Universidade de Aveiro Departamento de Química
2015

Bebiana Costa Sousa

ALTERAÇÕES NO PERFIL LIPÍDICO DE CÉLULAS E EXOSSOMAS NO ENFARTE AGUDO DO MIOCÁRDIO

LIPID PROFILE ALTERATION OF CELLS AND EXOSOMES IN ACUTE MYOCARDIAL INFARCTION

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, ramo Bioquímica Clínica realizada sob a orientação científica da Doutora Maria do Rosário Gonçalves Reis Marques Domingues, Professora Auxiliar com Agregação do Departamento de Química da Universidade de Aveiro e do Doutor Henrique Manuel Paixão dos Santos Girão, Investigador Auxiliar da Faculdade de Medicina da Universidade de Coimbra (IBILI).

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Dedico esta tese aos meus pais e irmãos pelo constante apoio.

Dedicatória Póstuma
Dedico esta tese a José Francisco, o meu avô.

Para ser grande, sê inteiro: nada
Teu exagera ou exclui.

Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.

Assim em cada lago a lua toda
Brilha, porque alta vive.

Ricardo Reis, in "Odes"
Heterónimo de Fernando Pessoa

O júri

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palavras-chave apoptose, autofagia, biomarcadores, cardiomiócitos, doenças cardiovasculares, espectrometria de massa, exossomas, fosfolípidos, infarte do miocárdio, isquémia, lipidómica

resumo

As doenças cardiovasculares são a principal causa de morte em todo o mundo. De entre estas, o enfarte do miocárdio é uma das doenças mais comuns, sendo caracterizado por isquemia que leva a morte de células cardíacas. A isquemia ocorre em consequência da privação simultânea de nutrientes e oxigénio. Enquanto a isquemia representa um dano celular, a privação de nutrientes está relacionada com efeitos cardioprotetores. A resposta das células a estes estímulos pode ser por indução de autofagia ou de apoptose, dependendo da sua capacidade de adaptação e resposta aos fatores indutores de isquemia. A autofagia é um processo auto-degradativo que permite à célula adaptar-se ao stresse e é, portanto, um processo associado à sobrevivência celular. A libertação de exossomas pelas células é também um mecanismo de adaptação cujas funções estão relacionadas com a comunicação intercelular. Por outro lado, a apoptose é um processo de morte celular programada. A regulação destes processos é de extrema importância para a sobrevivência e recuperação nos episódios de enfarte do miocárdio.

Hoje em dia sabe-se que os lípidos têm um papel importante no desenvolvimento de doenças cardiovasculares embora o seu papel ainda não esteja completamente esclarecido. Os lípidos são os componentes maioritários da membrana celular e desempenham funções a nível estrutural e de sinalização. Quando exposto a diversas condições fisiopatológicas, o conteúdo lipídico das células e dos exossomas é modificado. No entanto, existem ainda poucas publicações sobre a avaliação do lipidoma de cardiomiócitos em patologias cardiovasculares. Assim, o objetivo principal deste trabalho é identificar alterações no perfil lipídico de cardiomiócitos e exossomas libertados por estes sob privação de nutrientes e de oxigénio, de forma a melhor compreender o enfarte do miocárdio e se possível identificar novos biomarcadores para esta patologia.

Nas células cardíacas verificamos que algumas espécies moleculares de fosfatidilcolina (PC34:1 e PC36:2), fosfatidiletanolamina (PE34:1), fosfatidilserina (PS36:1), fosfatidilinositol (PI36:2, PI38:3 e PI38:5) e esfingomielina (SM34:1) variam em isquemia e em privação de nutrientes em comparação com o controlo. Algumas variações foram específicas da privação de nutrientes como a diminuição de SM(34:1) e o aumento de PS(36:1) e outras foram específicas da isquemia como a diminuição de PC(36:2) e de LPC(16:0). A espécie molecular PC(34:1) foi a que se mostrou alterada de forma diferente em cada condição sendo que aumenta em caso de isquemia e diminui em caso da privação de nutrientes.

No caso dos exossomas, os resultados obtidos permitiram verificar que houve um maior desvio entre o lipidoma de exossomas libertado em isquemia e privação de nutrientes em todas as classes de lípidos. Algumas alterações foram coincidentes com as observadas para as células, por exemplo a diminuição PC(34:1) em starvation mas outras foram diferentes. Uma vez que apenas se realizaram análises lipdómicas para uma dimensão reduzida de amostra de exossomas, serão necessários estudos futuros para a validação dos resultados obtidos.

Em conclusão, a privação de nutrientes e a isquemia induzem alterações na homeostasia dos lípidos. Este trabalho sugere que os lípidos são potenciais ferramentas para avaliar se os cardiomiócitos estão a optar pela morte celular ou pela recuperação, que serão úteis para melhorar o diagnóstico e prognóstico de doenças cardiovasculares.

Keywords

apoptosis, autophagy, biomarkers, cardiomyocytes, cardiovascular diseases, exosomes, ischemia, lipidomics, mass spectrometry, myocardial infarction, phospholipids, starvation

Abstract

Cardiovascular Diseases are the most significant cause of death. Myocardium infarction is one of the most common of this type of diseases and it is characterized by myocardium ischemia. Ischemia occurs in consequence of simultaneous starvation and hypoxia. While ischemia represents a cellular damage, starvation is associated with a cardioprotective effect. The cell response to this injury includes either autophagy or apoptosis depending on the ability to adapt and respond to the injury and is very important for the evolution and recovery of the myocardium infarction. Autophagy is a self-degradative process that allows cell to adapt to stress and so it is associated with cell survival. The exosomes release by cardiomyocytes is also an adaptive process which functions are related with intercellular communication. On the other hand, apoptosis is a process of programmed cell death.

It is well known that lipids play an important role in cardiovascular disease although their role is not completely understood. Lipids are the major component of a cell membrane and play structural and signaling roles. Under several physiopathological conditions, the cell and exosomes lipid content can be modified. However reports on lipidome of cardiomyocytes under cardiovascular diseases are scarce. Thus, the primary aim of this work is to identify lipid profile changes in cardiomyocytes and exosomes released by them under starvation and ischemia, in order to better understand myocardial infarction and if possible to recognize new biomarkers for myocardial infarction.

Cardiac cells showed that molecular species alterations in phosphatidylcholine (PC34:1 and PC36:2), phosphatidylethanolamine (PE34:1), phosphatidylserine (PS36:1), phosphatidylinositol (PI36:2, PI38:3 and PI38:5) and sphingomyelin (SM34:1) were changed in ischemia and in starvation in comparison with control group. Some differences were specific of starvation as the decrease in SM(34:1) and the increase in PS(36:1) while

others were specific of ischemia as the decrease in PC(36:2) and LPC(16:0). The molecular specie PC(34:1) showed different alterations in each condition increasing in case of ischemia and decreasing in case of starvation.

For exosomes, our results showed a deviation between the lipidome of exosomes released upon ischemia and starvation for all lipid classes. Some differences matched the ones observed in cells, for example the decrease in PC(34:1) in starvation, but others were different. Since we have only performed lipidomic analysis for a smaller sample of exosomes, it requires further studies to validate the results.

In conclusion, ischemia and starvation induced changes in lipid homeostasis. Our work suggests that lipids are potential tools for evaluation of cell fate, either cell death or recovery, that will be useful to improve diagnosis and prognostic of cardiovascular diseases.

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ABBREVIATIONS

Akt - PKB – protein kinase B
AMI - acute myocardial infarction
AMP - adenosine monophosphate
AMPK - adenosine monophosphate kinase
ANOVA - analysis of variance
AP - autophagosome
Apo - apolipoprotein
AST - aspartate transaminase
Atg - autophagic gene
ATP - adenosine triphosphate
ATPase - ATP monophosphatase
Bcl - beclin
BM - bone marrow
BNP - b-type natriuretic peptide
$CD4^+$ - cluster of differentiation 4
CDase - ceramidase
CE - cholesterol esterified
Cer - ceramide
CerS - dihydroceramide synthase
CHF - chronic heart failure
CK - creatine kinase
CL - cardiolipin
COX - cyclooxygenase
CRP - c-reactive protein
CT - x-ray computed tomography
CVD - cardiovascular disease
CXCL - CXC chemokine ligand
CXCR - CXC chemokine receptor
DAG - diacylglycerol
DC - dendritic cells
DNA - deoxyribonucleic acid
EBV - Eppstein-barr virus
ECG - electrocardiographic
ER - endoplasmic reticulum
ESCRT - endosomal sorting complexes required for transport
EV - extracellular vesicles
FA - fatty acid
FAME - fatty acid methyl esters
FOXO - forkhead box O protein
GC - gas chromatography
GTP - guanosine triphosphate

HHD - hypertensive heart disease
HILIC - hydrophilic interaction liquid chromatography
HPLC - high performance liquid chromatography
HSP - heat shock protein
IAP - inhibitors of apoptosis proteins
IDH - ischemic heart diseases
ILV - intraluminal vesicles
IMM - inner mitochondrial membrane
INE - instituto nacional de estatística
IRGM - immunity-related GTPase M
JNK - c-jun n-terminal kinase
LD - lipid droplet
LDH - lactate dehydrogenase
LDL - Low density lipoprotein
LOX - lipoxygenases
LPC - lysophosphatidylcholine
LPE - lysophosphatidylethanolamine
LPS - lysophosphatidylserine
MHC - histocompatibility complex
MI - myocardium infarction
miR - microRNA
MOMP - mitochondrial outer membrane permeabilization
MPS - myocardial perfusion scintigraphy
MRI - magnetic resonance imaging
mRNA - messenger RNA
MS - mass spectrometry
mTOR - mammalian target of rapamycin
MVB - multivesicular body
oxPL - oxidized phospholipid
PA - phosphatidic acid
PC - phosphatidylcholine
PE - phosphatidylethanolamine
PET - positron emission tomography
PG - phosphatidylglycerol
PI - phosphatidylinositol
PI3K - phosphatidylinositol-3-phosphate kinase
PI3P - phosphatidylinositol-3-phosphate
PIP - phosphatidylinositides
PKC - protein kinase c
PL - phospholipids
PLA_2 - phospholipase A_2
PLD1 - PX domain containing phospholipase D1

PPAR	-	peroxisome proliferator-activated receptor
PS	-	phosphatidylserine
OMM	-	outer mitochondrial membrane
RNA	-	ribonucleic acid
ROS	-	reactive oxygen species
S1P	-	sphingosine-1-phosphate
SC	-	stem cells
SK1	-	sphingosine kinase 1
SM	-	sphingomyelin
SMase	-	sphingomyelinase
SNARE	-	soluble NSF attachment receptor
SPECT	-	single proton emission computed tomography
SPK	-	sphingosine kinase
SPP1	-	sphingosine-1-phosphate phosphohydrolase
TFR	-	transferrin receptor
TG	-	triglycerides
TLC	-	thin layer chromatography
Tn	-	troponin
TNF	-	tumor necrosis factor
UC	-	unesterified cholesterol
UV	-	ultraviolet light
WHO	-	world health organization

CHAPTER I:

INTRODUCTION

1. Cardiovascular Diseases

Cardiovascular diseases are the most significant cause of death all over the world. An accurate diagnosis is essential to early therapeutic intervention and to prevent further complications. Due to the lack of specific and sensitive tests, it is somehow difficult to do an early diagnosis to be beneficial to those presenting this debilitating condition.

The mortality rate of cardiovascular diseases has decreased in the last two decades (Figure 1) due to contribution of three conjugated factors [1]. First is the adoption of preventive strategies such as reduced smoke tobacco, exercise and a healthy diet. Second is the improvement of diagnosis and adjustment of modifiable risk factors [4]. The third is the significant advance in treatment not only by the increased availability of drugs but also by the upgrade of administrative conditions allowing better target locations where treatment can be administered [1]. Even though the morbidity and mortality associated to this group of disease is still very high and need to be reverted.

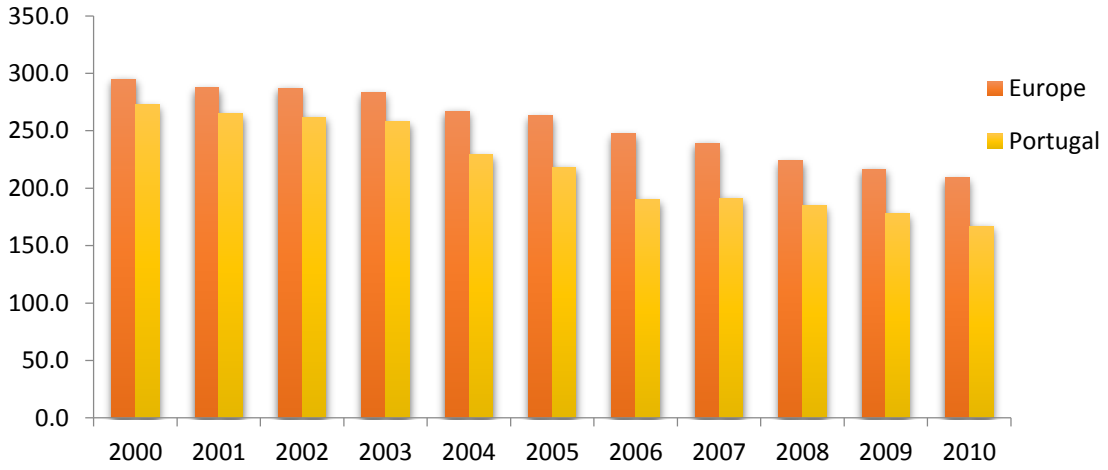


Figure 1. Cardiovascular Disease Mortality Rate in Europe and Portugal (INE).

Cardiovascular diseases include two major groups of diseases: cerebrovascular disease and ischemic heart diseases (IDH). IDH are the most prevalent in world population and myocardial infarction is the most relevant disease among this category (Figure 2).

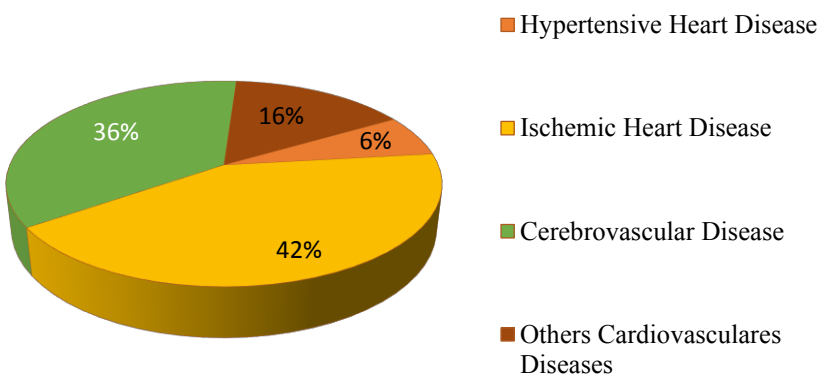
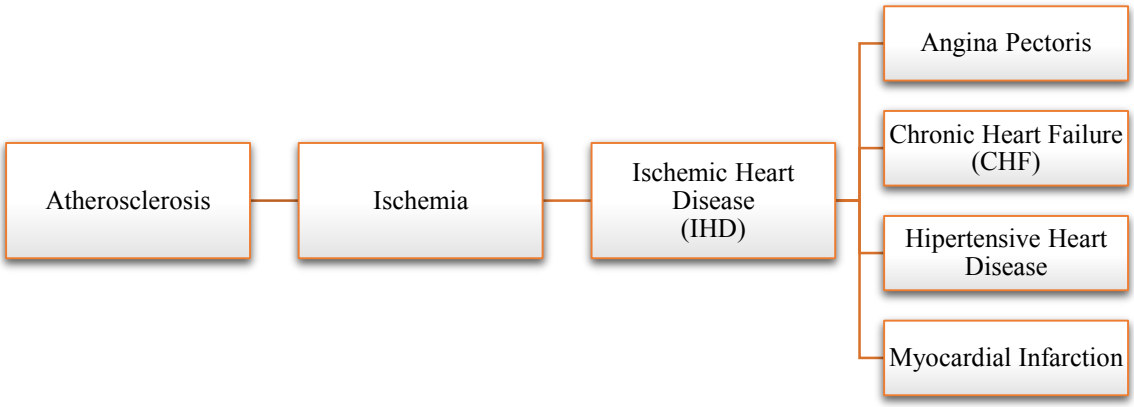


Figure 2. Prevalence of different cardiovascular diseases in the world (WHO).

Myocardium infarction is one of the most common IHD, which are characterized by ischemia. Ischemia is described as the reduced blood, and thus in a shortage of oxygen and nutrients, supply to the heart due to narrowing of the arteries. The narrowing of the arteries is a characteristic of atherosclerosis which is described as the thickening and hardening of the artery walls caused by deposits of cholesterol-lipid-calcium plaque. Therefore, atherosclerosis is a major cause of ischemia and consequently ischemic heart diseases [2]. The atherosclerosis is an inflammatory chronic disorder wherein endothelial lesions and accumulation of fat and cells obstruct the vascular lumen and weaken the underlying smooth muscle causing severe damages. Ischemia can be the cause of angina pectoris, chronic heart failure (CHF), hypertensive heart disease (HHD) and myocardial infarction (MI) (Scheme 1) [3].



Scheme 1. Different ischemic heart diseases with atherosclerosis as a cause.

Angina Pectoris, referred as cardiac chest pain, is not simply one type of pain but a group of symptoms related to cardiac ischemia. It usually begins gradually and last only a few minutes. Angina can either occur in rest or be brought by effort [4]. Chronic Heart Failure (CHF) is referred to some structural or functional cardiac disorders that impair the ability of the ventricle to fill with or eject blood. CHF may result from disorders of the heart walls, such as myocardium, or from disorders of the great vessels. However, most of the cases are due to left ventricular dysfunction. Hypertensive Heart Disease is a term referring to heart diseases caused by direct or indirect effects of elevated blood pressure. It increases the amount of work of the ventricle resulting in hypertrophy and dilatation, increasing the arterial pressure and enhancing hypertension. Myocardial Infarction (MI) is defined as necrosis of myocardium due to prolonged ischemia. Pathologically it includes acute, healing and healed myocardial infarction [2].

The diagnostic of the IHD related diseases, including myocardial infarction, the most common IHD and thus will be considered herein, are associated with difficult early diagnostic due to the inexistence of very sensitive and specific markers in the first hours of the events. Several enzymes are released from the injured tissues that lead to increased serum levels. Thus, it can be used as markers for this pathology. Despite the lack of tissue specificity, few proteins have clinical significance in the cardiac muscle and thus in MI, such as lactate dehydrogenase (LDH), aspartate transaminase (AST), creatine kinase (CK) and troponin (Tn) [5]. These marks only show up hours after the cardiac event and allow confirming MI diagnosis and evaluate it prognosis. Sometimes this prevent to perform a correct diagnosis and to implement a correct therapeutics. Thus, there is a need for searching new cellular markers to improve the early diagnosis of IHD. Lipids have emerged as a plausible hypothesis to this lack of primary markers. It is therefore necessary understand the alterations in cardiomyocytes at a molecular level and the underlying signaling process.

