.3	38:6	18:2/20:4
	808.3	38:5	18:1/20:4
	810.4	38:4	18:0/20:4
	812.4	38:3	18:0/20:3
	814.4	38:2	18:1/20:1
	816.4	38:1	18:0/20:1
	818.3	38:0	18:0/20:0
	830.4	40:8	20:4/20:4
	832.4	40:7	18:1/22:6
	834.4	40:6	18:0/22:6
	836.4	40:5	20:1/20:4
	838.4	40:4	18:0/22:4
	840.5	40:3	18:0/22:3
	842.4	40:2	18:0/22:2
	844.3	40:1	18:0/22:1
	846.3	40:0	18:0/22:0
	862.3	42:6	20:0/22:6
	864.3	42:5	20:1/22:4
	866.3	42:4	20:0/22:4
	868.3	42:3	20:1/22:2
	870.3	42:2	20:0/22:2
	872.3	42:1	20:0/22:1
	874.3	42:0	20:0/22:0
	886.4	44:8	22:2/22:6
	888.5	44:7	22:1/22:6
	890.3	44:6	22:0/22:6

	892.3	44:5	22:1/22:4
	894.3	44:4	22:0/22:4
	896.3	44:3	22:1/22:2
	900.3	44:1	22:0/22:1
	902.4	44:0	22:0/22:1

1.3.6 Analysis of PI profile

PIs are a group of phospholipids widely distributed in nature and which are involved in secretory events and in intercellular signalling. The metabolism of inositol lipids is involved in the signal transduction of many hormones, neurotransmitters and growth factors [94]. PI classes are precursors of important signalling molecules that modulate cell growth, proliferation and death [70].

PIs were analysed also in the negative mode forming $[M-H]^-$ ions and the identified species are resumed in Table 8. As we can see by the Figure 30 and Table 8 the most abundant PI in both samples (CTL and stress) is the one at *m/z* 885.5, which corresponds to PI (18:0/20:4). PI's were identified taking into account the characteristic product ion observed in MS/MS, formed by the loss of the polar head group leading to the ion at *m/z* 241, identified as an inositolphosphate. Even though PI showed a significant decrease in its relative abundance (Figure 20), the comparison of the spectra of control and stress do not demonstrate significant changes in the molecular ion species of PIs. The decrease in relative abundance may suggest that the signalling cascades in which PIs are involved may be activated and also they may be forming more signalling molecules, since they are precursors of PIPs, which have an important role in many diseases, modulating cell death [118].

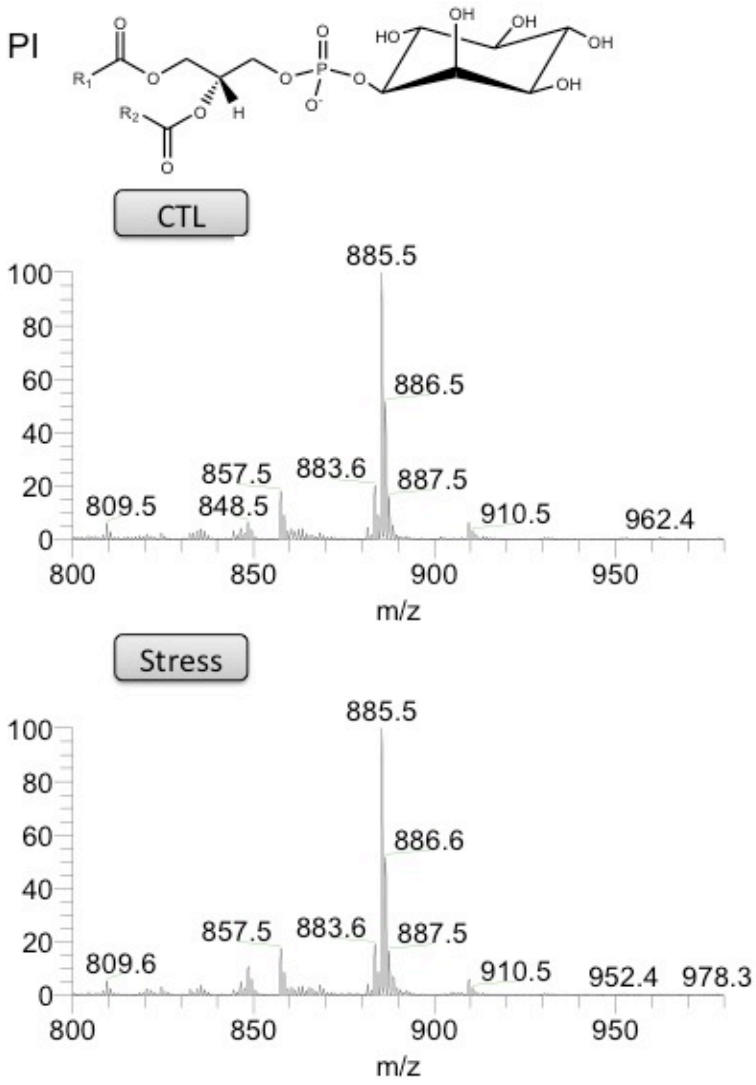

Figure 30. General structure of PI class; HPLC-MS spectra of PI class in the negative mode with formation of $[M-H]^-$ ions, in control (CTL) and in chronic stress situations (Stress). Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: *m/z* for each ion.

Table 8. Identification of $[M-H]^-$ ions observed in the MS spectra of PI. Phospholipids are designated as follows: diacyl 38:4 PI, where 38 indicates the summed number of carbon atoms at both the *sn-1* and *sn-2* positions and 4 designates the summed number of double bonds at both positions.

Class	*m/z* $[M-H]^-$	C:N	Fatty Acyl Chains
PI	809.5	32:0	16:0/16:0
	825.5	34:6	16:2/18:4
	827.6	34:5	16:2/18:3
	833.5	34:2	16:0/18:2
	835.5	34:1	16:0/18:1
	837.7	34:0	16:0/18:0
	853.5	36:6	16:2/20:4
	857.5	36:4	16:0/20:4
	859.5	36:3	18:1/18:2
	861.6	36:2	18:1/18:1
	863.7	36:1	18:0/18:1
	865.4	36:0	18:0/18:0
	881.5	38:6	16:0/22:6
	883.5	38:5	18:1/20:4
	885.5	38:4	18:0/20:4
	887.5	38:3	18:1/20:2
	889.5	38:2	18:0/20:2
	891.7	38:1	18:0/20:1
	893.3	38:0	18:0/20:0
	907.5	40:7	18:1/22:6
	909.5	40:6	18:0/22:6
	911.7	40:5	20:1/20:4
	913.7	40:4	20:0/20:4
	915.4	40:3	20:1/20:2
	917.3	40:2	20:0/20:2
	931.5	42:8	20:4/22:4
	939.4	42:5	20:1/22:4
	941.4	42:4	20:0/22:4
	945.3	42:2	20:0/22:2
	961.5	44:8	22:2/22:6

1.3.7 Analysis of PA profile

PA phospholipid class is a very important and determinant phospholipid class. In addition to the role as intermediate in lipid biosynthesis, PAs have signalling functions, being involved with many aspects of cell biochemistry and physiology, including cell proliferation and differentiation, and tumour progression. PAs regulate also some membrane trafficking events, and it is involved in activation of the enzyme NADPH oxidase, which operates as part of the defense mechanism against tissue damage during inflammation [119].

This class was also analysed by HPLC-MS in negative mode (Figure 31) and the species identified are presented in Table 9.

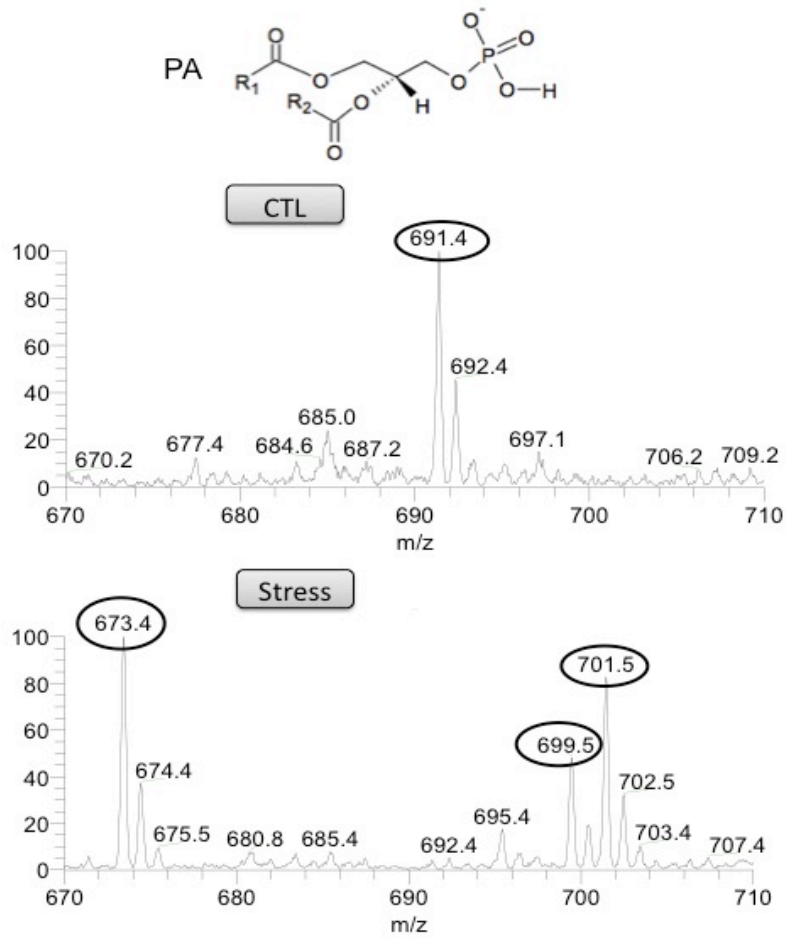

Figure 31. General structure of PA class; HPLC-MS spectra of PA class in the negative mode with formation of $[M-H]^-$ ions, in control (CTL) and in chronic stress situations (Stress). Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: *m/z* for each ion. The ions marked are the ones that show variation in chronic stress situations, compared with the standard of PA that we add to the sample before the HPLC-MS analysis.

Table 9. Identification of $[M-H]^-$ ions observed in the MS spectra of PA. Phospholipids are designated as follows: diacyl 36:6 PA, where 36 indicates the summed number of carbon atoms at both the *sn-1* and *sn-2* positions and 6 designates the summed number of double bonds at both positions.

