UNIVERSITÀ DEGLI STUDI DI MILANO

DOTTORATO DI RICERCA IN SCIENZE BIOCHIMICHE

Ciclo XXXI

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LIPIDS AND MASS SPECTROMETRY APPLICATION

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SUMMARY

Abbreviations:

- HMG-CoA R, 3-hydroxy-3-methylglutaryl-coenzyme A reductase;
- 3a-diol, 5alpha-androstane-3alpha, 17beta-diol;
- 3β-diol, 5alpha-androstane-3beta, 17beta-diol;
- 7a-OH, 7a-hydroxycholesterol;
- 7β-OH, 7β-hydroxycholesterol;
- 7-keto, 7-ketocholesterol;
- 24(S)-OH, 24(S)-hydroxycholesterol;
- 27-OH, 27-hydroxycholesterol;
- 5a-DHP, 5a-dihydroprogesterone;
- 5a-DHT, 5a- dihydrotestosterone
- 3b-diol, 3b-androstenediol
- 3a-diol, 3a-androstenedio
- 17b-E, 17b-Estraiol;
- 17a-E, 17a-Estraiol;
- P450scc, cytochrome P450 side chain cleavage;
- ACN, Acetonitrile;
- AD, Alzheimer disease
- APOE, apolipoprotein E;
- ABCA1, ATP-binding cassette A1;
- ABCG1, ATP-binding cassette G1;
- CL, cardiolipin;
- CE, cholesterol ester;
- CER,Ceramides;
- CPS1, carbamoyl-phosphate synthase 1
- CTRL, non-diabetic rats;
- DG, diacylglycerol ;
- DHA, Docosahexaenoic acid
- DHEA, dehydroepiandrosterone;
- DRG, dorsal root ganglia;
- ELOVL, elovl fatty acid elongase 5

- FAs, fatty acids;
- GC, gas chromatography;
- HCl, chloridric Acid;
- HSL, hormone-sensitive lipase;
- IDDM, insulin dependent diabetes mellitus;
- ISOPREG, 3b,5a- Tetrahydroprogesteroide ;
- Lc, lung cancer;
- LC, liquid chromatography
- LC–MS/MS, liquid chromatography–tandem mass spectrometry;
- LDLR, low density lipoprotein receptor;
- Lyso-PLs, lysophospholipids,
- LysoPC, lysophosphatidylcholines;
- MeOH, methanol;
- MG, glycerolipids monoacylglycerol;
- MS, multiple sclerosis;
- NIDDM, non-insulin dependent diabetes mellitus;
- NCV, nerve conduction velocity;
- PREG, pregnenolone;
- PROG, progesterone;
- PLs, phospholipids;
- PA, phosphatidic acid, ;
- PC,phosphatidylcholine
- PE, phosphatidylethanolamine;
- PG. phosphatidylglycerol;
- PI. phosphatidylinositol;
- PS, phosphatidylserine;
- SUL,Sulfatidis;
- PUFAs, n-3 polyunsaturated fatty acid;
- SCAs, Spinocerebellar Ataxias;
- SIM/SIM, selective ion monitoring;
- SM. Sphingomyelin;

- SNC, central nervous system;
- SNP, peripheral nervous system;
- SREBP, sterol regulatory element-binding protein ;
- StAR, steroidogenic acute regulatory protein;
- SOAT1, sterol O-acyltransferase 1;
- STZ, streptozotocin;
- T, testosterone
- TG, triacylglycerol
- THP, tetrahydroprogesterone;
- TSPO, translocator protein- 18 kDa;
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1.PUBLICATIONS

CLINICAL OUTCOMES OF ORAL METRONOMIC VINORELBINE IN ADVANCED NON-SMALL CELL LUNG CANCER: CORRELATIONS WITH PHARMACOKINETICS AND MDR1 POLYMORPHISMS.

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ORAL METRONOMIC VINORELBINE (OMV) IN ELDERLY OR PRETREATED PATIENTS WITH ADVANCED NON SMALL CELL LUNG CANCER: OUTCOME AND PHARMACOKINETICS IN THE REAL WORLD.

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2. LIPIDS

2.1 INTRODUCTION

Lipids are a very heterogenic class of biomolecules with a wide range of structures and functions. In 2005, the International Lipid Classification and Nomenclature Committee on the initiative of the LIPID MAPS Consortium developed and established a comprehensive classification system for lipids based on well-defined chemical and biochemical principles and using a framework designed to be extensible, flexible, scalable and compatible with modern informatics technology. On this basis the lipids are defined as hydrophobic or amphipathic small molecules that may originate entirely or in part by carbanion based condensations of ketoacyl thioesters and/or by carbocation based condensations of isoprene units Based on this classification system, lipids have been divided into eight categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids and polyketides (derived from condensation of ketoacyl subunits); and sterol lipids and prenol lipids (derived from condensation of isoprene subunits). (Fahy et al., 2011). The term lipidome refers to the complete profile of lipid species present in cell, organelle, tissue or body fluid, while lipidomics has been defined as the study of cellular lipidomes in biological systems including the complete characterization of lipid molecular species and the comprehension of their biological roles (Gross et al., 2011; Wenk, 2010). In the last years, lipidomics has emerged as an extremely valuable tool for biosciences. An increasing number of research paper are focused towards experimental protocols mapping the lipidome of biological models to better understand the role of lipid in the different pathway or pathologies.

2.2 ROLE OF THE LIPIDS IN THE NERVOUS SYSTEM

The lipids are fundamental constituents of the nervous tissue to guarantee physiological function, such as in myelin, in the plasma membrane of neurons where they have regulatory functions. In particular, have been identified steroids, cholesterol and its derivatives, fatty acids and its metabolites such as prostaglandins, endocannabinoids.

Myelin sheaths represent a highly specialized form of plasma membrane present in the central and peripheral nervous system. The myelin sheaths are enriched in lipids and in according to recent paper, the lipidome human of central nervous system (CNS) myelin consists of about 700 different lipids, majority known and classified like phosphatidylcholines, phosphatidylethanolamines, sphingomyelins, cerebrosides, sulfatides and they represent 60%, while the remaining species have not been previously associated with myelin. The mainly components that are present in myelin are cholesterol, glycerophospholipids and glycosphingolipids with a molar ratio well defined (4:4:2). Similarly, lipid to protein ratio is maintained at about 1:1 in CNS myelin and 5:1 in peripheral nerves. Qualitative or quantitative alterations of these ratios are associated with myelin defect (Gopalakrishnan et al., 2013).

2.2.1 CHOLESTEROL, OXYSTEROLS AND NEUROACTIVE STEROIDS.

Defects in lipid homeostasis in the central nervous system (CNS) o peripheral nervous system (PNS) lead to structural and functional alterations affecting different pathway (i.e. cholesterol biosynthesis, fatty acid biosynthesis). The brain represents the most cholesterol rich organ; in the cerebral tissue, cholesterol is necessary for both cell functions and membrane structure of neurons and glial cells. Similar to other tissues in the brain, the cholesterol homeostasis is regulated by LXRs, whose activation induces the expression of a plethora of genes involved in cholesterol efflux. (Cermenati et al., 2013).

Liver X Receptors (LXR) are members of the nuclear receptors family. LXR have the classical structure of a nuclear receptor: a DNA binding domain, a ligand binding domain, and a ligand independent activation function 2 that, through the recruitment of coactivators and corepressors, regulate the activity of the receptor. LXR natural ligands are represented by oxysterols, an oxidized form of cholesterol produced from the cells as intermediates in steroid hormones or bile acids biosynthesis (Janowski et al., 1996). In according to the nature of the physiological ligands, LXR play an important role in cholesterol and lipid metabolism.

The brain's cholesterol is mainly present in myelin and low cholesterol levels in CNS results in reduced myelination in oligondedrocytes. Thus, the maintenance of cholesterol homeostasis is crucial in the CNS. On the other hand, high cholesterol levels can lead to detrimental effects (Saher et al., 2005).

Alterations in cholesterol metabolism in the SNC are linked to several diseases such as Alzheimer's disease, Parkinson disease, Multiple sclerosis (Cai et al., 2012; Teune et al., 2014).

Cholesterol is the precursor of signaling molecules or hormones such as neuroactive steroids and oxysterols. In the nervous system

neuroactive steroids exert their action by activating gene transcription, through the classic nuclear receptor-dependent mechanism involving different signaling pathways (Nadal et al., 2001; Melcangi and Panzica, 2006).

Several studies have highlighted the important roles of neuroactive steroids in the control of central nervous system functions during physiological and pathological conditions, suggesting that they may represent good candidates for the development of protective strategies against neurodegenerative and psychiatric disorders. In fact neuroactive steroid levels are modified by neurodegenerative conditions (i.e., Alzheimer's and Parkinson's diseases, multiple sclerosis) or by mental diseases (i.e,. schizophrenia) and may have an important role in physiological conditions, as the reorganization of grey and white matter during human puberty (Luchetti et al., 2011).

Oxysterols are a class of cholesterol and represent important intermediates in a number of hepatic and extrahepatic metabolic pathways. The term "oxysterol" was used to refer to derivatives of cholesterol obtained by introduction of a hydroxyl group in the molecule by autoxidation or by action of specific monooxygenases (Crosignani et a., 2011). The conversion of cholesterol into bile acids follows different pathways and starts enzymatically from the introduction of a hydroxyl group in different cholesterol positions giving origin at 24S-hydroxycholesterol, 7a-hydroxycholesterol 27-hydroxycholesterol and 25-hydroxycholesterol (Crosignani et a., 2011). The biological role of oxysterols under physiological conditions is strictly related to the entire cholesterol metabolism, ranging from regulation of cholesterol synthesis to regulation of cholesterol elimination through bile acid synthesis (Javitt NB.,2002). Also, cholesterol uptake and cholesterol transport are influenced by the signaling pathways between oxysterols and individual proteins, such as liver X receptor (LXR) (Javitt NB.,2002). One of the most important

enzymatic derived cholesterol is 24S-hydroxycholesterol. The conversion of cholesterol in 24S-hydroxycholesterol is the only way to eliminate excess cholesterol from brain (Bjorkhem I, 2006). Recent papers have investigated oxysterols in different animal model. Altered levels or distribution of oxysterols in brain areas have been associated with several neurodegenerative diseases (Jeitner et al.,2011) supporting that these molecules play crucial role in signaling function. J.L. Goldstein et al have demonstrated as the oxysterol negatively influence the regulation of cholesterol biosynthesis (J.L. Goldstein et al.,2006).

2.2.2 FATTY ACIDS

Fatty acids (FAs) represent another important component widely diffused in all living organisms. They are essential as substrate in both CNS and PNS for the synthesis of more complex lipids such as glycerophospholipids, cerebrosides, gangliosides and for post translational modification of proteins.

The role of fatty acids as a mitochondrial substrate for ATP production and as an inhibitor of pyruvate dehydrogenase has been investigated since the 1960s, and it is now well described (Lopaschuk et al., 2010). Fatty acid regulation of gene expression via activation of various nuclear receptors was extensively studied over the past 30 years, and it is now well established that stimulation of peroxisome proliferator-activated receptors up-regulates the expression of genes involved in mitochondrial fatty acid metabolism (Madrazo et al., 2008). In particular, dietary fatty acids affect the composition of mitochondrial phospholipids, which in turn impacts mitochondrial function. Mitochondrial dysfunction plays a causal role in different pathologies, such as heart failure, neurodegenerative disorders and diabetes. Thus, there is great interest in understanding how dietary long chain fatty acids can be used to prevent or reverse mitochondrial dysfunction in human disease. Supplementation with n-3 PUFA increases membrane phospholipid DHA and depletes arachidonic acid, and can increase cardiolipin, a tetra-acyl phospholipid that is unique to mitochondrial and essential for optimal mitochondrial function (Stanley et al., 2012). Cardioprotection observed in pathological states such as heart failure may be due in part to mitochondrial phospholipid remodeling and improved mitochondrial function (Stanley et al., 2012).

In recent years, polyunsaturated fatty acids like DHA have gained much attention due to promising results in a number of

neurodegenerative conditions (Hacioglu et al., 2012; Yassine et al., 2017). In this contest, several studies have demonstrated that oral intake DHA improves cognitive performance and protecting against synaptic degeneration (Lim et al 2005; Green et al 2007).

Moreover, polyunsaturated fatty acids are required for the normal development of the central nervous system. The PLs containing ω-3 PUFAs seem to provide beneficial effects towards inflammation related disorders through specific mechanisms and a plethora of bioactivities including their ability to modulate the eicosanoid pathway (Burri et al 2012; Hishikawa, et al 2017; Murray et al 2015). These PLs include plasmalogens, PLs and PL carriers of FA precursors of eicosanoids (Tessaro et al 2015; Reis 2017). It has been demonstrated that when ω-3 PUFAs (such as EPA and DHA) are constituents of membrane phospholipids (PLs), they are more efficiently incorporated into tissue membranes (Bazan., 2007). Indeed, long chain polyunsaturated fatty acids, such as arachidonic acid, and various ω-3 fatty acids, like docosahexaenoic acid and eicosahexenoic acid, are commonly found at the sn-2 position of glycerophospholipids and represent the substrates for the synthesis of classical (prostaglandins leukotrienes, thromboxanes, lipoxins) and nonclassical eicosanoids (endocannabinoids, neuroprotectins and resolvins) (Bazan, 2007).

2.3 NEUROPROTECTIVE ACTIVITIES OF LIPIDS

As mentioned above, alteration of lipid metabolism or homeostasis is the cause of some neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, Parkinson's disease, diabetes-associated cognitive loss, as demonstrated in animal models and human subjects (Vance, 2012; Cai et al., 2012; Teune et al., 2014).
In addition, alterations of lipid profile in both CNS and PNS myelin are observed in model of peripheral neuropathy (Cermenati et al., 2012)
In principle, restoration of lipid species to physiological levels and composition could represent a direct and simple goal of therapeutic strategies. Notably, some lipid species such as n-3 PUFAs beneficially affecting the brain functions, are commonly present in the diet, hence consumption of foods enriched or supplemented with n-3 PUFAs could represent an alternative strategy for prevention of neurodegenerative disorders. This field has received great attention and a high number of studies have been published in latest years (Figueroa et al., 2013; Zárate et al., 2017). Nevertheless, strong evidences of neuroprotective effects by dietary n-3 PUFAs in randomized controlled trials are still lacking (Gillette et al., 2013; Agostoni et al., 2017).
Low blood levels of DHA and EPA have been shown to be associated with ADHD symptoms such as inattention and hyperactivity (Crippa et al., 2016). Based on this evidence, an increasing number of intervention studies have been conducted to evaluate the efficacy of ω-3 PUFAs supplementation for the treatment of children with ADHD (Crippa et al., 2016).
To concern neuroactive steroids several studies have demonstrated the protective action of these molecules in peripheral neuropathy

associated with pathological condition (Melcangi et al., 2006; Cermenati et al., 2017).

Diabetes is one of the major causes of peripheral neuropathy therefore the protective effects of neuroactive steroids have been investigated in deep. It has been demonstrated in several studies that diabetes-induced peripheral neuropathy in animals is characterized by reduced levels of neuroactive steroids in both CNS and PNS and unbalanced fatty acid pattern (Caruso et al., 2008; Cermenati et al., 2012;) The restoration of neuroactive steroid levels could represent a possible therapeutic approach, also applicable to humans. Indeed, promoting steroidogenesis, by increased cholesterol translocation into mitochondria or by acting on cholesterol metabolism, translates into improvement of neuropathy in diabetic animals (Giatti et al., 2009; Cermenati et al., 2010). Given the role of lipids in CNS and PNS, lipid lowering therapies could in principle affect the brain homeostasis and peripheral myelin function.

2.4 SEX DIMORPHISM IN LIPIDOMIC

Experimental evidence underlines that sex is one of the most relevant biological variables significantly influencing lipidomic profiles. For many years, the researchers have considered the estrous cycle a confounding variable rather than a parameter to better understand the differences between the sex (Shansky et al., 2016). Sex specific lipidomics and genomics integration represents a powerful tool to understand gene regulation.

Mittelstrass et al., (2011) have found a correlation between a single nucleotide polymorphism in carbamoyl-phosphate synthase 1 (CPS1) locus in plasma of patients affected by coronary artery diseases (CD). Specifically, he has been demonstrated a significant difference in 102 out of 131 metabolites between males and females, namely sphingomyelin (SM, C22:2-OH), several lysophosphatidylcholines (LysoPC), phosphatidylcholines (PC) and acylcarnitines. (Mittelstrass et al., 2011). Plasma sphingolipids levels were associated with an increased risk of cardiovascular events in subject affected by type 2 diabetes as reported in Alshehry et (2016).

Alteration of the lipids in cancer is related with a sexual dimorphysm (Guo, et al., 2012). Specifically, differences in plasma levels of lipid as phosphatidilcoline (PC) (C34:2), PC (C36:3) and PC (C36:2), in females affected by lung cancer (Lc) compared to LC males, are detected (Guo, et al., 2012). These different sex metabolic behaviors could imply different clinical treatments for the lung cancer in females and males in the future. In addition, these results strongly indicated that the serum metabolites found in this study may have great clinical potential in early prognosis and progression of Lc (Guo et al., 2012).

Lipidomic studies in mice have also allowed to understand the metabolic mechanisms underlying CNS pathophysiological

conditions. For instance, lipidomic analysis in an Alzheimher disease mouse model showed sex- related differences of several metabolites. Specifically, Gonzales- Dominguez and collaborators, studying peripheral tissues, such as liver and kidney in AD mouse model, have found several alterations in various lipid species such as PC, PE, plasmalogens, phosphoinositols, phosphoserines, phosphoglycerols and lysophospholipids (Gonzales- Dominguez R. et al., 2015). These results, compared with those obtained in another study by Rappley and collaborators, who showed a greater decrease of LysoPC (C22: 6) in males than in females confirm the relation between the sex and changes in the brain lipidome (Rappley et al, et al., 2009).

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In this contest, neuroactive steroids play an important role; sex differences have been observed in the incidence of psychiatric and neurodegenerative disorders. For instance, as reported in the acute phase of experimental autoimmune encephalomyelitis model (an experimental model of MS), male rats showed decreased levels of PREG and PROG in the cerebral cortex and cerebellum and increased levels of THP in the cerebral cortex and spinal cord. However, these changes in steroid levels did not occur in female rats (Giatti et al., 2010).

Finally, lipidomic studies offer a powerful tools and therapeutic strategies useful for the integration with other omics data (i.e, transcriptomics and proteomics) to better understand pathophysiological mechanisms. In this context, the characterization of sex, age and race-related features among different population or during distinct phases of development and aging is now considered extremely relevant for the attainment of personalized therapeutic approaches.

3. MASS SPECTROMETRY

3.1 INTRODUCTION

Mass spectrometry is an essential analytical tool in all fields of sciences. It is used to analyze combinational data, sequences of biomolecules, to explore single cells and for quality control of drugs (De Later et al., 2006; Budzikiewicz et al., 2006;).

The basic principle of mass spectrometry is to measure the mass to charge ratio (m / z) of ions. The first step in the mass spectrometric analysis of compounds is the ion source. Then in the analyzer these ions will be manipulated and separated according to their mass to charge ratio. Finally, the detector detects the m / z and the abundance of each ion population. Thank to tandem mass spectrometry, It is easily used to elucidate the structure and chemical properties of molecules and complex. Due to its sensitivity, its selectivity and its ability to perform rapid analyzes, mass spectrometry plays an important role in all sciences.

Lipidomics, defined as the large-scale study of cellular lipids (i.e., the lipidome), has recently emerged as a rapidly expanding research field: the systems biology (Han X et al.,2003). In the development of lipidomics study, mass spectrometry is the leading techniques in the characterization, identification, and quantitation of lipids.

Lipidomics, as the metabolomics, could be classified in Untargeted and Targeted approaches.

Lipidomic profiling studies the lipidome in a holistic approach and from a point of limited a priori biological knowledge. The study is designed so to acquire data on a large subset of metabolites followed by interrogation of these data to define biological differences. Data interrogation is performed with the application of univariate and multivariate analysis tools. These are discovery-phase studies, otherwise referred to as hypothesis-generating or inductive sometimes by the non-cognoscenti as a 'fishing expedition' and

have the goal to discover new biology or biomarkers. Untargeted approaches allow that a huge number of metabolites can be measured. To this end, different lipidomics-based platforms have been developed and extensively used to analyze diverse pathways and networks associated with lipid metabolism, trafficking, and homeostasis.