1.1. Myocardial Infarction: Physiopathology

The myocardial infarction is a major cause of death and disability worldwide. Formerly, there was a consensus for the syndrome myocardial infarction. The World Health Organization (WHO) defined MI based on symptoms, ECG changes and cardiac enzymes. However, with the improvement of more sensitive cardiac markers and more sensitive imaging techniques it was possible to detect small amounts of myocardial

injury. For that reason, it was required an updated definition of MI. In 2000, the First Global MI Task Force presented a new definition of MI suggesting that any necrosis in the setting of myocardial ischemia should be considered MI [6]. In 2007, these principles were refined by the Second Global MI Task Force. The universal definition of myocardial infarction consensus document highlighted the different conditions which might lead to a MI. This document was well accepted by the medical community and was adopted by the WHO [7]. Though, the development of even more sensitive markers mandates additional revision, particularly when necrosis occurs in the setting of the critically ill, after coronary procedures or after cardiac surgery. Thus in 2012, the Third Global MI Task Force integrate this new insights and recognize that very small amounts of myocardial injury can be detected by biochemical markers, preferentially troponin, and imaging [8].

Nowadays, the MI diagnosis is based on clinical features, including symptoms, electrocardiographic (ECG) deviations, and elevated values of biochemical markers and by imaging. The symptomatology of MI includes chest pain, at effort or at rest, dyspnea, fatigue, and usually takes less than twenty minutes. Frequently, the discomfort is diffuse and it may be accompanied by diaphoresis, nausea or syncope. MI may even occur without symptoms, called silent myocardial infarction. Dynamic changes in the ECG waveforms occur during acute myocardial ischemic episodes and often require the acquisition of multiple ECG performed at fifteen to thirty minutes intervals, but these changes are not always easily perceptible, making difficult the MI diagnosis [8]. The serum biomarkers in acute cardiac diseases can be classified as established, outdated and developing. Among these, troponin (cTn) has been established as fundamental for diagnosis and also provides strong prognostic information [9]. This protein is responsible for the regulation of muscle contraction. Whether cTnI or cTnT, both are highly specific to myocardial muscle and so sensitive for cardiac injury. The cTn elevations begin two to four hours after onset of symptoms. There are novel data suggesting that re-elevations of cTn and an increasing pattern can be used to estimate whether a given MI event is acute or is chronic. Other markers are creatine kinase-MB (CK-MB), myoglobin and aspartate transaminase (AST). CK-MB is a protein that could be used to define infarct timing although clinicians become more comfortable with cTn. Myoglobin has been used with the intent of shortening the time to a more definitive

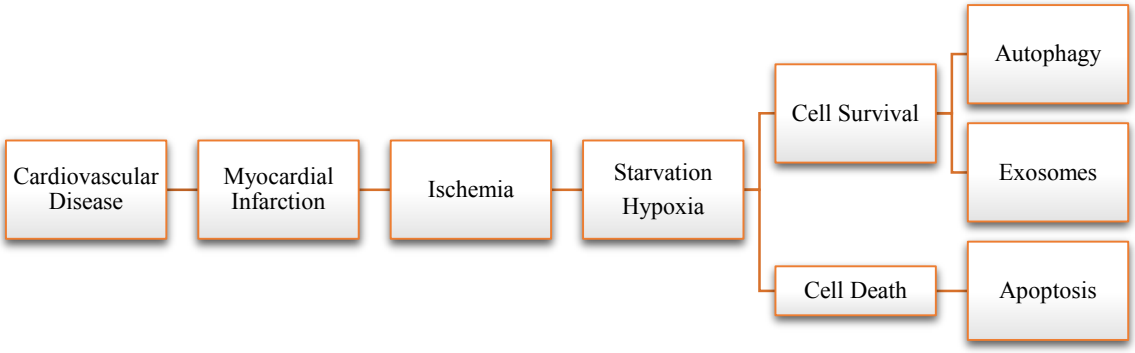
diagnosis in patients with chest pain, but it lacks specificity, since it is also abundant in muscle and can be elevated in muscle lesions or other myopathies. AST levels begin to rise within six to eight hours, peak at twenty-four hours and return to normal within 5 days. However, because of the extensive tissue distribution, these levels are not useful in diagnosis of myocardial infarction. The c-reactive protein (CRP) and the B-type natriuretic peptide (BNP) are two developing markers. Some authors argue that, in the absence of acute illness, including myocardial injury, levels of CRP are stable. BNP is a peptide released in response to cardiac stretch. It is helpful for the detection of congestive heart failure in patients with uncertain cause of dyspnea. The routine use of this to developing markers is not supported [10]. Commonly used imaging techniques in acute and chronic infarction are echocardiography, radionuclide ventrilography, myocardial perfusion scintigraphy (MPS) using single proton emission computed tomography (SPECT) and magnetic resonance imaging (MRI). Positron emission tomography (PET) and x-ray computed tomography (CT) are less common. Each of the techniques can assess myocardial viability, perfusion and function [11].

Pathologically MI is categorized as acute, healing and healed. Acute MI is characterized by the presence of polymorphonuclear leukocytes. Healing MI is characterized by the presence of mononuclear cells and fibroblasts and absence of polymorphonuclear leukocytes. Healed MI is characterized by scar tissue without cellular infiltration [2].

According to pathological and clinical features, and prognostic differences, along with different treatment strategies, myocardial infarction is classified into five types. Type 1 is spontaneous myocardial infarction which is related to atherosclerotic plaque with resulting thrombus leading to decreased myocardial blood flow. Type 2 is myocardial infarction secondary to an ischemic imbalance. There is an imbalance between myocardial oxygen supply and demand. Type 3 is myocardial infarction resulting in cardiac death but when biomarker values are unavailable. There are symptoms suggestive of myocardial ischemia and ECG changes but death occurred before blood samples could be obtained, before cardiac markers could rise or cardiac markers were not collected. Type 4 and type 5 are related to myocardial infarction associated with revascularization procedures. Periprocedural myocardial injury or infarction may occur at some stages in the instrumentation of the heart that is required

during mechanical revascularization procedures. Following this procedures are elevated troponin values since various insults may occur that can lead to myocardial injury with necrosis [8].

Myocardium infarction is one of the most common IHD. These diseases are characterized by ischemia, a biological conditions that includes starvation and hypoxia. Cells exposed to these conditions have two alternatives: survive, and for that initiates the autophagy process; or die and in this case initiates apoptosis.



Scheme 2. Adaptative and non-adapatative cell response to an ischemic event.

2. Ischemia: a cross talk between starvation and hypoxia

Ischemia may occur in consequence of simultaneous nutrient and oxygen deprivation, also called starvation and hypoxia respectively. But food is required to life so organisms and cells developed several mechanisms to survive to the lack of nutrients. Thus, cell response to ischemia induces either autophagy or apoptosis depending on the ability to adapt to the injury (Scheme 2) [12]. Autophagy is a mechanism that allows cell survival. It is a self-degradative process that balances sources of energy at critical times in response to nutrient stress [13]. Autophagy is also associated with exosomes release. Exosomes physiologic functions are not fully understood however it is suggested that they are responsible for intercellular communication [14]. Thus, autophagy and exosomes release mostly allows cell to adapt to stress enabling them to recycle nutrients at times of nutrient scarcity and to communicate with other cells, respectively. Even though massive autophagy can also induce cell death [15]. In contrast, apoptosis is a process of programmed cell death with biochemical events leading to morphologic cell changes and death. Wherein the cell is not able to adapt to

stress, and increase of reactive oxygen species (ROS) production may occur being one of the plausible mechanism that underlie cell apoptosis [16].

Starvation of specific nutrient triggers different responses in tissues [12]. Cell detects levels of energy by sensing ATP and aminoacids levels. ATP is mainly produced by mitochondrial oxidative phosphorylation and during starvation, with the intention of meet cellular energy requirement. It is reported an increase in mitochondrial proliferation along with decrease in maturation. This indicates that mitochondrial defect cannot be compensated by the increase in mitochondrial number [17]. So the lack of glucose, that is necessary to maintain ATP levels, leads to the activation of AMPK, which is sensitive to AMP:ATP ratio. This activation increases the translocation of glucose transporter to the plasma membrane and inhibits promoters of glucose-repressed genes. Additionally it activates p53 in order to inhibit cell proliferation [18]. The lack of aminoacids such as glutamine and leucine leads to the inhibition of mTORC resulting in inhibition of protein synthesis. Moreover, it leads to the decrease of cdc2 activity stopping cell cycle and inducing autophagy, a way to help the cell survive. In conclusion, cell response when confronted with lack of nutrients by shifting to catabolism in hope to survive [12].

2.1. Autophagy as a way of cell survival

Autophagy is seen as a self-degradative process important not only for housekeeping but for balancing sources of energy in response to nutrient stress. It initiates with a phagophore derived from lipid bilayer that expands to engulf intra-cellular cargo. The loaded double-membrane autophagosome matures through fusion with the lysosome, leading to the degradation of autophagosomal contents by acid proteases. This process promotes cellular senescence, protects against genome instability and prevents necrosis, giving it a key role in preventing diseases such as neurodegeneration, cardiomyopathy and diabetes [13].

Two hypotheses have emerged: is autophagy assisting in the death of cells or is it protecting the cell from it? Since autophagy promotes cell survival during nutrient starvation and it may also indirectly promote cell survival by retarding or preventing apoptosis, and it seems that the correct hypothesis in these circumstances is the second one. In the heart, autophagy is low under normal conditions but it is enhanced in

pathophysiological conditions, such as in the case of ischemia that is associated with ATP depletion and increased ROS and permeability transition pore opening [19].

Autophagy can be classified as micro-autophagy, macro-autophagy and chaperone-mediated autophagy. In micro-autophagy the lysosome itself directly take up the cargo. In chaperone-mediated autophagy the cargo makes a complex with a chaperone and this complex is recognized by the lysosomal membrane. In macro-autophagy an autophagosome delivers cargo to the lysosome and it will be referred as autophagy only [13].

This process appears to randomly degrade cargo but there is evidence that the phagophore can recognize proteins in the target cargo. It acts as a receptor interacting with an adaptor promoting a selective degradation. Selective autophagy is significant for diseases and it seems to be correlated with normal growth conditions and not associated with disease while non-selective autophagy is associated with starvation.

Autophagy plays a housekeeping role removing damaged organelles such as mitochondria. The oxidative phosphorylation occurs in mitochondria generating ATP by transfer electron to oxygen. The reactive oxygen species (ROS) production is inevitable in this metabolism and it can cause mitochondrial damage [20]. The removal of mitochondria results in a decrease of these species. The damage caused by ROS production can induce the opening of pores with high permeability leading to ATP depletion and promoting apoptosis [21]. So, mitophagy is a term used to refer to autophagy-dependent degradation of mitochondria and it is a mechanism of self-defense of the cell. It is relevant to limit ROS production and prevent the oxidation of biomolecules such as lipids and proteins. Starvation is also a mitophagy inducing factor [22]. It causes depolarization of mitochondria that move into vacuoles where they are subjected to acidic conditions. The membrane that surrounds and sequesters mitochondria after nutrient deprivation is LC3-containing [21]. Several molecules are implicated in yeast mitophagy, which are Uth1 a protein required to mitochondrial clearance and Atg32 that interacts with Atg8 and Atg11, all LC3 proteins, that act as a receptor, but there are no known homologues in mammals [23,24]. Although there is an amino acid motif required for the interaction that is conserved in p62. The existence of Parkin, an E3 ubiquitin ligase, in the mitochondria outer membrane and ability of the

UBA domain of p62 to bind to ubiquitin chains suggests that ubiquitinated molecules are required to promote selective autophagy [25].

2.1.1. Lipids in Autophagic Signaling

Lipids and particularly phospholipids have been also considered key players in autophagy. Phosphoinositides (PI) and their derivatives of PIPS formed by phosphorylation/desphosphorylation of the inositol group, have important role in cell signaling and also in autophagy. Phosphatidylinositol-3-phosphate (PI3P) for example is required for autophagy [26]. It is localized in autophagic structures and promotes negative curvature and thus controls autophagosome (AP) size. It is also highly enriched on the inner surface of phagophores and coordinate specific effectors necessary for downstream transduction [27]. However, PI(3,4,5)P3 and class I phosphatidylinositol 3-kinase (PI3K) have been shown to inhibit autophagy through activation of Akt pathway. Akt activates Rheb which in turn promotes mTOR that suppresses autophagy [28]. By contrast, in starvation, mTOR is inactivated and autophagy is positively regulated by PI(3,4,5)P3 phosphatase [29]. Class I PI3K, however, was recently shown to positively regulate autophagy by association with class III PI3K, independently of the Akt-TOR pathway, leading to increase in PI3P production [30]. So, different PI3Ks tightly regulate autophagy.

Apart from being the main precursor for most phospholipids, the phosphatidic acid (PA) has also important signaling functions. Its cone-shape is thought to directly affect membrane dynamics inducing negatively curvature, facilitating the budding or fusion of vesicles [31]. PA has been shown to activate mTOR signaling and thus inhibit autophagy by direct interaction. It was reported that the PX domain containing phospholipase D1 (PLD1), which makes PA from PC, is part of an aminoacid-sensing mTOR pathway. PLD1 binds primarily to PI3P and PI(3,4,5)P3 on lysosomes thereby stimulating PLD1 activity. This binding occurs in lysosomes to ensure that PA is made in the proximity of mTOR [32]. On the contrary, it was reported that starvation induces PLD1 activity, increasing PA that activates mTOR and inhibits autophagy. It was also found that PLD1, under starvation, relocalize to autophagosome membrane suggesting that PA would be generated on autophagosomal membrane [33]. Additional investigations are required to find out if the role of PA is through its conversion in other

lipids, if it is required for membrane curvature or if PA recruits proteins involved in autophagy [26].

PA acts as mTOR activator and for that inhibits autophagy. But on the other hand, PLD1 produces PA and it is required for autophagy. These contradictory facts could be explained by a rapid turnover of PA to other lipids. Diacylglycerol (DAG) for example has been shown to induce autophagy [34]. DAG activates protein kinase C δ (PKCδ), which in turn activates c-Jun N-terminal kinase (JNK). JNK phosphorylates Beclin1 inhibitor Bcl-2 to cause Bcl-2 dissociation resulting in activation of autophagy by the class III PI3K [35].

Ceramide (Cer) and Sphingosine-1-phosphate (S1P) that can be formed by hydrolysis of sphingomyelin both induce autophagy [36]. They inhibit mTOR but at distinct steps resulting in different outcomes. Ceramide induces cell death with autophagic features [37], while S1P induces autophagy that protects from cell death [38]. Ceramide acts upstream of S1P and triggers a stronger autophagic response not affected by starvation. It inhibits class I PI3K/Akt pathway causing Bcl2 to dissociate from Beclin1 by JNK [39]. Several autophagy genes are regulated by transcription factor FOXO3, which is inhibited by Akt. Ceramide relieves this inhibition promoting autophagy [40]. The role of ceramide could also be structural since it favors negatively membrane curvature [27]. Sphingosine kinase 1 (SK1) produces S1P which increases autophagy by inhibition of mTOR, independently of Akt [38]. The activity of SK1 increases during starvation. This leads to starvation-induced autophagy. Depletion of sphingosine-1-phosphate phosphohydrolase (SPP1) was also shown to increase S1P levels and induce autophagy independently of mTOR [41].

Cardiolipin (CL) is mainly found in the inner membrane of mitochondria being important not only for its normal function but also to apoptosis [42]. The BH3-only protein ApoL1 causes cell death by sequestering PA, which activates mTOR, and CL, which activates mitochondria-mediated apoptosis. This alters the balance between survival and death leading to autophagic cell death [43]. Apoptosis will be discussed later. The human immunity-related GTPase M (IRGM) is another CL-binding protein. IRGM affects mitochondrial fission to induce autophagy. It can either confer autophagic protection or cell death. It is known that knockdown of IRGM causes abnormal

elongation of mitochondria thus inhibiting autophagy [44]. However, a recent study reports that mitochondria are elongated in the course of starvation [45].

2.1.2. Lipid alteration related to autophagy

Lipids are also targets for autophagic degradation, a process called lipophagy. Lipids are stored in the cell in the form of lipid droplets (LDs) that consist of cholesterol and triglycerides (TG) surrounded by a phospholipid monolayer. These lipids stored in LDs can be mobilized to generate energy when needed. In fact, LDs tend to be accumulated as a cause of disruption of autophagy. The degradation of LDs through lipophagy can also be stimulated by starvation. It is known that lipid metabolism is affected in the course of starvation but it is yet to know if lipophagy depends on a LD-specific cargo receptor as in other selective autophagy processes [46]. A study using hepatocyte-specific Atg7-knockout mice revealed that high lipid loads seem to inhibit autophagy resulting in a further lipid accumulation. Since these mice have a very similar phenotype to that of human fatty liver disease, this negative feedback contributes to diet-induced fatty liver disease [47]. The accumulation of LDs is also associated with several other metabolic diseases including diabetes [48].

LDs are the major site of cholesterol storage that in the form of cholesteryl esters are associated with atherosclerosis. Recent studies show that autophagy is activated upon exposure to oxidized LDL. This stimulates delivery of cholesterol to lysosomes where it is degraded thereby generating free cholesterol for efflux and transport to the liver. Autophagy is important in cholesterol efflux because Atg5 macrophages have a reduced ability to clear accumulated cholesterol [49].

In contrast to the liver, inhibition of autophagy in adipocytes prevents the accumulation of TG. Mice with adipocyte-specific knock-out of Atg7 have decreased white adipose tissue mass and a switch to production of brown adipocytes. The increased brown adipocytes prevent accumulation of lipids in other organs such as the heart. Thus, inhibition of autophagy in adipocytes may be beneficial. However, the dual role need more studied before autophagy can be targeted as a potential therapy for disorders such as atherosclerosis [50,51].

2.2. Apoptosis: a programmed cell death

Apoptosis was first described in the twenty century as a mechanism of controlled cell deletion, which appears to be complementary but opposite to mitosis in the

regulation of cell population [52]. In current days we know apoptosis as the most meticulously form of programmed cell death. It is considered a central component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death [16].

There are two major apoptotic signaling pathways: the intrinsic pathway and the extrinsic pathway. The main difference between them is the involvement of Bcl-2 protein family that only regulates the intrinsic pathway. The Bcl-2 family have been gathered in three classes: the first inhibits apoptosis as Bcl-2, Bcl-x and Mcl-1; the second induces apoptosis as Bax and Bak; the third is BH3-protein as Bad and Bid, which enhances apoptosis by regulation the anti-apoptotic class relieving the inhibition of Bcl-2 leading to the promotion of apoptosis [53].