Class	*m/z* $[M-H]^-$	C:N	Fatty Acyl Chains
PA	671.4	34:2	16:0/18:2
	673.4	**34:1**	**16:0/18:1**
	675.1	34:0	16:0/18:0
	691.4	**36:6**	**16:2/20:4**
	693.4	36:5	16:1/20:4
	695.4	36:4	16:0/20:4
	697.4	36:3	18:1/18:2
	699.5	**36:2**	**18:0/18:2**
	701.5	**36:1**	**18:0/18:1**
	703.4	36:0	18:0/18:0
	719.4	38:6	16:0/22:6
	721.4	38:5	18:1/20:4
	723.4	38:4	18:0/20:4
	725.4	38:3	18:1/20:2
	727.4	38:2	18:0/20:2
	729.6	38:1	18:0/20:1
	731.5	38:0	18:0/20:0

As we can see in Figure 31, this class shows alterations in the relative abundance of molecular species after chronic stress. We can see an increase in the relative abundance of the $[M-H]^-$ ions at *m/z* 673.4 (C16:0/18:1), 699.5 (C18:0/18:1) and 701.5 (C18:1/18:1) in the MS spectrum of chronic stress. On the other hand we see a very significant decrease of the specie at *m/z* 691.4 (C16:2/20:4). The increase in these specific species may suggest a remodelling or even a *de novo* synthesis of some phospholipid species in chronic stress situations, favouring the formation of fatty acyl chains shorter and with less unsaturation's. This fact is corroborated by the decrease in the ion at *m/z* 691.4, which has fatty acids with more unsaturation's and longer in length (20:4). This remodelling is very interesting since some published studies pointed out for a decrease in the incorporation of PUFAs into phospholipids from individuals with schizophrenia and bipolar disorders, specially a decrease in arachidonic acid (C20:4) and docosahexenoic acid (C22:6) [32]. This fact is

very important since chronic stress is indicated as a risk factor for neurological disorders, like depression, among others. In this class we also observed a decrease in the relative abundance of the total class, which can be correlated with the increase in PCs and PEs, which are produced by reactions involving PAs. Furthermore, they are important signalling molecules, which can mean that some signalling events involving PAs are activated in chronic stress.

1.3.8 Analysis of CL profile

CL is found predominantly in the mitochondrial inner membrane playing a central role in mitochondrial processes and energy metabolism [120]. CL content is about 2-11% in brain [121]. It is associated with several mitochondrial proteins and is essential for the optimal function of numerous enzymes that are involved in mitochondrial energy metabolism and in ATP production. Changes in its structure, due to oxidation, have been implicated in the process of apoptosis. CL interacts and is essential for normal functioning of many intrinsic proteins, including major electron transport complexes.

CL is a dimeric phospholipid in which two phosphatidyl residues are linked by a central glycerol group, corresponding to a structure that contains four fatty acyl chains [120]. In brain, as the other phospholipid classes, CL has predominantly long polyunsaturated fatty acyl residues. The predominance of unsaturated fatty acyl chains in CL is responsible for the increased susceptibility of this phospholipid to oxidative damage by ROS [95]. Furthermore its preferential location in mitochondria, a site for ROS production, increases the probability of occurring oxidative damage and it has been suggested to be a preferred oxidation substrate in neuronal injury [36]. CL oxidized products are responsible for mitochondrial dysfunction, which has been correlated with a variety of pathological situations like aging, diabetes, and also neurodegenerative diseases [120].

CL has four alkyl groups and two phosphate groups, thus potentially can have two negative charges resulting in the possibility to form $[M-H]^-$ and $[M-2H]^{2-}$ ions, respectively mono charged or double charged ions appear in the mass spectrum. Due to its low abundance in the total brain lipid extract and possible co-elution of CL with PG, in HPLC, we analysed cardiolipins by HPLC-MS and also by direct ESI-MS of the cardiolipin content, obtained by TLC separation. Thus, the mass spectra presented in Figure 32 are

those obtained by TLC. In Table 10 we listed the CLs identified in control and chronic stress situations.

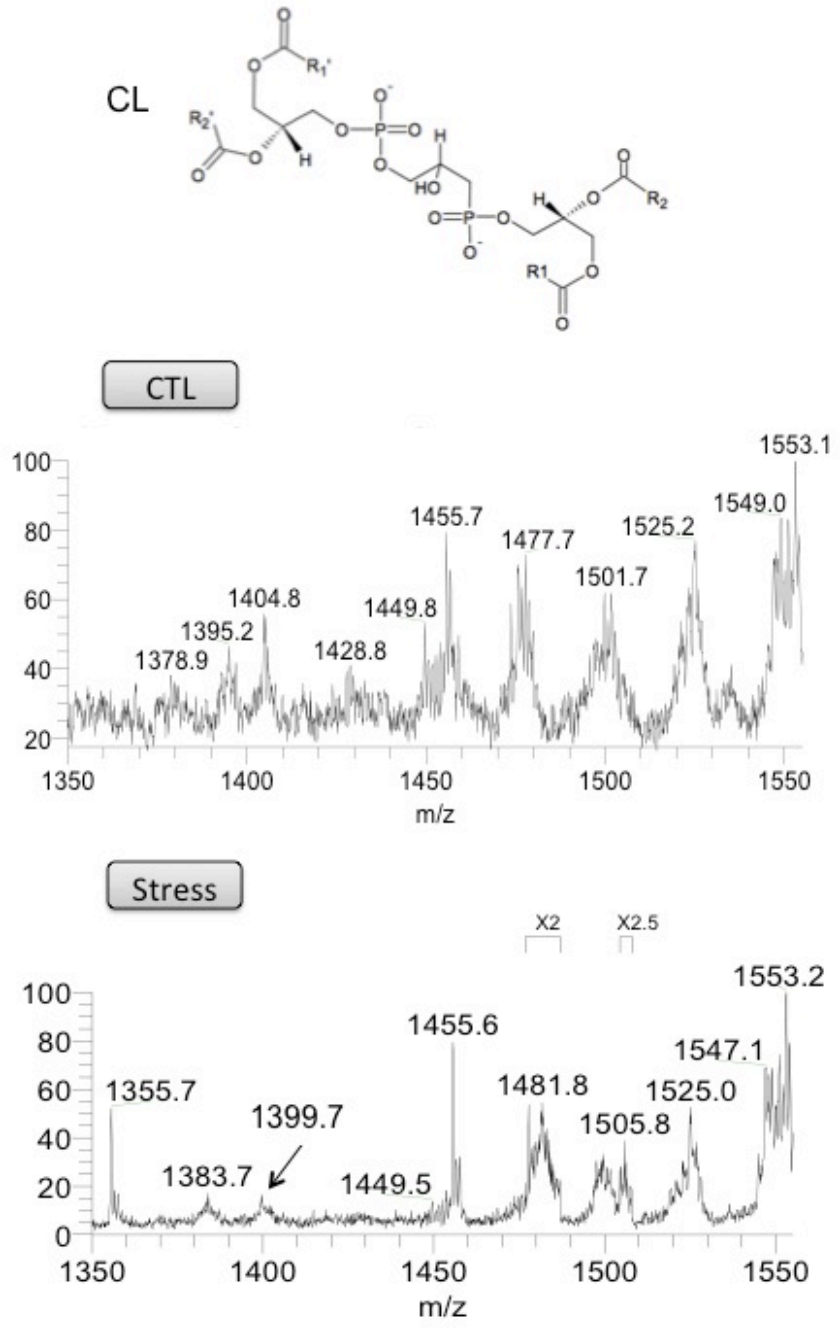

Figure 32. General Structure of CL class; Mass spectra of CL class in the negative mode with formation of $[M-H]^-$ ions, in control (CTL) and in chronic stress situations (Stress), obtained by ESI-MS. Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: *m/z* for each ion.

Table 10. Identification of $[M-H]^-$ ions observed in the MS spectra in CL. Phospholipids are designated as follows: tetra-acyl 76:9 CL, where 76 indicates the summed number of carbon atoms at both *sn-1, sn-2, sn-1'* and *sn-2'* positions and 9 designates the summed number of double bonds at the four positions.

Classe	*m/z* $[M-H]^-$	C:N	Fatty Acyl Chains
CL	1401.8	68:3	$(C18:1)_2/(C16:1)/(C16:0)$
	1403.8	68:2	$(C18:1)_2/(C16:0)_2$
	1405.9	68:1	$(C18:0)/(C18:1)/(C16:0)_2$
	1407.5	68:0	$(C18:0)_2/(C16:0)_2$
	1421.7	70:7	$(C20:4)/(C18:1)/(C16:1)_2$
	1423.9	70:6	(C20:4)/(C18:1)/(C16:1)/(C16:0)
	1425.6	70:5	$(C16:1)/(C18:2)/(C18:1)_2$
	1427.7	70:4	$(C16:1)/(C18:1)_3$
	1429.7	70:3	$(C16:0)/(C18:1)_3$
	1431.6	70:2	$(C18:0)/(C18:1)_2/(C16:0)$
	1433.9	70:1	$(C18:0)_2/(C18:1)/(C16:0)$
	1435.6	70:0	$(C18:0)_3/(C16:0)$
	1445.7	72:9	$(C16:1)/(C18:2)_2/(C20:4)$; (C16:1)/(C16:0)/(C18:3)/(C22:5)
	1447.6	72:8	$(C18:2)_4$; (C16:1)/(C18:1)/(C18:2)/(C20:4); (C16:0)/(C18:1)/(C18:3)/(C20:4)
	1449.8	72:7	$(C18:1)/(C18:2)_3$; (C16:1)/(C18:1)/(C18:2)/(C20:3)
	1451.6	72:6	$(C18:1)_2/(C18:2)_2$; $(C16:1)/(C18:1)_2/(C20:3)$
	1453.7	72:5	$(C18:0)/(C18:1)/(C18:2)_2$; $(C16:0)/(C18:1)_2/(C20:3)$;$(C18:1)_3/(C18:2)$
	1455.7	72:4	$(C18:1)_4$; $(C16:0)/(C18:1)_2/20:2$
	1473.7	74:9	$(C18:1)/(C18:2)_2/(C20:4)$; (C16:0)/(C18:1)/(C18:2)/(C22:6)
	1475.6	74:8	(C16:1)/(C18:2)/(C20:3)/(C20:2); $(C16:1)/(C18:1)_2/(C22:5)$; $(C16:0)/(C18:1)_2/(C22:6)$; (C16:0)/(C18:1)/(C20:4)/(C20:3)
	1477.7	74:7	$(C18:1)_3/(C20:4)$; (C16:1)/(C18:2)/(C20:3)/(C20:1)
	1479.7	74:6	$(C18:1)_2/(C18:0)/(C20:4)$; (C16:0)/(C18:1)/(C20:4)/(C22:4)
	1485.7	74:3	$(C18:0)_3/(C20:3)$
	1487.8	74:2	$(C18:0)_3/(C20:2)$
	1489.6	74:1	$(C18:0)_3/(C20:1)$
	1491.7	74:0	$(C18:0)_3/(C20:0)$
	1493.8	76:13	(C16:1)/(C18:2)/(C20:4)/(C22:6)
	1495.7	76:12	(C16:1)/(C18:2)/(C20:3)/(C22:6)

	1497.7	76:11	$(C18:1)/(C18:2)/(C20:4)_2$
	1499.7	76:10	$(C18:1)_2/(C20:4)_2$
	1501.8	76:9	$(C18:1)_2/(C20:4)/(C20:3)$
	1503.8	76:8	(C18:1)/(C18:2)/(C20:3)/(C20:2)
	1521.8	78:13	$(C18:1)_1/(C20:4)_3$
	1523.8	78:12	$(C18:1)_2/(C20:4)/(C22:6)$

By the analysis of the MS spectra both in control and chronic stress conditions, we can see that changes occur in the relative abundance of the molecular species in this class. In addition, new ions were observed at *m/z* 1355.7, 1383.7, 1399.7, 1481.8 and 1505.8.

A detailed analysis of MS/MS spectra allows the structural characterization of phospholipids. The MS/MS spectra of the $[M-H]^-$ ions of CL can be divided in distinct zones, as described by Hsu and Turk [122], being some of them: the phosphatidic acid (PA - $[PA-H]^-/[PA_2-H]^-$); the $[(PA-H)+136]^-/[(PA_2-H)+136]^-$ and the loss of the fatty acyl chains $[M-RCOO^-]^-$, as it is schematically represented in Figure 33.