The second approach of MS techniques focuses on the "targeted" lipidomic analysis of one or a limited number of lipids. The lipidome targeted analysis is driven from known biology, where a limited number of metabolites are characterized and biochemically known with biological importance at the start of the study before data acquisition is performed. Quantification of the metabolites is performed through the use of internal standards and authentic standards for the preparation of calibration curves for each of the metabolite. LC-MS and LC-MS/MS based methods have been extensively utilized for this purpose.

3.2 MASS SPECTROMETRY IN LIPIDOMICS

3.2.1 TECHNIQUE AND APPROACH IN LIPIDOMIC STUDY

As described above, the main strategies used for lipidomics approaches are "untargeted lipidomics" and "targeted lipidomic". One of the important steps in a lipidomic experiment is represented by sample collection and preparation for the analysis (Haukaa et al., 2016). The optimization of sample extraction procedures must guarantee experimental reproducibility, precision, accuracy for the best quality of the analysis. Therefore, the extraction method should be chosen depending on the type of lipidomic approach. Indeed, for "untargeted approach" a minimal or no sample preparation should be emplayed to avoid loss of metabolites from the biological samples. On the other hand, "targeted approach" requires a specific extraction and purification method of selected metabolites should be carried out (Astarita et al., 2013). In addition, another parameter that should be considered is the nature of metabolites. They can be grouped in water soluble or hydrophilic compounds and in water insoluble or hydrophobic compounds, requiring different extraction procedures.

Several works report a simple procedure to extract lipids and hydrophilic metabolites with a single method. These protocols is able to separate and recover the water soluble and lipid metabolite fractions by adding to samples a mixture of chloroform:methanol (1:1, v/v) or chloroform:- water (2:1, v/v), followed by vortex-mix and then centrifugation. Subsequently, a phase containing polar metabolites (upper phase) and a phase containing lipids (bottom phase) are obtained (Paglia et al., 2017). After the extraction protocol, it is common to perform a sample pretreatment step before carrying out the analysis. Gas chromatography (GC) or liquid

chromatography (LC) coupled to mass spectrometry (MS) allow the simultaneous acquisition and, if any, quantification of metabolites with high specificity, resolution and in most cases with high-throughput capacity.

Mass spectrometers with atmospheric pressure ionization source (API) such as electron spray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were used. ESI is a soft ionization technique used in mass spectrometry that uses an electrospray produced by applying an electric field to the eluent forced to pass through a capillary to create a fine aerosol from which ions are formed by desolvation. Soft ionization refers to the processes which imparts little residual energy onto the subject of molecules and as a result in little fragmentation. APCI does not generate multicharged ions and does not produce (or in a very small amount) fragmentation; it operates at higher temperatures and is commonly used to analyze polar and nonpolar compounds of relatively low molecular weight and thermostable. The principle of APCI is to ionize the primary ions (produced from the gas in the atmosphere, such as nitrogen, and oxygen); these ions exchange the charge with solvent molecules that in turn exchange with the analytes, the reactions are carried out at atmospheric pressure. Usually, APCI is less sensitive compared to ESI, but for polar compounds such as lipids, it may offer some advantages over electrospray.

However, it is extremely challenging to identify large numbers of lipids from MS experiments of biological samples where analysis is complicated by the presence of a huge amount of interferering with similar mass-to-charge (m/z) ratios.

in the last decades efficient improvement has been made in mass analyzers. The very first LC-MS was single stage quadruple (SSQ) that gives data on molecular ion peaks and fragment ion. However, SSQ has been replaced by triple stage quadruple (TSQ), Ion trap, Time of Flight (TOF), Hybrid Ion Trap (Q-Trap), Hybrid TOF (Q-TOF), Fourier Transform Inductive Couple Resonance (FT-ICR) and Orbitrap analyzers. In such a system, parent ion is selected and then it is fragmented to get complete spectrum. There are various methods for fragmenting molecules for tandem mass spectrometry, including collision-induced dissociation (CID), electron capture dissociation (ECD), electron transfer dissociation (ETD).

The introduction of Ultra High-Performance Liquid Chromatography (UHPLC) has significantly improved separation as well as High resolution mass spectroscopy (HRMS) such as Orbitrap MS, have allowed to detect ions with high resolution (Zhang et al., 2007). The data also permits the calculation of accurate mass of the metabolites and in determination of their molecular formula (Han et al., 2008).

In addition, it's well known, the use of mass spectrometry to quantify specific lipids by targeted approach (Gowda et al., 2008). MS like Q-Trap, TSQ are designed to perform single reaction monitoring (SRM) and multiple reaction monitoring (MRM). MRM mode involves that the first analyzer allows only a single mass through and the second analyzer monitors for multiple user-defined fragment ions. Precursor ion scanning refers to monitoring for a specific loss from the precursor ion. thanks to this characteristic these experiments are used to increase specificity of detection of known molecules permitting detection of low levels analytes (Yao et al., 2008).

Recently another approach used for the determination of lipids in biological samples is the MS shotgun. The technique involves the acquisition of data by direct infusion. This approach is able to identify many metabolites by distinguish isobaric precursor ions and fragments (Sadowski et al., 2017).

In addition, mass spectrometry imaging (MSI) is an emerging tool for mapping the distribution, in a label-free mode, from a thin, flat samples, biological tissue sections, ranging from small metabolites to large proteins. The ambient mass spectrometry include the desorption electrospray ionization (DESII, direct analysis in real time (DART), desorption atmospheric pressure chemical ionization (DAPCI), electrospray-assisted laser desorption/ionization (ELDI), atmospheric solids analysis probe (ASAP) and jet desorption ionization (JeDI) can be used as a rapid analysis tool with little to no sample preparation and allows repeated measurements on samples from different biological sources (Gowda et al., 2008; Hyotylainen et al., 2015).

3.2.2 LIPIDOMIC DATA ANALYSES

The large amount of data obtained from a lipidomic analysis requires tools to manage results in a clear and unequivocal way. The choice of the appropriate chemometric methods is essential to extract valuable information from the crude data as well as to allow the interpretation of the lipidomic results. Because of the wide set of data generated, the study of lipidomic data use statistic particular methods. There are different approaches to apply: from the application of univariate tests for the comparison of two populations (i.e., control vs. treated) to the multivariate tests.

The most used tool in the treatment of lipidomic data is the analysis of hypothesis testing the difference between two groups of samples, distribution of the population or correlation between variables (*t*-tests) (Checa et al., 2015). Depending on the assumptions about the probability distribution of the data, statistics could be parametric (based on assumptions about the parameters of probability distributions) or nonparametric (without these assumptions regarding the distribution) (Checa et al., 2015).

Analysis of Variance (ANOVA) is a family of statistical models that can be used to separate and estimate the different causes of variation. ANOVA is used to provide a statistical test for the equality of the means of several groups and it can be seen as a generalization of the t-test to more than two groups (Miller et al., 2010). Millet explains as different ANOVA approaches can be considered: from the classical one-way ANOVA (a single measured variable and one factor) to two-way ANOVA (a single measured variable and more than one factor). In this case, more information can be obtained as not only significance of the factors is tested but also the interaction between them (in this case replicates of the measurements are needed) (Miller et al., 2010).

The most explorative multivariate method used is Principal Component Analysis (PCA). The primary goal of PCA is to explain the variation in the original data, defined by variables called principal components, which retain most of the relevant information from the original data. These principal components are linear combinations of the original variables that hierarchically describe the directions of maximum variation in the data without repeating information which is guaranteed by the orthogonality between them (Harrington et al., 2005). Finally, even all these statistic methods are used for lipidomic data analysis, it is evident that a critical evaluation needs to selecte the best tests to apply. Before to analysis, data needs to be inspected to check if it fulfils the requirements of the chemometric method of choice.

4. Sterol regulatory element binding protein-1C knockout mice show altered level of neuroactive steroid levels in sciatic nerve

Mitro N, Cermenati G, Audano M, Giatti S, Pesaresi M, Pedretti S, **Spezzano R**, Caruso D, Melcangi RC. J Neurochem. 2017 Aug;142(3):420-428. doi: 10.1111/jnc.14063. Epub 2017 May 29.

4.1 INTRODUCTION

Peripheral neuropathy is one of the most common disorders with a prevalence of about 2.4% that rises with aging to 8% in the general population. Peripheral neuropathy may be inherited, such as those referred to Charcot– Marie–Tooth (CMT) or acquired, for instance those occurring during aging process, after physical injury, in systemic or metabolic disorders (i.e., diabetes mellitus, vitamin deficiencies, alcoholism, kidney failure, cancer, etc.) (England and Asbury, 2004). In the context of PNS, it is important to highlight the role of neuroactive steroids, cholesterol-derived molecules that function as protective agents in central and peripheral nervous system (Giatti et al., 2010). Peripheral nerves and Schwann cells not only synthesize and metabolize neuroactive steroids but are also targets for these molecules. Classical intracellular steroid receptors, such as PROG (PR), estrogen, glucocorticoid, and mineralocorticoid receptors, have been detect (Melcangi et al., 2001; Groyer et al., 2006). In according to neurosteroidogenic capacity of PNS, several studies have demonstrated altered level of neuroactive steroid in different experimental models of peripheral neuropathy (Melcangi et al., 2016; Giatti et al., 2015). In addition, it has been observed a link between neuroactive steroids and FA synthesis in the PNS. LXR activation in diabetic peripheral neuropathy induced by streptozotocin, exert protective effect, restoring alteration in the levels of fatty acid and neuroactive steroids in sciatic nerve (Cermenati et al., 2010 and 2012). According with this finding, it has been, demonstrated that 5a-dihydroprogesterone (5a-DHP) and 3-alpha-diol are protective agents against diabetic peripheral neuropathy by regulating the de novo lipogenesis pathway, which positively influences myelin fatty acid profile and consequently improved myelin structure and function (Mitro et al., 2014).

In addition, there are evidences that peripheral neuropathy could be induced by deficiency of the sterol regulatory element binding protein-1c (SREBP-1c) (Cermenati et al., 2015). SREBP-1c is a lipogenic transcription factor of the basic helix-loop-helix-leucine zipper family that controls the synthesis of fatty acids (FA) and triglycerides (Liang et al. 2002). SREBP-1c belongs to the family of membrane-bound transcription factors, called Sterol regulatory element binding proteins (SREBPs). Roles of SREBPs have been established as lipid synthetic transcription factors for cholesterol and fatty acid synthesis. SREBPs are able to regulate a wide range of lipid genes. Specifically, several studies have highlighted that SREBP-1 is involved in both the metabolism of fatty acids and of insulin and glucose, while SREBP-2 is a regulator of the synthesis of cholesterol (Shimano, 2001). Horton et al have demonstrated that lack of the SREBP-1c factor induces alteration of the "bundle of Remak" and hypermyelination of the small-caliber fibers that compromise the functions of the nerves; peripheral nerves with a lack in the SREBP-1c show the decrease of the fatty acid synthesis and glycolytic flow and an accelerate catabolism of fatty acids and mitochondrial functions. (Horton et al., 2002). Cermenati et al have, also, found that knock-out (KO) SREBP-1c mice at 2 months of age shows a neuropathic phenotype with reduced expression of genes and altered levels of fatty acids such as palmitic, stearic, oleic and linoleic in the sciatic nerve (Cermenati et al., 2015).

Based on these observations, to prove that neuroactive steroids and fatty acid synthesis are two metabolic pathways sensitive each other, aim of this work was to evaluate, the levels of neuroactive steroids in sterol regulatory-binding protein-1c (SREBP-1c) knock-out (KO) male mice, an experimental model of peripheral neuropathy and to compare with observations in wild type animals. Neuroactive steroids levels have been evaluated by liquid chromatography tandem mass

spectrometry in plasma and sciatic nerve at two and ten months of age (Caruso et al., 2013). These analyses were complemented by the gene expression profile of crucial steroidogenic enzymes in Srebf-1cKO sciatic nerve of Srebf-1cKO and relative littermate control mice.

4.2 MATHERIALS AND METHOD

All solvents and reagents were liquid chromatography–tandem mass spectrometry (LC-MS) grade (CARLO ERBA Reagents, Milano, Italy). All steroids PREG, PROG, 5a-DHP, 3a,5a-THP, 3b,5a-THP, T, 5a-DHT, 3a-diol, 3b-diol, dehydroepiandrosterone (DHEA), and 17b-E were purchased from Sigma-Aldrich, Milan, Italy. Further three internal standards 17b-Estradiol- 2,3,4-$13C_3$, PREG-20,21-$13C_2$, and PROG-2,3,4-$13C_3$ were used to quantitative analysis ((Sigma-Aldrich).

4.2.1 ANIMALS AND REAGENTS

The animals used in the experiment were purchased from The Jackson Laboratories, Bar Harbor, ME, USA (B6;129S6-Srebf1tm1Jdh, stock number: 004365) and they were bred to generate a colony of wild type, heterozygous and homozygous mice. All procedures were performed following institutional guidelines (D.L. No. 26, March 4, 2014, G.U. No. 61 March 14, 2014) and international laws and policies (EEC Council Directive 2010/63, September 22, 2010: Guide for the Care and Use of Laboratory Animals, United States National Research Council, 2011). The genotyping was performed, in agreement with The Jackson Laboratory instructions, with the GoTaq Green Master Mix (Promega, Milan, Italy). To aim of the study a minimal number of animals was used and a F-test ANOVA analysis was applied by setting the effect size (f) at 0.75, the alpha error probability at 0.05, the power of the test ≥ 80%. We obtained five animals per group. After the genotyping animals were assigned to the four experimental groups (wild type and SREBP-1c KO at 2 months of age and WT and SREBP-1c KO at 10 months of age). 10 animals were used to form the wild type group and 10 animals for the SREBP-1cKO. Subsequently 5 animals for genotype were arbitrarily assigned for each group. To aim

of the study the experiments were conducted in male mice at 2 and 10-months of age. At 2 months of age, SREBP-1cKO animals must show a reduction in thermal and mechanical nociceptive threshold and no effect on nerve conduction velocity (early sign of peripheral neuropathy) compared to relative age mates. For 10-month mice: SREBP-1cKO male mice must show a reduction in thermal and mechanical nociceptive threshold and nerve conduction velocity (a late sign of peripheral neuropathy) compared to relative age mates.

4.2.2 NEUROACTIVE STEROIDS ANALYSIS

Neuroactive steroids levels have been evaluated by liquid chromatography tandem mass spectrometry in plasma and sciatic nerve at two and ten months of age following the extraction protocol described in (Caruso et a., 2013).

Internals standards were added to sciatic nerve tissues (15 mg for samples), homogenized in 2 mL of methanol (MeOH)/acetic acid (99:1 v/v) using a tissue lyser (Qiagen, Milan, Italy). After an overnight extraction at 4°C, samples were centrifuged for 5 min and the pellet was extracted twice with 1 mL of MeOH/acetic acid (99:1 v/v). The organic phases were combined and dried with a gentle stream of nitrogen in a 40°C water bath. The samples were resuspended with 3 mL of MeOH/H20 (10:90 v/v) and concentrated through SPE cartridges, previously activated with MeOH (5 mL) and MeOH: H2O 1: 9 v/v (5 mL), the steroids were eluted in MeOH, concentrated and transferred in autosampler vials before the LC– MS/MS analysis. To concern plasma samples, internal standards were added to 250 µL of plasma, were first extracted with acetonitrile, the organic residues were resuspended with 3 mL of MeOH/H20 (10:90 v/v) and passed through SPE cartridges; the steroids were eluted in MeOH, concentrated and transferred in autosampler vials.

Quantitative analysis was performed on the basis of calibration curves prepared and analyzed daily by positive atmospheric pressure chemical ionization (APCI+) using Ion Trap Mass Spectrometer (LTQ, ThermoElectron Co., San Jose, CA, USA) equipped with a Surveyor liquid chromatography (LC) Pump Plus and a Surveyor Autosampler Plus (ThermoElectron Co.). The mass spectrometer was operated in in tandem mode (MS/MS) using helium as collision gas.

The LC mobile phases were: 0.1% formic acid in water (phase A) and 0.1% formic acid in MeOH (phase B). The gradient (flow rate 0.5 ml/min) was as follows:

minuti	A%	B%
0,0	70	30
1,5	70	30
2,0	55	45
3,0	55	45
35,0	36	64
40,0	25	75
41,0	1	99
45,0	1	99
45,2	70	30
55,0	70	30

Table 1: Elution gradient of the chromatographic analysis

The Hypersil Gold column C18 (100 x 3 mm, 3 µm; ThermoElectron Co) was maintained at 40°C.

Neuroactive steroids	Parent ions	Transitions detected	CID
17β-Estraiol (17 β -E)	255.20	133;159	35
17a-Estraiol (17 α -E)	255.20	133;159	35
TESTOSTERONE (T)	289.2	97;109	35
Dehydroepiandrosterone (DHEA),	271.25	197;213	35
progesterone (PROG)	315.20	97;109	35
5 α - dihydrotestosterone (5a-DHT)	291.20	255.20	35
3 β -androstenediol(3b-diol)	257.20	121;135;147;161.20;175.20	35
3 α -androstenedio (3a-diol)	257.20	121;135;147;161.20;175.20	35
Pregnenolone (Preg)	299.20	159.20;199.20	35
5 α - dihydroprogesterone (5a-DHP)	299.20	189.20	35
3 β,5 α-Tetrahydroprogesteroide (ISOPREG)	301.20	159;173;213;227	35
3 α,5 α -Tetrahydroprogesteroide (THP),	301.20	159;173;213;227	35
17β estradiolo $^{13}C_3$	258.00	135;162	35
Progesterone $^{13}C_3$	318.00	100;112	35
Pregnenolone $^{13}C_4$	303.00	159;175;185;203;229;243;255	35

Table 2: ion molecular, fragment ion and collision energy of the neuroactive steroids under evaluation

4.2.3 GENE EXPRESSION ANALYSES

Gene expression analysis was performed extracting mRNAs from wild type and Srebp-1c knockout sciatic nerves. To this aim Nucleospin RNA II kit (Macherey-Nagel, Duren, Germany) was used. The gene expression profile of the crucial enzymes involved in steroidogenesis was carried out with a TaqMan qRT-PCR instrument (CFX96 real-time system; Bio-Rad Laboratories, Segrate, Italy) using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories). The relative quantification of the genes of interest was carried out by comparing their quantity with that of 36B4 and setting up a standard curve. The gene expression analyses were performed for:

P450 side chain cleavage (P450scc): enzyme responsible for the conversion of cholesterol to pregnenolone.

5a-Reductase (5a-R): enzyme involved in the conversion of Progesterone into 5 alpha DHP and Testosterone into 5a-DHT.

3a-hydroxysteroid oxidoreductase (3a-HSOR): enzyme necessary for the synthesis of 3a,5a-Tetrahydroprogesteroide (THP) and 3a-androstenedio (3a-diol).

4.2.4 STATISTICAL ANALYSIS

Comparison were obtained by two-way ANOVA, with genotype and age as two independent variables, followed by Bonferroni post-test. All statistical analyses were performed using PRISM version 5 (GraphPad, La Jolla, CA, USA) considering a $p < 0.05$ as statistically significant.

4.3 RESULTS

4.3.1 NEUROACTIVE STEROID LEVELS IN SCIATIC NERVE

To study the effect of the lack srebp-1c, the levels of neuroactive steroid (PREG, DHEA, PROG and its derivatives such as 5a-DHP, 3 a, 5a-THP and 3β,5 a-THP, T and its derivatives such as 5a-DHT, 3a-diol, 3β-diol and 17β-E were evaluated by LC- MS/MS in sciatic nerve and plasma of SREBP-1c KO and WT animals 2 and 10 months old. The validation of the method and transition used were previously described (Caruso et al., 2013). To have better sensitivity LC analysis was divided into three time windows: in the first one (13C-estradiol as internal standard) four analytes are eluted: 17a-E, 17β-E, T and DHEA; in the second one PROG, 5a-DHT, 3a- and 3β- diol are eluted using 13C-progesterone as internal standard, in the last window the quantification of PREG, 5a-DHP, 3a,5a-THP, and 3β,5a-THP was obtained with 13C-pregnenolone as internal standard. (Caruso et al., 2013).