A wide variety of signals, including hypoxia, activate the intrinsic apoptotic pathway [16]. The signal starts to activate p53 which binds to DNA promoting the transcriptional upregulation of target genes, for instance apoptotic gene Bax. Bax oligomerization in the outer mitochondrial membrane (OMM) leads to mitochondrial outer membrane permeabilization (MOMP) and release of proteins from intermembrane space to the cytoplasm [54]. Several pro-apoptotic proteins are released such as AIF neutralizing inhibitors of apoptosis proteins (IAP). AIF translocate to the nucleus and causes DNA fragmentation and condensation of chromatin [55]. Another protein released is cytochrome c that is able to bind to apoptotic protease in the presence of ATP and activate factor-1 [53]. This process develops a complex named apoptosome which recruits procaspase 9 that after being activated to caspase 9 by cleavage of the prodomain recruits in turn the procaspase 3. Caspases (**c**ysteine-utilizing **asp**artate-cleaving prote**ases**), as described by their designation, are proteases with cysteine in the active site. Caspase 3 activated is the straight responsible for cell death. It activates others proteins which produce the characteristic changes in an apoptotic cell. Some of these changes are plasma membrane blebbing, cell shrinkage, DNA fragmentation, chromatin condensation and apoptotic body formation [56].

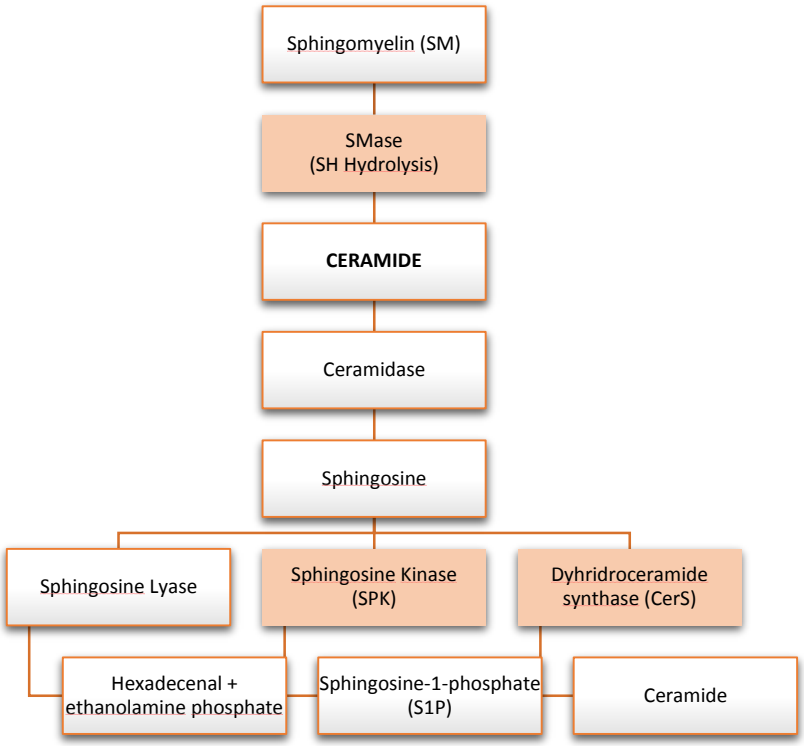
The activation of the extrinsic apoptotic pathway is the responsibility of death ligands with no involvement of Bcl-2 family. The death ligands interact with the respective receptor and trigger a cascade of events. Fas ligand and TNF-α are the death

ligands that interact with two different receptors on the plasma membrane. Although the ligand and the receptor are different, both recruit procaspase 8 that is autocatalytic activated. Caspase 8 in turn recruits procaspase 9 that is activated to caspase 9 which recruits procaspase 3. Caspase 3 activated produces the same results as in the intrinsic pathway resulting in characteristic changes in the apoptotic cell [56].

2.2.1. Apoptotic-induced Changes in Lipids

The apoptotic machinery is based on proteins. Additionally, it has been suggested crucial functions for lipids in apoptosis and cooperation between lipid metabolism and BCL-2 proteins [57]. It is reported a membrane important role in propagation of the cell death response [58]. The two key cases are cardiolipin and sphingolipid metabolism.

Ceramide is the hub of sphingolipid metabolism. The major pathways of ceramide biosynthesis are de novo synthesis, sphingomyelin hydrolysis and sphingosine salvage [59]. In order to know how sphingolipid metabolism accommodates key factors in apoptosis, it is important to exam the enzymes surrounding ceramide metabolism. The three fundamental enzymes are sphingomyelinase (SMase), dihydroceramide synthase (CerS) and sphingosine kinase (SPK), highlighted in Scheme 3.



Scheme 3. Sphingolipid biosynthesis. Ceramide is generated via three major pathways including SM hydrolysis by sphingomyelinase (SMase). Ceramidase (CDase) degrade ceramide to sphingosine, which can be irreversibly degraded by sphingosine lyase to ethanolamine phosphate and hexadecenal, phosphorylated by sphingosine kinase (SPK) to sphingosine-1-phosphate (S1P) or acylated by dihydroceramide synthase (CerS) to regenerate ceramide.

Bcl2 family-dependent apoptosis via endogenous SMase-mediated ceramide elevation has been observed in different cell types in response to diverse set of signals [57]. The antibiotic minocycline protected rat cortical neurons against bacterial sphingomyelinase (bSMase) by up-regulating BCL-2 expression [60]. Ceramide elevation and BAX translocation were also observed upon hypoxia/reoxygenation (H/R) of NT-2 human neuronal precursor cells [61]. Treatment of leukemia cells with the anticancer agent led to an increase in ceramide level where the first peak corresponded to SM hydrolysis and the second peak was attributable to CerS [62]. Mitochondria-specific ceramide pool is necessary for mediating the TNFα apoptotic response in MCF-7 cells [63]. SMase was also indispensable for UV-induced BAX conformational change at the mitochondria of several cell types. It was not observed any UV-induced BAX conformational change neither cyt c released in cells with SMase compromised activity [64].

Ceramidases (CDases) degrade ceramide to sphingosine, which can be irreversibly degraded by sphingosine lyase to ethanolamine phosphate and hexadecenal. This is phosphorylated by sphingosine kinase (SPK) to sphingosine-1-phosphate (S1P) or acylated by dihydroceramide synthase (CerS) to regenerate ceramide. There are six mammalian CersSs. BCL-2 family-regulated apoptosis via endogenous CerS-mediated ceramide elevation has been observed in multiple cell types in response to a wide range of stimuli. A requirement for CerS in BCL-2 protein-dependent caspase activation was also observed [65]. Ceramide elevation preceded BAX activation, and inhibition of CerS led to reduced BAX insertion, oligomerization and cyt c release, implying UV-induced ceramide synthesis is required for BAX-dependent apoptosis [66]. SPK has two known mammalian isoforms. SPK1 and 2 have opposing cellular functions. While SPK1 activation leads to pro-survival phenotypes, SPK2 enhances apoptosis in multiple cell types. Down-regulation of SPK2 lead to decreased sphingosine conversion to ceramide while down-regulation of SPK1 has the opposite effect. In endoplasmic reticulum (ER) SPK1 promoted apoptosis suggesting that S1P role depends on its localization [67].

Changes in the expression of BCL-2 proteins, particularly Bax, can alter apoptotic sensitivity by modulation of sphingolipid metabolism. BCL-2 overexpression inhibited SMase activity, ceramide production, cyt c release and caspase activation [68]. It was reported the assembly of ceramides into channels to facilitate protein release in the apoptotic program. Ceramide channels can interact with BCL-2 proteins demonstrating that ceramides and BAX acted synergistically to promote membrane permeabilization [69].

Cardiolipin is found exclusively in mitochondrial membrane. CL exerts control over the mitochondrial apoptotic pathway primarily through the BH3 domain-only messenger BID. An intact BID is necessary for interaction with the BH1 domain of either BAX or BCL-2, and induced BID expression promoted apoptosis. BID was identified as caspase-8-dependent cyt c releasing factor. BID can be cleaved in response to Fas and TNF signaling. The resulting fragment cooperates with Bax to promote membrane permeabilization and cyt c release [70]. Among several roles, CL acts as a docking site for Bcl-2 proteins and caspase-8 [71]. Additionally, CL acts as an anchor for cytochrome c. Cyt c is anchored to the inner mitochondrial membrane (IMM)

between complex III and IV of respiration chain [72]. CL peroxidation allows cyt c release from IMM and may occur due to ROS, for example. The superoxide generation is an essential step towards cyt c release and depends on Bcl-2 proteins [73].

2.3. Exosomes

Exosomes are becoming popular since they have been recognized as important mediators in cell-cell signaling events. First it was though that extracellular vesicle (EV) bud directly from the plasma membrane but in the early 80s a study described a more complex mode of EV secretion. It was shown that small vesicles were formed inside an intracellular endosome, leading to multivesicular body (MVB) formation with subsequent fusion with the plasma membrane [74]. In 1987s the word "exosomes" was proposed for these EVs of endosomal origin. The term exosomes will be used as defined by Rose Johnstone and not the more general use for any vesicle released by cells [75].

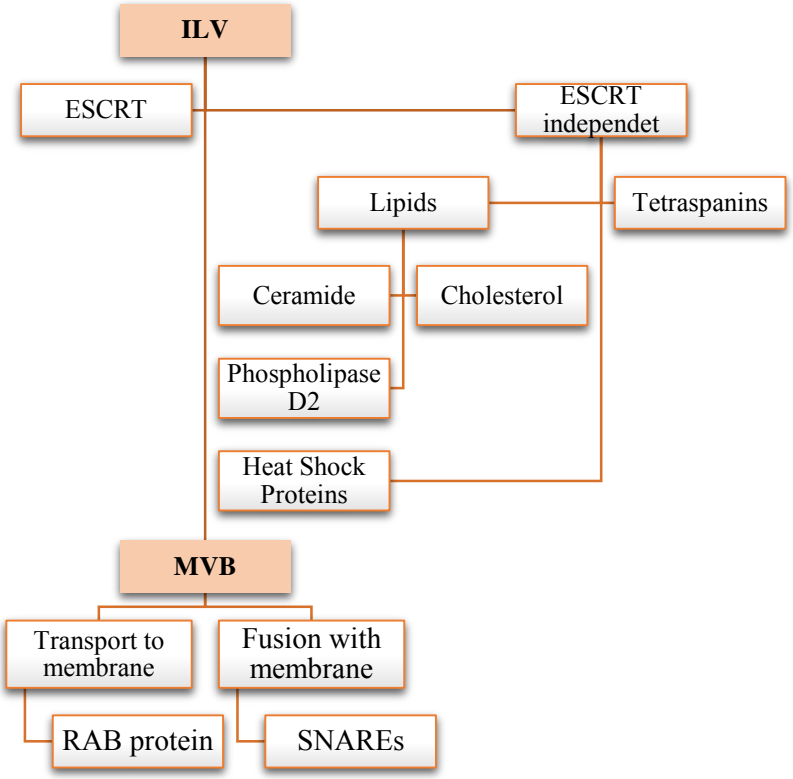
Secreted exosomes are biologically active entities that play specific biologic roles [76]. Exosomes are an alternative to lysosomal degradation of proteins that are resistant to degradation by lysosomal proteases since they are secreted to discard membrane proteins useless in the cell [14]. They play an immunological mediator role, for example, exosomes secreted by Eppstein-Barr virus (EBV)-transformed B cells stimulate human $CD4^+$ T-cell clones in an antigen-specific manner [77]. Meanwhile, exosomes produced by dendritic cells (DC) have also an immunological role. After the pathogen uptake, immature and mature DC, generate MHC complexes and some of these complexes could be secreted on exosomes [78]. These exosomes sensitize others DC that have not encountered the pathogen directly, increasing the number of DC that bear the relevant MHC complexes and thereby amplify the magnitude of immune response [14,78]. Exosomes are capable of intercellular transport of functional DNA and RNA. The horizontal transference of genetic material between cells induces exogenous expression and mediates RNA silencing [14,76,79].

Proteomic studies revealed that exosomes contain a specific subset of proteins from endosomes, the plasma membrane and the cytosol [80]. Different cell origin results in different composition. So, exosomes do not contain a random set of protein but represent a specific subcellular compartment [80]. Although investigations of the lipid composition of exosomes were not as recurrent as proteomic analyses, the few studies published so far revealed that exosomes have differences in lipid composition when

compared to the cell membranes. Thus, the lipid profile of exosomes were studied and reported to be constituted mainly by glycosphingolipids, sphingomyelin, cholesterol, ceramide and phosphatidylserine [81]. Some of these lipids and proteins are enriched in lipid rafts and, indeed, there is a link between endocytosis of lipid rafts and secretion into exosomes [82,83].

The exosomes biogenesis process is divided into MVB formation mechanisms, transportation of MVB to the plasma membrane and fusion of these MVB with the membrane (Scheme 4). The intraluminal vesicles (ILV) are formed by inward budding from the limiting membrane. The molecular mechanism involved in biogenesis includes endosomal sorting complex required for transport (ESCRT) machinery, lipids and tetraspanin. It is not known if act simultaneously on the same or on different MVBs. The ESCRT consists of four complexes in which ESCRT-0 is responsible for cargo clustering, ESCRT-I and II induce bud formation and ESCRT-III drives vesicle scission. The VPS4 ATPase, an accessory protein, allows the dissociation and recycling of the ESCRT machinery [82]. Several studies have showed that cells with depletion of ESCRT key component still form MVBs, thus this suggests an ESCRT-independent mechanism [84]. Lipids are key players in the molecular mechanism to exosomes biogenesis. Ceramide was proposed to induce inward curvature of the limiting membrane of MVBs to form ILVs. Cholesterol is an important component of MVBs and it accumulation was shown to increase the secretion of vesicles. Phospholipase D2, enriched in exosomes, is involved in hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA). PA, as described for ceramide, induces inward curvature and thus formation of ILV. Proteins are also involved in the molecular mechanism of exosomes biogenesis. Tetraspanins are four-transmembrane domain proteins enriched in ILVs of MVBs and exosomes, and are responsible for select cargos for exosome secretion. TSPAN8 is an example that modifies both the mRNA content and the protein composition of exosomes. The HSC70, a heat shock protein, allow the recruitment of the transferrin receptor (TFR) to exosomes [82].The RAB family of small GTPase protein controls different steps of intracellular vesicular trafficking such as vesicle budding and docking of vesicles to their target compartment ultimately leading to membrane fusion. These RAB proteins function is docking MVB to the plasma membrane, which is a required process for eventual fusion. After docking, soluble NSF-

attachment protein receptor (SNARE) complexes are instrumental in allowing fusion of the lipid bilayers. However, SNAREs do not necessarily mediate the fusion of MVBs with the plasma membrane during exosome secretion [82].



Scheme 4. Schematic representation of the machineries of exosomes biogenesis and secretion.

During stress conditions, the cell tends to segregate exosomes in order to protect the cell from apoptosis. A high level of exosomes is detected in cardiovascular diseases which make them potential indicators for early diagnostics [85]. While protein modulation in exosomes under cellular stress is known, the role of phospholipids is still overlooked and deserves to be explored.

2.3.1. Molecular Lipidomic of Exosomes

The molecular lipid composition of exosomes is largely unknown. Recent studies have been shown lipid profile of exosomes released from some cells, for example prostate cancer cells [81]. In this study, it was quantified approximately 280 molecular lipid species. Exosomes showed enrichment in distinct lipids such as glycosphingolipids, sphingomyelin, cholesterol and phosphatidylserine. Additionally, this study also shows that cholesterol, long-chain sphingolipids, and PS 18:0/18:1 are

enriched to a similar extent in exosome membranes. The analysis of lipid species helps to understand the biogenesis of exosomes, their stability in the extracellular environment and/or the interaction of exosomes with other cells. Thereby, the high content in glycosphingolipids, for example, is related to exosome role in cell-to-cell communication since it confers exosomes with the stability needed for extracellular environments [86]. The great enrichment of specific glycosphingolipids such as HexCer and LacCer in exosomes suggests that these lipids could potentially be used as cancer biomarkers [81].

It has also been shown that exosomes from mast cells have the ability to induce phenotypic and functional maturation of DCs [87]. Since the mechanisms underlying these immunological properties are not clearly understood, the characterization of lipids present on exosomes could help to elucidate them. Exosomes released from DC have phospholipids and cholesterol as major components of the membrane. It was also showed an increase in SM content in exosome compared to the cell. Molecular species of the major phospholipids, PC and PE, were analyzed. The results showed an increase in saturated species. The high content of SM and the di-saturated molecular species are known to affect lipid packing in membranes. In terms of cholesterol, it was identical with that of presented in cells [88]. It has also been observed in this study that the proportion of LPC is relatively high in exosomes. The presence of LPC might be essential for the activity *in vivo*, since LPC trigger DC maturation [89].

2.3.2. Exosome as an Agent in Cardiac Repair

It is known that myocardial infarction is a leading cause of death. Even though there are some therapeutically approaches to cardiac damage, there is a need for novel treatments to reduce subsequent cardiac remodeling that can adversely affect heart function. How the myocardium initiates the local repair process post-MI or how it manipulates the bone marrow (BM) environment to induce stem cell mobilization are question yet to respond. The exosomes seems to come as a possible response to the questions above [85].

A major component of the healing response is scar formation, rather than muscle regeneration. This is due to the limited ability of the heart to rapidly response [90]. However, the limited endogenous reparative mechanisms seem to depend more on the replenishment by progenitor cells than on replacement by cardiomyocytes proliferation

[91]. It was suggested that the release of stem and progenitor cells by BM could help in the turnover of vascular endothelium and myocardial repair response after MI [92]. MI induces rapid mobilization of BM-derived stem cells (BM-SCs). These are related to reparatory response and it was already reported an important role of BM-SCs in cardiac repair [92]. In MI, the levels of soluble inflammatory mediators, cytokines, and growth factors are significantly increased. They are release by ischemic tissue to mobilize BM-SCs to them. So, these molecules act as chemoattractants [92]. The complete mechanism of BM-SC mobilization is much more complicated because other signaling pathways and mechanisms, including exosomes and miRNAs, could promote trafficking and engraftment of BM and cardiac SCs.

Cardiomyocytes are generally not considered a typical secretory cell but in addition to several factors can release exosomes. This exosomes may provide the underlying mechanisms by which the damaged heart communicates with other tissues to initiate the repair process, and how stem/progenitor cells repair and regenerate the myocardium [93]. Once secreted, exosomes either interact with surrounding cells or can be released to the systemic circulation [94]. Exosomes from a specific cell of origin can selectively bind and be internalized by certain target cell types. However, the cellular and molecular basis for this targeting is still undetermined. After endocytosis, exosomes can fuse with the endosomal membrane or can be targeted to lysosomes for degradation. They mediate communication through transfer of proteins, mRNA, miRNA [95].