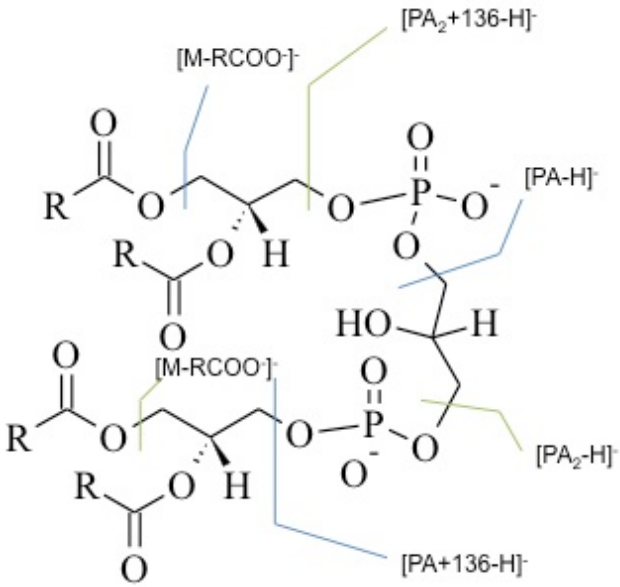

Figure 33.Schematic representation of the possible fragments produced by CL under MS/MS conditions.

Having the information provided by this fragmentation products we went looking for the formation of these ions in the MS/MS spectra of the new ions found in MS spectrum of chronic stress group.

Analysis of MS/MS of these ions allowed us to confirm that they are correspondent to oxidation products of CL. The ions at *m/z* 1481.8 and 1505.8 were identified as long chain CL oxidation products [123] and were identified as hydroperoxides of CL (C16:1)(C18:2)/(C18:1)(C20:3) and CL (C18:2)(C18:2)/(C18:1)(C20:4). The MS/MS spectrum of the $[M-H]^-$ ion at *m/z* 1481.8 is presented in Figure 34.

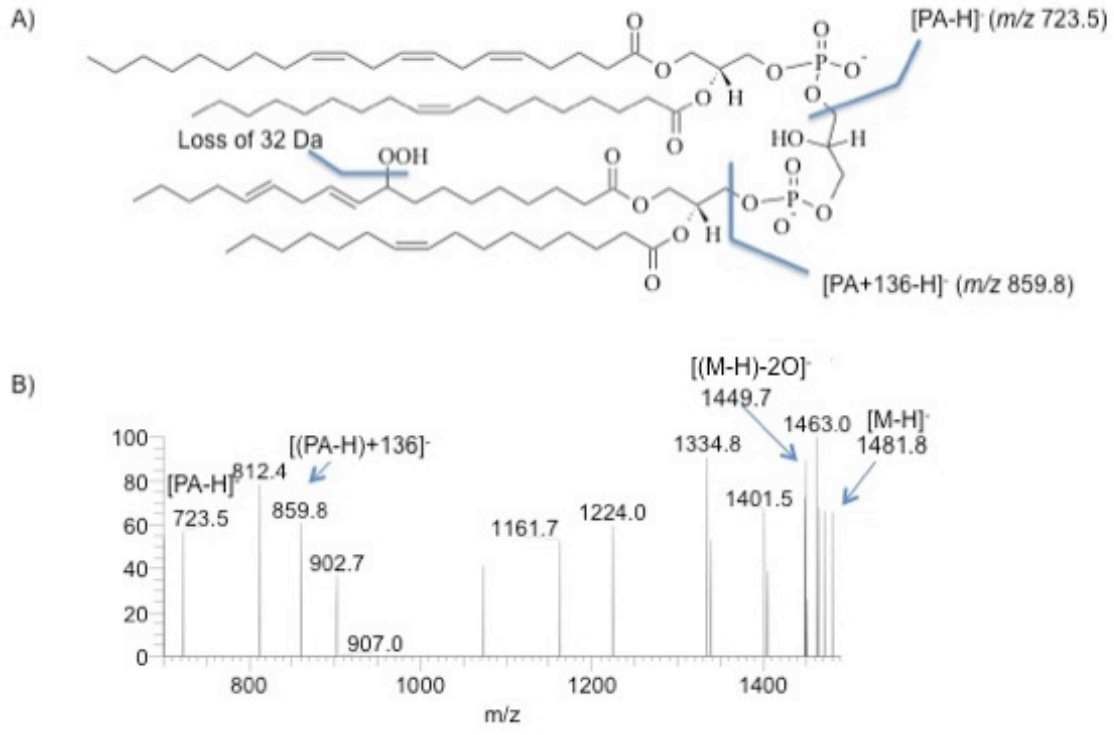

Figure 34. A) Proposed structure for the long chain oxidation product of CL, based in the MS/MS spectrum obtained for the ion at *m/z* 1481.8. B) MS/MS spectrum of the $[M-H]^-$ ion at *m/z* 1481.8.

By the analysis of the MS/MS spectrum of the ion at *m/z* 1481.8 we can see the ions at *m/z* 1463.0 and 1449.7 corresponding to the losses of 18 Da and 32 Da respectively, which were identified as the loss of water and the loss of two oxygen atoms, suggesting that the ion at *m/z* 1481.8 corresponds to the hydroperoxide product of the CL at *m/z* 1449.7 that is found decreased in chronic stress. The loss of water and two oxygen atoms, in hydroperoxide products, is very common in phospholipid peroxidation [61]. In addition we can see the ions at *m/z* 723.5 and 859.8 which may correspond to the $[PA-H]^-$ formed by the fatty acyl chains (C18:1)/(C20:3) and the same $[(PA-H)+136]^-$, respectively, suggesting that the CL structure has this two fatty acyl chains together in its structure.

Thus, by the analysis of Table 10, where we can see the fatty acyl chains found in CL structure of the ion at *m/z* 1449.7, and by the mass of the ions found in MS/MS spectra we hypothesize that the other PA of CL is formed by the fatty acyl chains (C16:1)/(C18:2). Furthermore, it is known that the fatty acyl chain (C18:2) is very susceptible to oxidation [124], suggesting that the hydroperoxide group is formed in this fatty acyl chain. Altogether, this data allowed us to propose the oxidation structure of CL presented in Figure 34 (CL - (C16:1)(C18:2(OO))/(C18:1)(C20:3)).

The MS/MS spectrum shows more ions than those that we identified. This may be due to the very low abundance of CL, especially in chronic stress, which makes more difficult to analyse the MS/MS spectra. In addition, different species of CL can exist with the same *m/z* ratio, masking the oxidation fragments, which can be less abundant and very unstable and can be metabolized/decomposed quickly.

The other ion identified to be a long-chain oxidation product of CL is the one at *m/z* 1505.8. The MS/MS spectrum for this ion is found in Figure 35.

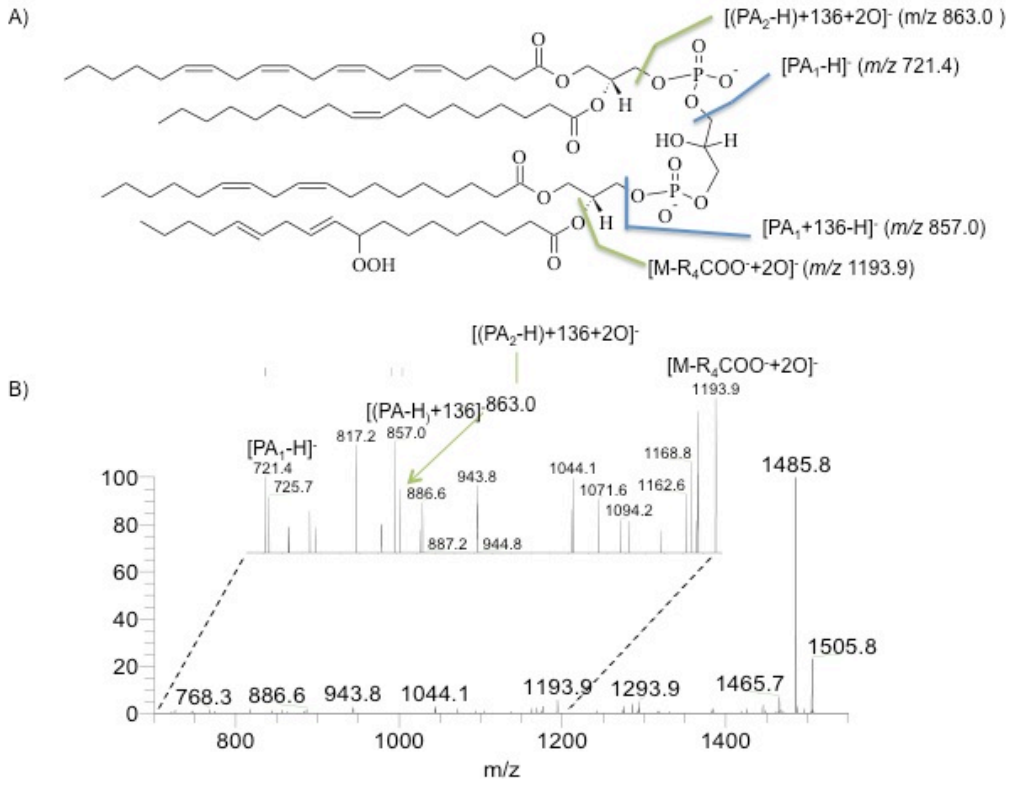

Figure 35. A) Proposed structure for the long chain oxidation product of CL, based in the MS/MS spectrum obtained for the ion at *m/z* 1505.8. B) MS/MS spectrum of the $[M-H]^-$ ion at *m/z* 1505.8.

By the analysis of the MS/MS spectrum of the ion at *m/z* 1505.8 we can see an ion at *m/z* 1193.9 identified as the loss of a fatty acyl chain (C18:2+2O) corresponding to a mass of 312 Da. Additionally, we can see the ions at *m/z* 721.4 and 857.0, identified as the $[PA_1\text{-}H]^-$ with (C18:1) and (C20:4) fatty acyl chains and the same $[(PA_1\text{-}H)+136]^-$, respectively. In the MS/MS spectra we were still able to see the ion at *m/z* 863.0 identified as the other $[(PA_2\text{-}H)+136+2O]^-$, constituted by the fatty acyl chains (C18:2) and (C18:2+2O). This data suggests that the ion at *m/z* 1505.8 corresponds to the long chain oxidation product of CL at *m/z* 1473.7, which is decreased in chronic stress group. The analysis of these data presented here we were able to propose a structure for the ion at *m/z* 1515.8 (Figure 35) (CL - (C18:2(OO))(C18:2)/(C18:1)(C20:4)).

Furthermore, in the mass spectra of the chronic stress group we were also able to identify the formation of the ions at *m/z* 1355.7, 1383.7 and 1399.7, which have a low *m/z* ratio for being CL species. During oxidation, oxygen atoms are inserted with formation of long-chain oxidation products. But when the alkoxyl radical is formed during the oxidative reaction (Figure 22), it may undergo posterior β-cleavage of the fatty acyl chain with formation of oxidation products with shortened fatty acyl chain, and with aldehyde or carboxylic terminal function. These products have lower molecular weight and are called short-chain phospholipid products [69]. Maciel *et al.* described the formation of these short-chain products of CL in *in vitro* and *in vivo* analysis [99]. Fatty acyl chain C18:2 has two double bonds in the carbon 9 and 12. The hydrogen found between the two double bonds is very unstable; in the presence of ROS this hydrogen is abstracted leading to the dislocation of the double bond for the 10 or 11 carbon, leaving a free radical in the carbon 9 or 13 respectively. The formation of linoleic acid shortened with C9 length is more favourable than the linoleic acid shortened with C13 length.