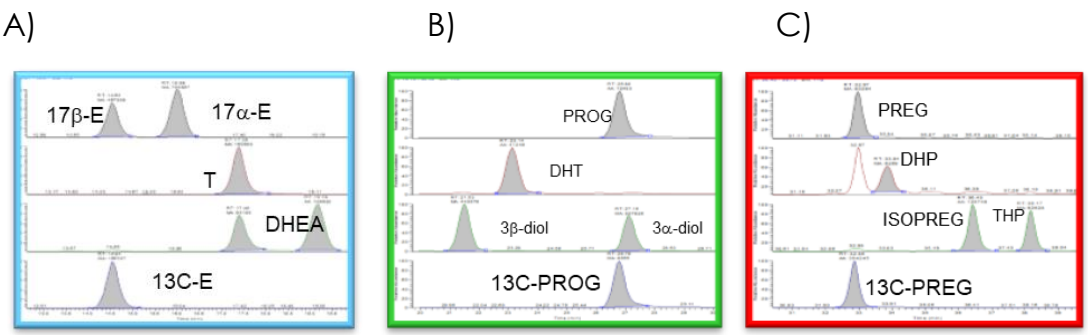


Fig.1: A)1° segment analysis; B) 2° window; C) the last segment in the analysis.

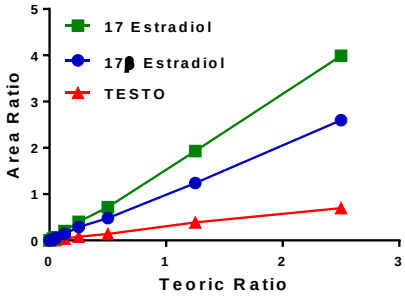


Fig.2: calibration curve of 3 neuroactive steroids evaluated

In the table 3, anova analysis has revealed a significant effect of genotype on the levels of PREG ($p<0.001$), PROG ($p<0.001$) and its metabolites 5α-DHP ($p<0.001$) and 3α,5α-THP ($p<0.05$). In the case of PREG levels, a significant effect of age and their interaction was also observed ($p<0.001$).

	METABOLITES	*PREG*	*PROG*	*5 α-DHP*	*3 α,5 α-THP*	*3 β,5α-THP*
***p* value summary**	INTERACTION	***	ns	***	ns	**
	GENOTYPE	***	***	***	*	ns
	AGE	***	ns	ns	ns	ns

Table 3: The effects of age, genotype, and their interaction in sciatic nerve of SREBP-1c KO and WT animals. The effects have been analyzed using the two-way ANOVA *p < 0.05; **p < 0.01, and ***p < 0.001

Bonferroni post-test has indicated that the levels of PREG depends on the age of the animals.

As shows in figure 3, at 2 months of age the levels of PREG are significantly higher in comparison to those observed in WT animals while lower levels of PREG was observed.

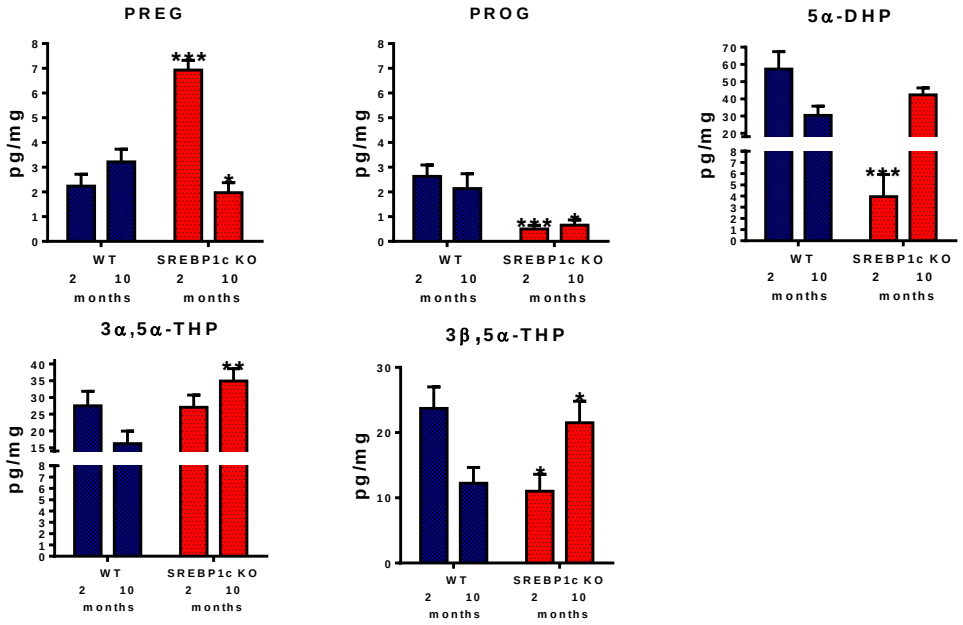


Fig.3: The levels of Pregnenolone (PREG), progesterone (PROG) and its derivatives in sciatic nerve of wild type (WT, blue bars) and sterol regulatory-binding protein-1c (SREBP-1c) knock-out (KO, red bars) mice at 2 and 10 months of age. Data are expressed as pg/mg tissue ± SEM. Bonferroni post-test: *p < 0.05; **p < 0.01, and ***p < 0.001 versus WT (n = 5 for each experimental group)

Changes in the concentration of PROG and 3a,5a-THP (THP) and 3β,5a-THP (ISOPREG) were reported at 2 and 10 months of age. With respect to WT mice, the levels of PROG are significantly decreased at 2 and 10 months of age in KO group, while its metabolites depending on the age of the mice. Indeed, the levels of 5a-DHP and ISOPREG were significantly lower in sciatic nerve of KO mice at 2 months of age, while at 10 months, a significant increase in 5 alpha DHP levels of SREBP-1C KO mice, THP and ISOPREG was observed in comparison with WT group.

Statistical analysis showed a significant effect of genotype, age and their interaction on the levels of testosterone, while the levels of 17 B estradiol were significant only for the interaction between genotype and age (Table 4).

	***p* value summary**		
METABOLITES	**INTERACTION**	**GENOTYPE**	**AGE**
DHEA	ns	ns	ns
TESTO	**	*	*
5α-DHT	ns	ns	ns
3α-DIOL	ns	ns	ns
3β-DIOL	ns	ns	ns
17β-E	**	ns	ns

Table 4: Effects of age, genotype and their interaction were analyzed by a two-way ANOVA test: * p <0.05, ** p <0.01, *** p <0.001.

Figure 4 showed the levels of androgen metabolites in sciatic nerve of SREBP-1c KO mice and WT animals at 2 and 10 months of age. Bonferroni post-test showed that T levels were significantly higher at 2 months of age in sciatic nerve of SREBP-1c KO in comparison to WT animals. The same effect was observed on the levels of 17β-E (Fig.4).

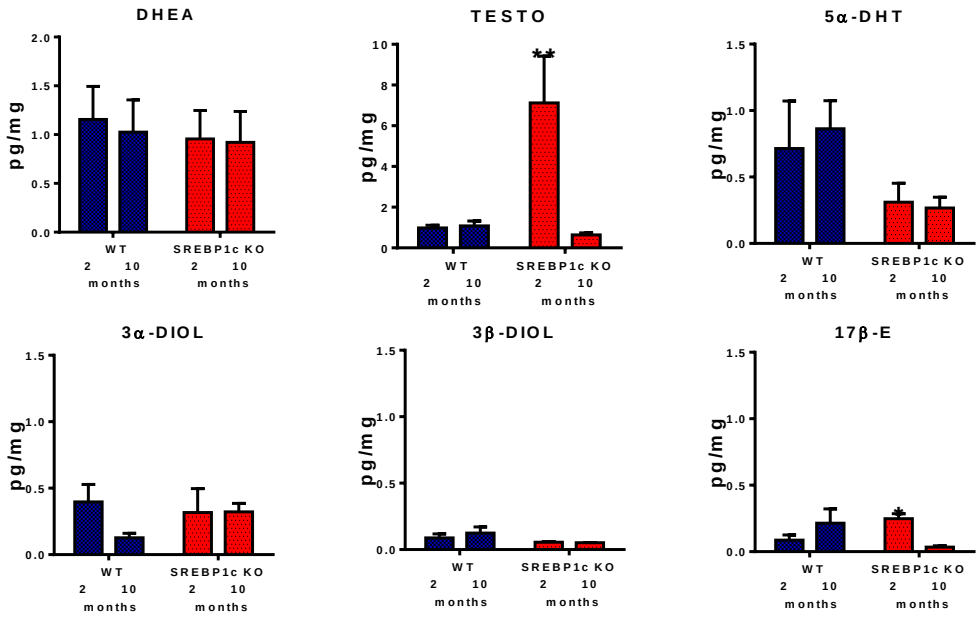


Fig.4: The levels of DHEA, T and its metabolites in sciatic nerve of wild type (WT, blue bars) and sterol regulatory-binding protein-1c (SREBP-1c) knock-out (KO, red bars) mice at 2 and 10 months of age. Data (n = 5 for each experimental group) are expressed as pg/mg tissue ± SEM. Bonferroni post-test: *p < 0.05; **p < 0.01, and ***p < 0.001 versus WT (see histogram).

4.3.2 NEUROACTIVE STEROID LEVELS IN PLASMA

As reported in table 5, Anova analysis has revealed a significant effect of genotype only on PREG ($p < 0.05$).

	METABOLITES	*PREG*	*PROG*	*T*	*17β-E*
***p* value summary**	**INTERACTION**	ns	ns	ns	ns
	GENOTYPE	*	ns	ns	ns
	AGE	ns	ns	ns	ns

Table 5: Effects of age, genotype and their interaction on the levels of some neuroactive steroid levels in plasma of SREBP-1c KO and WT animals. The effects were analyzed by a two-way ANOVA test: * p <0.05, ** p <0.01, *** p <0.001.

Figure 5 showed the levels of crucial metabolites in plasma of SREBP-1c KO mice and WT animals at 2 and 10 months of age. Quantitative analysis has indicated at 2 months of age, no significant difference in the levels of PREG and PROG and 17β-E, while Testosterone concentration was significantly higher in plasma of SREBP-1c KO compared with wild type group. At 10 months of age no significant difference was observed between WT and SREBP-1c KO groups for all the other neuroactive steroids (Fig.5).

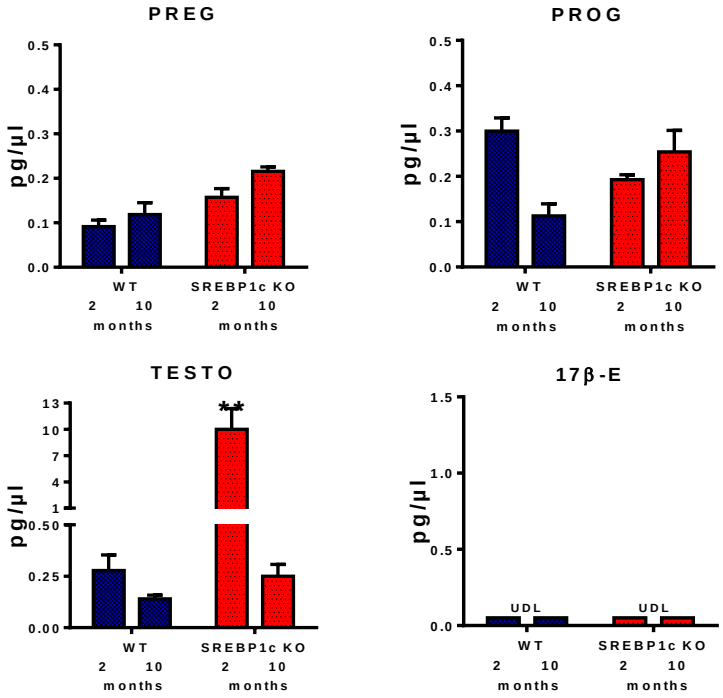


Fig.5: The levels of DHEA, T and its derivatives in plasma of wild type (WT) and sterol regulatory-binding protein-1c (SREBP-1c) knock-out (KO) mice at 2 and 10 months of age. Data (n = 5 for each experimental group) are expressed as pg/µL plasma T SEM. Bonferroni post-test: *p < 0.05; **p < 0.01, and ***p < 0.001 versus WT (see histogram). UDL: Under detection limit.

4.3.3 GENE EXPRESSION OF ENZYME IN STEROIDOGENESIS

The gene expression profile of crucial steroidogenic enzymes in sciatic nerve of Srebf-1cKO and control mice were detected at 2 and 10 months of the age.

Anova analyses showed significantly effect of the age on the levels of all enzymes analyzed. In addition, analyses have indicated an effect of genotype and age on the levels of 5a-R but no effect concerning the interaction between two parameters. To concern 3a-HSOR, a significant effect of genotype, age and interaction was observed.in the case of the enzyme P450scc, two way analysis has also revealed a significant interaction between two two parameters (table 6).

	METABOLITES	*P450scc*	*3α-HSOR*	*5 α-R*
***p* value summary**	INTERACTION	*	*	ns
	GENOTYPE	ns	*	**
	AGE	*	*	*

Table 6: Effects of age, genotype and their interaction on gene expression levels in sciatic nerve of SREBP-1c KO and WT animals. The effects were analyzed by a two-way ANOVA test: * p <0.05, ** p <0.01, *** p <0.001

Figure 7 shows the levels of the enzymes in sciatic nerve of Srebf-1cKO and wild type mice in the described experimental conditions. At two months of age, only P450scc enzyme shows a significant increase of the gene expression in Srebf-1cKO with respect to WT. In contrast, at 10 months of age, a significant increase in gene expression of the enzymes 5a-R and 3a-HSD enzymes were detected in SREBP-1c KO while no difference was observed in the gene expression of the enzyme P450scc (Fig.7).

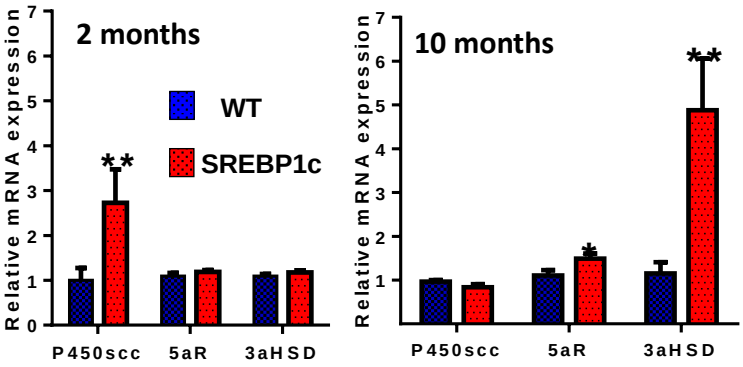


Fig.7: Levels of P450scc, 5a-R, 3a-HSD enzymes in the sciatic nerve of WT and SREBP-1c KO mice, respectively at 2 and 10 months of age. The data are expressed as relative mRNa ± SEM. Statistical analysis is performed using the Bonferroni test: * p <0.05, ** p <0.01, *** p <0.001 vs WT.

4.4 DISCUSSION

As described before, recently it has been demonstrated that the lack of SREBP-1c, the key lipogenic transcription factor lead to development peripheral neuropathy (Cermenati et., 2015). Several studies have observed changes in the levels of neuroactive steroids in sciatic nerve in different disease such as aging process, physical injury, diabetes (Melcangi et al., 2016; Giatti et al., 2015). According to previous paper, we found that SREBP-1c KO mice show alteration in the levels of some neuroactive steroids in sciatic nerve. Specifically, as reported in the figure 3. altered levels of PREG are observed depend on the evolution of the pathology. Indeed, at 2 months of age, we observed that SREBP-1c KO mice showed significantly higher levels of this neuroactive steroid in comparison to what was reported in WT animals, while at 10 months of age, lower levels of PREG was detected. According to this results, gene expression of the enzyme converting cholesterol into PREG followed this pattern, at least at 2 months of age. Specifically, P450scc enzyme shows a significant increase of the gene expression in Srebf-1cKO with respect to WT (Figure 7). This finding together the observation that PREG levels did not change in plasma, suggest a specific effect of SREBP-1c on steroidogenesis in the peripheral nerve. Changes in the levels of PREG induces, as reported in the figure 3, alterations in the levels of other metabolites of neurosteroidogenesis. Specifically, the levels of PROG are significantly decreased at 2 and 10 months of age in SREBP-1c KO mice when compared with WT animals. Concerning other metabolites of progestin pathway, the pattern depends on the age of the mice.

The levels of 5α-DHP significantly decreased at 2 months of age in sciatic nerve of SREBP-1c KO mice in comparison with control animals; in opposite, at 10 months of age, an increased in the levels

of 3α,5α-THP, metabolite of 5α-DHP, was observed when compared with WT animals. The increase of 3α,5α-THP levels is in agreement with the high gene expression of the enzyme 3 α-HSD, which convert DHP in 3α,5α-THP (Figure 7). In addition, also the levels of other metabolite of 5α-DHP, 3β,5α-THP were significantly modified in opposite mode at 2 and 10 months, namely decreased and increased respectively. With respect to androgens pathway, at 2 months of age only testosterone levels were significantly higher in sciatic nerve of SREBP-1c KO mice than in WT animals. This modification is not a peculiarity of sciatic nerve, but is present also in plasma. In addition, the levels of a metabolite of T, 17β-E, are also significantly higher in sciatic nerve of SREBP-1c KO mice than in WT animals but no variation in plasma levels is observed. Taken together, these results could be interpreted as an endogenous response to the first signs of peripheral neuropathy. These data are in agreement with what observed in other papers. Indeed, Patte-Mensah et al. have demonstrated that neuropathic pain (i.e., an important component of peripheral neuropathy) has an important impact on neurosteroidogenesis with a transient up-regulation of the PREG levels (Patte-Mensah et al. 2014). The high levels of neuroactive steroids as a response to the first stage of the peripheral neuropathy might be interpreted like a mechanism for coping with neurodegeneration. For instance, PREG has been reported to counteract the decrease of the amounts of myelin membranes induced by cryolesion in sciatic nerve as well as to increase, in guided regeneration of the rabbit facial nerve, the number and g-ratio of myelinated nerve (Koenig et al. 1995). At 10 months, when the peripheral neuropathy was evident, we found low levels of PREG and PROG and high levels of 3α,5α-THP and 3β,5α-THP. SREBP-1c KO sciatic nerve at 10 months of age experience energy depletion and mitochondrial dysfunction from a metabolic point of view. (Cermenati et al. 2015). To this regard, it well known that

Schwann cells and neurons rely on glycolysis as fuel source. The lack of SREBP-1c imposes cells to use fatty acid instead of glucose and lactate leading mitochondrial dysfunction in sciatic nerve of SREBP-1c null mice. On this basis, it might be possible that at 2 months of age, at the onset of the peripheral neuropathy, mitochondria try to counteract the first stage of neurodegeneration by inducing P450scc expression and consequently the levels of its product PREG.

On the other hand, at 10 months of age, the peripheral nerve boots the mitochondria to cope energy depletion, the levels of PREG are decreased as result of altered mitochondrial function. To compensate this altered mitochondrial function, SREBP-1c KO sciatic nerve increased both 5a-R and 3a-HSOR expression levels and relative metabolites. Several papers have, indeed, demonstrated that also PROG derivatives are effective in protecting peripheral nerve by aging damage, physical injury ((Melcangi et al. 2000;) and diabetic peripheral neuropathy (Cermenati et al. 2012; Mitro et al. 2014).

In conclusion, this research has highlighted one of application of LC-MS/MS; specifically, this study has indicated the ability of method to identify and quantify simultaneous main neuroactive steroids involved in steroidogenesis. These findings suggest that during the development of peripheral neuropathy because of the blunted FA biosynthesis (Cermenati et al., 2015), at the 2 months of age, the PNS try to counteract the early stages of neurodegeneration modulating the neuroactive steroids synthesis. During the progression of the pathology, the peripheral nerve boosts mitochondria to cope energy depletion due to the reduced glycolysis, and the levels of mitochondria-produced hormone such as PREG are decreased and downstream neuroactive steroids are instead increased. To summarize, these experiments have demonstrated that impair lipogenesis due to lack of SREBP-1c induces alteration on

steroidogenesis altering different levels of neuroactive steroids. These data further support the idea that neuroactive steroids, by a cross-talk with FA biosynthetic pathway, may represent a possible therapeutic strategy for peripheral neuropathy.

5. Diabetes induces mitochondrial dysfunction and alters cholesterol homeostasis and neurosteroidogenesis in the rat cerebral cortex

Romano S, Mitro N, Giatti S, Diviccaro S, Pesaresi M, **Spezzano R**, Audano M, Garcia-Segura LM, Caruso D, Melcangi RC. J Steroid Biochem Mol Biol. 2017 Nov 26. pii: S0960- 0760(17)30358-8.