It is suitable to propose that exosomes can be important communicators of ischemic signaling and myocardial repair. Few reports have suggested that cardiomyocytes secrete exosomes under healthy and ischemic conditions and that they are involved in communication in the heart [96]. For the first time, it was provided evidence that exosomes are secreted by progenitor cells. Moreover, it was also shown that cardiomyocytes are able to uptake exosomes [96]. An *in vivo* study [85] suggested that exosome secretion from the cardiomyocytes was significantly increased under hypoxic stress. Exosomes are known to target via the transfer of proteins or genetic materials such as mRNA and miRNAs. This suggests that exosomes generated by cardiomyocytes are able to transfer proteins and genetic material to other cells. However, it is not clear whether exosomes have any physiological function for example, in heart remodeling and repair [85].

Recent studies demonstrate that cardiac and circulating miRNAs are markedly altered after MI [97]. The muscle-specific miR-1 and miR-133, for example, are increased in the serum of patients with acute coronary syndrome. These miRNA levels correlate with serum cardiac troponin T levels. They have shown that the origin of this miRNA is the infarct zone and that this miRNA released in exosomes from H9C2 cells [98]. Additionally, a cardioprotective miR-214 has been shown to be upregulated in the heart after ischemia and to be secreted via exosomes from human endothelial cells [99]. As these miRNAs are released even before the release of cardiac troponin T, these could function as biomarkers for the early detection of acute MI [85]. Interestingly, several reports suggest circulating miR-126 to be an important miRNA to indicate the damage and repair mechanisms in acute MI patients [100]. Circulating human $CD34^+$ stem cell–derived exosomes are enriched with miR-126 [101]. The miR-126 is reported to induce the expression of CXCL12, promoting the recruitment of progenitor cells by a feedback mechanism [102]. Further experimental studies are necessary to explore the mechanism behind of which MI affect circulating exosomal miRNA levels.

Recent study proposed a novel mechanism by which circulating exosomes from a tumor could crosstalk, reprogram, and permanently educate the BM progenitor cell to mobilize out of the BM [103]. This shows the complexity of the cargo carried by exosomes and its enormous potential to directly influence the biology of distant microenvironments. Although this work relies on cancer, it has obvious potential implications in cardiology. Several recent reports demonstrate that acute MI modulates the miRNA expression of BM cells both in humans and in mice [104]. Other reports suggest that cardiac ischemia mobilizes BM mononuclear cells via downregulating the expression of miR-150 activating CXCR4 in BM mononuclear cells [105]. Collectively, this evidence strongly supports that cardiac exosomes released after ischemic insults affect the BM microenvironment to reprogram the BM cells and initiate a repair process [89].

Exosomes are vesicles recognized as an important intercellular system of communication between cells and tissues. The biological role of exosomes seems to be dependent not only on the protein and RNA content, but also on the bioactive lipid content. They have been reported in all biological fluids and can be associated with specific pathophysiological situations [106]. Thus, it is important to recognized lipid

profile of exosomes and it variations depending on the environmental and pathophysiological conditions.

3. Lipid profile and Lipid Oxidation in Cardiovascular Diseases

Lipids are a group of biomolecules that embraces a diversity of molecules with different structural features and different functions. The International Lipid Classification and Nomenclature Committee describes lipids as "hydrophobic or amphipathic small molecules that may originate entirely or in part by carbanion based condensation of thioester (fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, and polyketides) and/or by carbocation-based condensations of isoprene units (prenol lipids and sterol lipids)". These eight lipid categories are further subdivided on the basis of chemical structures (Figure 3) [107].

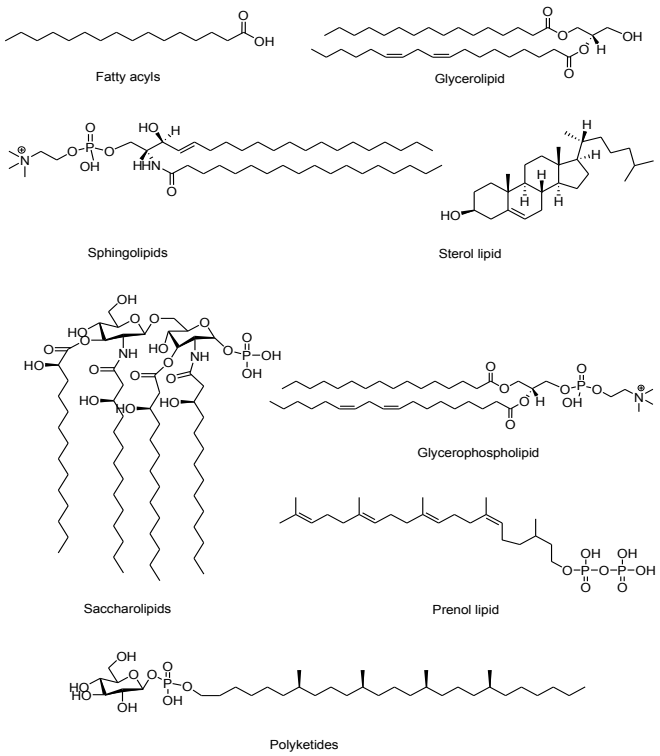


Figure 3. Lipid categories according to the LIPID MAPS lipid-classification system.

Lipids, namely phospholipids (PL), are the major component of a cell membrane. In addition to that structural function, lipids play multiple crucial roles within cells, such as energy storage and as substrates for the synthesis of signaling mediators. Several studies reported an importance of a specific lipid environment to a proper function of membrane protein [108]. Different types of assemblies have been described for the interaction of membrane proteins with lipids. Although some proteins depend on being

surrounded by a specific lipid shell, some multimeric proteins specifically bind individual lipids that are important for the structure of these protein complexes [109]. Recent studies suggest that lipids can also act as protein co-factors modulating their activity [110].

However under several physiopathological conditions, lipids can be modified by reactive oxygen species (ROS) that are generated from life processes and can cause disturbance of pro and antioxidant systems. This oxidative stress is responsible for basic oxidation reaction mechanisms. Lipids are primary targets of ROS attack [111]. Lipid peroxidation leads to the formation of a variety of oxidized products mainly due to the modifications of polyunsaturated fatty acyl chains. When the oxidation is nonenzymatic it is expected as the result several new products with new bioactivities, some deleterious to cells and tissues. On the contrary, when the oxidation is enzymatic it is a highly regulated physiological event involving receptors and intracellular signaling. These oxidized products are generated by proteins such as lipoxygenases (LOX) and cyclooxygenases (COX) existing in all mammalian species. In this case lipids have a role in immunity and homeostasis [112].

Oxidative stress, which is associated with lipid peroxidation, is a key factor in atherosclerosis. Oxidized phospholipids (OxPL) play a significant role in atherosclerosis [113]. An important marker of oxidative stress is the association of oxidized phospholipids with the apolipoprotein B-100. Increased levels of OxPL/apoB are implicated in coronary artery disease, progression of atherosclerosis and prediction of new cardiovascular events [114]. Other studies have demonstrated increased levels of auto-antibodies against oxPL in patients with hypertension and myocardial infarction [115]. Thus, oxPL could be used as a biomarker for cardiovascular diseases.

Biologically active oxidized phospholipids can initiate and modulate many cellular events. In aortic endothelial cells, OxPL modulate the expression of a variety of genes related to angiogenesis, atherosclerosis and inflammation [116]. Additionally, oxPL can activate platelets, inducing differentiation of monocytes and de-differentiation of smooth muscle cells. These are all processes related to atherosclerotic plaque formation. They also can modulate the fate of an inflammatory response by intervening in the processes by removing apoptotic cells [117]. Nowadays it is recognized the importance

of oxidized lipids as a key players in the onset of cardiovascular diseases and oxidized lipids have been considered as possible biomarkers for cardiovascular disease [115].

4. Lipidomic Approach in Diagnosis of Cardiovascular Diseases

Lipidomic is the analysis of molecular lipid species, including their quantitation and metabolic pathways [118]. It may be used to risk assessment and therapeutic monitoring due to the diversity of roles that lipids play in distinct cellular functions. Lipid imbalance or dysregulation result in consequences for multiple biological functions, culminating in the development of disease. Since lipids are involved in the pathophysiology of several diseases, lipidomics has the potential to improve prediction of future disease risk, inform on mechanisms of disease pathogenesis, identify patient groups responsive to particular therapies and more closely monitor response to therapy [119]. Lipidomic came as an alternative to genetic, epigenetic and gene expression measures which are further upstream in disease aetiology. The method of choice for identification of lipidomic biomarkers is mass spectrometry, either by direct infusion or coupled with liquid chromatography [118]. In what concern cardiovascular disease, the majority of studies reported alteration in lipids from plasma as toll for the identification of biomarkers of CVD [120]. Plasma lipidomics provide a detailed picture of the dysregulation of lipid metabolism. It has been used to identify lipidomic biomarkers associated with a variety of diseases including type 2 diabetes and cardiovascular diseases [120]. However the work concerning lipid changes in cardiomyocytes and exosomes released by them are absent.

Dysfunctional lipid metabolism is associated with atherosclerotic cardiovascular diseases. The stability of atherosclerotic plaque is important since its disruption is an important cause of vascular events. Lipids may act to promote plaque instability and rupture and whether plasma lipid analysis may provide insight into this process [119]. A plaque at risk of rupture has a marked abundance of both extra- and intra-cellular lipid, mainly cholesterol either esterified (CE) or unesterified (UC). The phospholipid content is similar to stable plaques and include essentially PC, PI, LPC and SM. The total classes of lipids observed are quite different as it were observed in unstable plaques approximately half of the classes observed in stable plaques [121].

Most of the published works are concerned with finding new plasmatic biomarkers associated with cardiovascular disease. However there are few who report changes at

the cellular level, including cardiomyocytes, and there are no studies on the lipid signaling carried by exosomes.

5. Aims

Phospholipids and phospholipid oxidation products have been considered important players in cardiovascular diseases that are associated with ischemia, which occurs in consequence of hypoxia and deprivation of nutrient to the cardiomyocytes. However, there is a lack of knowledge on complete picture of the physiopathological process that underlies ischemia and also how recovery occurs, by selected processes such as autophagy. Since lipids, and particularly phospholipids, are important components of cells membranes involved in cell death and survival and as signaling molecules, it is important to understand lipid profile alterations in cell during starvation and hypoxia.

The possible role of phospholipids in cardiomyocytes under these conditions encouraged us to explore and thus the major aims of this work will be:

a) to identify the change in lipid profile of a myoblast cell line H9c2 under starvation and under ischemic conditions;
b) to identify the changes in lipid profile of exosomes, released by myoblast cell line H9c2, under starvation and under ischemic conditions.

Mass spectrometry based approaches are becoming increasingly important in lipid research allowing us to assess the changes on phospholipids caused by stress or pathological conditions. It will be used a lipidomic approach to identify the above reported variation in lipid profiling. The results that will be achieved intend to clarify the mechanism of modulation of lipid profile during starvation and ischemia and to evaluate the cell fate under such conditions. This will allow us to recognize new lipid biomarkers for ischemia and starvation associated with disease such as myocardium infraction.

CHAPTER II:

MATERIAL and METHODS

1. Chemicals

Phospholipid internal standards (1',3'-bis[1,2-dimyristoyl-*sn*-glycero-3-phospho]-*sn*-glycerol (CL), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (dMPC), 1-nonadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (dMPE), 1,2-dimyristoyl-*sn*-glycero-3-phosphate (dMPA), 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (dMPG), 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine (dMPS) and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1'-myo-inositol) (dPPI)) were purchased to Avanti polar lipids, Inc (Alabaster, AL, USA). Primuline were purchased from Sigma (St Louis, MO, USA), triethylamine and potassium hydroxide were purchased from Merck (Darmstadt, Germany) and perchloric acid from Panreac (Barcelona, Spain). Acetonitrile; chloroform, methanol and hexane from Fisher Scientific (Leicestershire, UK) were of HPLC grade and were used without further purification. All other reagents and chemicals used were of highest grade of purity commercially available. Mili-QH_2O used was filtered through a 0.22-mm filter and obtained using a Milli-Q Millipore system (Milli-Q plus 185).

2. Cell Culture

The myoblast cell line H9c2 (Sigma-Aldrich, St. Louis, MO) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA), supplemented with 10% FBS, 1% Penicillin/Streptomycin (100 U/mL:100 µg/mL) and 1% GlutaMAX (Life Technologies, Carlsbad, CA), at 37ºC under 5% CO2. Metabolic ischemia was induced by a buffer exchange to an ischemia-mimetic solution (in mM: 118 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 1.2 CaCl2, 25 NaHCO3, 5 calcium lactate, 20 2-deoxy-D-glucose, 20 Na-HEPES, pH 6.6) and by placing the dishes in hypoxic pouches (GasPakTM EZ, BD Biosciences), equilibrated with 95% N2/5% CO2. Cells were stimulated with, unless otherwise stated, 2.5 µg/mL of exosomes in exosome-depleted medium, for the indicated time periods. Starvation was induced by growing cells during 24 hours in a complete medium without serum.

3. Exosomes Extraction

The exosomes extraction was performed using an adaptation of two experimental protocols [122,123]. Serum was depleted of exosomes by ultracentrifugation at 154.000 g, for 16 hours, in a 1:1 dilution to minimize serum proteins loss due to its viscosity.

Exosomes derived from cultured cells were isolated from conditioned medium after culture in either exosome-depleted medium or ischemia-mimetic solution, for x hours. Afterwards, the medium was collected and exosomes were isolated by ultracentrifugation64. In brief, the supernatants were subjected to differential centrifugation at 4°C, starting with a 300 g centrifugation, for 10 min followed by a 16.500 g centrifugation for 20 min. To remove cellular debris and larger particles, the supernatants were filtered with a 0.22 μm filter unit, and then ultracentrifuged at 120.000 g, for 70 min. The resultant pellet was washed with phosphate buffered saline (PBS) (1.5 mM KH2PO4, 155 mM NaCl and 2.7 mM Na2HPO4.7H2O pH 7.4) and, after a second ultracentrifugation, exosomes were ressuspended in PBS (sterile when used for biological assays).

4. Lipid Extraction

The pellet of cells was re-suspended in 1 mL of Ultra-pure water (Mili-QH_2O). Thereafter, total lipids were extracted using the Bligh and Dyer method [124]. Briefly, 3.75 mL of a mixture of chloroform/methanol 1:2 (v/v) was added to the cell homogenate, vortexed very well and incubated on ice for 30 min. An additional volume of 1.25 mL chloroform was added followed by vortex. Then, 1.25 mL mili-QH_2O were added also followed by strong vortex. The samples were centrifuged at 2000 rpm for 5 min at room temperature to obtain a two-phase system: an aqueous top phase and an organic bottom phase. The lipid extract was recovered from the organic phase (bottom phase). In order to guarantee full extraction of lipid phase, 1.88 mL of chloroform were added to the aqueous phase followed by vortex and new centrifugation. The organic phase was recovered to the same tube as before and dried in the speedvac (UNIVAPO 100H, Reagente5 – Química e Electrónica Lda.). After drying, the total lipid extracts were re-suspended in 300 μL of chloroform, transferred to a smaller tube, dried under nitrogen stream and stored at -20ºC. Three or different cultures were extracted and analyzed in order to verify the reproducibility of the results.

5. Quantification of Phospholipids

In order to determine the PL amount in each extract, the phosphorus measurement was performed according to Bartlett and Lewi [125]. Briefly, 125 μL of percloric acid (70%) was added to standards and samples. Samples were incubated for 1h at 170°C in

a heating block (Stuart, U.K.), followed by cooling to room temperature. Phosphate standards from 0.1 to 2 µg of phosphorous (P) were prepared from a phosphate standard solution of dihydrogenphosphate dihydrated ($NaH_2PO_4.2H_2O$) with 100 µg/mL of P. Thereafter, 825 µL of MilliQ water, 125 µL of ammonium molybdate (2.5 g ammonium molybdate/ 100 mL MilliQ water) and 125 µL of ascorbic acid (10 g ascorbic acid/ 100 mL MilliQ water) were added to samples and standards, vortexed well following the addition of each solution and incubated for 10 min at 100°C in a water bath. After cool in a cold water bath, the standards and samples absorbance were measured at 797 nm in a microplate reader (Multiscan 90, ThermoScientific). These experiments were performed at least in triplicate, from different cell culture extracts (at least n=3 independent experiments).

6. Thin-Layer Chromatography (TLC)

Separation of several PLs classes from the total lipid extracts was achieved by TLC. Prior to separation, TLC plates (silica gel 60 with concentration zone 2.5x20cm) were washed in a methanol: chloroform mixture (1:1, v/v) and left in the safety hood for 15 min, then plates were sprayed with 2.3% (m/v) boric acid and dried in an oven at 100ºC during 15 min. Then 30 µg of total lipid extract dissolved in chloroform was applied on the TLC plates. The plates were dried with a nitrogen flow and developed with a mixture chloroform/ethanol/water/triethylamine (30:35:7:35, v/v/v/v). Lipid spots on the silica plate were observed and identified by spraying the plate with 50µg/100mL primuline solution dissolved in a mixture of acetone and mili-QH_2O (acetone:water; 80:20(v/v)), and visualized with a UV lamp (λ=254nm). Identification of PL classes was carried out with the use of phospholipid standards (SM, PC, PI, PS, PE, and CL), which run side by side in the TLC plate. Following separation the spots were scraped from TLC plates and used for quantification by phosphorus assay as previously described.

7. High Performance Liquid Chromatography Mass Spectrometry (HPLC-MS)

HPLC-MS was performed with an internal standard to confirm and quantify the ions variations observed in the spectra. The PL standards used were PC (14:0/14:0), LPC (19:0), PI (16:0/16:0), PE (14:0/14:0), PA (14:0/14:0), PG (14:0/14:0), PS (14:0/14:0), SM (17:0/d18:1) and CL (14:0/14:0/14:0/14:0). PL classes were separated by HPLC–MS, performed on an HPLC system (Waters Alliance 2690) coupled to an electrospray

linear ion trap mass spectrometer LXQ (ThermoFisher, San Jose, CA, USA). The mobile phase A consisted of 10% water and 55% acetonitrile with 35% (v/v) methanol. The mobile phase B consisted of acetonitrile 60%, methanol 40% with 10mM ammonium acetate. Total lipid extract were diluted in the mobile phase B (90 µL) and 10 µL of reaction mixture was introduced into an 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 100% of B. It linearly increased to 60% of A and decreased to 40% of B during 15 min, and held isocratically for 22 min, returning, to the initial conditions in 3 min. The flow rate through the column was 60 µL/min and it was redirected to a LXQ linear ion trap mass spectrometer (ThermoFinningan, San Jose, CA, USA) by a capillary (0.350 × 0.150 mm) of 70 cm length using a home-made split. The LXQ linear ion trap mass spectrometer was operated in negative-ion mode. Typical ESI conditions were as follows: electrospray voltage, - 4.7 kV; capillary temperature, 275 ºC; and the sheath gas flow of 8 U. To obtain the product-ion spectra of the major components during LC experiments, cycles consisting of one full scan mass spectrum and three data-dependent MS/MS scans were repeated continuously throughout the experiments with the following dynamic exclusion settings: repeat count 3; repeat duration 15 s; exclusion duration 45 s. Data acquisition was carried out on an Xcalibur data system (V2.0). Relative quantification of each individual phospholipid specie was obtained by the ratio between the area of reconstructed ion chromatogram of a given *m/z* value against the area of the reconstructed ion chromatogram of the respective standard. At least three independent samples were analyzed.