In the Figure 36 is presented the MS/MS spectrum of the $[M\text{-}H]^-$ ion at m/z 1355.7.

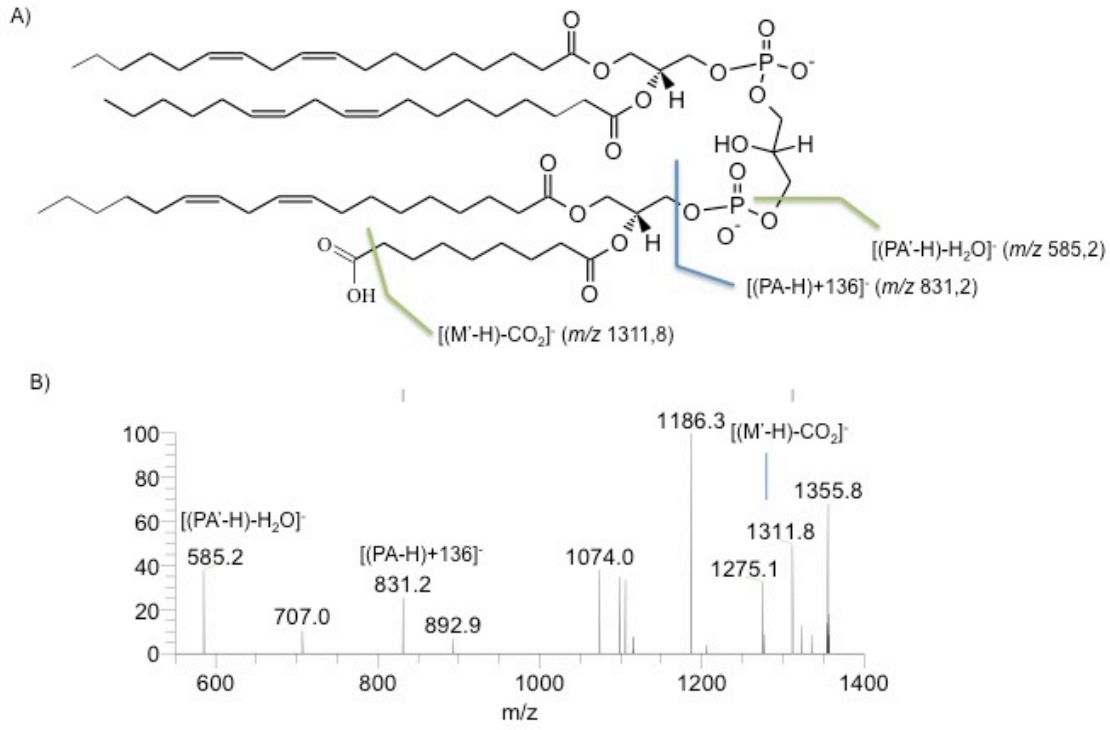

Figure 36. A) Proposed structure for the short chain oxidation product of CL, based in the MS/MS spectrum obtained for the ion at *m/z* 1355.7. B) MS/MS spectrum of the $[M-H]^-$ ion at m/z 1355.7.

By the analysis of the MS/MS spectrum of the ion at *m/z* 1355.7 we found ion at *m/z* 1311.8, correspondent to the loss of 44 Da identified as the loss of CO_2, suggesting the existence of a carboxilic terminal. We found also the ions at *m/z* 831.2 identified as the $[(PA-H)+136]^-$, formed by the presence of two fatty acyl chains (C18:2), and 585.2 the PA' (PA with the modified chain) with loss of water, formed by a fatty acyl chain (C18:2) and other shortened with C9 length, due to β-cleavage of a fatty acyl chain (C18:2). Due to these findings we suggest that the ion at *m/z* 1355.8 is a short chain product of CL at *m/z* 1447.7, which is decreased in chronic stress situations. The high abundance found for this ion in MS of CL in chronic stress may be due to the presence of the carboxilic terminal, which may increase the susceptibility to ionization. We considered this product a CL product due to the fact that we fractionated the lipid extracted by TLC and this product co-elutes in the spot of CL molecular species, and in fact the MS/MS analysis suggests that the short is present Figure 36 (CL - (C18:2)(C18:2)/(C18:2)(C_8COOH)).

By the analysis of the CL MS spectrum for the chronic stress group we were able to identify the formation of two more ions at *m/z* 1383.7 and 1399.7. These ions caused much

attention, since the mass difference between them is 16 Da, which could correspond to an oxygen atom. However, due to their very low abundance it was not possible to perform the MS/MS analysis of these ions. Still, due to the fact that we were able to identify the formation of a short chain oxidation product of CL, we hypothesized that these ions could be short chain oxidation products too. In fact, by the analysis of the MS spectra we can see a very significant decrease of the ion at *m/z* 1475.7. By the analysis of Table 10 we can see that one of the combinations for the fatty acyl chains constituting this CL has a linoleic acid. In the study of the short chain oxidation products of CL, Maciel *et al.* described the formation of products due to β-cleavage of the fatty acyl chain of linoleic acid, with the formation of fatty acyl chains with C9 length. In this study, they observed the formation of isomers with carboxylic and aldehyde terminals. In addition, to the short acyl chains formed due to oxidation they reported that many times the other fatty acyl chains of CL can suffer oxidation, at the same time [99]. By the analysis of the data provided by this study we were able to propose the structure for the ions at *m/z* 1383.7 and 1399.7 (Figure 37). Nevertheless, these structures are only proposals, which need to be confirmed.

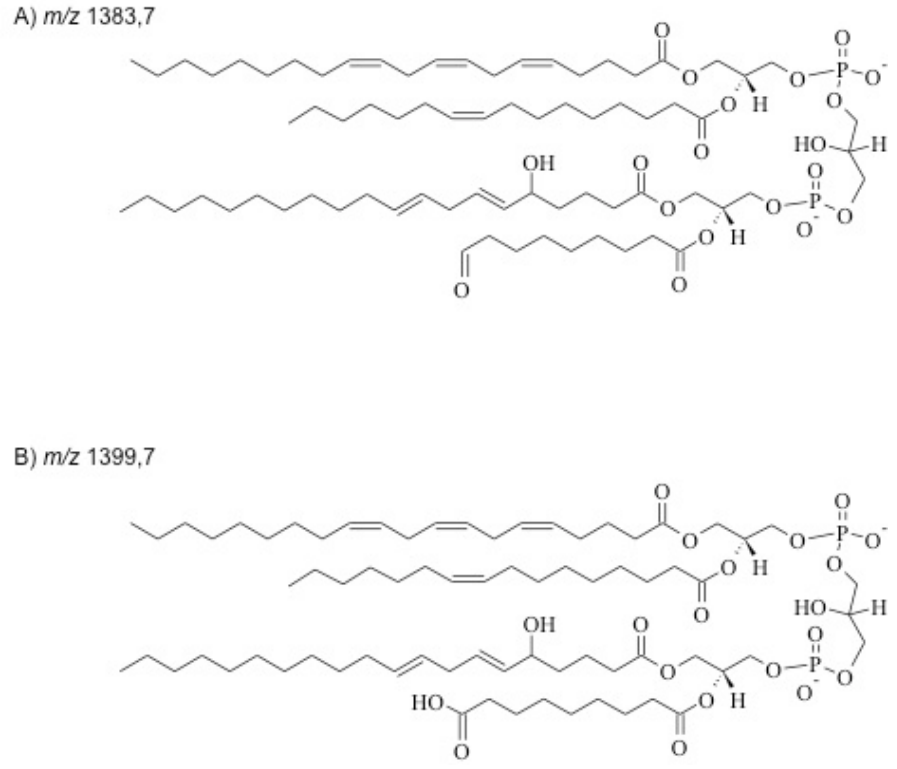

Figure 37. Proposed structures of short chain oxidation products of CL for the ions at m/z 1383.7 and 1399.7.

In Table 11 are resumed the fatty acyl chain composition of the oxidation products identified by MS of CL from mice brain under chronic stress conditions.

Table 11. Oxidized species identified in CL spectra of brain from mice under chronic stress conditions.

m/z $[M-H]^-$	Oxidation products
1481.8	(C18:1)(C20:3)/(C18:2+OO)(C16:1)
1505.8	(C20:4)(C18:1)/(C18:2)(C18:2+OO)
1355.7	(C18:2)(C18:2)/(C18:2)(C_8COOH)
1383.7	(C20:3)(C16:1)/(C20:2+O)(C_8COH)
1399.7	(C20:3)(C16:1)/(C20:2+O)(C_8COOH)

It was already reported by many studies the increase in oxidative stress produced in chronic stress situations [71]. With the measure of hydroperoxides by FOX II assay, we have confirmed this hypothesis. These oxidized species were found only in CL class and not in the other classes analysed. Since CL is an exclusive phospholipid of mitochondria, where ROS formation occurs preferentially, it is expected that CL is more susceptible to oxidation. Thus, it is natural that CL is the first to suffer oxidation, and not the other classes. Lipid hydroperoxides exist in low abundance, are fast metabolized/removed from the system by defense mechanisms, they have some chemical and thermal instability, but at the same time this very low abundance have critical consequences [62]. Because of the structural diversity of CL in brain, detecting hydroperoxide species of CL by MS is challenging, since masses associated with oxidized species are likely to overlap the natural brain CL species present [62].

In addition to the observed oxidation of CL, we also reported a decrease in the total content of CL in chronic stress situations. The loss of CL content is associated with many pathophysiological conditions such as aging, diabetes and neurodegenerative diseases. This loss can also lead to the decline in mitochondrial respiratory functions and to ROS accumulation. At this point it is still unknown whether the ROS cause the CL loss or if it is

the loss of CL that triggers ROS generation. However, it is clear that during cell death processes ROS and loss of CL are closely linked in a cycle of CL peroxidation [63].

The loss of CL content and its oxidation are related with the development of many diseases. Several studies indicate that peroxidized CL is unable to support the reconstituted activity of mitochondrial respiratory enzymes. Furthermore, CL peroxidation may lead to an overall loss of detectable CL content, either by preferential hydrolysis of peroxidized acyl chains by PLA_2, direct decomposition of lipid peroxides, or the formation of CL-protein complexes [43].

In summary chronic stress has been suggested as a risk factor for the development of many neurological and neurodegenerative diseases. Many studies have been made in brain with chronic stress conditions [4, 6, 9, 12, 24, 125]. However, these studies reported changes related to DNA and proteins, and not changes related with phospholipids. Our results show that chronic stress has a wide range of consequences in brain phospholipid profile. As we showed chronic stress causes changes in relative abundance of some PLs classes in neuronal cells. These changes can be related with the triggering of many signalling cascades and may induce changes in structure and function of cellular and sub-cellular membranes, which can be involved with the development of neurological and neurodegenerative diseases. Besides, we found an increase in oxidative stress in CL class, which can cause the impairment of mitochondrial function, related with apoptosis. Finally, we found also some changes in PAs, suggesting the existence of a remodelling in PLs classes, with an increase in species with fatty acyl chains with less unsaturation's and shorter in length, which was already proved to be related with the development of some neurological disorders.

Pereira and colleagues have already described that several changes in brain in chronic stress conditions are accompanied by some modifications in several cardiac functions. These changes range from functional to structural alterations of this organ [18]. Thus, it is important to evaluate the modifications that may occur in heart phospholipids under chronic stress conditions.