5.1 INTRODUCTION

Diabetes mellitus is a metabolic disease where improper glycemic control may induce several complications in different organs.
This pathological situation can be the result of the lack or reduced insulin secretion from pancreas or an improper insulin function on target tissues such as liver, skeletal muscle, adipose tissues and others; a phenomenon known as insulin resistance (American Diabetes Association, 2009). The type 1 diabetes mellitus or insulin dependent diabetes mellitus, IDDM, is usually diagnosed in children and young adults, as a result of an autoimmune destruction of the insulin producing beta cells in the pancreas. Type 1 diabetes accounts for 5–10% of all diabetic patients around the world.
Another type of diabetes, non-insulin dependent diabetes mellitus (NIDDM) or type 2 diabetes, represents the most common form of diabetes (85–90% of all cases of diabetes). An important complication of diabetes mellitus is represented by the damage in the PNS (i.e., diabetic peripheral neuropathy) and CNS (i.e., diabetic encephalopathy).
Diabetic peripheral neuropathy occurs in more than 50% of all diabetic patients (Zochodne, 2007) involving a spectrum of functional and structural changes in peripheral nerves. These include slowing in nerve conduction velocity (NCV) followed by axonal degeneration, paranodal demyelination and loss of myelinated fibers (Sugimoto et al., 2000).
In addition, as shown in an experimental model of type 1 diabetes (i.e., rats raised diabetic by injection with streptozotocin, STZ), peripheral nerve alterations are represented by myelin invaginations in the axoplasm (infoldings) and myelin evaginations in the Schwann cell cytoplasm (outfoldings) as well as alterations in myelin compaction such as abnormally wide incisures and abnormal

separation of myelin lamellae (Veiga et al. 2006). Important myelin components, such as proteins (i.e., myelin protein zero, P0, and peripheral myelin protein 22, PMP22) and lipids (i.e., phospholipids, fatty acids, and cholesterol content), are also strongly affected by diabetes mellitus (Cermenati et al., 2012).

In addition, in a mouse model of type 1 diabetes, prolonged hyperglycemia leads to accumulation of small fragmented mitochondria in DRG probably as a consequence of altered mitochondrial dynamics, such as fission and fusion and/ or trafficking (Vincent et al. 2010). In addition, neuropathological events, including diabetic encephalopathy (i.e., damage induced by diabetes in the brain) and diabetic peripheral neuropathy (i.e., damage in the peripheral nervous system), affect neuroactive steroid levels.

Indeed, it has been observed decreased levels of several neuroactive steroids in plasma of streptozotocin (STZ) induced diabetes mice at three months (M. Pesaresi, et al., 2010).

In addition, long-term type 1 diabetes has been shown to alter cholesterol homeostasis not only in the peripheral nervous system but also in cerebral cortex (Cermenati et al., 2017). Furthermore, STZ diabetes in rats also affect mitochondrial function in the cerebral cortex (Mastrocola, et al., 2005).

In the last years, a growing experimental evidence suggests that cholesterol oxidation products, known as oxysterols, play an important role in the development of major chronic diseases, including atherosclerosis, alzheimer's disease, and inflammatory bowel disease (Testa G et al., 2018). In addition, several studies have indicated that oxysterol may be the link between altered cholesterol metabolism and human diseases (Testa et al., 2018). Oxysterols are involved in various key steps of these complex processes. They are signaling molecules and interact with different types of protein targets exhibiting regulatory function on cholesterol metabolism and

homeostasis (Javitt NB, 2002). Oxysterols, also, act as ligands to nuclear receptors; indeed, as reported in Theofilopoulos et al (2012), activation of LXR by oxysterols can induce neurogenesis of dopaminergic neurons.

On the basis of previous data and taking into account that functional and biochemical changes may already occur in short-term diabetes (Suzuki, et al., 2010), in this study by means of LC-MS/MS tecnique, levels of cholesterol, oxysterols and neuroactive steroids in the cerebral cortex after one month of diabetes, were evaluated.

5.2 MATERIALS AND METHODS

All solvents and reagents were liquid chromatography–mass spectrometry (LC-MS) grade (CARLO ERBA Reagents, Milano, Italy). PREG, PROG, 5α-DHP, 3α,5α-THP, 3β,5α-THP, T, 5V-DHT, 3α- diol, 3 β -diol, dehydroepiandrosterone (DHEA), 17 β -E and the internal standards 17b-Estradiol- 2,3,4-$^{13}C_3$, PREG-20,21-$^{13}C_2$, and PROG-2,3,4-$^{13}C_3$ such as cholesterol and cholesterol-2,2,3,4,4,6-d_6 its internal standard were purchased from Sigma-Aldrich, Milan, Italy.

(S.I) 7a-hydroxycholesterol, 7β-hydroxycholesterol were bought from Steraloids (Newport, RI, USA), 24(S)-hydroxycholesterol, 27-hydroxycholesterol, 7-ketocholesterol were obtained from ResearchPlus (Barnegat, NJ, USA).

5.2.1 ANIMALS

Male Sprague-Dawley rats (175–200 g at arrival, Charles River Laboratories, Lecco, Italy) were housed in the animal care facility of the Dept of farmacological and biomolecular sciences (DiSFeB). All animals were kept in standard rat cages with food and tap water available ad libitum and under controlled temperature (21 ± 4 ° C), humidity (40–60%), room ventilation (12.5 air changes for h) and light cycles (12 - hour light / dark cycle; on 7 am / off 7 pm).

The animals were randomly divided into two experimental groups: 1) control non-diabetic rats (CTRL) and 2) diabetic rats (STZ) following institutional guidelines (D.L. No. 26, March 4, 2014, G.U. No. 61 March 14, 2014) and international laws and policies (EEC Council Directive 2010/63, September 22, 2010: Guide for the Care and Use of Laboratory Animals, United States National Research Council, 2011). Diabetes was induced by a single injection of freshly prepared STZ (60

mg/kg body weight; Sigma-Aldrich, Milano, Italy) in citrate buffer (0.09 M pH 4.8). (Pesaresi et al.,2010). After 48 h only animals with glucose level above 300 mg/dl were considered as diabetic. After one month, CTRL and STZ rats were sacrificed, the cerebral cortex and plasma were collected, weighed and stored at−80 °C until analysis.

5.2.2 NEUROACTIVE STEROIDS ANALYSIS

Neuroactive steroids (table 2) levels have been evaluated by liquid chromatography tandem mass spectrometry (LC-MS/MS) in plasma and cerebral cortex at one months of age in non-diabetic and STZ animals, after extraction and purification as described in the chapter 4.2.2.

5.2.3 CHOLESTEROL AND OXYSTEROLS ANALYSIS

The levels of free and total cholesterol as well as of its mainly metabolites, reported in the table were assessed by LC–MS/MS in the cerebral cortex of non-diabetic and STZ rats.

Analytes	**Parent ions**	**Transitions detected**	**CID**
Cholesterol d_6	375.5	135,149,161, 243, 257	35
Cholesterol (CHOL),	369.5	135, 161, 175, 233, 243	35
27-hydroxycholesterol (27-OH)	385.5	135, 147, 161, 175, 189, 203, 215, 229, 243, 257	35
7α-hydroxycholesterol (7α-OH)	385.5	145, 149, 173, 187, 201, 213, 219, 227, 241, 255	35
7β-hydroxycholesterol (7β- OH)	385.5	145, 149, 173, 187, 201, 213, 219, 227, 241, 255,	35
7-ketocholesterol (7-keto)	401.0	365, 383	35
24(S)-hydroxycholesterol (24(S)-OH)	385.5	135, 147, 161, 175, 189, 203, 215, 229, 243, 257	35

Table 7: parent ion, transition detected and collision energy of the cholesterol and oxysterols under evaluation

Cerebral cortex of CTRL and STZ rats (60mg), were weighted, added with internal standards and homogenized in 1 ml of MeOH/ACN (1:1, v/v) using the TissueLyser (Qiagen, Italy). After centrifugation at 12,000 rpm for 5 min, supernatant was divided in two aliquots: one for the analysis of free cholesterol and oxysterols and the other for total cholesterol. To analyze total cholesterol levels, aliquots were added

in chloroform-MeOH 1:1, v/v and 1M HCl:MeOH (1:1, v/v). The mixture was shaken for 2 hours with mechanical agitator at room temperature. After addition of a volume of chloroform-water (1:1, v/v), the lower organic phase was collected, transferred into new tubes and dried under nitrogen flow (Taguchi et al. 2010).

Quantitative analysis was performed using calibration curves prepared and analyzed in the same day of the biological samples ranging 0.5ng-5ng/sample and and 10-100µg/sample for oxysterols and cholesterol respectively.

The analyses were performed using a linear Ion Trap Mass Spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source (LTQ, ThermoElectron Co., San Jose, CA, USA, a Surveyor liquid chromatography (LC) Pump Plus and a Surveyor Autosampler Plus (ThermoElectron Co.). The mass spectrometer was operated in positive-ion mode and in tandem mode (MS/MS) using helium as collision gas.

The LC mobile phases were: ACN/MeOH/H_20 (76:20:4) (phase A) and Isopropanol (phase B). The gradient (flow rate 1 ml/min) was as reported in the following table:

Time	A%	B%
0.0	100	0
15.0	100	0
15.5	50	50
35.0	50	50
35.5	100	0
50.0	100	0

Table 8: Elution gradient of the chromatographic analysis

An Intersil ODS column C18 (150 x 3 mm, 3 µm; GL sciences) was used for chromatographic separation and maintained at 40°C.

5.2.4 QUANTITATIVE REAL TIME PCR (RT-QPCR)

Gene expression analysis was performed extracting mRNAs from cerebral cortex of control and diabetic rats. The extraction was performed using Directzol™ miniprep kit (Zymo Research, Irvine, California, USA) following specific manucfaturing protocol. The gene expression of enzyme was carried out with TaqMan quantitative real-time PCR using a CFX96 real-time system (Bio-Rad Lab, Segrate, Italy). The relative quantification of the genes was carried out by comparing their quantity with that of 36B4 and setting up a standard curve. The gene expression analyses were performed for:

Sterol regulatory element-binding protein 2 (SREBP2): enzyme involved in the regulation of the biosynthesis of cholesterol.

3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA R): rate-controlling enzyme of the mevalonate pathway

Low density lipoprotein receptor (LDLR): internalizes cholesterol-rich lipoproteins into the cellular compartment.

Steroidogenic acute regulatory protein (StAR,): enzyme involved in the transport of cholesterol in the mitochondria.

Translocator protein- 18 kDa (TSPO,): enzyme involved in the transport of cholesterol in the mitochondria.

P450 side chain cleavage (P450scc): enzyme responsible for the conversion of cholesterol to pregnenolone.
Sterol 27-hydroxylase (Cyp27A1): a gene encoding a cytochrome P450 oxidase.

Sterol O-acyltransferase (SOAT1): enzyme that favors the conversion of free cholesterol into cholesteryl esters.

Hormone sensitive lipase (HSL): enzyme that hydrolyzes cholesteryl esters into free cholesterol

apolipoprotein E (APOE): protein involved in the metabolism of fats in the body

ATP- binding cassette G1 (ABCG1) and ATP- binding cassette A1 (ABCA1): enzyme involved in cholesterol efflux

5.2.5 STATISTICAL ANALYSIS

Student's t-test was used to compare results obtained by the analysis of plasma and cerebral cortex of CTRL and STZ rats. A p-value < 0.05 was considered significant. All statistical analyses were per- formed with GraphPad PRISM version 6 (San Diego, CA, USA).

5.3 RESULTS

5.3.1 SHORT-TERM DIABETES ALTERED NEUROACTIVE STEROID LEVELS IN THE CEREBRAL CORTEX AND PLASMA.

The levels of different neuroactive steroid were evaluated in cerebral cortex and plasma of control non-diabetic and diabetic rats by LC–MS/MS.

Specifically, in the figure 8 the levels of progestin neuroactive steroids in cerebral cortex are reported. As shown, we found that the levels of PREG, PROG and 3a,5a-THP significantly decreased in diabetic animal when compared with non-diabetic group (Fig.8).

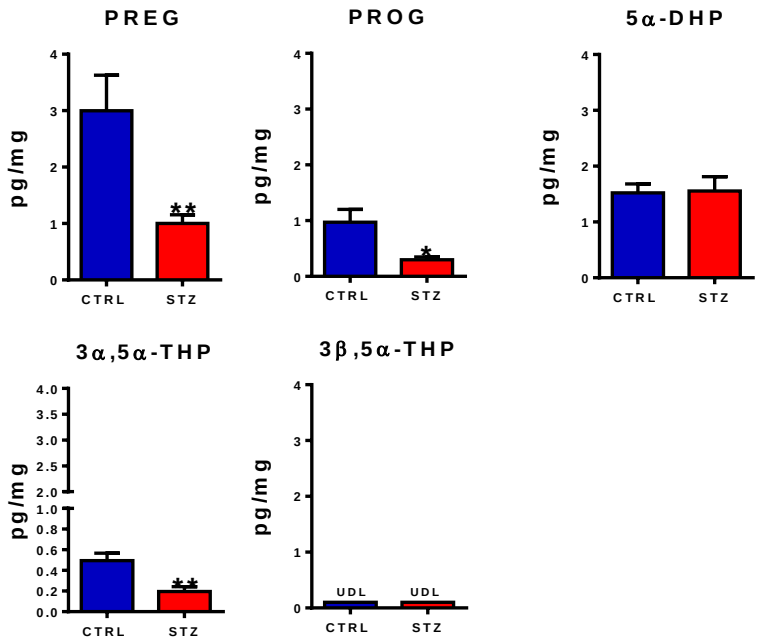


Fig.8: The levels PREG and its derivatives in cerebral cortex of control (CTRL) and diabetic rats (STZ) at 1month of age. Data (n = 5 for each experimental group) are expressed as pg/mg tissue ± SEM; t-Student: *p < 0.05; **p < 0.01, and ***p < 0.001 versus CTRL. UDL: Under detection limit.

To concern androgen pathway, neuroactive steroids analysis has indicated significant difference in some neuroactive steroids. We found that the levels of T and two metabolites such as 5a-DHT and 3a-diol, were significantly decreased in the cerebral cortex after short- term diabetes when compared with control group (Fig.9).

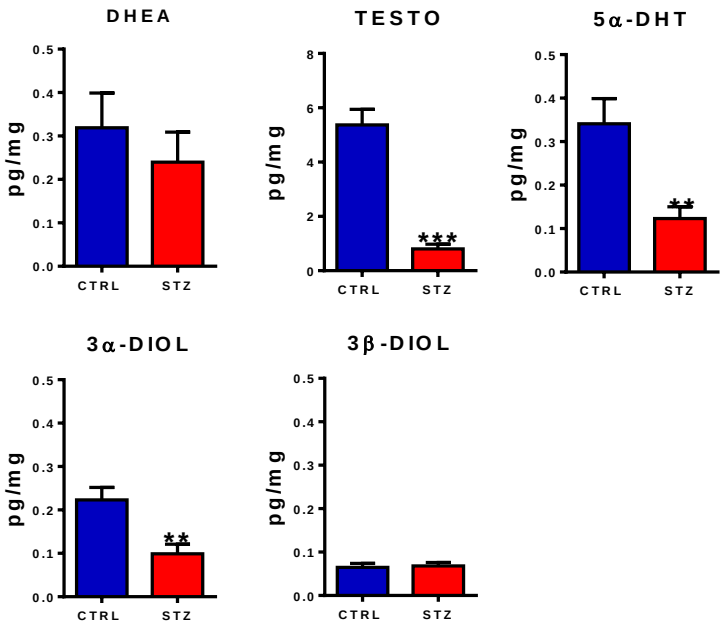


Fig.9: The levels DHEA and its derivatives in cerebral cortex of control (CTRL) and diabetic rats (STZ) at 1month of age. Data (n = 5 for each experimental group) are expressed as pg/mg tissue ± SEM; t student: *p < 0.05; **p < 0.01, and ***p < 0.001 versus CTRL. UDL: Under detection limit.

Neuroactive steroid levels were, also, evaluated in plasma of STZ and control animals at one month of age. The levels of neuroactive steroids were assessed by LC-MS/MS. As reported in the figure 10, a significant decrease in the levels of metabolites 3b,5a-THP were observed.

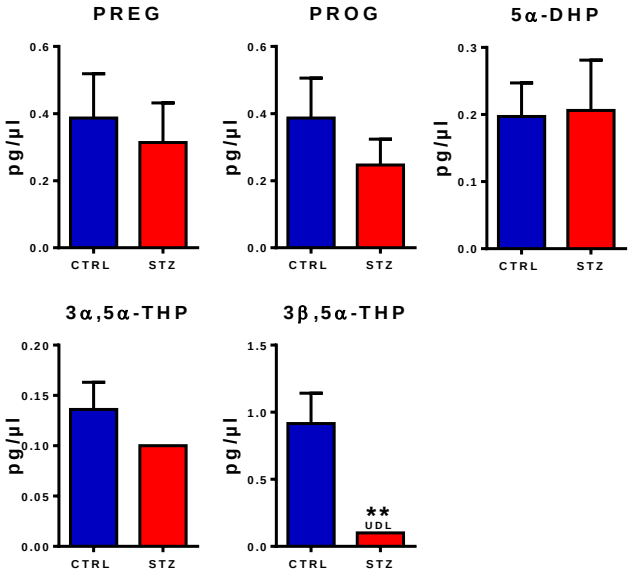


Fig.10: The levels PREG and its derivatives in plasma of control (CTRL) and diabetic animals (STZ) at 1month of age. Data (n = 5 for each experimental group) are expressed as pg/µL plasma ± SEM; t-sudent: *p < 0.05; **p < 0.01, and ***p < 0.001 versus CTRL. UDL : Under detection limit.

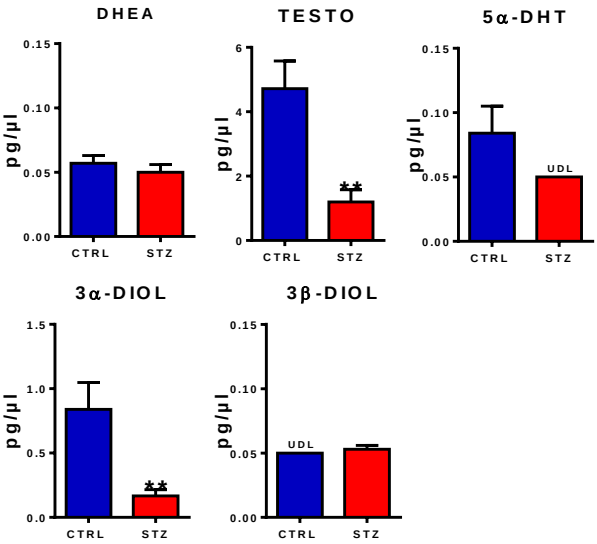


Fig.11: The levels DHEA and it derivatives in plasma of control (CTRL) and diabetic animals at a1month of age. Data (n = 5 for each experimental group) are expressed as pg/µL plasma ± SEM; t student: *p < 0.05; **p < 0.01, and ***p < 0.001 versus CTRL. UDL : Under detection limit (<0.05 pg/µL)

In the figure 11, analysis of androgens is reported. Analysis showed a significant decrease in the levels of Testosterone (**p < 0.01) and its metabolite 3a-DIOL ((**p < 0.01) were observed in STZ group.

5.3.2 SHORT-TERM DIABETES ALTERED THE LEVELS OF CHOLESTEROL AND OXYSTEROLS IN THE CEREBRAL CORTEX

The levels of free cholesterol and oxysterols as well as total cholesterol were evaluated in cerebral cortex in both experimental groups. The analyzes were carried out by LC-MS/MS and the chromatographic profile obtained is shown in the following figure:

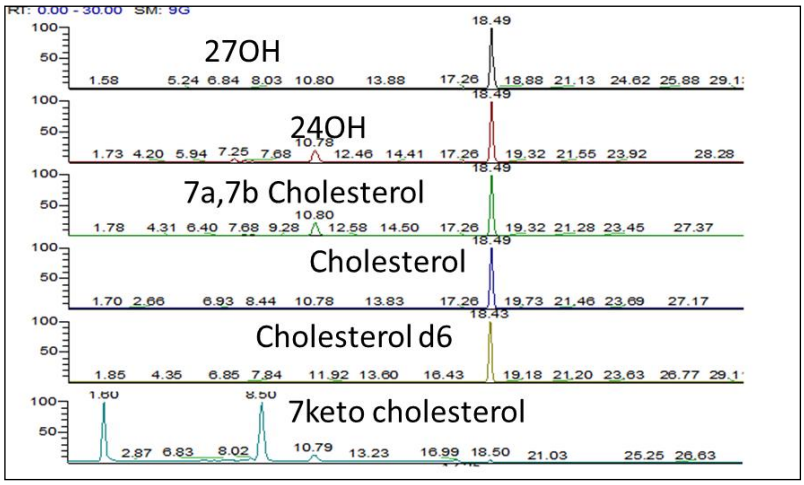


Fig.12: Chromatogram of cholesterol and its derivates.