8. Gas Chromatography Mass Spectrometry (GC-MS)

Fatty acid methyl esters (FAMEs) were obtained after transesterification of lipid extracts according to the method described by Aued-Pimentel et al. [126]. Briefly, dried lipid extracts (30 µg of total phospholipid) were dissolved in 1 mL n-hexane. A methanolic KOH solution (2 M) was added (200 µL), followed by intense vortex-mixing for 1-2 min. Saturated NaCl solution (2 mL) was added. After centrifugation at 2000 rpm for 5 min, the organic phase was collected and dried under a nitrogen stream. The resulting FAMEs were dissolved in n-hexane and volumes of 2.0 µL were analyzed by gas chromatography–mass spectrometry (GC–MS) on an Agilent Technologies 6890

N Network (Santa Clara, CA) equipped with a DB-FFAP column with 30 m of length, 0.32 mm of internal diameter, and 0.25 μm of film thickness (J&W Scientific, Folsom, CA). The GC equipment was connected to an Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the range m/z 50–550 in a 1 s cycle in a full scan mode acquisition. The oven temperature was programmed from an initial temperature of 80 °C, a linear increase to 160 °C at 25 °C min^{-1}, followed by linear increase at 2 °C min^{-1} to 210 °C, then at 30 °C min^{-1} to 250 °C and stays here for 10 min. The injector and detector temperatures were 220 and 280 °C, respectively. Helium was used as carrier gas at a flow rate of 0.5 mL min^{-1}. The relative amounts of FAs were calculated by the percent area method with proper normalization considering the sum of all areas of the identified FAs.

9. Statistics

The results are presented as mean values and their standard deviations (mean ± SD) for each experimental group. Two-way analysis of variance (ANOVA) was used with the Bonferroni pos-test to evaluate significant differences among samples. A value of $p<0.05$ were considered to be statistically significant. Statistical analysis were performed using GraphPad Prism 5 software.

CHAPTER III:

RESULTS

1. Unraveling the modifications of myoblast cell line H9c2 lipidome towards starvation and ischemia

In this work the changes in the phospholipid (PL) profile of myoblast cells cultured with deprivation of nutrients (starvation) and in conditions mimicking ischemia were evaluated, as physiopathological conditions associated with myocardium infarction. Comparison with control group allowed us to observe changes in the phospholipid profile that can be important to unravel cardiac cells death or recovery.

In order to evaluate the alterations in PL profile, total lipid extracts were fractionated by thin layer chromatography (TLC) and each PL class was identified by comparison with PL standards applied into the TLC plate. The relative amount of each PL class was determined by phosphorous quantification of each spot attribute to each PL class and results obtained are shown in Figure 4.

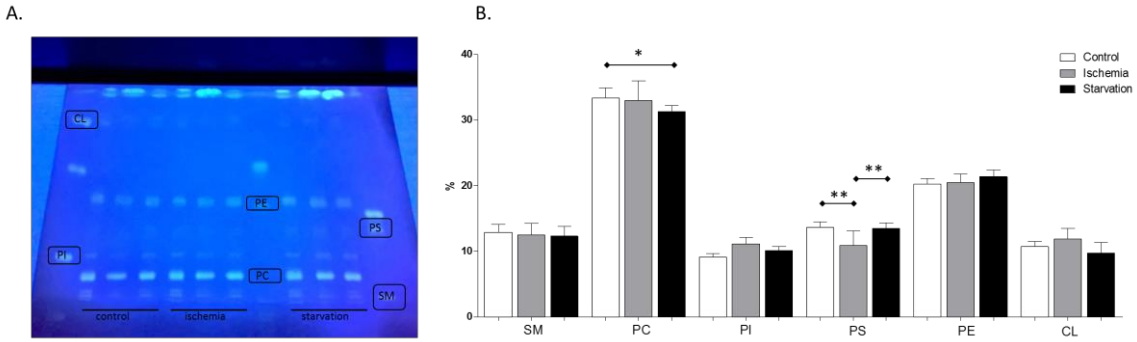


Figure 4. Thin-layer chromatography of total lipid extract obtained from untreated myoblast cells (control) and ischemic and starved cells. A- TLC plaque with the signalized spots for each PL class. Phospholipid standards were also applied: (PC) - Phosphatidylcholine; (PS) - Phosphatidylserine; (PE) - Phosphatidylethanolamine; (SM)- Sphingomyelin; (PG) - Phosphatidylglycerol; (PI)-phosphatidylinositol; (CL) - Cardiolipin. B-Relative phospholipid content of myoblast cells (control) and for ischemic and starved cells. 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.

The most abundant class in all conditions was phosphatidylcholines (PC) followed by phosphatidylethanolamines (PE), phosphatidylserines (PS), sphingomyelins (SM) and cardiolipins (CL) and phosphatidylinositols (PI). We have identified significant alterations in the relative amount of PC and PS classes. It was observed a decrease in PC content in starved cells and a decrease of PS in ischemic cells, in comparison with control group.

The identification of the fatty acid profile of the total lipid extract in all conditions and the quantification of the relative content of fatty acid composition were performed by GC–MS analysis of fatty acid methyl esters. In the total lipid extracts, the major fatty acids identified were: C14:0, C16:0, C16:1n-9, C18:0, C18:1n-9, C18:2n-6, C20:3n-9, C20:3n-6 and C20:4n-6 (Figure 5). An increase in the fatty acid C16:0 and C18:0 and a decrease in the fatty acid C18:1 was observed in ischemia while a decrease in fatty acid C20:4 was observed in starvation.

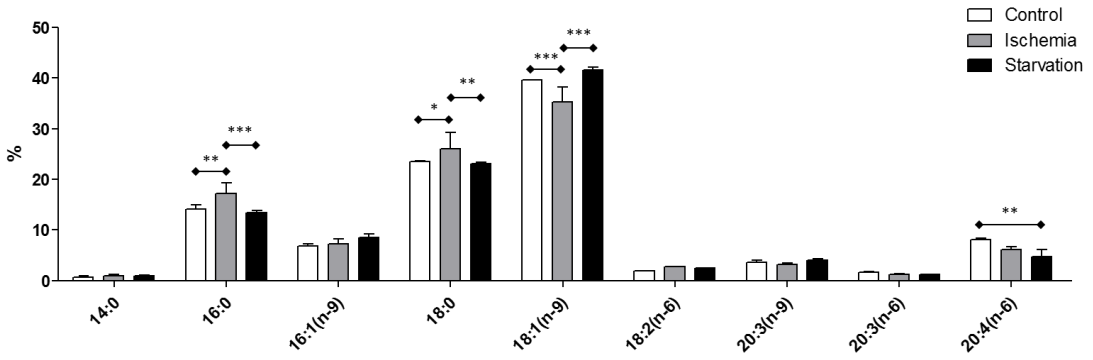


Figure 5. Fatty acid profile of untreated myoblast cells (control) and associated with ischemia and starvation.

Specific molecular composition of each PL class and information about the alterations in their profile at molecular level were achieved by using HILIC-LC-MS and MS/MS lipidomic approaches. In the LC-MS run we were able to identify all the six PL classes previously identified after TLC analysis but also two more PL classes: phosphatidylglycerols (PGs) and lysophosphatidylcholines (LPCs) (Table A.1). To ensure the reproducibility of the results, data were obtained, at least in triplicate, from different cell culture passages (at least $n = 3$).

PC were identified as $[M+CH_3COO]^-$ in LC-MS spectra and in all conditions the most abundant PC molecular specie identified was PC (34:1) at *m/z* 818.3, followed by PC molecular species including PC (32:1), PC (34:2), PC (36:2) and PC (36:1) (Figure S.1). Significant alterations in PC molecular profile were observed after comparing starvation and ischemia conditions with the control group and also between each other (Figure 6). A statistically significant increase of PC(34:1) and a decrease in PC(36:2) were observed for ischemia in comparison with control while a higher decrease of PC(34:1) was observed in starvation. Interestingly dissimilar deviations were observed for PC molecular specie PC(34:1) when the effect of ischemia and starvation are

compared. In fact, PC(34:1) decrease in ischemia and increase in starvation. Also the decrease in PC(36:2) was observed exclusively for ischemia.

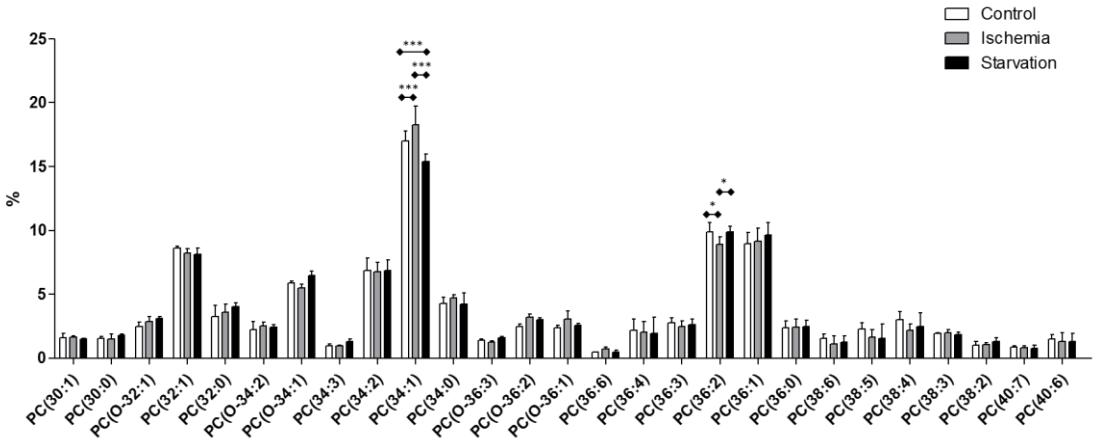


Figure 6. Phosphatidylcholine (PC) molecular species content of untreated myoblast cells (control) and associated with ischemia and starvation analyzed by HPLC-MS in negative mode. Identification of PC molecular species with the indication of the C:N (carbon:unsaturation).

In the LC-MS spectra of all conditions, five LPC molecular species, identified by their $[M+CH_3COO]^-$ ions, were observed. In all conditions, the most abundant LPC molecular specie was LPC(18:0) at *m/z* 582.1, followed by LPC (16:1), LPC(16:0), LPC(18:1), LPC(18:2) and an alkylacyl LPC molecular specie (LPC(O-18:0)) (Figure S.2). Comparison of LPC profile between all conditions with control allowed us to identify a significant increase in LPC(18:0) and a decrease in the LPC(18:1) molecular species for ischemia and for starvation. LPC(16:0) only decrease in ischemia (Figure 7). No differences were observed in LPC profile between starvation and ischemia.

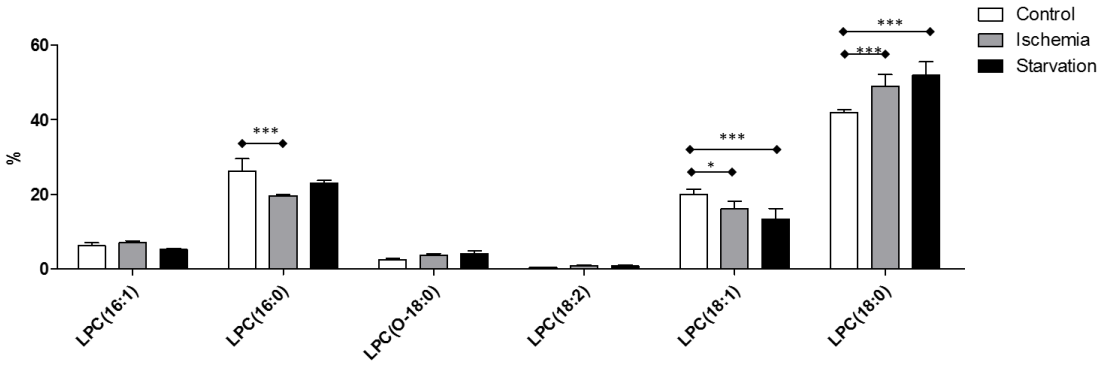


Figure 7. Lysophosphatidylcholine (LPC) molecular species content of untreated myoblast cells (control) and associated with ischemia and starvation analyzed by HPLC-MS in negative mode. Identification of LPC molecular species with the indication of the C:N (carbon:unsaturation).

The molecular species of SM were also identified in negative mode as $[M+CH_3COO]^-$ ions. The ion at *m/z* 761.2 corresponding to SM(34:1) is the major molecular specie identified in all conditions (Figure S.3). For starvation, a significant decrease in the SM(34:1) was observed in comparison with control and ischemic cells (Figure 8).

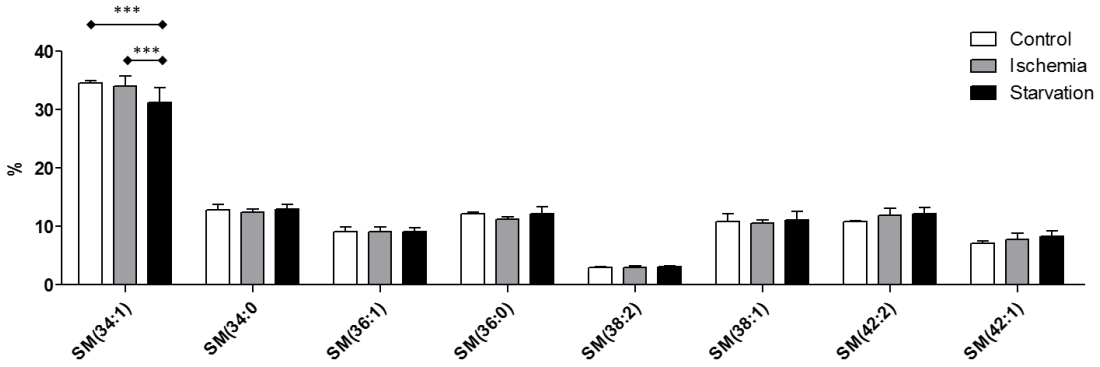


Figure 8. Sphingomyelin (SM) molecular species content of untreated myoblast cells (control) and associated with ischemia and starvation analyzed by HPLC-MS in negative mode. Identification of SM molecular species with the indication of the C:N (carbon:unsaturation).

The molecular species of PE were analyzed in negative mode and identified as $[M-H]^-$ ions. The molecular specie at *m/z* 716.2 (PE(34:1)) was the most abundant molecular specie identified in all conditions (Figure S.4). We observed an increase of PE(34:1) for ischemia and starvation in comparison with control (Figure 9).

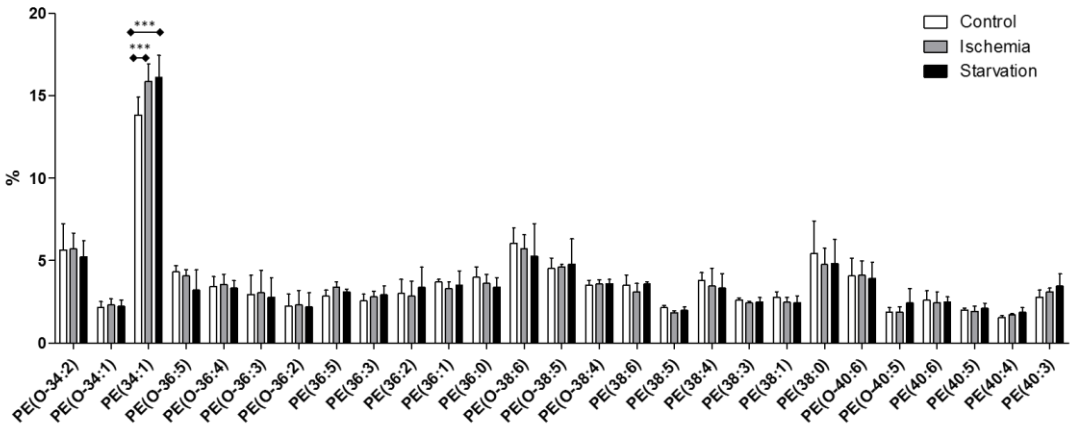


Figure 9. Phosphatidylethanolamine (PE) molecular species content of untreated myoblast cells (control) and associated with ischemia and starvation analyzed by HPLC-MS in negative mode. Identification of PE molecular species with the indication of the C:N (carbon:unsaturation).

PS was identified in negative mode as $[M-H]^-$ ions. The most abundant PS in both conditions was PS(36:1) at *m/z* 788.4 (Figure S.5). An increase in the levels of PS(36:1) were found for starvation in comparison with control and with ischemia (Figure 10).

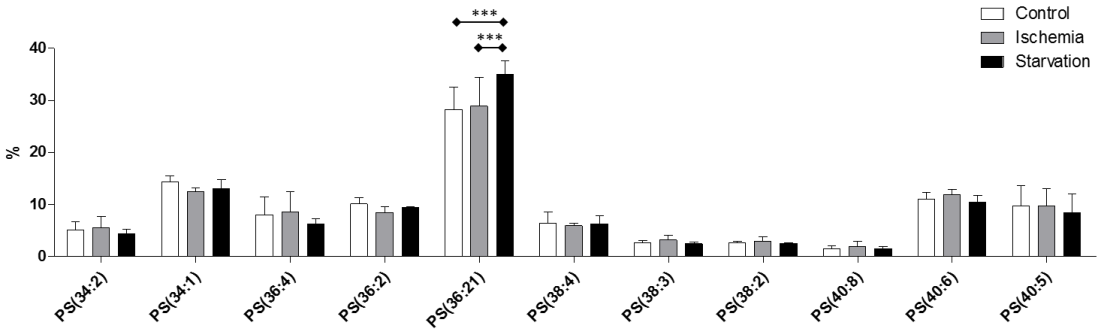


Figure 10. Phosphatidylserine (PS) molecular species content of untreated myoblast cells (control) and associated with ischemia and starvation analyzed by HPLC-MS in negative mode. Identification of PS molecular species with the indication of the C:N (carbon:unsaturation).

The PI molecular species were identified as $[M-H]^-$ ions and the major PI molecular specie were PI(38:4) at *m/z* 885.4 and PI(38:3) at *m/z* 887.4 (Figure S.6). A significant increase of PI (38:3) and a decrease of PI(38:5) were observed for ischemia and starvation in comparison with control. An increase of PI(36:2) was observed for starved cells when compared with ischemic (Figure 11).