2 Analysis of heart phospholipid profile of chronic stressed mice

In order to evaluate possible changes in heart phospholipid profile between chronic stress and controls, lipid extracts were obtained from the total heart of mice. To know the amount of phospholipids and proteins per µg of tissue each organ was weighted and quantified the total lipid and protein content in each extract (Table 12). This analysis was carried out with an n=3 for the two groups.

Table 12. Percentage of heart phospholipids (PLs) and proteins in each tissue (µg) in controls and chronic stressed mice and relation between µg of PLs per µg of tissue.

	µg of PLs per µg of Tissue	µg of Proteins per µg of Tissue	µg of PLs per µg of Proteins
CTL	4.49E-03 ± 4.68E-04	1.90E-01 ± 2.68E-02	2.36E-02 ± 5.75E-03
Stress	1.10E-02 ± 2.34E-03	1.84E-01 ± 4.69E-02	6.02E-02 ± 1.01E-02

Results represent mean± SD of three brains analysed (n=3) in each group.

By the analysis of data presented in Table 12 we show that, as we verified in brain, there is a tendency for an increase in the total amount of phospholipids in chronic stress and, on the other hand, a tendency to a decrease in proteins. However, these differences are not statistically significant. This tendency for the increase in PLs/proteins ratio may suggest that some metabolic changes occur in heart under chronic stress conditions.

2.1 Changes in relative abundance of different classes of phospholipids in heart

In the study of heart phospholipid profile we evaluated the differences between the relative content in each class of PLs. For that total lipid extracts were fractionated by TLC and each class was identified by comparison with pure standards applied in the same TLC plate. This approach allowed the separation of eight classes: lysophosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and cardiolipin (CL) (Figure 38).

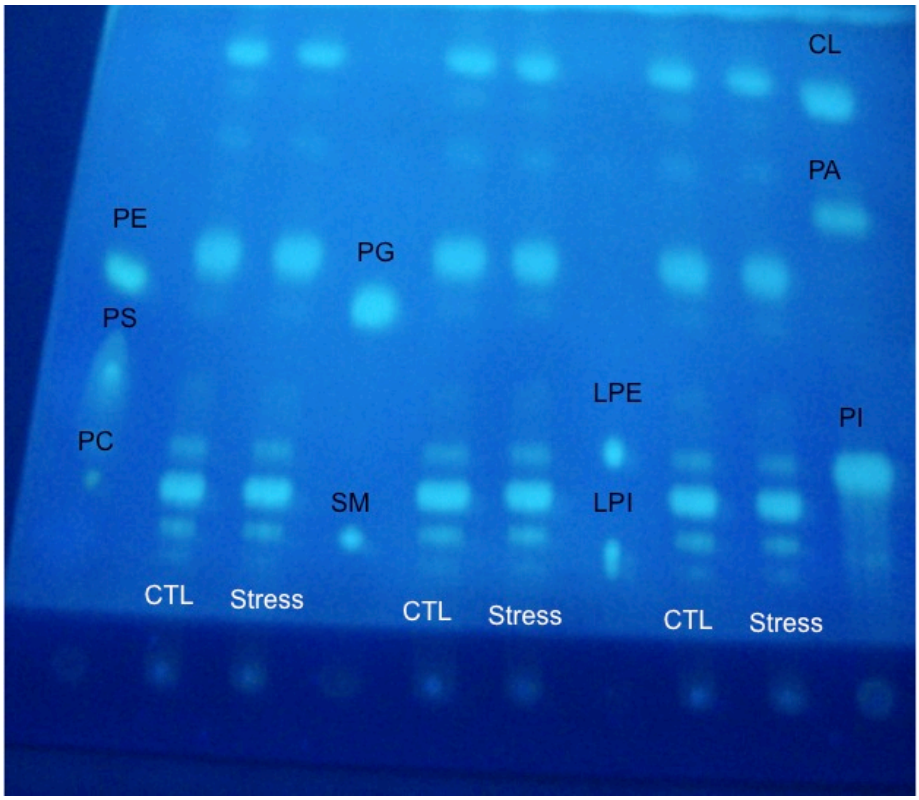

Figure 38. Thin-layer chromatography of total lipid extract obtained from mice heart control (CTL) and with chronic stress (Stress). Phospholipid standards were also applied: (PC) - Phosphatidylcholine; (PS) - Phosphatidylserine; (PE) - Phosphatidylethanolamine; (SM) - Sphingomyelin; (PG) - Phosphatidylglycerol; (LPI) - Lysophosphatdylinositol; (LPE) - Lysophosphatidylethanolamine; (PI) - Phosphatidylinositol; (PA) - Phosphatidic Acid; (CL) - Cardiolipin.

Relative quantification of each class was made by phosphorous assay. This analysis was carried out in triplicate for each sample and three different samples were analysed for each experimental group.

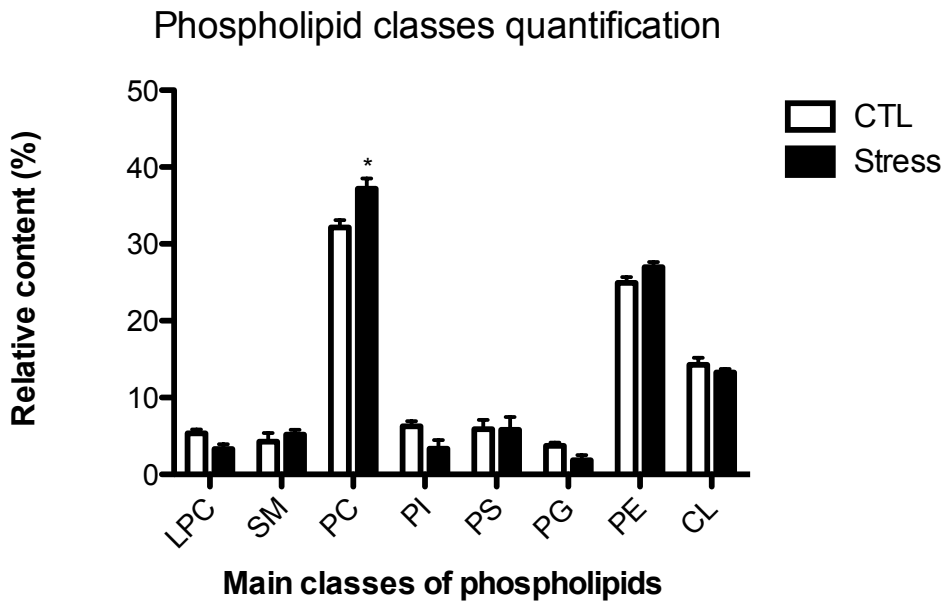

Figure 39. Relative abundance of phospholipids, in controls (CTL) and chronic stress situations (Stress), from mice heart. The phospholipid classes were separated by thin-layer chromatography and the phosphorous content of each spot was calculated taking in account the amount of phosphorous in the total lipid extract. In this case we were able to separate eight different classes and quantified them: (LPC) - Lysophosphatidylcholine; (SM) - Sphingomyelin; (PC) - Phosphatidylcholine; (PI) - Phosphatidylinositol; (PS) - Phosphatidylserine; (PG) - Phosphatidyglycerol; (PE) - Phosphatidylethanolamine; (CL) - Cardiolipin. * $p < 0.05$ versus control, n=3 independent experiments.

By the analysis of the data presented in Figure 39, we show that PC, SMs and PE classes are increased, while PI, CL, LPCs and PGs are decreased. PS class did not show variation. In the case of the control group the relative abundance of classes follows the order PC>PE>CL>PI>LPC≈PS>SM>PG. However, this order is changed in chronic stress group, becoming PC>PE>CL>PS>SM >PI≈LPC>PG (Figure 39). Both in the control group (CTL) as in the chronic stress group (Stress), phospholipid classes more abundant in heart were phosphatidylcholines (CTL=33%; Stress=35%) and phosphatidylethanolamines (CTL=26%; Stress=28%), which was already expected, since these are the most abundant classes found in cardiomyocytes [42]. Although PCs and PEs increased with chronic stress, only the increase verified in PCs is statistically significant. However, we can also see that cardiolipin class is also very abundant in heart, being quite abundant in the total lipid extract, which was also expected since heart is an organ that needs much energy and, consequently, is extremely rich in mitochondria [121].

Taking into account the possible biosynthesis pathways of PCs and PEs (Figure 21), the increase in these classes can be explained by an increase in the synthesis of these classes by its precursors. Also the decrease in LPCs can be related to the increase in these classes.

Despite the increase in SMs content not be statistically significant, it may be related to the increase in the activity of the pathways that lead to the formation of SM.

PI class is decreased in heart of chronic stressed mice, which can be explained by an increase in the degradation or conversion of these molecules or, on the other hand, it may be due to a lower production of these species. It is very important in this case to remember that PIs are very important precursors of signalling molecules, which are involved in many signalling events, especially in cardiomyocytes [46, 73].

CL class is decreased in chronic stress situations, suggesting the possibility of a mitochondrial dysfunction. The decrease in CL content has been reported in many diseases, including diseases involving the heart, like diabetic cardiomyopathy, among others [74].

PG class suffers a decrease in chronic stress group. PGs are synthesised in mitochondria of animals and it is used as the precursor for cardiolipin [120]. So, the decrease verified can be due to the decrease in PG biosynthesis, suggesting metabolic changes in mitochondria of chronic stressed mice. Moreover, the decreased verified in PG class could also be due to an increase in the synthesis of CL (of which PGs are precursors) to try to contradict the decrease in the total CL content showed in chronic stress.

By the data found in Figure 20 and Figure 39 we can see that the changes that occur in heart PLs classes content occurs in a less statistically significant way than those showed in brain. Nevertheless, it is important to notice that many changes found in PLs relative abundance in brain occur also in a similar way in heart PL classes

2.2 Quantification of lipid hydroperoxides in mice heart after exposure to chronic stress

Although no studies reported an increase in oxidative stress in heart, in chronic stress, we evaluated the possible increase in heart lipid peroxidation by FOX II assay. This is a well-established method for quantification of lipid hydroperoxides, (LOOH) primary products of lipid oxidation (Figure 22).

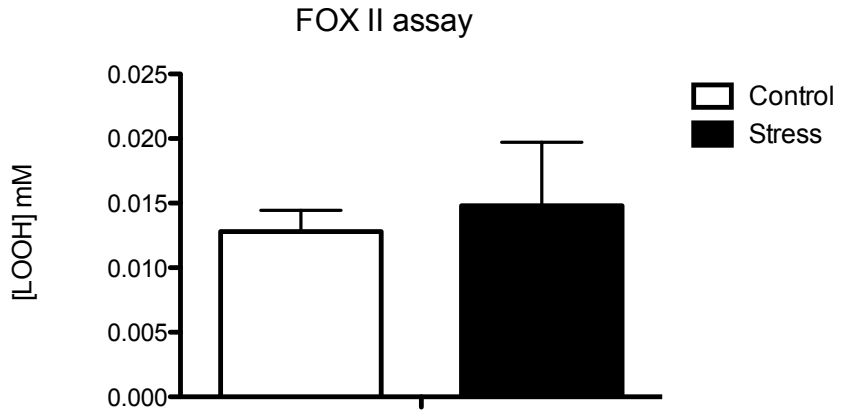

Figure 40. Concentration of lipid hydroperoxides in samples of control group and chronic stress group, evaluated by FOX II assay. n=3 independent experiments.

Our results (Figure 40) showed that there is no significant increase in LOOH in chronic stress group. It is important to notice that heart is less susceptible to oxidation than brain, since PLs possess less unsaturated fatty acids.