Quantitative analysis was performed on the basis of calibration curves prepared daily. The figure shows a calibration curve obtained for cholesterol analysis:

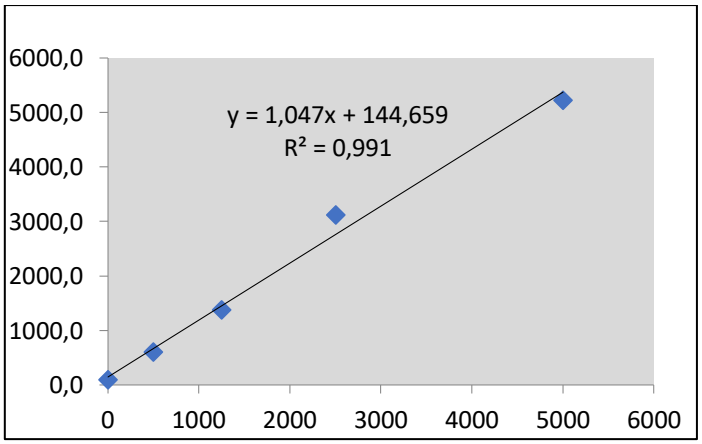


Fig.13: calibration curve of cholesterol

Figure 14 shows free and total cholesterol levels in STZ and control rats at one month of age. As reported, the levels of free cholesterol in the cerebral cortex was significantly decreased by short-term diabetes, while no difference in the levels of total cholesterol was observed.

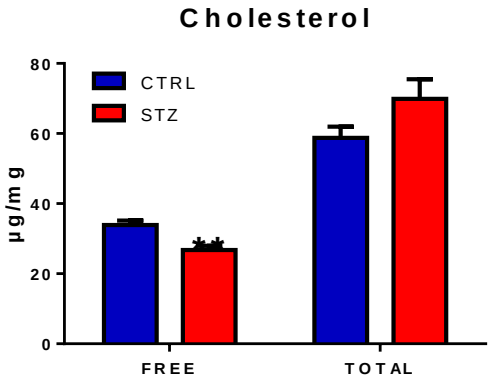


Fig.14: The levels of free and total cholesterol in STZ (red bar) and control (blue bar) at 1month of age. Data (n = 5 for each experimental group) are expressed as µg/mg ± SEM; t student: **p < 0.01versus CTRL.

As reported in fig. 15, the levels of 24(S)-hydroxycholesterol (24(S)-OH) and 27-hydroxycholesterol (27-OH), two enzymatic metabolites of cholesterol, were significantly decreased in the cerebral cortex of diabetic animals. In opposite, the levels of 7a-hydroxycholesterol (7a-OH), 7β-hydroxycholesterol (7β- OH) and 7-ketocholesterol (7-keto), three non-enzymatic oxysterols, were significantly increased in diabetic animals when compared with control group.

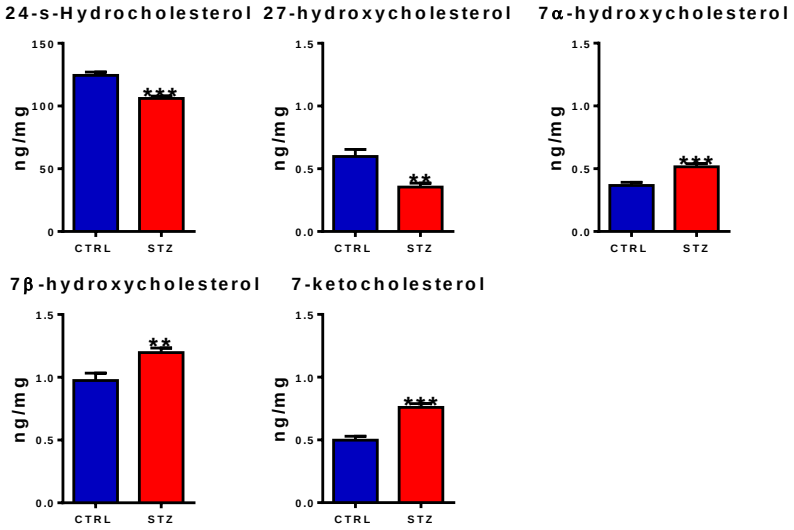


Fig.15: The levels of free oxysterols in diabetic and control rats at a1month of age. Data (n = 5 for each experimental group) are expressed as ng/mg ± SEM; t student: **p < 0.01, ***p < 0.001versus versus CTRL.

5.3.3 GENE EXPRESSION OF ENZYME INVOLVED IN BIOSYNTHESIS OF CHOLESTEROL AND STEROIDOGENESIS

To support alteration in the levels of cholesterol derivates and neuroactive steroids, the gene expression profile of enzymes involved in the biosynthesis of cholesterol and in metabolism of neuroactive steroids were evaluated (see chapter 5.2.4) The analyses were performed in cerebral cortex of diabetic and control animals at 1 month of age.

In the figure 16 (panel A) the gene expression of enzymes involved in cholesterol biosynthesis was reported. Results indicated that gene expression levels of sterol regulatory element-binding protein 2 (SREBP2), which is involved in the regulation of the biosynthesis of cholesterol, significantly decreased in STZ group when compared with control rats. In addition, the gene expression of HMG-CoA R (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase) and LDLR (low density lipoprotein receptor) were down regulated in the cerebral cortex of diabetic rats. The first one is the rate-controlling enzyme of the mevalonate pathway, , while LDLR internalizes cholesterol-rich lipoproteins into the cellular compartment.

In the figure 16 (panel B) the expression levels of mitochondrial cholesterol carriers such as Steroidogenic acute regulatory protein (StAR) and Translocator protein- 18 kDa (TSPO). According to the low levels of PREG observed in cerebral cortex of STZ animals, also gene expression of Star and TSPO decreased significantly while the expression of P450scc, the first neurosteroidogenic enzyme that convert cholesterol in PREG, remained unchanged. In addition, according to the decrease in the levels of 27 OH observed in the cerebral cortex of diabetic animals, a downregulation of Cyp27A1 was observed in STZ rats.

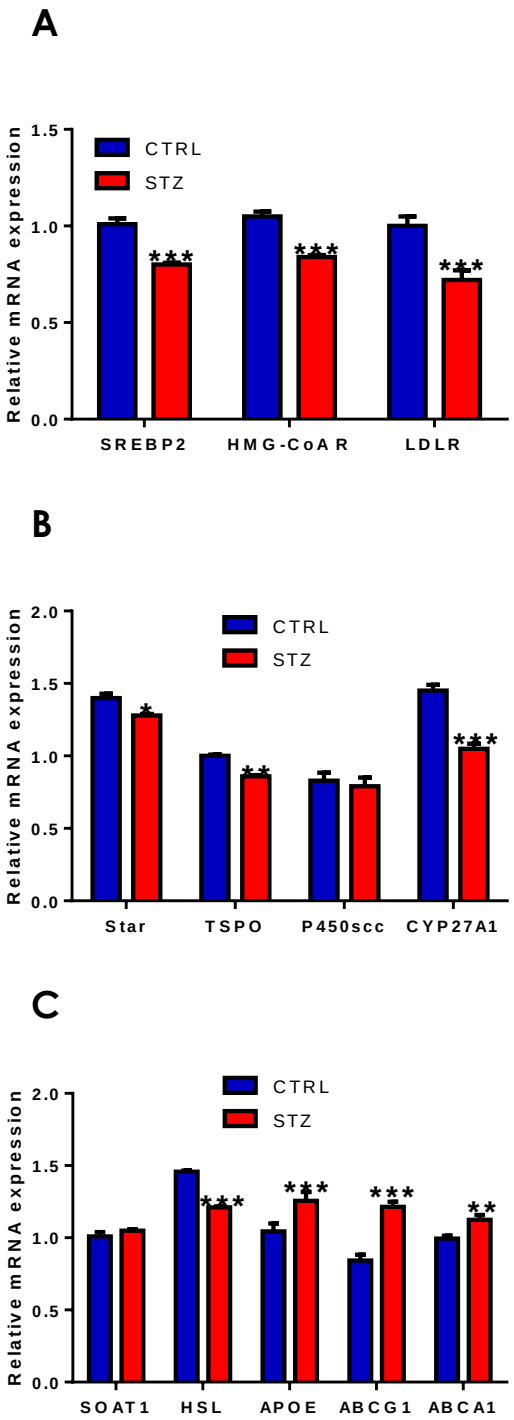


Fig.16: A) the gene expression of enzyme involved in cholesterol biosynthesis in the cerebral cortex of diabetic and control animals. **B)** the mRNA levels of molecules correlated to mitochondrial metabolism of cholesterol in the cerebral cortex of both experimental groups. **C)** One month after the STZ induction, diabetes modifies mRNA levels of molecules involved in cholesterol bioavailability in the rat cerebral cortex. Data (n = 10 animals for each experimental group) are expressed as mean ± SEM; t student: **p < 0.05**p < 0.01 p < 0.001 versus CTRL.

To evaluate bioavailability of cholesterol, the expression levels of sterol O-acyltransferase (SOAT1), which favors the conversion of free cholesterol into cholesteryl esters, and hormone sensitive lipase (HSL), which hydrolyzes cholesteryl esters into free cholesterol were assessed in both experimental groups. As showed in figure 16c SOAT1 mRNA remained unchanged in the cerebral cortex, while, HSL mRNA levels were down-regulated by short-term diabetes.

In addition, efflux another important pathway in the regulation of cholesterol homeostasis. We have observed an increase in apolipoprotein E (APOE) gene expression in the cerebral cortex of STZ rats. Finally, the effect of diabetes on two transporters highly expressed in the CNS and involved in cholesterol efflux like the ATP-binding cassette G1 and A1 (i.e., ABCG1 and ABCA1) were detected. As reported in figure 16c, mRNA levels of ABCG1 and ABCA1 were increased in the cerebral cortex after short-term diabetes confirming that diabetes affects cholesterol efflux.

5.4 DISCUSSION

The results have indicated that neuroactive steroid levels are changed in the cerebral cortex after one month after the induction of diabetes. Specifically, significantly decreased levels of neuroactive steroids such as PREG, PROG, THP, T, DHT and 3a-diol were observed in diabetic rats when compared with control animals (Figure 8 and 9).

In addition, it has been observed that short-term diabetes doesn't affect PREG plasma levels, suggest a specific effect of short-term diabetes on neurosteroidogenesis (Figure 10 and 11.

Furthermore, because cholesterol is precursor of steroidogenesis and it has been observed that diabetes affects the free cholesterol content of the brain (Cermenati et al, 2017), changes in the levels of PREG could be due to the availability of cholesterol.

On the basis of this considerations, we want to confirm that the level of free cholesterol, evaluated by LC-MS/MS, was significantly decreased in the cerebral cortex of STZ rats in comparison with control animals (Figure 14).

Previous papers have demonstrated that two months after the induction of diabetes, the biosynthesis of cholesterol decreased, probably due to the decrease in the levels of cholesterols, accordling with this evidence, the gene expression of SREBP2, HMG-COA R and LDLR was reduced in the cerebral cortex of STZ rats suggesting that decreased cholesterol synthesis may be involved in the lower levels of free cholesterol (Figure 16A). In addition, we reported that the gene expression of HSL, enzymes that plays a key role in the regulation of intracellular free cholesterol amount and in steroidogenesis, was decreased in the cerebral cortex of diabetic rats, suggesting that also this mechanism may contribute to the reduced amount of free cholesterol. Furthermore, to support the

results observed by LC-MS/MS analyses after one month after the injection of STZ, an upregulation of APOE gene expression and a downregulation of LDLR were observed in the cerebral cortex, suggesting an increase of cholesterol efflux from cellular compartment without an efficient uptake. These data is in agreement with previous study reporting an increase in APOE immunoreactivity in the hippocampus STZ mice short term diabetes (Revsin et al., 2005). In addition, the increase in the mRNA levels of APOE in the cerebral cortex of STZ rat was correlated with an increase of ABCG1 and ABCA1 gene expression, (Karten et al., 2006).

Cholesterol homeostasis also includes active metabolism into different oxysterols, like 24(S)-OH and 27-OH cholesterol. LC-MS/MS analyses have indicated that short- term diabetes significantly reduced levels of these oxysterols (24(S)-OH and 27-OH cholesterol) in the cerebral cortex (Figure 15). According to these evidences, a decrease in mRNA levels of the enzyme Cyp27A1, which catalizes 27-OH cholesterol synthesis at the inner mitochondrial membrane, was observed (Figure 16B). Moreover, a significant increase of the oxysterol levels (i.e., 7α-OH, 7β-OH and 7-keto) was detected (Figure 15). These molecules may have a crucial role in the down-regulation of HMG-CoA R. Indeed, as demonstrated in cultures of rat astrocytes, the treatment with 7β-OH or 7-keto inhibits the activity of HMG- CoA R (Behr et al.,1991).

The data, here reported, highlights that the decrease in PREG levels detected in the cerebral cortex of diabetic rats could be due to a decrease in cholesterol bioavailability. Indeed, mRNA levels of HMG-CoA R and HSL were down-regulated while those formed by oxidative activity (7β-OH and 7-keto) were upregulated in the cerebral cortex of STZ rats. In addition, the gene expression of SOAT1 (i.e., the enzyme producing cholesteryl esters) remained unmodified.

These findings suggest that short-term diabetes has specific effects depending on the brain region considered. This consideration is in agreement with what observed in the hippocampus, where free cholesterol levels were increased while 27-OH was unmodified (S. Romano et al., 2017).

Regarding the LC-MS / MS analysis on neuroactive steroids, the decrease in PREG concentration observed in the cerebral cortex of STZ rats may be due to an impairment of steroidogenic machinery.

Indeed, short-term diabetes causes a decrease in StAR and TSPO mRNA levels.

The observed reduction of StAR gene expression, associated with the decrease of TSPO gene expression, is in agreement with previous observations in previous paper as well as in cerebral area, as in the hippocampus (Kraemer et al.,2004; Romano et al.,2017). In contrast to observation conducted in hippocampus (Romano et al.,2017), in the cerebral cortex the gene expression of P450scc (i.e., the enzyme that converts cholesterol into PREG) was not altered. It is important to highlight that mitochondrial P450scc uses electrons from NADPH via the ferredoxin (FDX) and ferredoxin reductase (FNR) (Wada et al., 1999). Furthermore, P450scc and CYP27A1 in adipocytes can compete for the common redox partners, because CYP27A1 binds with higher affinity to FDX than P450scc (Miller et al., 2011) Therefore, since Cyp27A1 gene expression and the levels of 27-OH, which promotes metabolism of cholesterol to more polar compounds that are more efficiently exported from cells than the parent compound, were diminished in the cerebral cortex of STZ rats, a functional shift of cholesterol metabolism into the mitochondrial compartment to maintain sufficient amount of PREG could be hypothesized.

The present study has highlighted as LC-MS/MS technique, using a linear ion trap mass spectrometer, allow simultaneous quantification of 13 neuroactive steroids in a single analysis. In addition, 6 cholesterol

derivatives by LC-MS/MS analyses in cerebral cortex and plasma of short-term diabetes and control animal were detected. As reported in different papers, alterations in the levels of neuroactive steroids could be considered as important functional diagnostic markers for diabetic encephalopathy (Lavaque, et al., 2004: Melcangi et al., 2014). In addition, treatments with neuroactive steroids or with inducers of steroidogenesis are effective in reducing neural damage in STZ experimental models (Cermenati et al., 2017; Pesaresi et al., 2010). Indeed, diabetic encephalopathy is characterized by neurophysiological and structural changes and this complication may increase the risk of cognitive deficits and dementia especially in type-1 diabetic patients (Gispen et al., 2000) On this point of view, the finding that type 1 diabetes seems to affect neurosteroidogenesis before than peripheral steroidogenesis suggests that a therapeutic approach based on these molecules against diabetic encephalopathy could be more appropriate and effective in the early stages of the pathology.

6. Lipidomic studies in a mice model of SCA38, a newly identified form of spinocerebellar ataxia.

Manuscript in progress

6.1 INTRODUCTION

Spinocerebellar Ataxias (SCAs) are a group of autosomal dominant neurological disorders. SCAs are phenotypically characterized by gait and limb ataxia, incoordination of eye movements and speech and hand disturbances. Cerebellar hypometabolism is well documented and considered a main diagnostic marker (Durr, 2010). Ataxia is defined as a disturbance of balance and incoordination occurring in the absence of muscle weakness and can arise from dysfunction of the cerebellum (Fogel et al., 2006). The cerebellum plays a critical role in this process through the integration of multimodal sensory data with motor output predictions to yield smooth well-timed movement (Manto, et al., 2012).

The cerebellar syndrome is often associated with other neurological signs such as pyramidal or extrapyramidal signs, ophthalmoplegia, and cognitive impairment (Harding, 1983). Onset is usually during the third or fourth decade of life, but it can occur in childhood or old age (Harding, 1983). Atrophy of the cerebellum and brainstem are most often the prominent features, but other structures can be affected, leading to a substantial range of phenotypes (Yamada et al., 2008).

Recently it has been identified a new form of Spinocerebellar Ataxia type 38 (SCA38) caused by mutations in the ELOVL fatty acid elongase 5 gene (Di Gregorio et al., 2014). SCA38 is a rare form of inherited ataxia and the disease onset is in the fourth decade of life, characterized by slowly progressive gait ataxia and associated in most of the cases with *pes cavus* and hyposmia. The disease progresses with limb ataxia, dysarthria, dysphagia, ophthalmoparesis, and, in the later stages, sensory loss. Brain imaging documented cerebellar hypometabolism with sparing of cerebral cortex (Borroni et al., 2016).

ELOVL5 encodes an elongase enzyme involved in the synthesis of long-chain polyunsaturated fatty acids (lc-PUFA) and is highly expressed in murine and in human Purkinje cells (Di Gregorio et al., 2014). They are inhibitory neurons that regulate the complex and coordinated movements, preventing an abrupt movement. In humans, Purkinje cells can be damaged by a variety of causes: toxic exposure, for example alcohol or lithium; autoimmune diseases; genetic mutations that cause spinal-cerebellar ataxias (Marmolino et al., 2010).

Polyunsaturated fatty acids are required for the normal development of the central nervous system, indeed in recent years, polyunsaturated fatty acids like DHA have gained much attention due to promising results in a number of neurodegenerative conditions such as Parkinson disease (Hacioglu et al., 2012; Yassine et al., 2017). In this contest, several studies have demonstrated that oral intake DHA reduces brain pathology improving cognitive deficits and protecting against synaptic degeneration (Lim et al 2005; Green et al 2007). Given the specific expression of ELOVL5 in Purkinje cells, a reduction in the fatty acid synthesis could damage cerebellar function. PLs containing ω-3 PUFAs seem to show beneficial effects in different disorders through specific mechanisms and a plethora of bioactivities including their ability to modulate the eicosanoid pathway (Burri et al., 2012; Hishikawa et al., 2017; Murray et al., 2015). On this premisess, the aim of the study was to better understand the pathophysiological mechanisms underlying this new human SCA38. ELOVL5 KO mouse model was generate in collaboration with the Department of Medical Sciences and of Neurosciences (University of Turin). To characterize the phenotype features, behavioral tests were performed by University of Turin at three different ages in order to track the progressive development of the disease.

To investigate lipidomic pattern we have evaluated fatty acids profile and phospholipids family. The analysis was conducted in Purkinje cells and in several tissues (plasma, cerebellum, sciatic nerve and olfactory bulb) obtained from mouse (control and KO mice model) at 15 months of age. The analysis of the metabolic profile and of specific metabolites in biological samples provide an insight into the metabolic state and the biochemical processes of the organism and, therefore, may indicate the onset and the stage of the disease. Lipidomic profile analyses was carried out by means liquid chromatography tandem mass spectrometry (LC-MS/MS).

6.2 MATERIAL AND METHODS

6.2.1 ANIMALS AND REAGENTS

All animal experimental procedures have been approved by the Ethical Committee of the University of Torino and authorized by the Italian Ministry of Health (authorization number: 161/2016-PR). ELOVL5 knockout (KO) mice have been kindly provided by Dr. Moon and Dr. Horton of the UT Southwestern Medical Center (Moon et al.,2009) and bred in Animal Facility at NICO (Univesity of Turin).