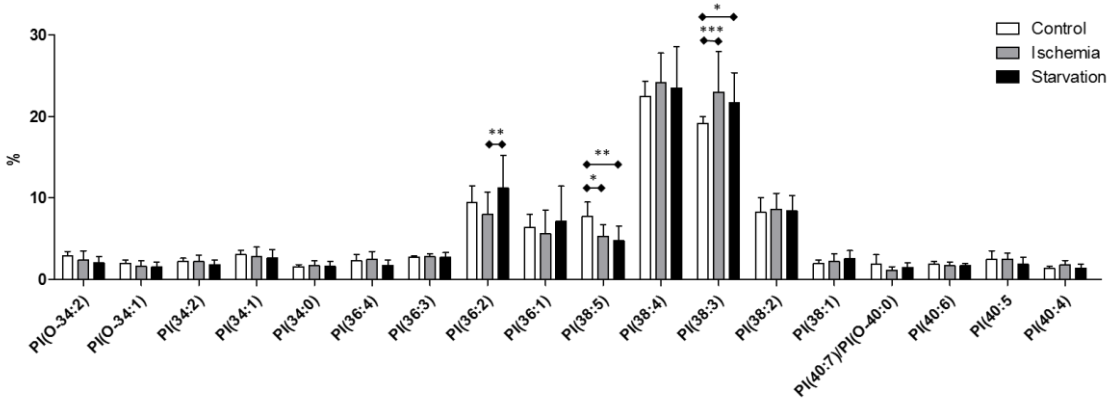


Figure 11. Phosphatidylinositol (PI) molecular species content of untreated myoblast cells (control) and associated with ischemia and starvation analyzed by HPLC-MS in negative mode. Identification of PI molecular species with the indication of the C:N (carbon:unsaturation).

PGs were also analyzed in negative mode as $[M-H]^-$ ions. Analysis of the MS and MS/MS spectra allowed us to identified several PGs molecular species being the most abundant the PG(36:2) at *m/z* 773.4 followed by PG(34:1) at *m/z* 747.4 and PG(36:1) at *m/z* 775.4 (Figure S.7). No significant alterations in PG molecular species were observed between conditions (Figure 12).

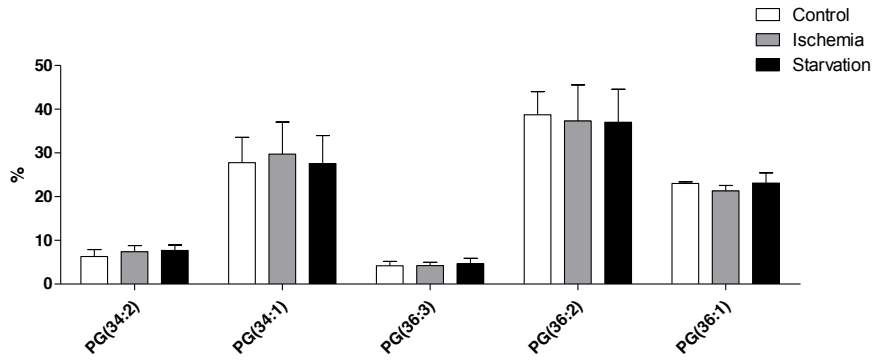


Figure 12. Phosphatidylglycerol (PG) molecular species content of untreated myoblast cells (control) and associated with ischemia and starvation analyzed by HPLC-MS in negative mode. Identification of PG molecular species with the indication of the C:N (carbon:unsaturation).

Overall the same PL molecular species were found for each PL class in control and either in ischemia and starvation. Interestingly, significant differences in the relative amount of specific molecular species were establish for all PL classes - except for PE and PG - between control and ischemia or starvation, as is summarized in Table 1. Some deviations were common to starvation and ischemia, while others seems to be correlated exclusively to starvation or ischemia.

Table 1. Resume of the alterations observed in the molecular species of PC, LPC, SM, PE, PS and PI comparing control with ischemia, control with starvation and starvation with ischemia.

Class	Molecular species	Control vS Ischemia	Control vS Starvation	Starvation vS Ischemia
PC	34:1	↑	↓	↓
	36:2	↓		↑
LPC	16:0	↓		
	18:1	↓	↓	
	18:0	↑	↑	
SM	34:1		↓	↓
PE	34:1	↑	↑	
PS	36:1		↑	↑
PI	36:2			↑
	38:5	↓	↓	
	38:3	↑	↑	

2. New insights on the lipidome of the exosomes released by myoblast cell line H9c2 upon ischemia and starvation

Identification of the phospholipid (PL) profile of exosomes released by myoblast cells was identified and the deviation in lipidome towards starvation and ischemia were also evaluated. Comparing conditions with control and between each other allowed us to observe changes in the phospholipid profile that can be important to unravel the role of exosomes in death or recovery.

A lipidomic analysis was performed based on the results gathered by the HILIC-LC-MS and LC-MS-MS to identify the specific molecular composition of each PL class and evaluate alterations in their profile at a molecular level. Due to the lower amount of lipid samples it was not possible to perform TLC or fatty acid analysis by GC-MS in these samples. Analsyis of LC-MS data allowed us to identify nine PL classes: phosphatidylcholines (PCs), lysophosphatidylcholines (LPCs), phosphatidylserines (PSs), lysophosphatidylserines (LPSs), phosphatidylethanolamines (PEs), lysophosphatidylethanolamines (LPEs), phosphatidylinositols (PIs), phosphatidylglycerols (PGs) and sphingomyelin (SM) (Table A.2).

PC were identified as $[M+CH_3COO]^-$ ions in LC-MS spectra of all conditions. The most abundant PC molecular specie identified was PC(34:1) at *m/z* 818.2 (Figure S.8). Interestingly this PC specie was also observed as the most abundant in the lipidome of myoblast cells. Significantly alterations in PC molecular profile were observed after comparing PC profile from exosomes of starvation and ischemia conditions, with control and also between both conditions (Figure 13). A significant increase of PC(30:1) and PC(32:1) and a decrease of PC(38:6) were observed for ischemia in comparison with control. Comparing exosomes released by starved cells with control we observed an increase of PC(32:5), PC(34:4), PC(38:6) and PC(38:2) and a decrease of PC(30:1), PC(34:1), PC(36:2) and PC(36:1). We have also observed alterations in alkylacyl PC molecular species namely an increase of PC(O-32:2and a decrease of PC(O-34:1) and PC(O-36:0). The alkylacyl PC molecular species PC(O-40:6) and PC(O-40:5) increase in exosomes from starved cell in comparison with exosomes from ischemic myoblast cells.

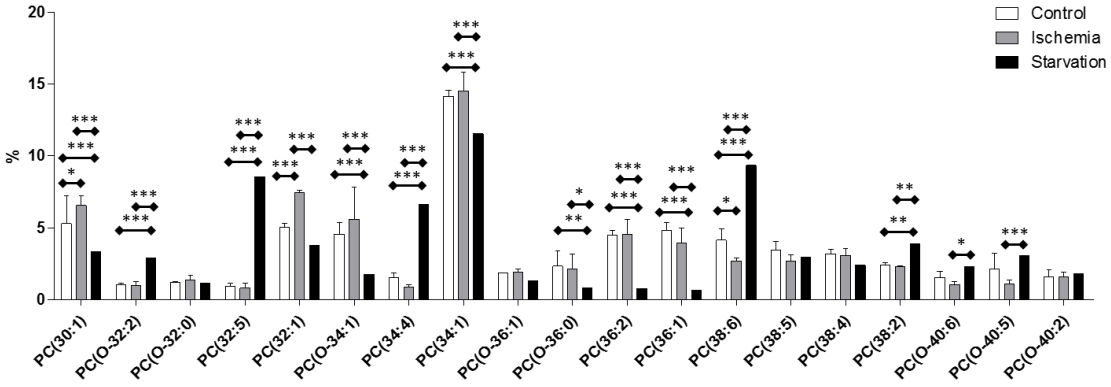


Figure 13. Phosphatidylcholine (PC) molecular species content of exosomes released by untreated myoblast cells (control) and associated with ischemia and starvation analyzed by HPLC-MS in negative mode. Identification of PC molecular species with the indication of the C:N (carbon:unsaturation).

Seven LPC molecular species were identified as $[M+CH_3COO]^-$ ions. For control and starvation groups the most abundant LPC molecular specie was LPC(18:0) at *m/z* 582.2 while for starvation the most abundant LPC molecular specie was LPC(16:1) at *m/z* 552.2 (Figure S.9). Comparing LPC profile of exosomes release during starvation with control and with ischemia, it was possible to see a significant increase in LPC(16:1) and a decrease in LPC(18:4) and LPC(18:0) (Figure 14). No differences were observed in LPC molecular profile between ischemia and control.

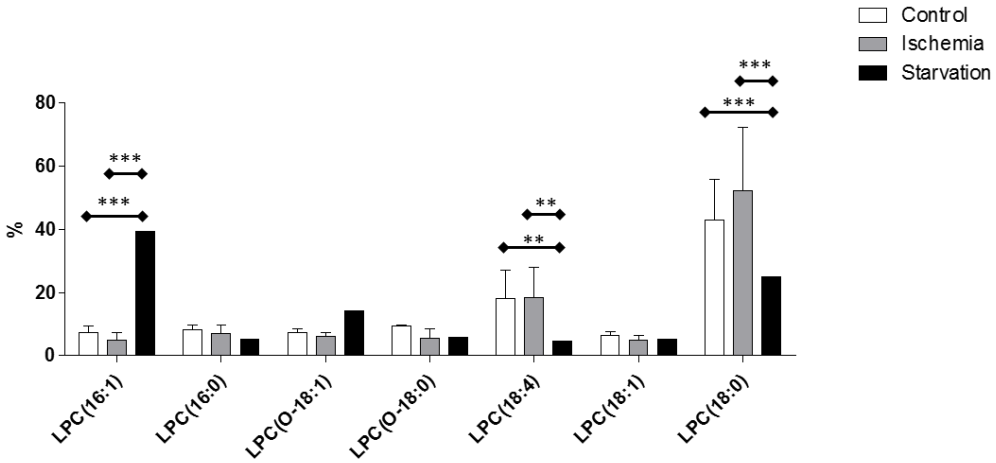


Figure 14. Lysophosphatidylcholine (LPC) molecular species content of exosomes released by untreated myoblast cells (control) and associated with ischemia and starvation analyzed by HPLC-MS in negative mode. Identification of LPC molecular species with the indication of the C:N (carbon:unsaturation).

The SM were also identified by $[M+CH_3COO]^-$ ions. The ion at *m/z* 761.2 corresponding to SM(34:1) is the major molecular specie identified in all conditions (Figure S.10). An increase of SM(34:1) and a decrease of SM(42:1) were observed for ischemia in comparison with control and with starvation. We have also observed for ischemia a decrease in SM(40:1) and SM(42:2) only in comparison with control. For starvation, we observed an increase in SM(38:2) in comparison with control and with ischemia. We have also observed an increase in SM(36:1) together with a decrease in SM(34:0) for starvation in comparison with ischemia (Figure 15).

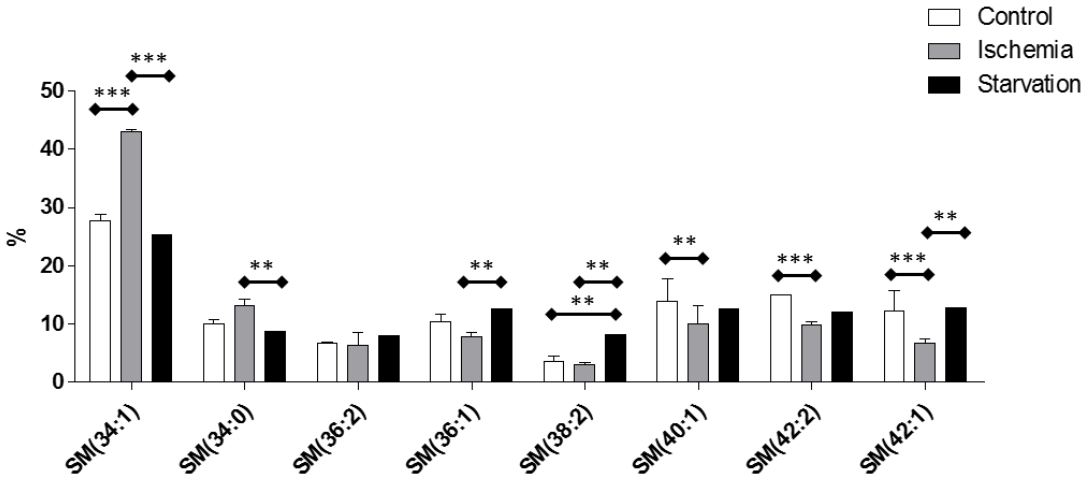


Figure 15. Sphingomyelin (SM) molecular species content of exosomes released by untreated myoblast cells (control) and associated with ischemia and starvation analyzed by HPLC-MS in negative mode. Identification of SM molecular species with the indication of the C:N (carbon:unsaturation).

The PI molecular species were analyzed in negative mode as $[M-H]^-$ ions. The major PI molecular specie identified for exosomes from control conditions was PI(40:7) while for exosomes released from starved cells the major PI molecular specie identified was PI(34:4) at *m/z* 829.8. For exosomes in ischemia, both ions presented the same abundance (Figure S.11) A significant increase of PI(34:4), PI(40:4) and a decrease in PI(38:4) and PI(40:7) were observed for exosomes in ischemia and starvation in comparison with control. Additionally, we observed an increase in PI(36:3) for ischemia and an increase in PI(34:3) together with a decrease in PI(38:1) for starvation in comparison with control. The molecular specie PI(34:5) showed a decrease in starvation in comparison with ischemia (Figure 16).

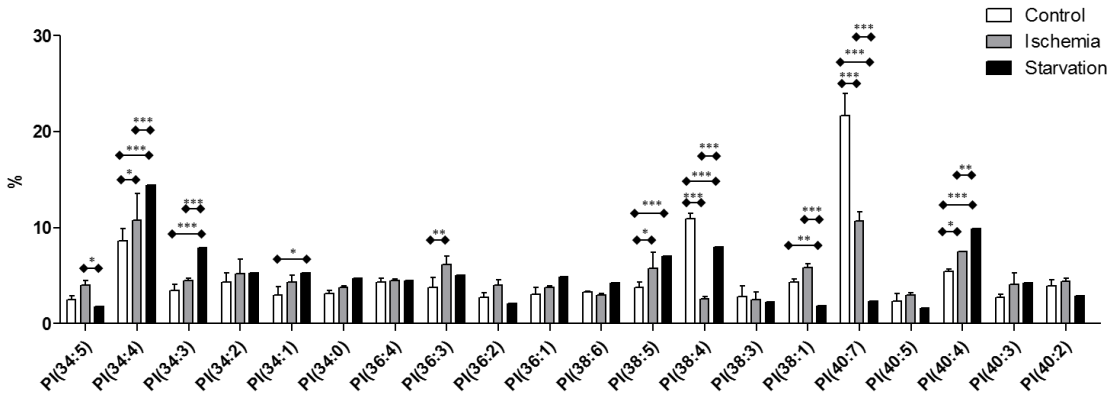


Figure 16. Phosphatidylinositol (PI) molecular species content of exosomes released by untreated myoblast cells (control) and associated with ischemia and starvation analyzed by HPLC-MS in negative mode. Identification of PI molecular species with the indication of the C:N (carbon:unsaturation).

The PG molecular species were identified as $[M-H]^-$ ions and PG(38:6) at m/z 793.4 was the major PG molecular specie in exosomes from control while for ischemia and starvation the major PG was PG(36:2) at m/z 773.6 (Figure S.12). An increase in PG(34:2) and PG(36:4) and a decrease in PG(38:6) and PG(38:5) were observed for exosomes released by ischemic cells in comparison with exosomes from control and from starved cells. The molecular species PG(34:0) and PG(36:6) decrease for both conditions in comparison with control. An increase of PG(32:1), PG(34:2), PG(36:2) and PG(38:2) and a decrease of PG(34:1) and PG(36:3) were observed for exosomes from starved cells in comparison with control. Interestingly PG(34:3) showed opposite deviation, namely increasing for ischemia while decreasing for starvation (Figure 17).

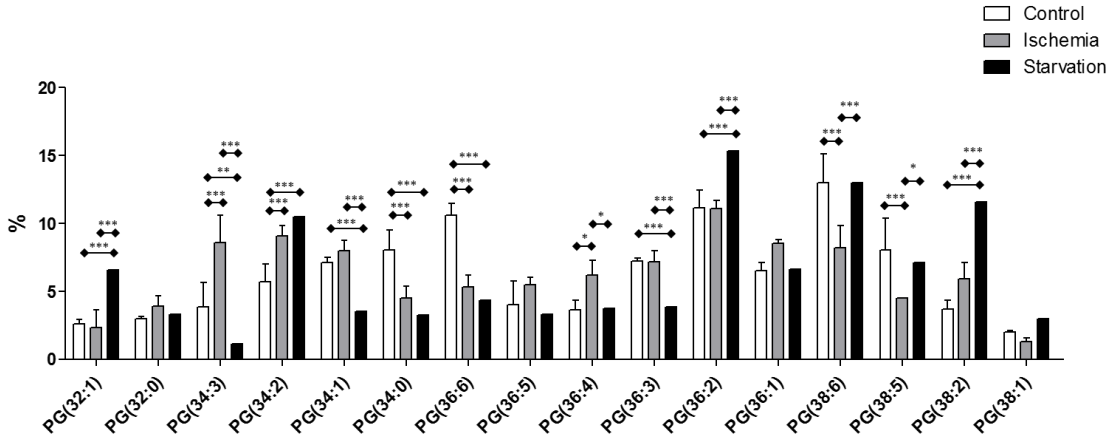


Figure 17. Phosphatidylglycerol (PG) molecular species content of exosomes released by untreated myoblast cells (control) and associated with ischemia and starvation analyzed by HPLC-MS in negative mode. Identification of PG molecular species with the indication of the C:N (carbon:unsaturation).

The molecular species of PE and LPE were identified as [M-H]- ions, but quantification was umpired by the low peak intensity in these classes identify some molecular species represented in Table A.2.

The molecular species of PS and LPS were also identified as [M-H]- ions but for the same reason were not quantified. However they were identified and are showed in Table A.2.

Overall, the same molecular species were found for each PL class from exosomes in control and either in ischemia and starvation. The differences identified so far are summarized in Table 2. It must be reported that data from exosomes need to be confirmed with more replicates.

Table 2. Resume of the alterations observed for exosomes in the molecular species of PC, LPC, SM, PI and PG comparing control with ischemia, control with starvation and starvation with ischemia.