These results are in accordance with the literature, where is suggested that the changes associated with chronic stress in heart are related with impairment of heart functions and no increase in oxidative stress was reported [18]. Although our results indicate that there is not an oxidative modification in PLs this does not mean that there are not structural and metabolic changes in PL classes, like those that we already showed in classes' relative content.

2.3 Analysis by HPLC-MS of heart phospholipid classes

In phospholipid profiling of heart total lipid extract, we did also the analysis of the phospholipid classes by HPLC-MS and HPLC-MS/MS, in order to evaluate possible differences in molecular composition of each class. PC, SM and LPC classes were analysed in the positive mode, while PE, PI and CL were analysed in the negative mode (chromatogram of classes elution is presented in Figure 24, in the anterior section).

2.3.1 Analysis of PC profile

By the analysis of Figure 41 we see that PC class is the most abundant in heart. The mass spectra of PCs was obtained by HPLC-MS in the positive mode, forming ions $[MH]^+$, presented in Table 13.

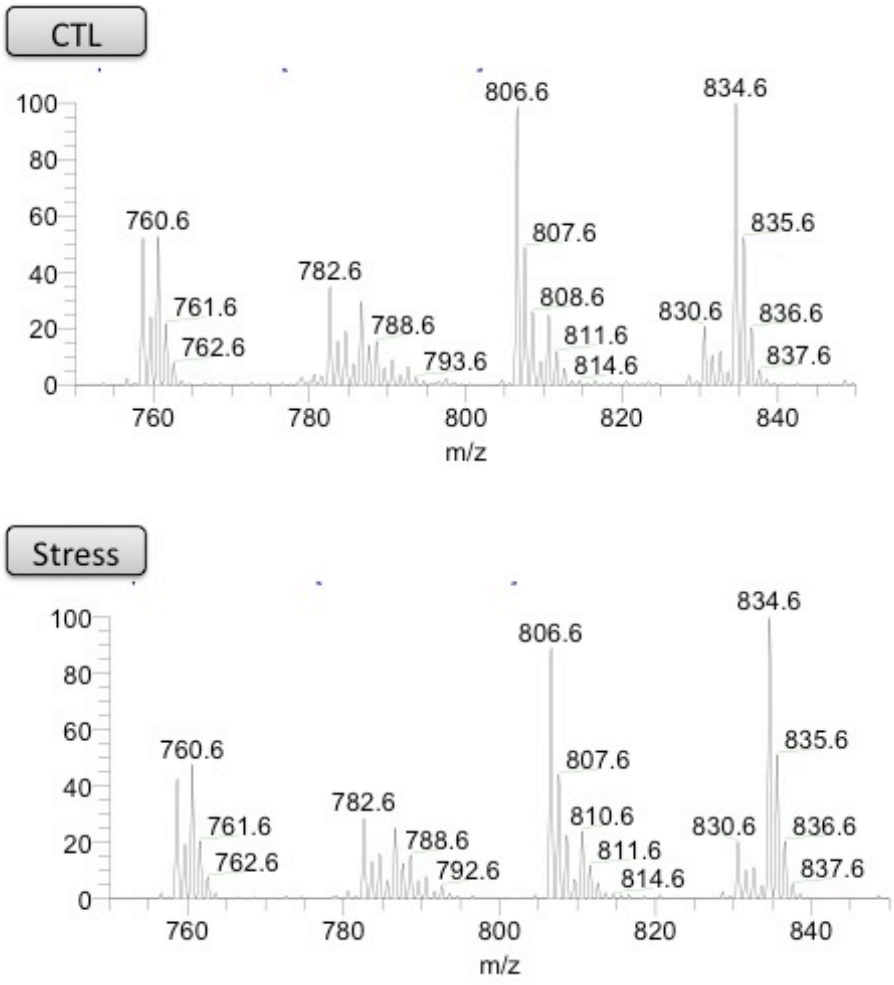

Figure 41. HPLC-MS spectra of PC class in the positive mode with formation of $[MH]^+$ ions, in control (CTL) and in chronic stress situations (Stress). Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: *m/z* for each ion.

The molecular species identified in Table 13 were analysed by MS/MS for the identification of the acyl chains present in the structure of each molecular species identified as PCs.

Table 13. Identification of $[MH]^+$ ions observed in the MS spectra in PC; p - an *sn-1* vinyl ether (alkenyl- or plasmalogen) linkage. Phospholipids are designated as follows: diacyl 34:1 PC, where 34 indicates the summed number of carbon atoms at both the *sn-1* and *sn-2* positions and 1 designates the summed number of double bonds at both positions.

PC Class	*m/z* $[MH]^+$	C:N	Fatty Acyl Chains
Diacyl Species	758.6	34:2	16:0/18:2
	760.6	34:1	16:0/18:1
	782.6	36:4	16:0/20:4 18:2/18:2
	784.6	36:3	16:0/20:3
	786.6	36:2	18:1/18:1
	788.6	36:1	18:0/18:1
	806.6	38:6	18:2/20:4
	808.6	38:5	16:0/22:5
	810.6	38:4	18:0/20:4
	832.6	40:7	18:1/22:6
	834.6	40:6	18:0/22:6
	836.6	40:5	20:1/20:4
Alkenyl species	830.6	40:0p	20:0p/20:0

By the analysis of the mass spectra obtained for the two study groups (Figure 41) we can see that the phospholipid profile is not altered in this phospholipid class. However, our results show a significant increase in the total phospholipid content of PC class. Since PCs are important constituents of cellular membranes, this increase may be related with changes in heart membranes, in chronic stress.

2.3.2 Analysis of SM profile

SM have important functions in the cytoplasmic membrane structure and cell signaling [100]. The MS spectra of SMs were obtained in the positive mode, with the formation of ions $[MH]^+$. The MS spectra of the two study groups are presented in Figure 42 and the major ions are identified in Table 14.

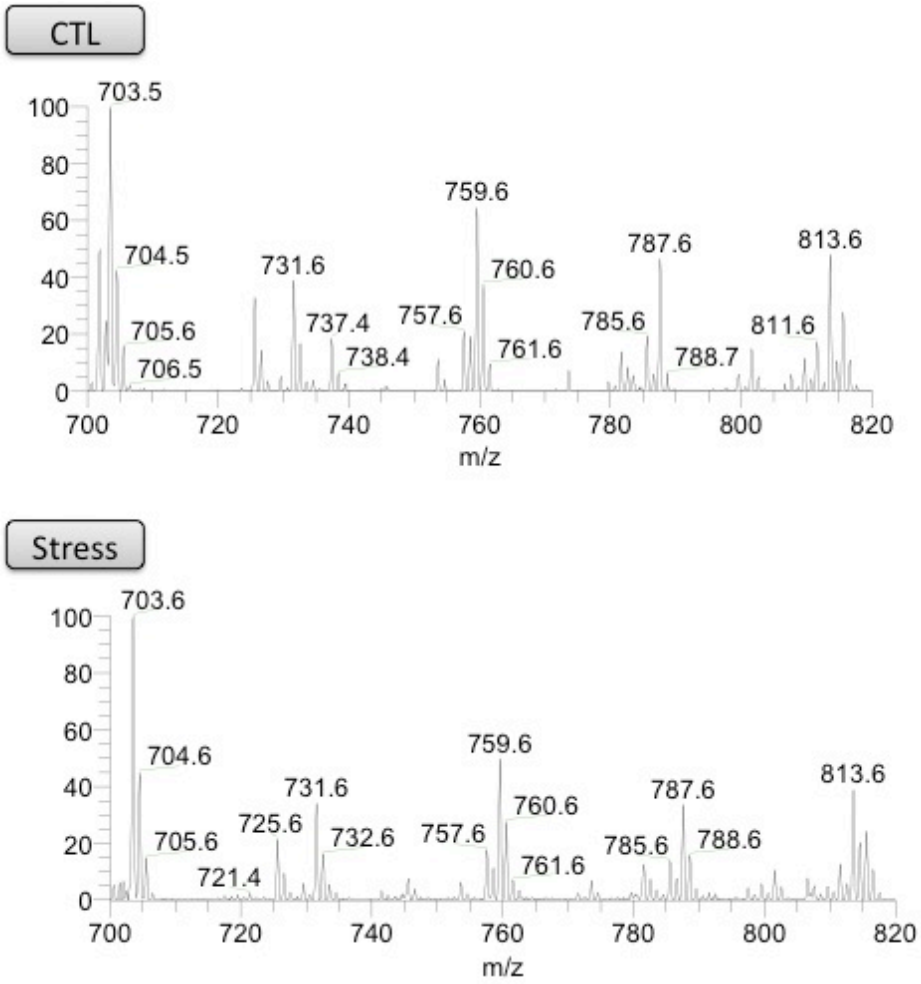

Figure 42. HPLC-MS spectra of SM class in the positive mode with formation of $[MH]^+$ ions, in control (CTL) and in chronic stress situations (Stress). Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: *m/z* for each ion.

MS/MS analysis allowed the identification of the acyl chains present in each molecular species of SM identified (Table 14).

Table 14. Identification of $[MH]^+$ ions observed in the MS spectra in SM. Phospholipids are designated as follows: d18:1/16:0 SM, where d18:1 indicates the sphingosine chain and 16:0 indicates the fatty acyl residue.

	m/z $[MH]^+$	C:N	Fatty Acyl Chains
SM Class	701.5	34:2	d18:1/16:1
	703.5	34:1	d18:1/16:0
	725.6	36:4	d18:1/18:3
	731.6	36:1	d18:1/18:0
	757.6	38:2	d18:1/20:1
	759.6	38:1	d18:1/20:0
	787.6	40:1	d18:1/22:1
	813.6	42:2	d18:1/24:1
	815.6	42:1	d18:1/24:1

The MS spectra of SMs class did not showed significant differences between control and chronic stress. Nevertheless, we found an increase in the SMs total content in chronic stress. This increase may be due to an increase in the activation of the biosynthetic pathways of this class.

2.3.3 Analysis of LPC profile

LPCs were analysed in the positive ion mode forming $[MH]^+$ ions and the obtained mass spectra are presented in Figure 43. The most abundant ions are identified in Table 15.

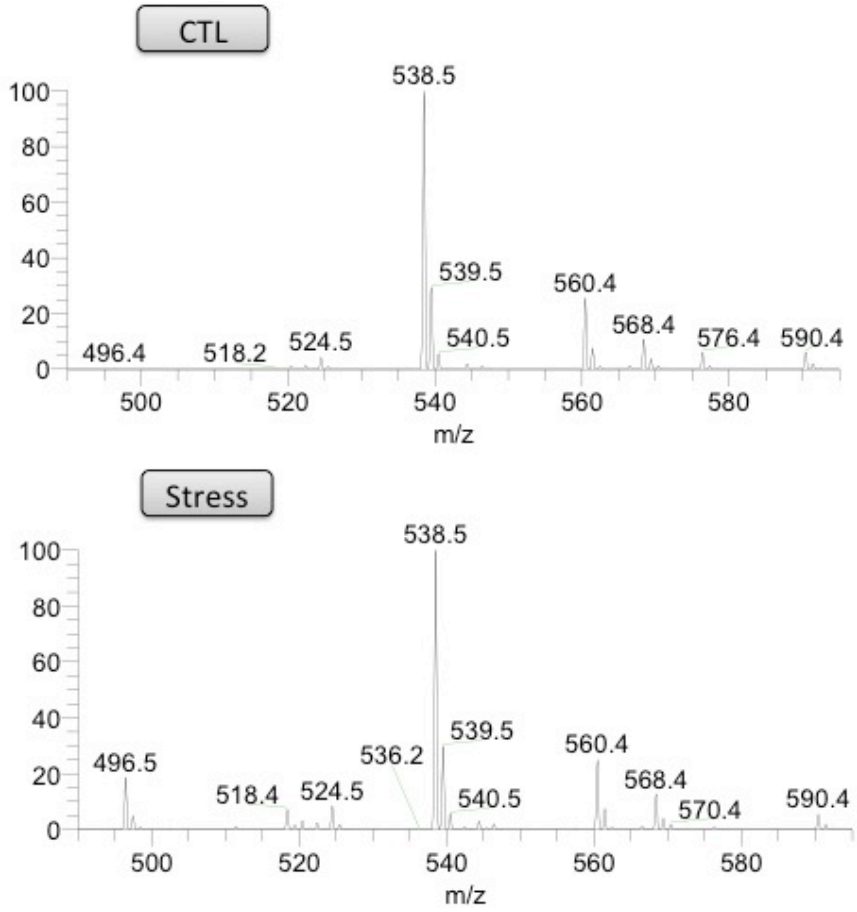

Figure 43. HPLC-MS spectra of LPC class in the positive mode with formation of $[MH]^+$ ions, in control (CTL) and in chronic stress situations (Stress). Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: *m/z* for each ion.