ELOVL5KO and wild type mice were used for all the experimental paradigms, while heterozygous mice were only used as breeders. Both ELOVL5KO mice and their wild type littermates were kept on natural diet without animal derivatives, containing essential PUFAs like linoleic and a-linolenic acids, but excluding the presence of omega-3 and omega-6 PUFAs downstream Elovl5, such as DHA, EPA and AA. The Lipidomic analyses was carried out on purkinje cells isolated by cerebellar cortex and in different tissues at 15 months of age: plasma, cerebellum, sciatic nerve and olfactory bulb. The lipidomic analysis was performed at 15 months because at this age, Motor Deficits and Cerebellar Atrophy in ELOVL5 Knock Out Mice was observed (Hoxha et al., 2017).

REAGENTS AND STANDARDS

Fatty acids standards were purchased from Sigma Aldrich. All Phospholipids standards were purchased from Avantpolar Lipids. All solvents used were purchased from Sigma Aldrich with mass spectrometry grade.

6.2.2 LIPIDOMIC ANALYSIS

The levels of total fatty acids and phospholipids were evaluated by means of Liquid Chromatography-tandem mass spectrometry (LC-MS/MS) according to Cermenati et al. (2012). Briefly, internal standards (^{13}C-labeled linoleic acid and ^{13}C-labeled palmitic acid) were added to samples (50µL for plasma and 10 mg for tissues), and lipid extraction was performed using 1 mL of methanol (MeOH)/Acetonitrile (1/1; v;v). After centrifugation at 12,000 rpm for 5 minutes, the supernatant was divided in two aliquots:

- A: phospholipid analysis;
- B: total fatty acid profile;

PHOSPHOLIPIDS ANALYSIS

ESI analysis of the major phospholipid classes (table 8) was accomplished by utilization of either positive or negative ionization modes. The identity of the different phospholipid families was confirmed using pure standards, namely one for each family comparing the acquired mass spectra. Methanolic extracts were analyzed by a 5 minutes run in both positive and negative ion mode with a 268 multiples reaction monitoring (MRM) transition in positive mode and 88 MRM transition in negative mode. A specific transition or specific neutral loss was used for each phospholipid family (see table 9). To establish the correct transition, infusions of a standard for each family was carried out to optimize all parameters

An API 4000 triple quadrupole instrument (AB Sciex, USA) equipped with ESI source was used. The mobile phase was 0.1% formic acid in MeOH for positive analysis using a Synergie Hydro RP column and 5 mM ammonium acetate pH 7 in MeOH for negative analysis using a Luna CN column.

MultiQuantTM software version was used for data analysis and peak review of chromatograms. Changes between detected phospholipid families were calculated as percent of single phospholipid species normalized to total phospholipid analyzed.

PHOSPHOLIPIDS FAMILY	Q1	Q3	DP	CE
LYSO-PHOSPHATIDILCHOLINE (LYSO-PC)	454.6	184	95	30
PHOSPHATIDILCHOLINE (PC)	636.7	184	95	30
LYSO-PHOSPHATIDYLETHANOLAMINE (LYSO-PE)	596.4	133	95	30
PHOSPHATIDYLETHANOLAMINE(PE)	596.4	133	95	30
PLASMALOGENS (PC ae+ PE ae)	636.7	184	95	30
SPHINGOMYELIN (SM)	647.8	184	95	30
PHOSPHATIDYLSERINE (PS)	639.4	186	95	30
PHOSPHATIDYLINOSITOL (PI)	712.4	241	115	51
PHOSPHATIDYLGLYCEROL (PG)	624.4	153	115	51
LYSO-PHOSPHATIDIC ACID (LYSO-PA)	550.4	153	115	51
PHOSPHATIDIC ACID (PA)	550.4	153	115	51
CERAMIDES (CER)	793.5	97	115	51
SULFATIDES (SUL)	482.7	264	95	30

Table 9: Parent ion (Q1) and fragment ion (Q3) of internal standards for each phospholipids family, potential difference (DP) and collision energy (CE) obtained after single infusion.

FATTY ACID ANALYSIS

Total FAs were obtained from samples by acid hydrolysis (Taguchi, R et al. 2010). Fraction B was resuspended in chloroform-MeOH 1:1, v/v. 1M HCl:MeOH (1:1, v/v) was added to the total lipid extract and shaken for 2 h. A mixture of chloroform:water (1:1, v/v) was added and the lower organic phase was collected, divided in two aliquots and dried under nitrogen flow. The residue was resuspended in 1 ml of MeOH and 10µL injected for LC-MS/MS analysis.

Quantitative analysis was performed on the basis of calibration curves prepared and analyzed daily

The mobile phases were: water/10 mM isopropylethylamine/15 mM acetic acid (phase A) and MeOH (phase B). The gradient (flow rate 0.5 ml/min) was as follows:

Time (min)	A%	B%
0.00	20	80
20.00	1	99
25.00	1	99
25.10	20	80
30.00	20	80

Table 10: Elution gradient of the chromatographic analysis

The Hypersil GOLD C8 column (100 mm × 3 mm, 3 µm) was maintained at 40°C. The mass spectrometer was operated in selective ion monitoring (SIM/SIM) mode. Peaks area were evaluated using a Dell workstation by means of the software Analyst release 4.1. The mass spectrometer was operated in negative-ion mode.

Fatty acid	Q1	Q3	DP	CE
PALMITIC ACID (C16:0)	255	255	-50	-10
PALMITOLEIC ACID (C16:1)	253	253	-50	-10
MARGARIC ACID (C17:0)	269	269	-50	-10
STEARIC ACID (C18:0)	283	283	-50	-10
OLEIC ACID (C18:1)	281	281	-50	-10
LINOLEIC ACID (C18:2)	279	279	-50	-10
γ-LINOLENIC ACID (C18:3)	277	277	-50	-10
ARACHIC ACID (C20:0)	311	311	-50	-10
ARACHIDONIC ACID (C20:4)	303	303	-50	-10
EPA (C20:5)	301	301	-50	-10
BEHENIC ACID (C22:0)	339	339	-50	-10
ERUCIC ACID (C22:1)	337	337	-50	-10
DHA (C22:6)	327	327	-50	-10
NERVONIC ACID (C24:1)	365	365	-50	-10
IS Linoleic 13C18	297	297	-50	-10

Table 11: parent ion (Q1), fragment ion (Q3), potential difference (DP) and collision energy (CE) of fatty acids under evaluation

QUANTITATIVE PROTEIN BCA

The pellets obtained after extraction were resuspended in RIPA 1X, lysed using tissue lyser (Qiagen, Milan, Italy) and centrifuged for 3 minutes. 5 µL of supernatant were transferred to the plate, diluted 1: 1 with water and added with 190 µL of BCA Bicinchoninic Protein Assay Kit (Euroclone). The samples were incubated for 30 minutes at 37 ° C and their absorbance was then evaluated using an Envision® MultilabelPlate Reader (Perkin Elmer) spectrophotometer, set at 570 nm. To quantify proteins a calibration curve was prepared at a known concentration of BSA (range 0.0-1.0 mg / mL.

6.2.3 STATISTICAL ANALYSIS

Data from female and male mice were pooled together because they did not show significant difference.

Comparisons between two experimental groups (Elovl 5 KO and WT) were done with unpaired two tailed Student's *t*-test or by two way or one way analyses of variance (ANOVA), followed by Holm-Sidak *post hoc* correction to concern motor test.

Unpaired Student's t-test was performed for lipidomic analysis and all statistical analyses were performed using GraphPad PRISM version 6.

6.3 RESULTS

6.3.1 LIPIDOMIC PROFILE IN PURKINJE CELLS OF ELOVL5KO MICE MODEL AND of WILD TYPE.

TOTAL FATTY ACID PROFILE IN PURKINJE CELLS OF ELOVL5KO MICE MODEL AND of WILD TYPE.

Total fatty acids were evaluated in both experimental groups out by LC-MS/MS and the chromatographic profile obtained is shown in the following figure:

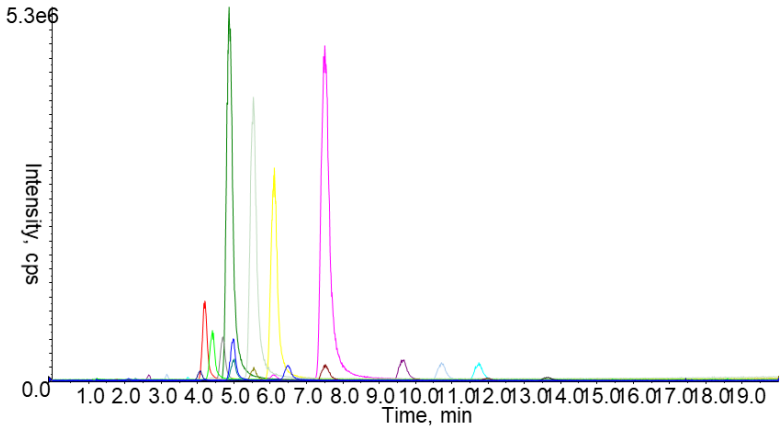


Fig.17: Chromatografic separation of fatty acids under evaluation.

Quantitative analysis was performed using calibration curves prepared daily. The figure shows a calibration curve obtained for Linoleic acid.

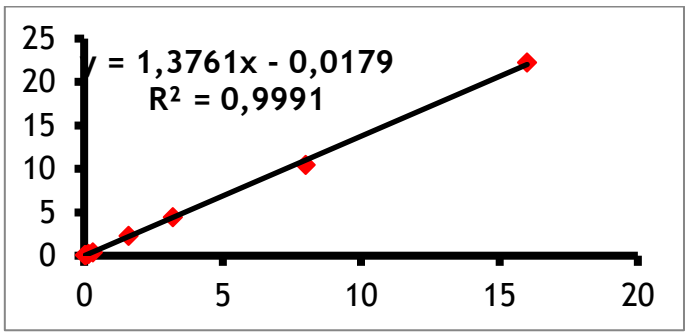


Fig.18: calibration curve of Linoleic acid.

To study the specific function of ELOVL5 in Purkinje cells, in collaboration with the Department of Medical Sciences and Neurosciences of the University of Turin, we compared the levels of the total fatty acid in Purkinje cells isolated from ELOVL5*KO* mice and from control animals.

Total fatty acid profile, as shown in figure 19, are mainly unchanged except a decrease for both Arachidonic Acid and Docosahexaenoic Acid that does not reach the statistic significance. It is important to underline that two fatty acids are the metabolites product by the action of the ELOVL5 enzyme in FAs biosynthesis.

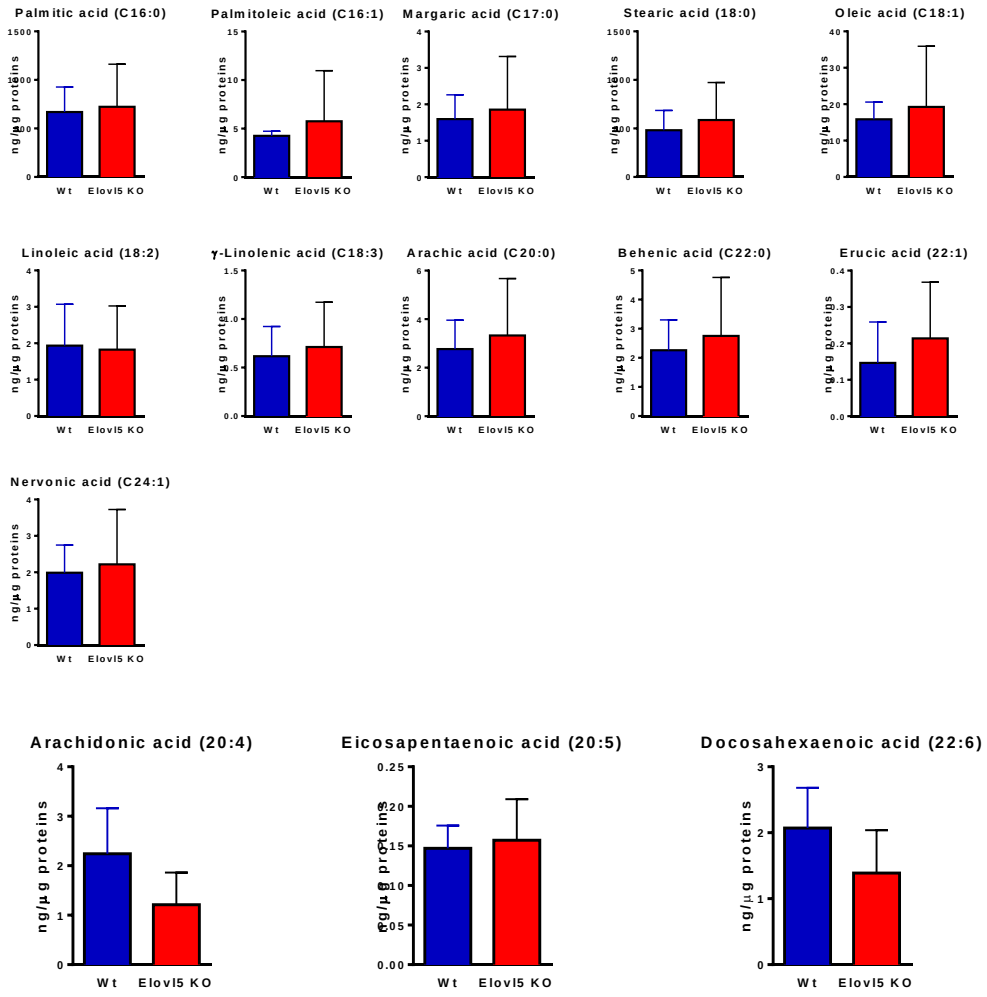


Fig.19: Total fatty acid levels in purkinje cells isolated from ELOVL5 KO (red bars) and wild type mice (blue bars). Data are expressed as ng/ug proteins (mean± SEM).

PHOSPHOLIPIDS ANALYSIS IN PURKINJE CELLS OF ELOVL5KO MICE MODEL AND of WILD TYPE.

Phospholipids were evaluated in Purkinje cells isolated from cerebellar cortex of ELOVL5 KO mice and wild type animals.

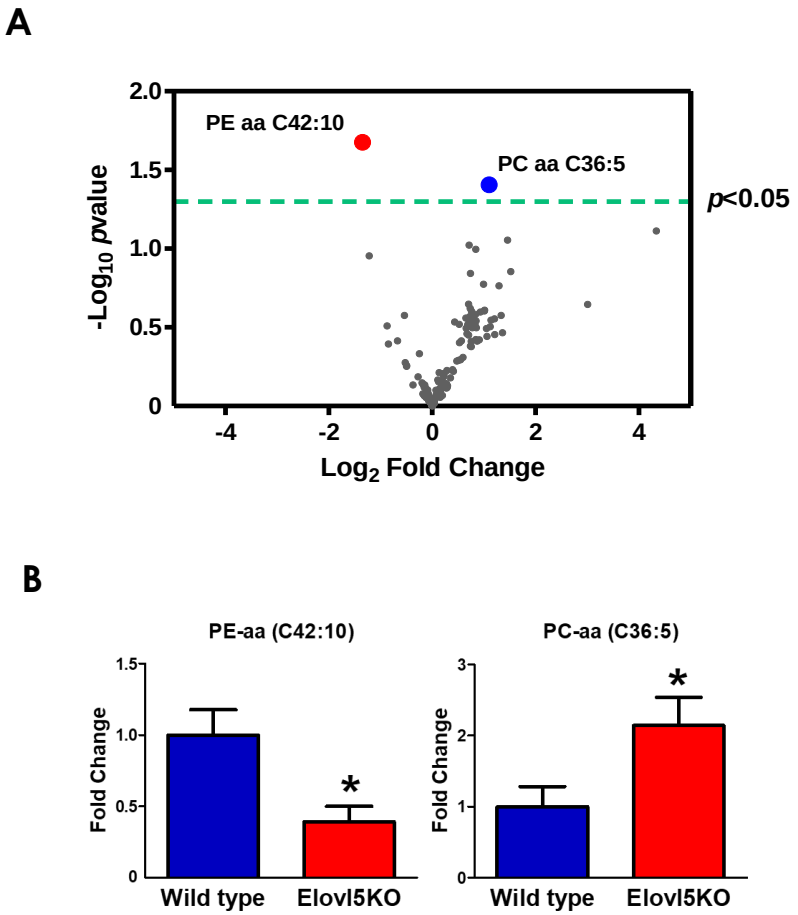


Fig.20: Effect of *ELOVL5* mutation on phospholipids family in Purkinje cell. **A)** Volcano plot reported all different phospholipids families detected. **B)** bar graph denoting fold change (in log2 base) of the statistically different levels of phospholipids between the experimental groups (n=6 for group). Data are expressed as mean± SEM.). *p* values for two groups were determined by Unpaired Student's t-test. * $p < 0.05$ vs Wild type.

Volcano plot (Figure 20A) in which are reported all phospholipids species evaluated expressed as log_2 Fold change, highlighted that only two phospholipids show significant differences in ELOVL5 KO group when compared with control group. These two phospholipids belongs to phosphatidylcholines (PCs) family and

phosphatidylethanolamines (PEs) family. In particular, PCaa 36:5, PC carrying different fatty acids bound to the glycerol by two ester linkages at *sn-1* and *sn-2* position (di-acyl form), significantly increased in ELOVL5 KO group while PEaa42:10 significantly decreased in ELOVL5 KO animals in comparison with wild type group (Fig 20B).

6.3.2 LIPIDOMIC PROFILE IN PLASMA OF ELOVL5KO MICE MODEL AND WILD TYPE.

TOTAL FATTY ACID PROFILE IN PLASMA OF ELOVL5KO MICE MODEL AND WILD TYPE.

Total fatty acids levels in plasma of ELOVL5 KO mice model and wild type at 15 months of age was reported in figure 21. A significant decrease in Arachidonic Acid (AA, 20:4, ω6), Eicosapentaenoic Acid (EPA, 20:5, ω3) and Docosahexaenoic Acid (DHA, 22:6, ω3) levels in ELOVL5 KO group when compared with wild type is evident.
This result is in agreement with trend observed in serum of patients affected by SCA38 (Di Gregorio et al., 2014).