Class	Molecular species	Control vS Ischemia	Control vS Starvation	Starvation vS Ischemia
PC	30:1	↑	↓	↓
	32:5		↑	↑
	32:1	↑		↓
	34:4		↑	↑
	34:1		↓	↓
	36:2		↓	↓
	36:1		↓	↓
	38:6	↓	↑	↑
	38:2		↑	↑
	O-32:2		↑	↑
	O-34:1		↓	↓
	O-36:0		↓	↓
	O-40:6			↑
	O-40:5			↑
LPC	16:1		↑	↑
	18:4		↓	↓
	18:0		↓	↓
SM	34:1	↑		↓
	34:0			↓
	36:1			↑
	38:2		↑	↑
	40:1	↓		
	42:2	↓		
	42:1	↓		↑
PI	34:5			↓
	34:4	↑	↑	↑
	34:3		↑	↑
	36:3	↑		

	38:5	↑	↑	
	38:4	↓	↓	↑
	38:1		↓	↓
	40:7	↓	↓	↓
	40:4	↑	↑	↑
PG	32:1		↑	↑
	34:3	↑	↓	↓
	34:2	↑	↑	↑
	34:1		↓	↓
	34:0	↓	↓	
	36:6	↓	↓	
	36:4	↑		↓
	36:3		↓	↓
	36:2		↑	↑
	38:6	↓		↑
	38:5	↓		↑
	38:2		↑	↑

CHAPTER IV:

DISCUSSION and CONCLUSION

This study evaluated the alterations in PL profile of myoblast cells induced by starvation and ischemia conditions, using a modern lipidomic approach. Both conditions induced significant alterations in PLs classes levels namely decrease in PCs in ischemia and PS related content in starvation conditions.

The fatty acid profile analysis of the studied myoblast cell line revealed an increase in FA 16:0 and 18:0 and a decrease in FA 18:1 in ischemic cells while in starved cells a decrease of FA 20:4 was observed. This dissimilar deviation of FA profile may reflect the different pathways adopted by the cells to cell death or cell survival namely to activate apoptotic pathway or autophagic pathways respectively. In fact an increase of serum FA 16:0 and 18:0 were reported in patients that have higher predisposition to have a coronary event in comparison with health volunteers [127]. Arachidonic acid FA 20:4 is the main precursor of pro inflammatory mediators, prostaglandins and leukotrienes, and thus the observed decrease of fatty acid 20:4 in starved cells may be an adaptive response of cell in order to unpair the release of these pro inflammatory mediators and thus preventing the enhance of pro inflammatory that triggers apoptosis and cell death [128]. This could be one of the routes that can be modulated by starvation towards cell. Decrease of arachidonic pro inflammatory mediators will reduce the inflammation and will have a beneficial effect in CVD. In fact, inflammation is recognized to be a major underlying mechanism of cardiovascular diseases [129].

In the case of ischemia, a severe form of starvation [12] due to simultaneous deprivation of oxygen and nutrients, we were not able to identify significant alterations in PC total content. However, a decrease in PC(36:2) - PC(18:0/18:2) and PC(18:1/18:1) - and an increase in PC(34:1) - PC (16:0; 18:1) - were observed. This shift is in accordance with the data reported from GC-MS analysis that in ischemia also showed an increase in C16:0 and C18:0 fatty acyl chains and a decrease in C16:1 and C18:1 in comparison with the control. Since PC is the most abundant PL class in mammalian cells, this lowering in the degree of unsaturation suggests modifications in cell membrane. The membrane fluidity is determined by the van der Waals forces between fatty acids incorporated into cell membrane phospholipids. These forces depend on the structure of the fatty acid and thereby the longer and saturated the fatty acid chain is, the higher the van der Waals forces are and the lower the membrane

fluidity is [130]. The decrease in fluidity interferes with the function of receptors in the membrane, affecting signal transduction [131].

In starvation, a significant decrease in the PCs levels together with a decreased in the levels of the molecular specie PC(34:1) - PC(16:0/18:1) - was observed in comparison with the control group. Under this condition, the cell tries to survive and adapts cellular lipid flow and storage to changing nutrient availability and metabolic need [132]. In starved cells, autophagy is strongly induced and represents the most important mechanism for survival and for maintenance of cardiac functions of the heart [133].

The molecular specie PC(34:1) – PC(16:0/18:1) - increased in ischemia and decreased in starvation. This is specific marker that could be used to accesses of the undergoing process in the cell. It was reported that a PC(16:0/18:1) is a physiologically relevant endogenous PPARα ligand [134]. This nuclear receptor is known for modulation of inflammatory pathways possessing relevant anti-inflammatory properties [135]. The decrease of PC(34:1) in starvation suggests that this molecular specie is being used to activate PPARα to promote cell survival.

LPC are derived from phosphatidylcholine degradation by phospholipase A_2 (PLA_2), which removes the fatty acid linked to the sn2 position. LPCs are quickly metabolized and last shortly in vivo. They can insert into the plasmatic membrane and influence several pathways, namely as immunomodulatory agents [136]. In this study, it is possible to see an increase in LPC species bearing C18:0, in both conditions, and a decrease in LPC(18:1) and LPC(16:0) decreased only in ischemia. The decrease of the molecular species LPC(16:0) and LPC(18:1) in ischemia may be associated with the increase of the molecular specie PC(34:1) once it is assigned as PC(16:0/18:1).

Since the activity of PLA_2 is usually upregulated in inflammatory environments [137], we suppose that PLA_2 acts upon PC(36:2) – PC(18:0/18:2) - releasing LPC(18:0) and contributing to the higher levels of this LPC molecular specie. This increase in LPC(18:0) as well as the increase in the molecular specie PC(34:1) are related with the reported anti-inflammatory role for PC and LPC [136].

We have also identified a decrease in the levels of SM(34:1) in starvation. In fact sphingolipids have been reported as important player in autophagy and also in apoptosis. The decrease of SM may downregulate the enzymatically conversion to ceramide (Cer), a well-known pro apoptotic molecule and thus preventing cell death

[138]. Sphingolipid, particularly in lipid rafts, are implicated in signal transduction from cell surface receptors to intracellular signaling machinery [139]. Hence alterations in SM metabolism will affect survival signaling.

PE is the second most abundant phospholipid in mammalian cell membranes and changes in PE may affect membrane structure and curvature [140]. PE plays an important role in the heart since a decrease in PE causes cell damage after periods of ischemia/reperfusion [141].While the decrease in PE accelerate cell death, the increase enhances autophagic flux and extends lifetime of mammalian cells in culture [142]. In our study, we observed an increase of PE(34:1) in ischemic and in starved myoblast cells.

PS is the most abundant negatively charged phospholipid in eukaryotic membranes [143]. Its extracellular exposure is, among other functions, essential in the recognition and clearance of apoptotic cells [144]. During apoptosis and the oxidative stress resulting from the inflammation, there is the release of cytochrome c from mitochondria that promote PS peroxidation followed by PS externalization [145]. In this study, we have observed a decrease in PS content of myoblast cells associated with ischemia. The observed decrease could be due to the PS peroxidation which triggers apoptosis. On the other hand, in starvation the molecular specie PS(36:1) showed an increase in comparison with control. Recently PS is being associated with anti-inflammatory roles, and thus considered key players in controlling inflammatory response [146]. In addition PS anti-inflammatory were associated with beneficial impact in myocardial infarction by attenuating the consequences and improve prognostic and cardiac repair [147].

PIs are important precursors of signaling molecules that regulate metabolic process. In this study we observed a decrease of PI(38:5) in both conditions in comparison with control and a decrease of PI(36:2) in ischemia in comparison with starvation. PI is a precursor of phosphoinositides (PIPs), and their decrease may be due to the upregulation of PIPs. That play a fundamental role in regulation of cell survival controlling membrane traffic and permeability [148]. It is known that PI3P is required for autophagy and among other roles is involved in the biogenesis of autophagosome [149].

In our study, we were not able to identify significant alterations in PG amounts between control and either ischemia or starvation conditions.

A communication between different cell types plays a central role in adaptative responses of the heart to stress. Recently, exosomes released by myoblast cells have been implicated as a new tool for cardiac cell communication allowing them to exchange biological messages to other cell types of the heart such as endothelial cells and fibroblasts [150]. Till now only DNA, miRNA and proteins were studied to try to understand this intercellular communication [151,152]. However lipids are well known signaling molecules and for sure have an important role in cell exosomes cross talk.

No study reported so far the lipid analysis of exosomes released by a myoblast cell line. Lipidomic analysis of exosomes were reported only for exosomes released by guinea-pig reticulocytes [153], by a B lymphocyte cell line [154], by human mast cells and dendritic cells [155], and by PC-3 prostate cancer cells [81].

In our work, we studied the lipidome of exosomes released by the myoblast cell line H9c2 and evaluated the alterations in PL profile of this exosomes associated with starvation and ischemia.

Due to the difficulty in obtaining exosomes we only studied the lipid profile of only one sample and were performed analytical replicates. The lipid classes identified by LC-MS for exosomes released by myoblast cells were SM, PC, PE, PS, PI, PG, LPC, LPE which is in agreement with what was already identified for exosomes released and isolated from other cell lines [81,155]. The analysis of LC-MS allowed us to identified alterations in specific phospholipid molecular species. It is noteworthy that these results are only preliminary and that the increase in the number of samples may change a bit the differences that we have seen so far.

Our results on exosomes showed a decrease in PS and PE together with an increase in LPE and LPS in exosomes in comparison with cells. This suggests that lyso species are somehow associated with exosomes role namely in the intercellular communication. In fact, lysophospholipid such as LPC, LPE and LPS exert functions as lipid mediators through G protein-couple receptors specific to each lysophospholipid [156]. We have also observed a deviation between the lipidome of exosomes released upon ischemia and starvation for all lipid classes. Some differences matched the ones observed in cells, for example the decrease in PC(34:1) in starvation, but others were different. Differences in lipidome of exosomes released upon ischemia and released upon starvation may be associated with the different responses that each one of them is going

to enhance after release. Since we only performed lipidomic analysis for a smaller sample of exosomes, it requires further studies to validate the results already obtained and to identify PE and PS molecular species, quantify lipid classes and analyze the fatty acids profile as performed for myoblast cell line lipidomic analysis.

In conclusion, our work showed changes of lipid homeostasis in ischemic and starved cells suggesting that lipids are key tools for evaluation of the cell fate, either cell death or recovery. This will be suitable to improve diagnosis and prognostic of cardiovascular diseases particularly acute myocardial infarction.

CHAPTER V:

ANNEXES

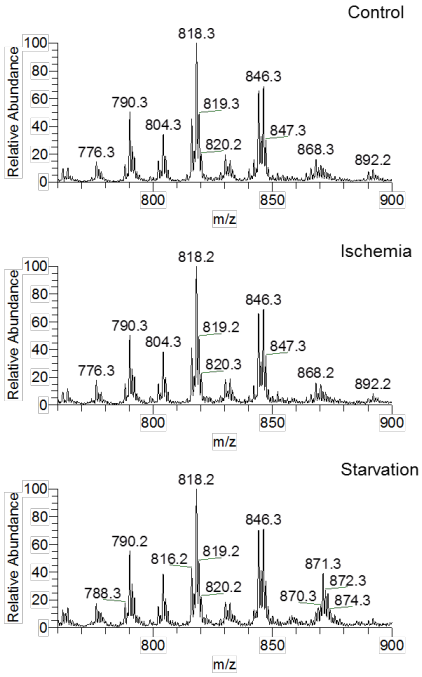


Figure S. 1. MS spectra of phosphatidylcholine (PC) molecular species obtained by HPLC-MS analysis in negative mode for untreated myoblast cells (control) and after being subjected to ischemia and starvation.

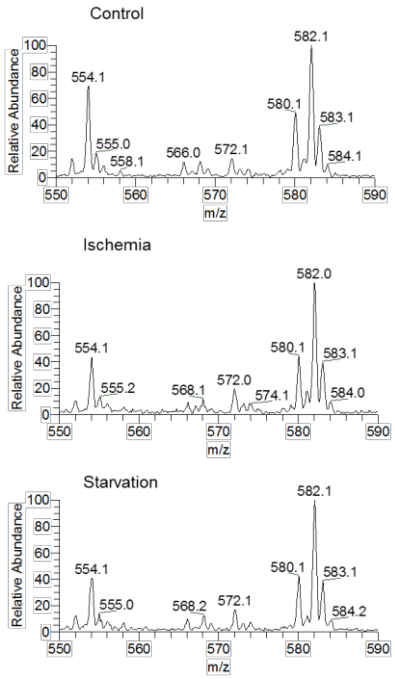


Figure S. 2. MS spectra of lysophosphatidylcholine (LPC) molecular species obtained by HPLC-MS analysis in negative mode for untreated myoblast cells (control) and after being subjected to ischemia and starvation.

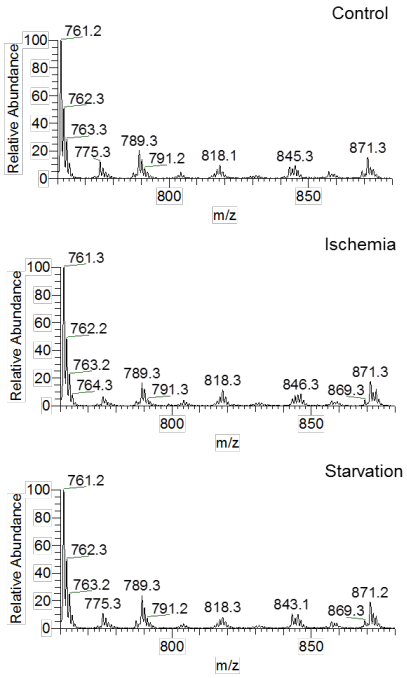


Figure S. 3. MS spectra of sphingomyelin (SM) molecular species obtained by HPLC-MS analysis in negative mode for untreated myoblast cells (control) and after being subjected to ischemia and starvation.

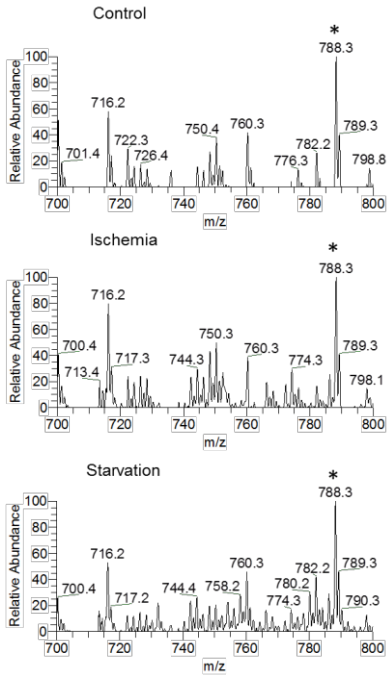


Figure S. 4. MS spectra of phosphatidylethanolamine (PE) molecular species obtained by HPLC-MS analysis in negative mode for untreated myoblast cells (control) and after being subjected to ischemia and starvation.

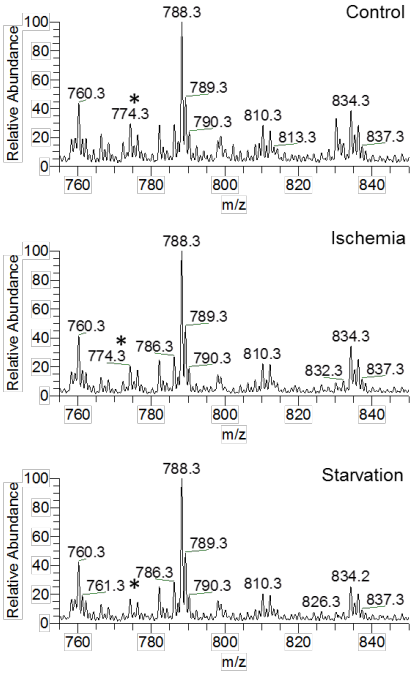


Figure S. 5. MS spectra of phosphatidylserine (PS) molecular species obtained by HPLC-MS analysis in negative mode for untreated myoblast cells (control) and after being subjected to ischemia and starvation.

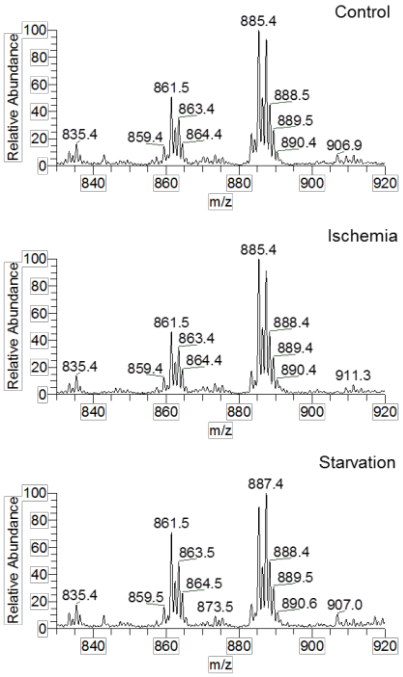


Figure S. 6. MS spectra of phosphatidylinositol (PI) molecular species obtained by HPLC-MS analysis in negative mode for untreated myoblast cells (control) and after being subjected to ischemia and starvation.

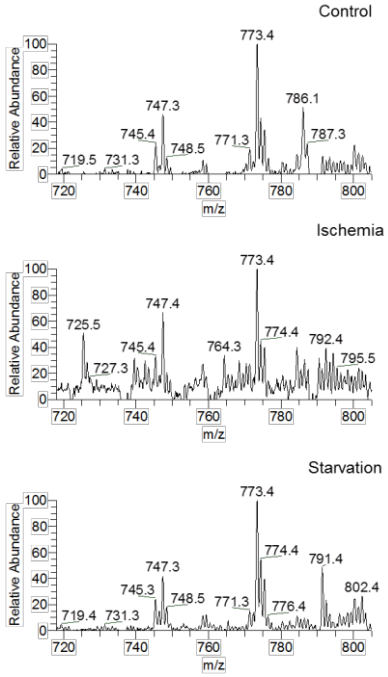


Figure S. 7. MS spectra of phosphatidylglycerol (PG) molecular species obtained by HPLC-MS analysis in negative mode for untreated myoblast cells (control) and after being subjected to ischemia and starvation.

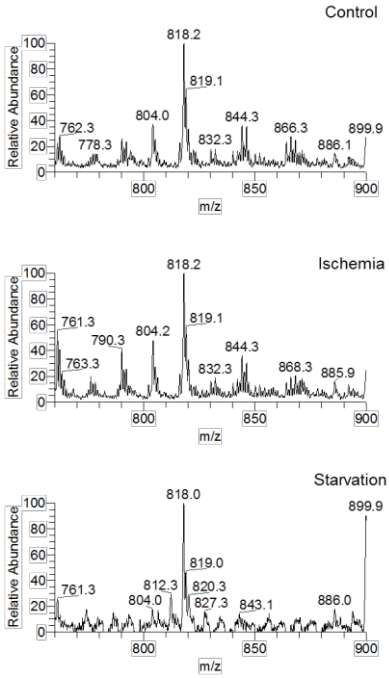


Figure S. 8. MS spectra of phosphatidylcholine (PC) molecular species obtained by HPLC-MS analysis in negative mode for untreated exosomes released by myoblast cells (control) and after being subjected to ischemia and starvation.