Table 15. Identification of $[MH]^+$ ions observed in the MS spectra in LPC; p - an *sn-1* vinyl ether (alkenyl- or plasmalogen) linkage. Lysophospholipids are designated as follows: diacyl 18:3 PC, where 18 indicates the number of carbon atoms at both the *sn-1* position and 3 designates the number of double bonds at this position.

LPC Class	*m/z* $[MH]^+$	C:N
Diacyl species	496.5	16:0
	518.4	18:3
	524.5	18:0
	560.4	20:4
	568.4	22:6
	570.4	22:5
Alkenyl species	538.5	20:4p

By the analysis of the mass spectra presented in Figure 43, there are no significant changes in the molecular species of the LPC class. However, we found that in chronic stress the total content of this class is decreased. This decrease may be due to the activation of the biosynthetic pathways for the formation of PC class, which is increased in chronic stress conditions.

2.3.4 Analysis of PE profile

The PE class is one of the most abundant classes in cellular membranes. It represents the second more abundant class of PLs in heart. The mass spectra obtained for the PE class was acquired by HPLC-MS in the negative mode, forming $[M-H]^-$ ions. The mass spectra for this class are presented in Figure 44 and show no differences between control and chronic stress conditions.

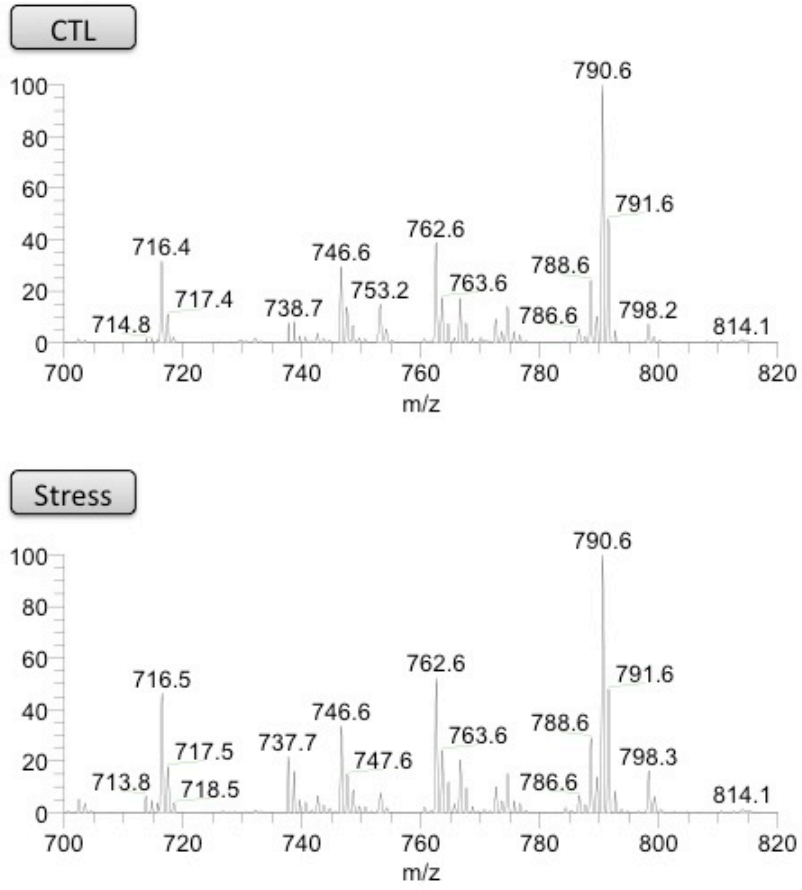

Figure 44. HPLC-MS spectra of PE class in the negative mode with formation of $[M-H]^-$ ions, in control (CTL) and in chronic stress situations (Stress) obtained. Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: *m/z* for each ion.

In order to identify the most abundant species both in control and chronic stress group, after the identification of the molecular species by MS, we analysed these ions by MS/MS to identify the acyl chains in *sn*-1 and *sn*-2 positions. The identification of species by MS/MS allowed us to identify two different species: diacyl- and alkenyl species.

Table 16. Identification of $[M-H]^-$ ions observed in the MS spectra of PE; p - an *sn-1* vinyl ether (alkenyl- or plasmalogen) linkage. Phospholipids are designated as follows: diacyl 40:6 PE, where 40 indicates the summed number of carbon atoms at both the *sn-1* and *sn-2* positions and 6 designates the summed number of double bonds at both positions.

PE Class	*m/z* $[M-H]^-$	C:N	Fatty Acyl Chains
Diacyl Species	716.5	34:2	16:0/18:1
	746.5	36:0	18:0/18:0
	762.4	38:6	18:2/20:4
	764.4	38:5	18:1/20:4
	766.5	38:4	18:0/20:4
	772.5	38:1	18:0/20:1
	788.4	40:7	18:1/22:6
	790.5	40:6	18:0/22:6
	792.5	40:5	18:0/22:5
Alkenyl Species	748.4	38:5p	18:1p/20:4
	774.5	40:6p	18:1p/22:6
	786.5	40:0p	20:0p/20:0

Analysis by MS/MS allowed the identification of diacyl and alkenyl species. In PE class, after the diacyl species, the alkenyl species are normally more abundant species in the heart. It was possible to distinguish the alkenyl species, because these species are characterized by a difference of mass of 12 Da in comparison with the correspondent diacyl species.

After the identification of the acyl chains present in each PE specie, it was possible to conclude that the most abundant fatty acyl chains in PE species is the stearic acid (C18:0), but it is also possible to see the existence of other fatty acyl chains with more unsaturation's, like the arachidonic acid (C20:4). Arachidonic acid is an extremely important fatty acid, especially in heart, since it is the precursor of eicosanoids, which are extremely important molecules in inflammation. The increase in eicosanoids is related with the development of many diseases, including cardiovascular diseases [126].

Although no changes were found in the molecular species of PE class, an increase in the total content of this class was showed in our results. This increase may be related with changes in the membrane structure, since PEs are most abundant in the inner leaflet of plasma membrane.

2.3.5 Analysis of PI profile

The MS analysis of PIs was carried out in the negative ion mode, with the formation of ions $[M-H]^-$. The mass spectra obtained for control and stress group is presented in Figure 45 and made possible the identification of the $[M-H]^-$ ions presented in Table 17.

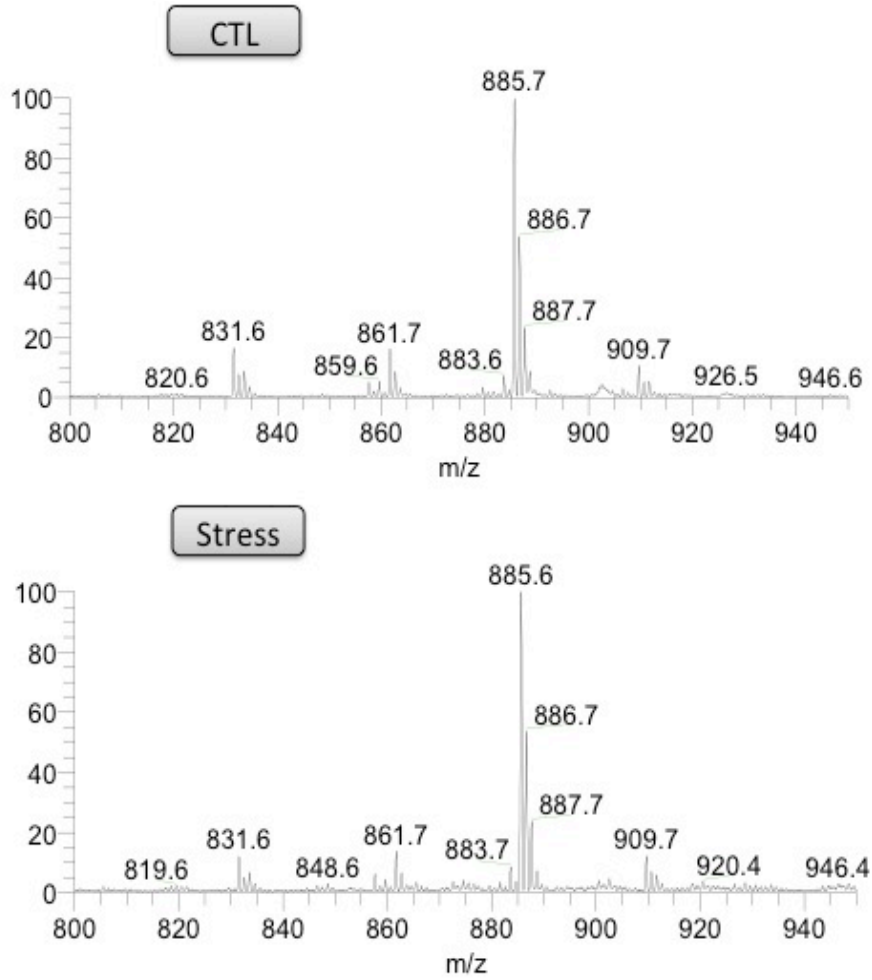

Figure 45. HPLC-MS spectra of PI class in the negative mode with formation of $[M-H]^-$ ions, in control (CTL) and in chronic stress situations (Stress). Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: *m/z* for each ion.

For the identification of the fatty acyl chains present in the ions identified in the MS spectra, we analysed all these ions by MS/MS. Through the interpretation of the MS/MS spectra and knowing the characteristic fragmentation of these class, it was possible to identify the molecular species corresponding to PIs presented in Table 17.