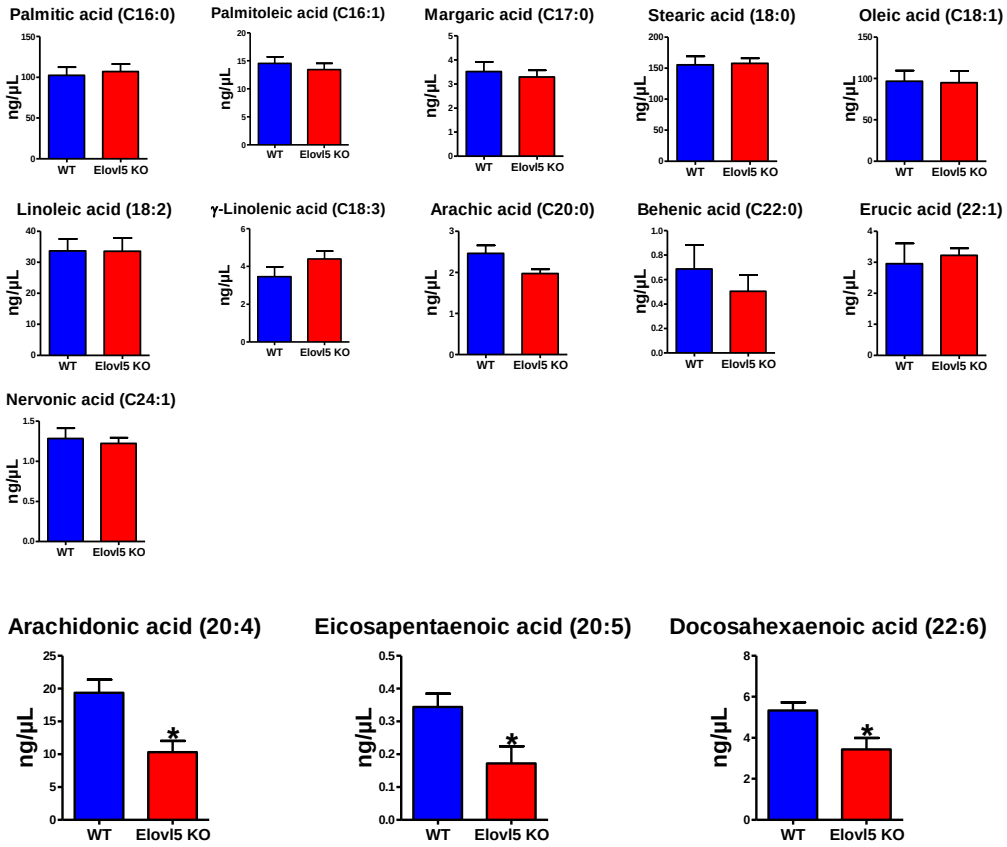


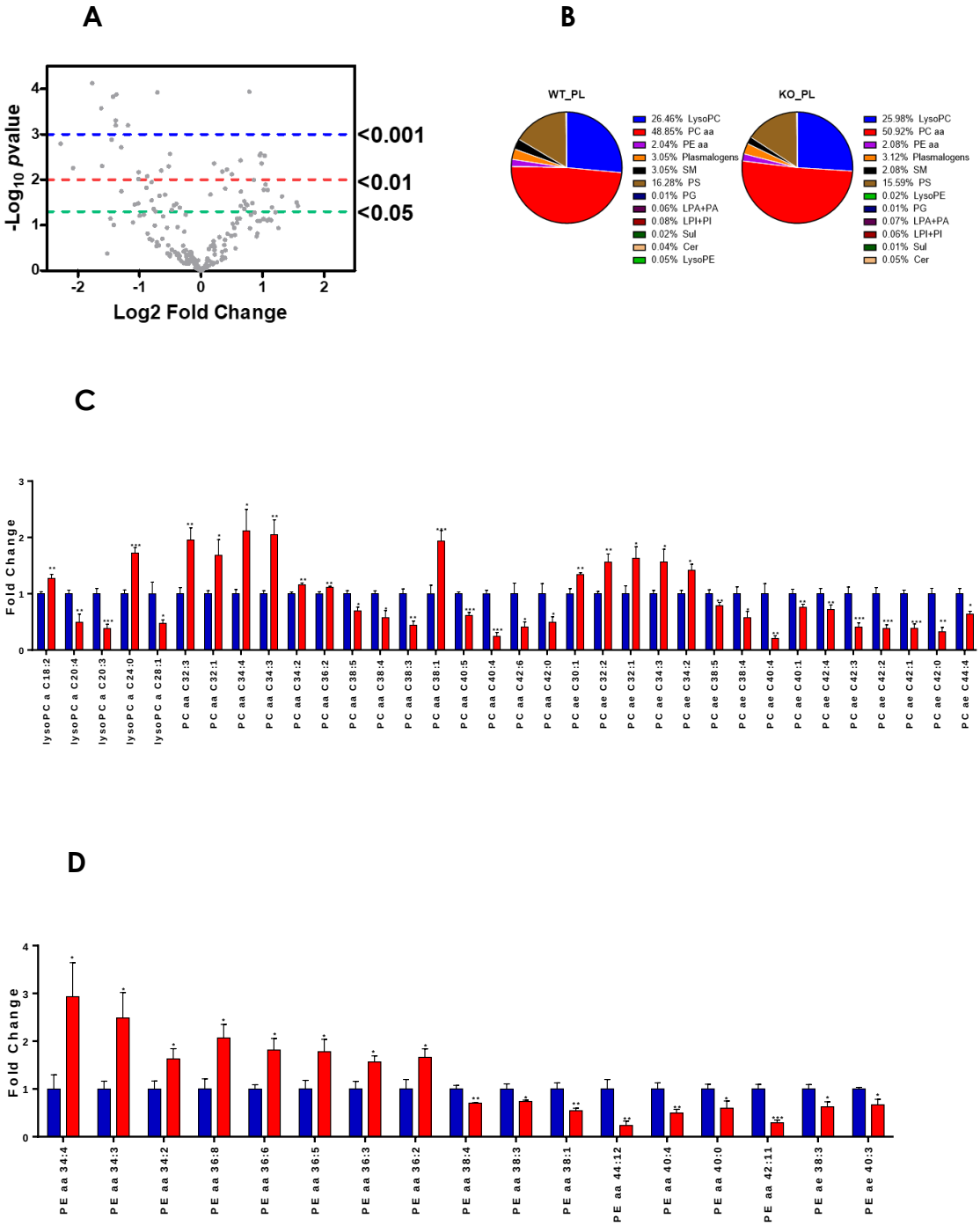
Fig.21: Total fatty acid levels in plasma of ELOVL5 KO and wild type group. Data are expressed as ng/µl (mean± SEM; n=6 each group); p values were determined by Unpaired Student's t-test. * p< 0.05 vs Wild type.

PHOSPHOLIPIDS ANALYSIS PROFILE IN PLASMA OF ELOVL5KO MICE MODEL AND WILD TYPE.

As shown (Fig.22B), the % composition in terms of phospholipids species detected in the two different experimental groups was comparable. However, Volcano plot (Fig.22A), in which are reported all phospholipids evaluated, highlighted that 61 phospholipids show significant difference in plasma of ELOVL5KO and wild type. Detailed analysis indicates that mutation of ELOVL5KO, induces significantly changed in many phospholipids of different species. As shown in Figure 22C, significant difference in five different lyso-phosphatidylcholines (LysoPCs), fourteen phosphatidylcholines (PCs) carrying different fatty acids bound to the glycerol by two ester linkages at *sn-1* and *sn-2* position (di-acyl form, PCaa) are detectable. PCs with less of 38 carbon atoms significantly increased in ELOV5 KO mice while PCs with more of 38 carbon atoms significantly decreased in ELOVL5 KO mice in comparison with wild type group. In addition, difference in fifteen phosphatidylethanolamines (PEs) are also evident. PEs carrying less than 38 carbon atoms in fatty acid composition displayed an increase in contrast PEs with a long chain fatty acid composition (> 38 total carbon) showed a significant decrease in ELOVL5KO with respect to control group (Fig.22D). We have, also, detected plasmalogens components, which are lipids characterized by the presence of a vinyl ether linkage at the *sn-1* position and ester linkage at the *sn-2* position of the glycerol (alkyl-acyl form, ae). The most common plasmalogens are ethanolamine (plasmenylethanolamines, PEae) or choline (plasmenylcholines, PCae).

In plasma analysis seventeen plasmalongens showed significantly changes: fifteen PCae and two PEae (Figure 22C and 22D). Similarly,

to what observed in PCs and PEs, also plasmalogens with less of 38 carbon atoms showed a significative increase in ELOVL5 KO when compared with control mice. Plasmalogens with more of 38 carbon atoms highlighted a significative decrease in ELOVL5 KO group in comparison with wild type group.



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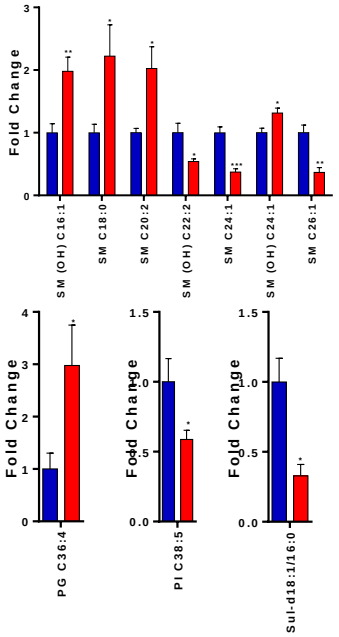


Fig.22: effect of ELOVL5 mutation in plasma (PL) phospholipids family. **A)** Volcano plot reported all different phospholipids families detected. **B)** composition of phospholipid species observed in PL of two experimental groups (% of total PLs). **C)** bar graph denoting fold change (in log2 base, red bar) of the statistically different levels of Lyso-PC and PC between the experimental groups (n=6 for group). **D)** bar graph denoting fold change (in log2 base, red bar) of the statistically different levels of PE between the experimental groups (n=6 for group. **E)** bar graph denoting fold change (in log2 base, red bar of SM, PG PI and Sulfatides between the experimental groups (n=6 for group). Data are expressed as mean± SEM.). *p* values by Unpaired Student's t-test. * $p < 0.05$, **<0.01, ***<0.001 vs Wild type.

We have, also, observed that seven sphingomyelin (SM) showed significative difference between two experimental groups, namely, four significantly increase and three significantly decrease (Figure 22E) In addition, one phosphatidylglycerol (PG) significantly increased in ELOVL5 KO mice when compared with wild type group while one sulfatides (SUL) and one phosphatidylinositol (PI) showed a significant decrease in ELOVL5 KO mice in comparison with control animals (Figure 22E).

Plasma phospholipids with less than 38 total carbons showed an

increase in ELOVL5KO, while in all species, phospholipids with a long chain fatty acid composition (> 38 total carbon) a significant decrease in ELOVL5KO mice in comparison with control mice is observed.

6.3.3 LIPIDOMIC PROFILE IN CEREBELLUM OF ELOVL5KO MICE MODEL AND WILD TYPE.

TOTAL FATTY ACID PROFILE IN CEREBELLUM OF ELOVL5KO MICE MODEL AND WILD TYPE.

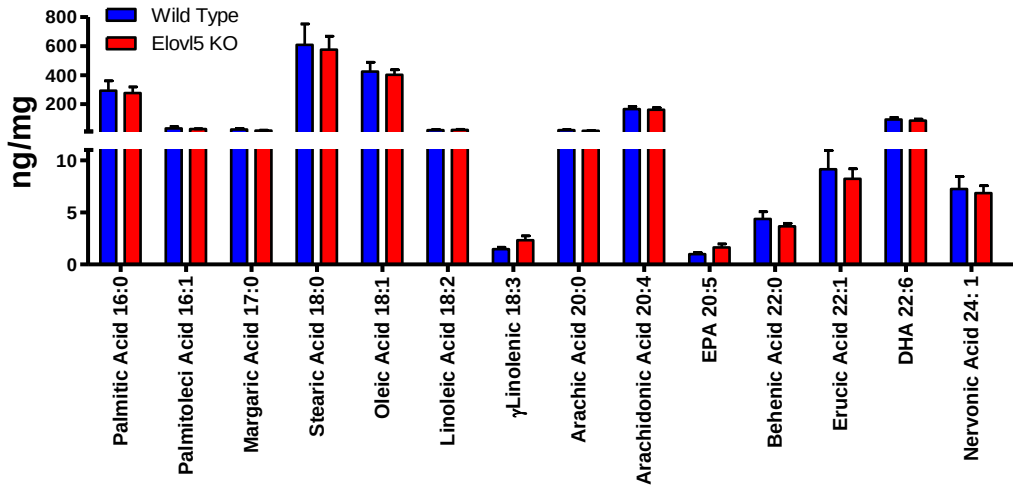


Fig.23: Total fatty acid levels in cerebellum of ELOVL5KO and wild type (n=6) group. Data are expressed as ng/mg tissue (mean± SEM).

In figure 23 are reported the levels of total fatty acids also in cerebellum of two experimental groups (ELOVL5KO and WT). Quantitative analysis does not show any significative difference in ELOVL5KO when compared with wild type.

PHOSPHOLIPIDS ANALYSIS IN CEREBELLUM OF ELOVL5KO MICE MODEL AND WILD TYPE.

Different species of phospholipids are evaluated in cerebellum of ELOVL5KO and WT groups. As shown (Figure24B), the % composition in terms of phospholipids species detected in the two different experimental groups in cerebellum was comparable. However, Volcano plot (Figure 24A), in which are reported all phospholipids evaluated, highlighted that phospholipids show significant difference in cerebellum of ELOVL5KO in comparison with wild type.

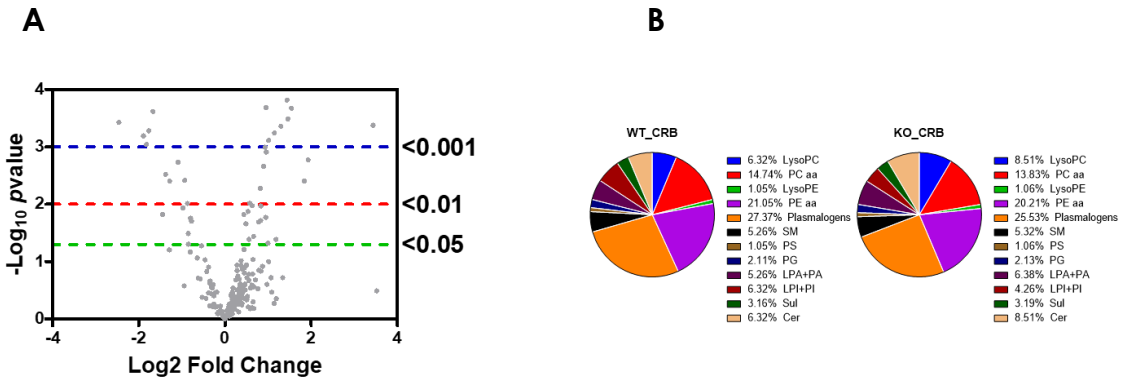
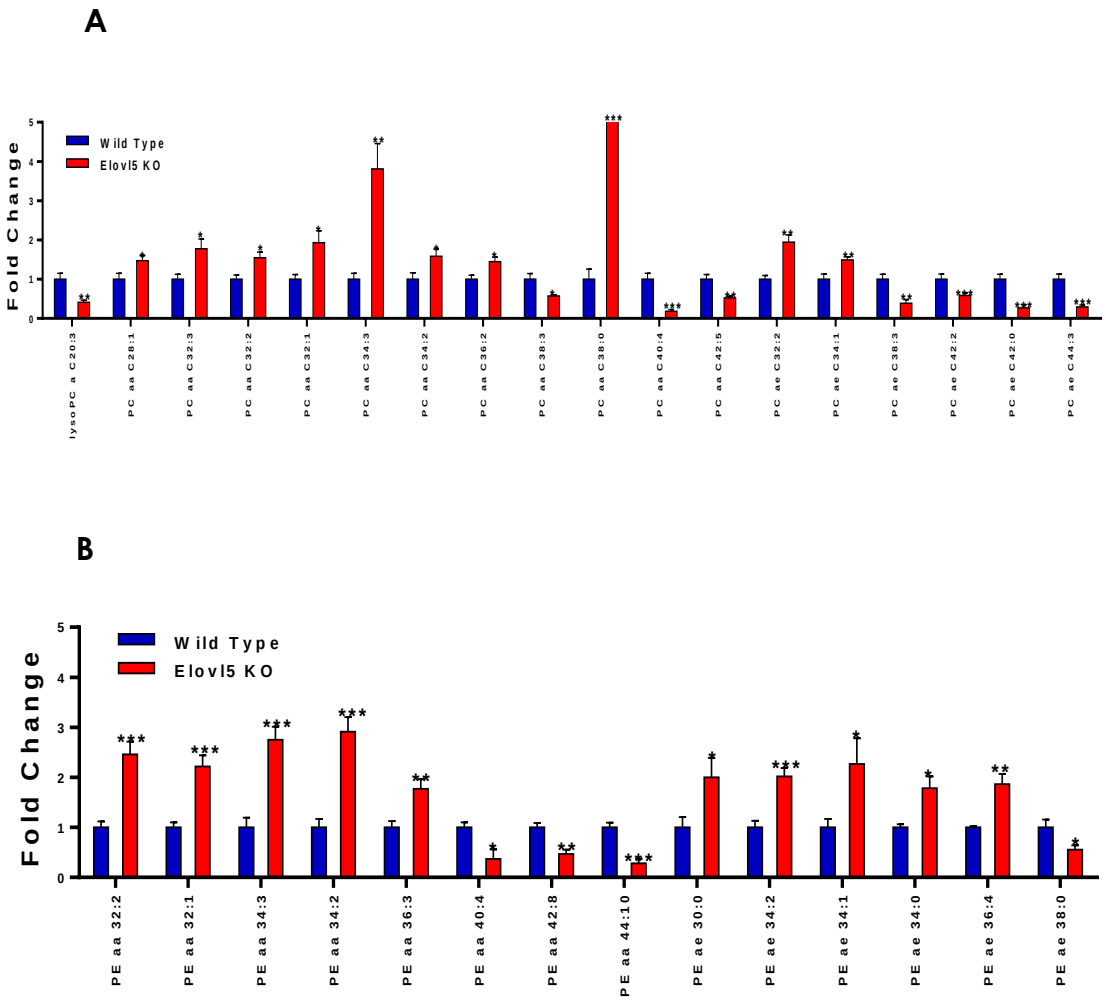


Fig.24: effect of ELOVL5 mutation on cerebellum (CRB) phospholipids family. **A)** Volcano plot reported all different phospholipids families detected. **B)** composition of phospholipid species observed in CRB of two experimental group (% of total PLs).

Therefore, a detailed analysis of each family and statistically difference between species are reported (Fig.25). Specifically, as shown in figure 25A, a significant difference in eleven different PCs carrying different fatty acids bound to the glycerol by two ester linkages at sn-1 and sn-2 position (di-acyl form, PC aa) were detected. PCs carrying less of 38 carbon atoms in fatty acid composition displayed an increase in cerebellum of ELOVL5KO in comparison with that of control mice, while PCs carrying > 38 total carbon atoms showed a significant decrease in ELOVL5KO when compared with wild type group.

Among PEs analyzed, species with less of 38 carbon atoms in the di-acyl form (PEaa) showed an increase statistically significative in ELOVL5KO group when compared with wild type group. In opposite, three PEs carrying more of 38 carbon atoms in fatty acid composition displayed a decrease in ELOVL5KO mice in comparison with control mice (Figure 25B). In addition, twelve plasmalogens showed significant difference; specifically, two PCae and five PEae with less of 38 carbon atoms show an increase in ELOVL5KO mice when compared with wild type animals (Figure 25A and 25B).

Furthermore, five plasmalogens (four PCae and one PEae) with more than 38 carbon atoms significantly decrease in cerebellum of ELOVL5KO with respect to control group (Figure 25A and 25B).



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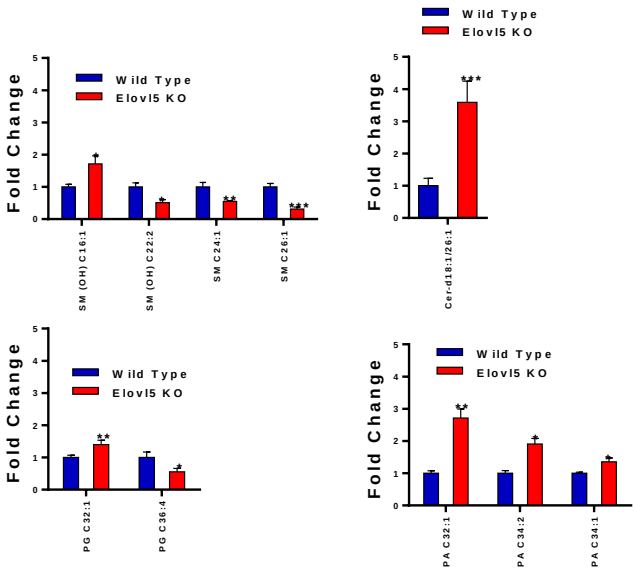


Fig. 25: bar graph denoting fold change (in log2 base) of the statistically different levels of phospholipids between the experimental group (n=6 for group). **A)** bar graph of statistically different levels of PC in cerebellum of ELOVL5KO (red bar) in comparison with WT (blue bar). **B)** bar graph statistically different levels of PE in cerebellum of ELOVL5KO (red bar) in comparison with WT (blue bar). **C)** bar graph statistically different levels of SM, Cer, PG and PA in cerebellum of ELOVL5KO (red bar) in comparison with WT (blue bar). Data are expressed as mean± SEM.). *p* values for two groups were determined by Unpaired Student's t-test. * $p < 0.05$, **<0.01, ***<0.001 vs Wild type.

Finally, four SM have highlighted a significative decrease in ELOVL5KO group while one ceramide (CER) and three phosphatidic acid (PA) showed a significant increase in ELOVL5KO in comparison with WT group (Figure 25C). Furthermore, two phosphatidylglycerols (PG) showed statistically significative changes between two groups. (Figure 25C).

Similarly, to that observed in plasma, even in cerebellum analysis, we found that phospholipids of different species showed a trend

depending of total carbon number of fatty acids. Phospholipids carrying less of 38 carbon atoms in fatty acid composition displayed an increase in ELOVL5KO mice in comparison with control mice.
In opposite, PLs with a long chain fatty acid composition (> 38 total carbon atoms) showed a significant decrease in ELOVL5KO group when compared with wild type group.

6.3.4 LIPIDOMIC PROFILE IN SCIATIC NERVE OF ELOVL5KO MICE MODEL AND WILD TYPE.

TOTAL FATTY ACID PROFILE IN SCIATIC NERVE OF ELOVL5KO MICE MODEL AND WILD TYPE.