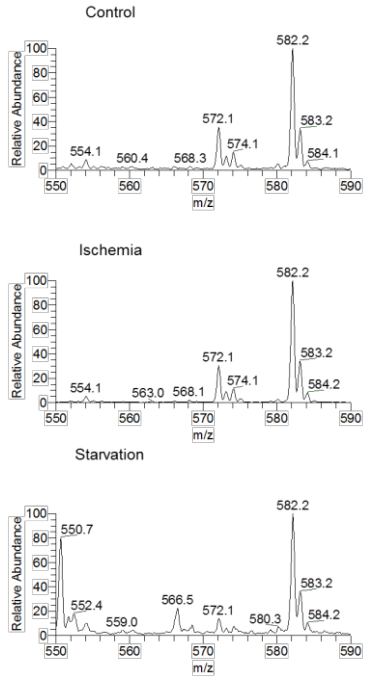


Figure S. 9. MS spectra of lysophosphatidylcholine (LPC) molecular species obtained by HPLC-MS analysis in negative mode for untreated exosomes released by myoblast cells (control) and after being subjected to ischemia and starvation.

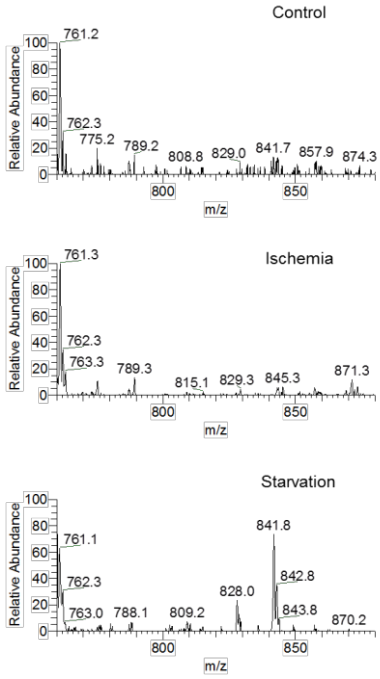


Figure S. 10. MS spectra of sphingomyelin (SM) molecular species obtained by HPLC-MS analysis in negative mode for untreated exosomes released by myoblast cells (control) and after being subjected to ischemia and starvation.

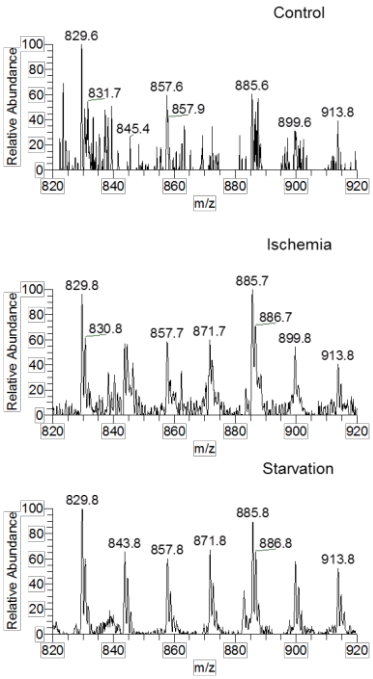


Figure S. 11. MS spectra of phosphatidylinositol (PI) molecular species obtained by HPLC-MS analysis in negative mode for untreated exosomes released by myoblast cells (control) and after being subjected to ischemia and starvation.

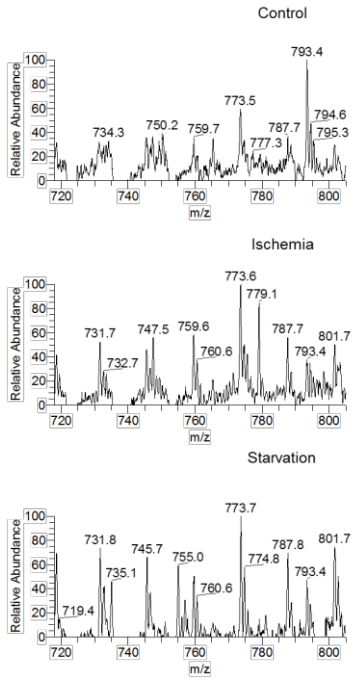


Figure S. 12. MS spectra of phosphatidylglycerol (PG) molecular species obtained by HPLC-MS analysis in negative mode for untreated exosomes released by myoblast cells (control) and after being subjected to ischemia and starvation.

Table A. 1 Lipid molecular species of PC, LPC, PE, PS, PI, PG and SM identified in myoblast cell line H9c2 submitted to ischemia and starvation and acquired using an automated HPLC-MS. Identification with m/z (mass/charge), corresponding C:N (carbon:unsaturation) and fatty acid composition.

Class	m/z	C:N	Fatty Acids
PC	**DIACYL**		
	762.3	30:1	14:0/16:1
	764.3	30:0	14:0/16:0
	790.3	32:1	14:0/18:1 and 16:0/16:1
	792.2	32:0	14:0/18:0 and 16:0/16:0
	814.2	34:3	16:0/18:3 and 16:1/18:2
	816.2	34:2	16:0/18:2 and 16:1/18:1
	818.3	34:1	16:0/18:1 and 16:/18:0
	820.2	34:0	16:0/18:0
	836.2	36:6	16:2/20:4
	840.3	36:4	16:0/20.4 and 18:1/18:3 and 18:2/18:2
	842.3	36:3	16:0/20:3 and 18:0/18:3 and 18:1/18:2
	844.3	36:2	16:0/20:3 and 18:0/18:2 and 18:1/18:1
	846.3	36:1	16:0/20:1 and 18:0/18:1
	848.3	36:0	18:0/18:0
	864.3	38:6	18:2/20:4
	866.1	38:5	18:1/20:4
	868.3	38:4	18:0/20:4 and 18:1/20:3 and 18:2/20:2 and 18:3/20:1 and 18:4/20:0
	870.3	38:3	18:0/20:3 and 18:1/20:2 and 18:2/20:1
	872.3	38:2	18:0/20:2 and 18:1/20:1
	890.3	40:7	20:3/20:4
	892.2	40:6	20:2/20:4 and 20:3/20:3
	ALKYLACYL		
	776.3	32:1	O-16:0/16:1
	802.2	34:2	O-16:0/18:2 and O-16:1/18:1
	804.3	34:1	O-16:0/18:1 and O-16:1/18:0
	828.3	36:3	O-16:0/20:3 and O-16:1/20:2 and O-18:0/18:3 and O-18:1/18:2
	830.3	36:2	O-16:0/20:2 and O-16:1/20:1 and O-18:0/18:2 and O-18:1/18:1

	832.3	36:1	O-16:0/20:1 and O-16:1/20:0 and O-18:0/18:1
LPC	**DIACYL**		
	552.0	16:1	16:1
	554.1	16:0	16:0
	578.2	18:2	18:2
	580.1	18:1	18:1
	582.1	18:0	18:0
	ALKYLACYL		
	566.0	18:0	O-18:0
PE	**DIACYL**		
	716.2	34:1	16:0/18:1 and 16:1/18:0
	736.4	36:5	16:0/20:5 and 16:1/20:4
	738.3	36:4	16:0/20:4 and 16:1/20:3
	740.4	36:3	16:0/20:3 and 16:1/20:2 and 18:0/18:3 and 18:1/18:2
	742.4	36:2	18:0/18:2 and 18:1/18:1 and 16:0/20:2 and 16:1/20:1
	744.3	36:1	16:0/20:1 and 18:0/18:1 and 16:1/20:0 and 14:0/22:1
	746.4	36:0	14:0/22:0 and 16:0/20:0 and 18:0/18:0
	762.3	38:6	18:1/20:5 and 18:2/20:4
	764.4	38:5	18:0/20:5 and 18:1/20.4 and 18:2/20:3
	766.4	38:4	16:0/22:4 and 18:0/20:4 and 18:1/20:3
	768.3	38:3	18:0/20:3 and 18:1/20:2 and 18:2/20:1
	772.4	38:1	18:0/20:1
	774.3	38:0	18:0/20:0 and 16:0/22:0
	792.4	40:5	18:0/22:5 and 18:1/22.4 and 20:1/20:4 and 20:2/20:3
	794.3	40:4	18:0/22:4 and 18:1/22:3 and 20:0/20:4 and 20:1/20:3
	796.2	40:3	18:0/22:3 and 18:1/22:2 and 20:0/20:3 and 20:1/20:2
	798.1	40:2	18:0/22:2 and 18:1/22:1 and 20:0/20:2 and 20:1/20:1
	ALKYLACYL		
	700.4	34:2	O-16:0/18:2 and O-16:1/18:1
	702.4	34:1	O-16:0/18:1 and O-16:1/18:0

	722.3	36:5	O-16:0/20:5 and O-16:1/20:4 and O-18:1/18:4
	724.4	36:4	O-16:0/20:4 and O-16:1/20:3 and O-18:0/18:4 and O-18:1/18:3
	726.4	36:3	O-16:1/20:2 and O-18:0/18:3 and O-18:1/18:2
	728.5	36:2	O-18:0/18:2 and O-18:1/18:1
	748.4	38:6	O-16:0/22:6 and O-16:1/22:5 and O-18:1/20:5
	750.4	38:5	O-16:0/22:5 and O-16:1/22:4 and O-18:0/20:5 and O-18:1/20:4
	752.4	38:4	O-16:1/22:4 and O-18:0/20:4 and O-18:1/20:3
	774.3	40:7	O-18:1/22:6
	776.3	40:6	O-18:0/22:6 and O-18:1/22:5
	778.4	40:5	O-18:1/22:4
PS	**DIACYL**		
	758.3	34:2	16:0/18:2 and 16:1/18:1
	760.3	34:1	16:0/18:1 and 16:1/18:0
	782.3	36:4	16:0/20:4 and 16:1/20:3
	786.3	36:2	16:0/20:2 and 18:0/18:2 and 18:1/18:1
	788.4	36:1	16:0/20:1 and 16:1/20:0 and 18:0/18:1
	810.4	38:4	16:0/22:4 and 18:0/20:4 and 18:1/20:3 and 18:2/20:2 and 18:3/20:1 and 18:4/20:0
	812.3	38:3	18:0/20:3
	814.1	38:2	16:1/22:1 and 18:0/20:2 and 18:1/20:1
	830.3	40:8	20:4/20:4
	834.3	40:6	18:0/22:6 and 18:1/22:5 and 18:2/22:4 and 20:1/20:5 and 20:2/20:4 and 20:3/20:3
	836.3	40:5	16:1/24:4 and 18:0/22:5 and 18:1/22:4 and 20:1/20:4 and 20:2/20:3
PI	**DIACYL**		
	833.4	34:2	16:0/18:2 and 16:1/18:1
	835.4	34:1	16:0/18:1 and 16:1/18:0
	837.4	34:0	16:0/18:0
	857.4	36:4	16:0/20:4 and 18:0/18:4 and 18:1/18:3

	859.4	36:3	16:0/20:3 and 18:0/18:3 and 18:1/18:2	
	861.5	36:2	16:0/20:2 and 18:0/18:2 and 18:1/18:1	
	863.4	36:1	18:0/18:1	
	883.4	38:5	16:0/22:5 and 18:0/20:5 and 18:1/20:4	
	885.4	38:4	16:0/22:4 and 18:0/20:4 and 18:1/20:3 and 18:2/20:2 and 18:3/20:1 and 18:4/20:0	
	887.4	38:3	18:0/20:3 and 18:1/20:2 and 18:2/20:1 and 18:3/20:0	
	889.5	38:2	18:0/20:2 and 18:1/20:1	
	891.4	38:1	18:0/20:1	
	907.0	40:7	18:1/22:6 and 18:2/22:5 and 18:3/22:4	
	909.4	40:6	18:0/22:6 and 18:1/22:5 and 20:2/20:4	
	911.3	40:5	18:0/22:5 and 18:1/22:4 and 20:0/20:5 and 20:1/20:4 and 20:2/20:3	
	913.4	40:4	18:0/22:4 and 20:0/20:4	
	ALKYLACYL			
	819.3	34:2	O-16:0/18:2 and O-18:1/16:1	
	821.3	34:1	O-16:0/18:1 and O-16:1/18:0	
	907.0	40:0	O-18:0/22:0	
PG	**DIACYL**			
	745.4	34:2	16:0/18:2 and 16:1/18:1	
	747.4	34:1	16:0/18:1 and 16:0/18:0	
	771.3	36:3	18:1/18:2	
	773.4	36:2	16:0/20:2 and 18:0/18:2 and 18:1/18:1	
	775.4	36:1	16:0/20:1 and 18:0/18:1	
SM	**m/z**	**C:N**	**Sphingoid Base**	**Fatty Acid**
	761.2	34:1	*d*18:1	16:0
	763.2	34:0	*d*18:0	16:0
	789.3	36:1	*d*18:0	18:1
			*d*18:1	18:0
	791.3	36:0	*d*18:0	18:0
	815.3	38:2	*d*18:1	20:1
	817.3	38:1	*d*18:1	20:0
	871.3	42:2	*d*18:1	24:1
	873.3	42:1	*d*18:0	24:1
			*d*18:1	24:0

Table A. 2. Lipid molecular species of PC, LPC, PE, LPE, PS, LPS, PI, PG and SM identified in exosomes released by myoblast cell line H9c2 submitted to ischemia and starvation and acquired using an automated HPLC-MS. Identification with m/z (mass/charge), corresponding C:N (carbon:unsaturation) and fatty acid composition.

Classes	m/z	C:N	Fatty Acid
PC		**DIACYL**	
	762.3	30:1	14:0/16:1
	782.4	32:5	14:1/18:4
	790.3	32:1	16:0/16:1 and 14:0/18:1 and 12:0/20:1
	812.3	34:4	14:0/20:4 and 16:0/18:4 and 16:1/18:3
	818.2	34:1	16:0/18:1 and 16:1/18:0
	844.3	36:2	16:0/20:3 and 18:0/18:2 and 18:1/18:1
	846.3	36:1	16:0/20:1 and 18:0/18:1
	864.3	38:6	18:2/20:4 and 18:4/20:2
	866.3	38:5	18:1/20:4
	868.3	38:4	18:0/20:4 and 18:1/20:3
	872.2	38:2	18:0/20:2 and 18:1/20:1
		ALKYLACYL	
	774.3	32:2	O-16:1/16:1
	778.3	32:0	O-14:0/18:0 and O-16:0/18:0
	804.2	34:1	O-16:0/18:1 and O-16:1/18:0
	832.3	36:1	O-16:0/20:1 and O-16:1/20:0 and O-18:0/18:1
	834.3	36:0	O—16:0/20:0
	878.3	40:6	O-18:0/22:6 and O-18:1/22:5
	880.3	40:5	O-18:0/22:5 and O-18:1/22:4
	886.1	40:2	O-18:0/22:2 and O-18:1/22:1
LPC		**DIACYL**	
	552.2	16:1	16:1
	554.2	16:0	16:0
	574.1	18:4	18:4
	580.2	18:1	18.1
	582.2	18:0	18:0

	ALKYLACYL		
	566.5	18:1	O-18:1
	568.3	18:0	O-18:0
PE	**DIACYL**		
	736.7	36:5	16:0/20:5 and 16:1/20:4 and 18:1/18:4 and 18:2/18:3
	738.3	36:4	16:0/20:4 and 16:1/20:3 and 18:0/18:4 and 18:1/18:3 and 18:2/18:2
	744.4	36:1	16:0/20:1 and 18:0/18:1
	762.3	38:6	18:1/20:5 and 18:2/20:4 and 18:3/20:3
	766.4	38:4	18:0/20:4 and 18:1/20:3 and 18:2/20:2
	ALKYLACYL		
	748.4	38:6	O-16:0/22:6 and O-16:1/22:5
LPE	**DIACYL**		
	424.1	14:0	14:0
	474.1	18:3	18:3
	500.1	20:4	20:4
	526.2	22:5	22:5
	532.1	22:2	22:2
	536.2	22:0	22:0
PS	**DIACYL**		
	838.3	40:4	18:0/22:4 and 18:2/22:2 and 18:3/22:1 and 18:4/22:0 and 20:0/20:4 and 20:1/20:3 and 20:2/20:2
	ALKYLACYL		
	748.3	34:0	O-16:0/18:0
LPS	**DIACYL**		
	468.3	14:0	14:0
	496.2	16:0	16:0
	518.2	18:3	18:3
	522.3	18:1	18:1
PI	**DIACYL**		
	827.6	34:5	16:2/18:3
	829.8	34:4	16:0/18:4 and 16:1/18:3
	831.7	34:3	16:0/18:3 and 16:1/18:2
	833.8	34:2	16:0/18:2 and 16:1/18:1
	835.4	34:1	16:0/18:1 and 16:1/18:0
	837.2	34:0	16:0/18:0
	857.7	36:4	16:0/20:4 and 18:0/18:4 and 18:1/18:3
	859.8	36:3	16:0/20:3 and 18:0/18:3 and 18:1/18:2
	861.8	36:2	16:0/20:2 and 18:0/18:2 and 18:1/18:1

	863.8	36:1	18:0/18:1	
	881.4	38:6	16:0/22:6	
	883.6	38:5	18:0/20:5 and 18:1/20:4	
	885.6	38:4	18:0/20:4 and 18:1/20:3	
	887.8	38:3	18:0/20:3 and 18:1/20:2 and 18:2/20:1	
	891.5	38:1	18:0/20:1	
	907.3	40:7	18:1/22:6	
	911.7	40:5	18:0/22:5 and 18:1/22:4 and 20:1/20:4 and 20:2/20:3	
	913.8	40:4	18:0/22:4 and 20:0/20:4	
	915.8	40:3	18:0/22:3 and 20:1/20:2	
	917.7	40:2	20:0/20:2	
PG	**DIACYL**			
	719.5	32:1	16:0/16:1	
	721.3	32:0	16:0/16:0	
	743.5	34:3	16:1/18:2 and 16:2/18:1	
	745.6	34:2	16:0/18:2 and 16:1/18:1	
	747.5	34:1	16:0/18:1 and 16:1/18:0	
	749.5	34:0	16:0/18:0	
	765.3	36:6	18:2/18:4 and 18:3/18:3	
	767.4	36:5	16:1/20:4 and 18:1/18:4 and 18:2/18:3	
	769.4	36:4	16:0/20:4 and 16:1/20:3 and 18:1/18:3 and 18:2/18:2	
	771.3	36:3	16:0/20:3 and 16:1/20:2 and 18:1/18:2	
	773.6	36:2	16:0/20:2 and 18:0/18:2 and 18:1/18:1	
	775.6	36:1	16:0/20:1 and 18:0/18:1	
	793.4	38:6	18:2/20:4 and 18:3/20:3	
	795.3	38:5	18:2/20:3 and 18:3/20:2 and 18:4/20:1	
	801.7	38:2	18:0/20:2 and 18:1/20:1	
	803.3	38:1	18:0/20:1	
SM	**m/z**	**C:N**	**Sphingoid Base**	**Fatty Acid**
	761.2	34:1	d18:1	16:0
	763.3	34:0	d18:0	16:0
	787.3	36:2	d18:1	18:1
	789.2	36:1	d18:1	18:0
			d18:0	18:1
	815.1	38:2	d18:1	20:1
	845.3	40:1	d18:1	22:0
	871.3	42:2	d18:1	24:1
	873.4	42:1	d18:1	24:0
			d18:0	24:1

CHAPTER VI:

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