Table 17. Identification of $[M-H]^-$ ions observed in the MS spectra of PI. Phospholipids are designated as follows: 38:4 PI, where 38 indicates the summed number of carbon atoms at both the *sn-1* and *sn-2* positions and 4 designates the summed number of double bonds at both positions.

	m/z $[M-H]^-$	C:N	Fatty Acyl Chains
PI Class	831.5	34:1	16:0/18:1
	833.5	34:0	16:0/18:0
	857.5	36:4	16:0/20:4
	859.6	36:3	18:1/18:2
	861.6	36:2	18:0/18:2
	863.6	36:1	18:0/18:1
	883.6	38:5	18:1/20:4
	885.6	38:4	18:0/20:4
	887.6	38:3	18:0/20:3
	889.5	38:2	18:0/20:2
	909.6	40:6	20:2/20:4
	911.5	40:5	20:1/20:4

By the analysis of the mass spectra presented in Figure 45 we can see that no changes occurred in the PI profile of heart of mice of chronic stress group. However, our results show a decrease in the total content of this class, although not statistically significant. It is important to notice that PIs form the structural basis for a complex interplay of signalling responses initiated, normally, by receptor activation and resulting in changes in Ca^{2+}, protein kinase cascades and ion channel/exchanger activity. PI can suffer phosphorylation in the inositol group. Phosphoinositol second messengers regulate responses ranging from immediate changes in vascular tone and hormone secretion to more prolonged responses such as cell growth and differentiation that require transcriptional changes [73]. The increase in PIP_2 contributes also for the development of arrhythmias [46]. PIP's are crucial determinants of cellular responses, including changes in cell survival, cardiac hypertrophy, cardiomyocytes contractile function, channel activity and cell metabolism. These molecules have complex signalling pathways in heart, and these pathways can be maladaptive when chronically activated [46].

2.3.6 Analysis of the CL profile

Cardiolipin has an important role in heart, since this organ has a high content of mitochondria and CL is an exclusive PL of this organelle. Our results in brain CL class showed oxidative changes in this class, suggesting that changes caused by chronic stress in brain are accompanied by mitochondrial deregulation. Also in brain we saw a decrease in the total content of CL class. However, in brain CL total content is much lower abundant than it is in heart. However, the molecular variability is much higher in brain, not only for CL class, but for all the other classes, has we can see by the number of molecular species identified in brain and the ones identified in heart.

CL is the more complex phospholipid class, since four acyl chains, three glycerol and two phosphate groups constitute it. The molecular analysis of CL by HPLC-MS was made in the negative ion mode, with formation of ions $[M-H]^-$ and/or $[M-2H]^{2-}$. The Figure 46 shows the mass spectra obtained for the two study groups and the ions found are identified in Table 18. In both spectra the most abundant molecular specie corresponds to the ion $[M-H]^-$ at *m/z* 1447.9, corresponding to the CL $(C18:2)_4$, already described in literature as the most abundant CL in mammalian heart [5].

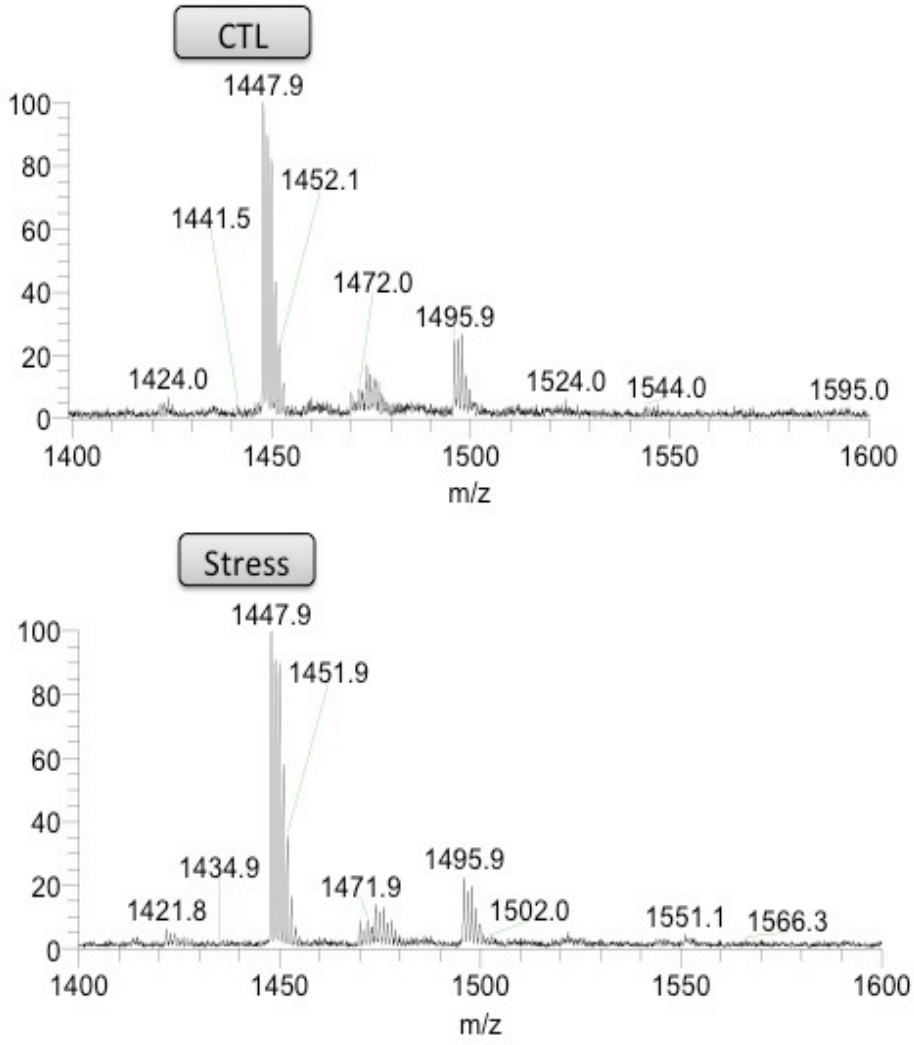

Figure 46. HPLC-MS spectra of CL class in the negative mode with formation of $[M-H]^-$ ions, in control (CTL) and in chronic stress situations (Stress). Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: *m/z* for each ion.

In Table 18 are represented the CL molecular species identified by MS/MS in the negative mode and their fatty acid composition.

Table 18. Identification of $[M-H]^-$ ions observed in the MS spectra in CL. Phospholipids are designated as follows: tetra-acyl 72:8 CL, where 72 indicates the summed number of carbon atoms at both *sn-1, sn-2, sn-1'* and *sn-2'* positions and 8 designates the summed number of double bonds at the four positions.

	m/z $[M-H]^-$	C:N	Fatty Acyl Chains
CL Class	1421.8	70:7	18:2/18:2/18:2/16:1
	1447.9	72:8	$(18:2)_4$
	1449.9	72:7	$18:1/(18:2)_3$
	1451.8	72:6	$(18:1)_2/(18:2)_2$
	1453.8	72:5	$(18:1)_3/(18:2)_1$
	1461.7	72:1	$(18:0)_3/(18:1)$
	1463.7	72:0	$(18:0)_4$
	1467.7	74:12	$16:2/(18:2)_2/22:6$
	1469.9	74:10	$16:1/(18:2)_2/22:6$
	1473.9	74:9	$(18:2)_3/20:3$
			$18:1/(18:2)_2/20:4$
	1475.9	74:8	(18:2)3/20:2
	1477.8	74:7	$18:1/(18:2)_2/20:2$
	1479.9	74:6	$18:1/(18:2)_2/20:1$
	1483.7	74:4	$(18:1)_3/20:1$
	1487.7	74:2	$(18:0)_2/(18:1)/20:1$
	1489.9	74:1	$(18:0)_3/20:1$
	1495.8	76:12	$(18:2)_3/22:6$
	1497.9	76:11	$18:1/(18:2)_2/22:6$
	1499.7	76:10	$(18:2)_3/22:4$
			$18:1/(18:2)_2/22:5$
	1501.9	76:9	$20:4/20:2/(18:2)_2$
	1507.8	76:6	$20:4/20:0/(18:1)_2$
	1511.8	76:4	$20:4/20:0/(18:0)_2$
	1521.9	78:13	$22:6/20:3/(18:2)_2$
	1533.8	78:7	18:0/22:6/20:1
	1565.9	80:5	$(20:0)_2/20:1/20:4$
	1579.9	82:12	20:4/20:0/20:2/22:6

In the MS spectra of CL class presented in Figure 46 we see that no changes occur in phospholipid profile in chronic stress conditions. Our results showed that in brain several changes occur in phospholipid profile of CL suggesting the possible impairment of mitochondrial function in brain. However, no changes were observed in profile of CL in heart, suggesting that the brain is the first target of chronic stress.

Nevertheless, our results show a decrease in the total content of CL class, although this difference was not statistically significant. The decrease of CL content was already associated with many heart diseases, like heart failure and diabetic cardiomyopathy [5, 43].

Several studies correlate chronic stress with alterations in heart functions, namely in the development of heart rate variability, heart failure and cardiovascular diseases [1, 20, 21].

In summary our results showed some changes in the total relative content of some phospholipid classes, being the increase in the PC content the more significant change. However, the analysis of PL profile showed no differences in the molecular species among all the classes analysed. In addition, no oxidation was identified after chronic stress in the heart.

Chronic stress has been related with the development of several pathophysiological disturbances, due to the deregulation of many biochemical and physical processes [9]. The close relationship between chronic stress and depression has drawn much attention in the scientific community, since depression is a major socioeconomic burden of disease. Several studies have correlated the biochemical changes occurred in central nervous system with impaired brain function and its consequences in heart diseases. In both of these organs phospholipids are very important biomolecules and their profile alterations have been related with many diseases of the brain and of the heart [18].

In this study we evaluated the phospholipid profile changes that occur during chronic stress in the brain and in the heart from mice. Our results suggested that the first target of chronic stress is the brain, causing the impairment of mitochondria functions, by the oxidation of cardiolipin. In fact, we did not found many significant changes in the heart of chronic stressed mice, after 21 days of chronic stress. However, the similar behaviour in the variation of the total phospholipid content of the classes, suggests the possibility that the changes found in brain may occur also in heart, when the chronic stress stimulus is longer.

The importance of oxidative modifications in CL is becoming increasingly evident and the study on these processes have been growing recently. Several studies have correlated oxidative changes in CL with neuronal diseases and brain injuries [32, 34, 36]. The reduction of CL level in the brain with aging was reported as being associated with an increase of lipid peroxidation in rat brain mitochondria exposed to oxidative stress [121]. CL oxidation has been associated with several pathologies, including the development of aging and neurodegenerative diseases [36].

IV. Conclusion

IV. Conclusion

The main goal of this work was to evaluate the changes in phospholipid profile of brain and heart from mice with chronic stress, which could explain the modifications that occur in these organs under these conditions. Also, this approach was used to understand the extent of the lesions that occur in the organism in chronic stress.

Our results showed changes in the relative abundance of phospholipids classes in brain. We verified an increase in phosphatidylcholines, a main class in cell membranes, suggesting a modification of neuronal membranes in chronic stress. On the other hand, other classes of PLs analysed were decreased (PIs and CLs). Since these classes are often involved in cellular signalling cascades and in apoptotic events, the decrease verified in chronic stress may be due to the activation of these signalling events. Furthermore, we found an increase in lipid peroxidation, which allowed us to identify some CL oxidised species. CL oxidation has been related with mitochondrial dysfunction and apoptosis. Thus our results suggest that there is a functional impairment of mitochondria in chronic stress. This fact together with the decrease in CL content can be associated with neuronal loss reported in chronic stress and consequently with the development of neurodegenerative diseases and depression. We were also able to identify changes in some PAs species.

Our results in the phospholipid profiling of the heart showed no significant changes among the molecular species of each class. However, in the evaluation of the phospholipid total content of each class, our results showed a very similar profile in the heart compared with the obtained in the brain, however the variations reported in the heart were shorter than the ones found in brain. In fact we only saw a significant increase in PCs. These data suggest that phospholipids of the brain suffer more changes in chronic stress, since this is where we found the most significant changes. However, the comparison of the total phospholipid classes content of the two organs suggests that the same changes may occur in heart when chronic stress conditions are prolonged.

V. Bibliography

V. Bibliography

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