Total fatty acids analysis is performed in sciatic nerve (SN) of ELOVl5 KO mice model compared with wild type animal. As shown in Figure 26, total fatty acids level in SN is unchanged in the two experimental groups.

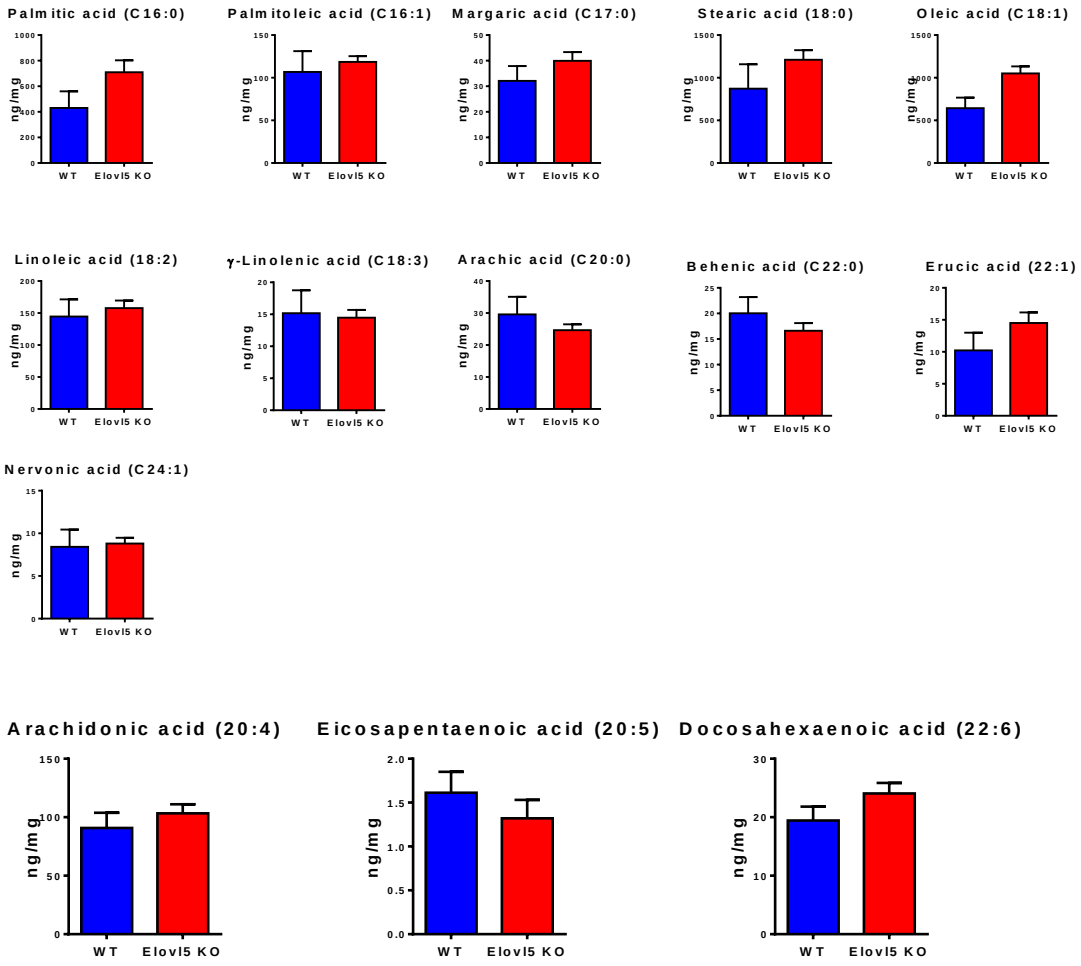


Fig.26: Total fatty acid levels in sciatic nerve (SN) of ELOVL5KO and wild type group. Data are expressed as ng/mg tissues (mean± SEM; n=6 each group

PHOSPHOLIPIDS ANALYSIS PROFILE IN SCIATIC NERVE OF ELOVL5KO MICE MODEL AND WILD TYPE.

Phospholipids analysis in sciatic nerve have indicated that the most abundant lipids present in sciatic nerve are PCs, PEs and plasmalogens. As shown in figure 27B, the % composition in terms of phospholipids species detected in the two different experimental groups was comparable. Volcano plot (Fig.27A), in which are reported all phospholipids evaluated, highlighted that phospholipids show significant difference in sciatic nerve of ELOVL5KO in comparison with wild type.

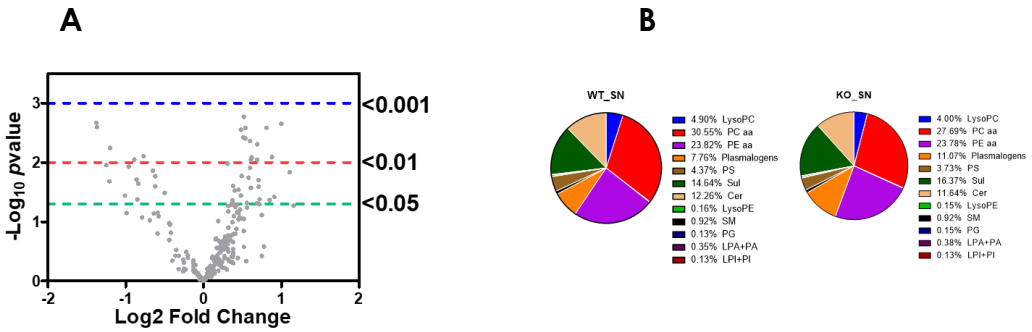
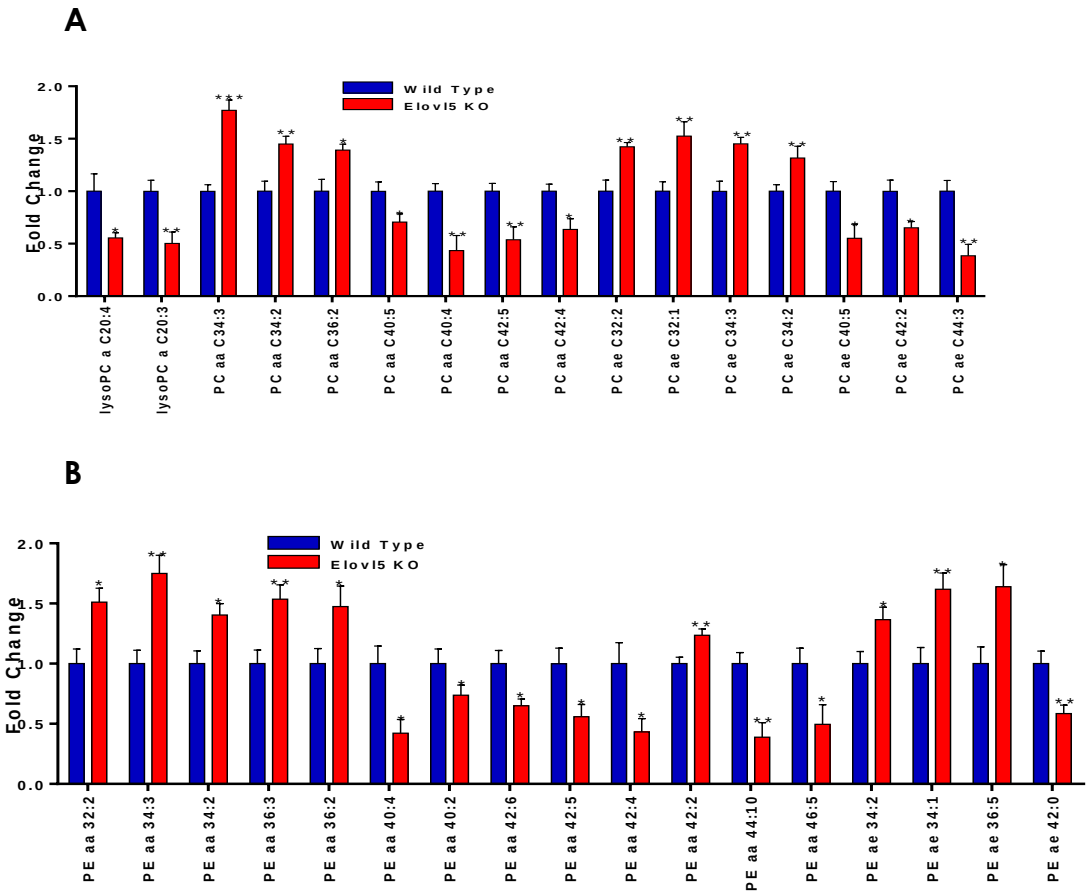


Fig.27: effect of ELOVL5 mutation on sciatic nerve (SN) phospholipids family. **A)** Volcano plot reported all different phospholipids families detected. **B)** composition of phospholipid species observed in SN of two experimental groups (% of total PLs).

Moreover, a detailed analysis of significative difference for each family is reported in figure 28. Specifically, as shown in figure 28A, a significant difference in two Lyso-PC and seven PCs with different fatty acids composition to the glycerol in the di-acyl form (aa) were obsrved. Three PCs with less of 38 total carbon atoms significantly increased in ELOVL5KO group in comparison with control group, while four PCs carrying more 38 carbon atoms in fatty acids composition showed a decrease in ELOVL5KO mice with respect to control mice. Furthermore, we found that thirteen PEs were significantly different in ELOVL5KO group with respect to control. Among these, five of them highlighted a decrease while eight

showed an increase in ELOVL5KO mice when compared with control group (Figure 28B). Similarly, to what observed with PCs, PEs carrying less than 38 carbon atoms in the fatty acid composition showed an increase in ELOVL5KO group in comparison with wild type. In opposite, PLs with more than 38 carbon atoms in composition of FAs has showed a significant decrease in ELOVL5KO mice. PLs analysis in sciatic nerve highlighted that eleven plasmalogens (seven PCae and four PEae) are statistically different between two groups. Plasmalogens with less of 38 total carbon atoms show an increase in ELOVL5KO mice when compared with wild type animals, while plasmalogens carrying long-chain polyunsaturated fatty acids composition (>38 carbon atoms) showed a significant decrease in in sciatic nerve of ELOVL5KO with respect to control group (Figure 28A, 28B).



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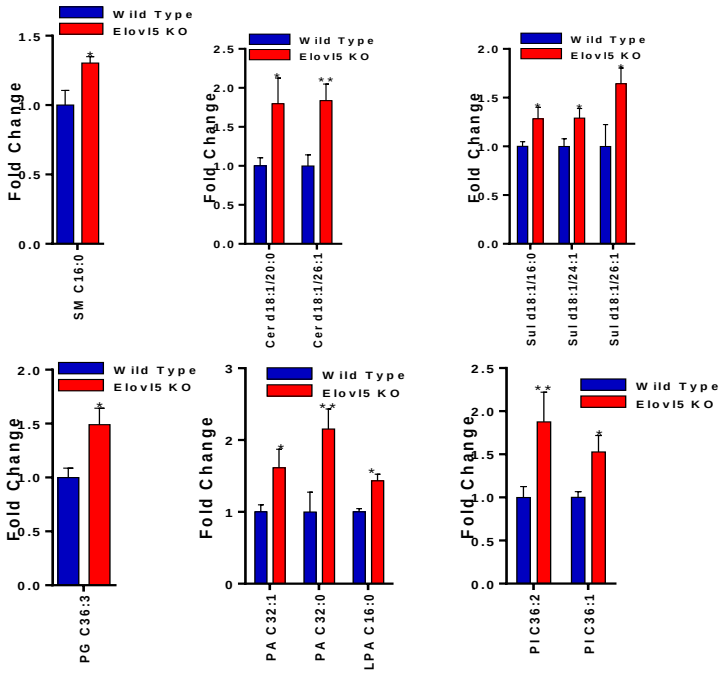


Fig. 28: bar graph denoting fold change (in log2 base) of the statistically different levels of phospholipids in sciatic nerve between the experimental group (n=6 for group). **A)** bar graph of statistically different levels of PC in sciatic nerve of ELOVL5KO (red bar) in comparison with WT (blue bar). **B)** bar graph statistically different levels of PE in sciatic nerve of ELOVL5KO (red bar) in comparison with WT (blue bar). **C)** bar graph statistically different levels of SM, Cer, Sul, PG, PA and PI in sciatic nerve of ELOVL5KO (red bar) in comparison with WT (blue bar). Data are expressed as mean± SEM.). *p* values for two groups were determined by Unpaired Student's t-test. * $p<0.05$, **<0.01, ***<0.001 vs Wild type.

Finally, other phospholipids (SM, CER, PG, PA, PI, SUL) showed a significant increase in ELOVL5KO mice when compared with wild type animals (Figure 28C).

Analysis of phospholipids in sciatic nerve of ELOVL5KO and wild type mice highlighted that PLs with less than 38 carbon atoms in the fatty acid composition were significantly increased in ELOVL5KO group in comparison with wild type. PLs with more than 38 carbon atoms in composition of FAs has showed a significant decrease in ELOVL5KO

mice.

6.3.5 LIPIDOMIC PROFILE IN OLFACTORY BULB OF ELOVL5KO MICE MODEL AND WILD TYPE.

TOTAL FATTY ACIDS PROFILE IN OLFACTORY BULB OF ELOVL5KO MICE MODEL

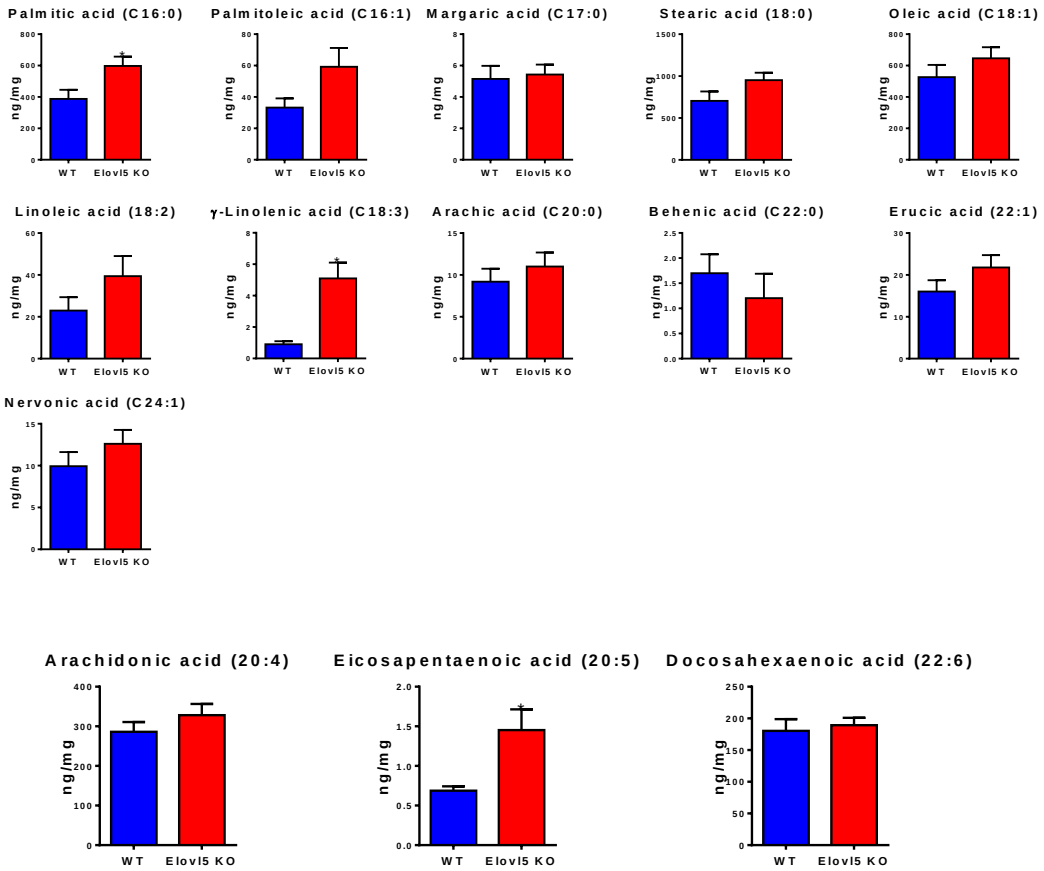


Fig.29: Total fatty acid levels in sciatic nerve of ELOVL5*KO* and wild type (n=6) group. Data are expressed as mean± SEM. *p* values for two groups were determined by Unpaired Student's t-test. * $p< 0.05$ vs Wild type

The analysis of total fatty acids is performed in olfactory bulb of ELOVL5KO animal compared with control mice at 15 months of age. In contrast with what observed in cerebellum and sciatic nerve, analysis highlighted some statistically difference between the two experimental groups. Specifically, as shown in figure 29, we have observed that the levels of palmitic acid (16:0), γ-linolenic acid (18:3)

and EPA (20:5) are significantly increased in ELOVL5KO mice in comparison with control animals.

PHOSPHOLIPIDS ANALYSIS IN OLFACTORY BULB OF ELOVL5KO MICE MODEL AND WILD TYPE

Analysis of different phospholipids family species in Olfactory Bulb (OB) indicated alterations in them composition. As shown in figure 30B, the % composition in terms of phospholipids species detected in the two different experimental groups was different for some species. To confirm this data, quantitative analysis showed significantly changes in the total amount of ELOVL5KO mice in comparison with control animal for the follow PLs family: Lyso-PC, PCs, PEs, plasmalogens, SM and PS (Figure 30C).

Volcano plot (Figure 30A), in which are reported all phospholipids evaluated showed that many phospholipids display differences in olfactory bulb of ELOVL5KO when compared with wild type.

A detailed analysis of each family and statistically difference between species are reported in figure 30.

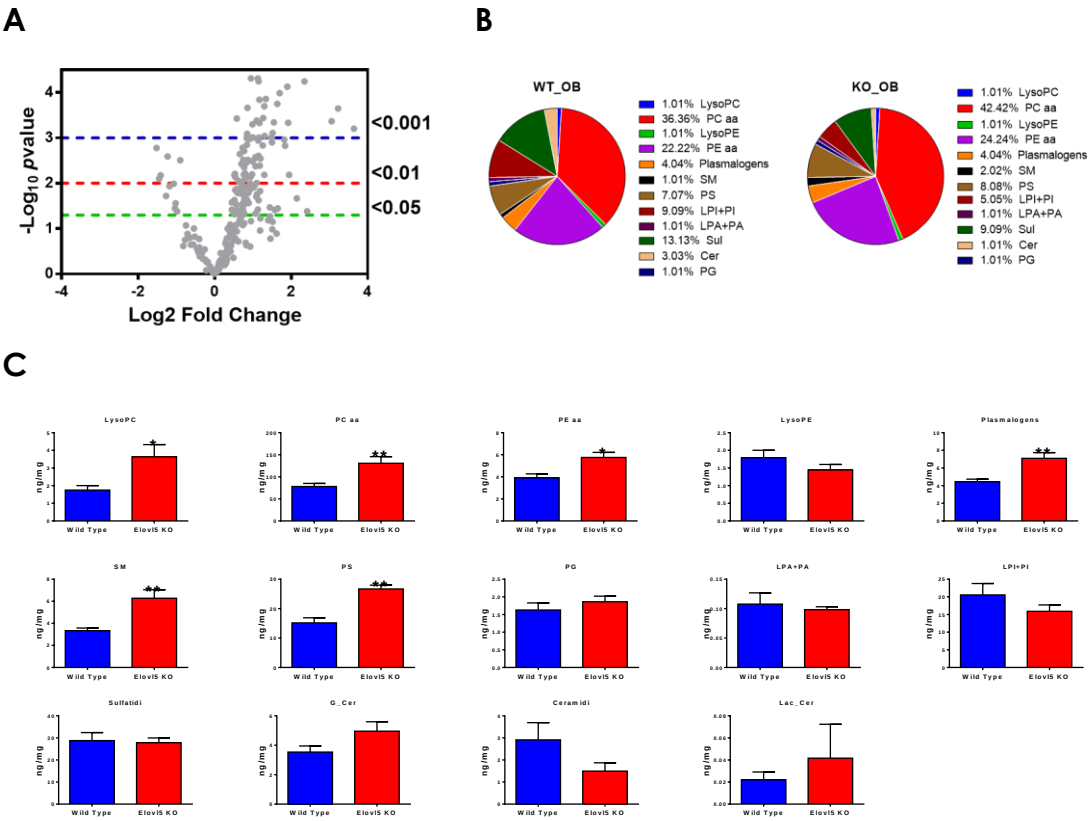
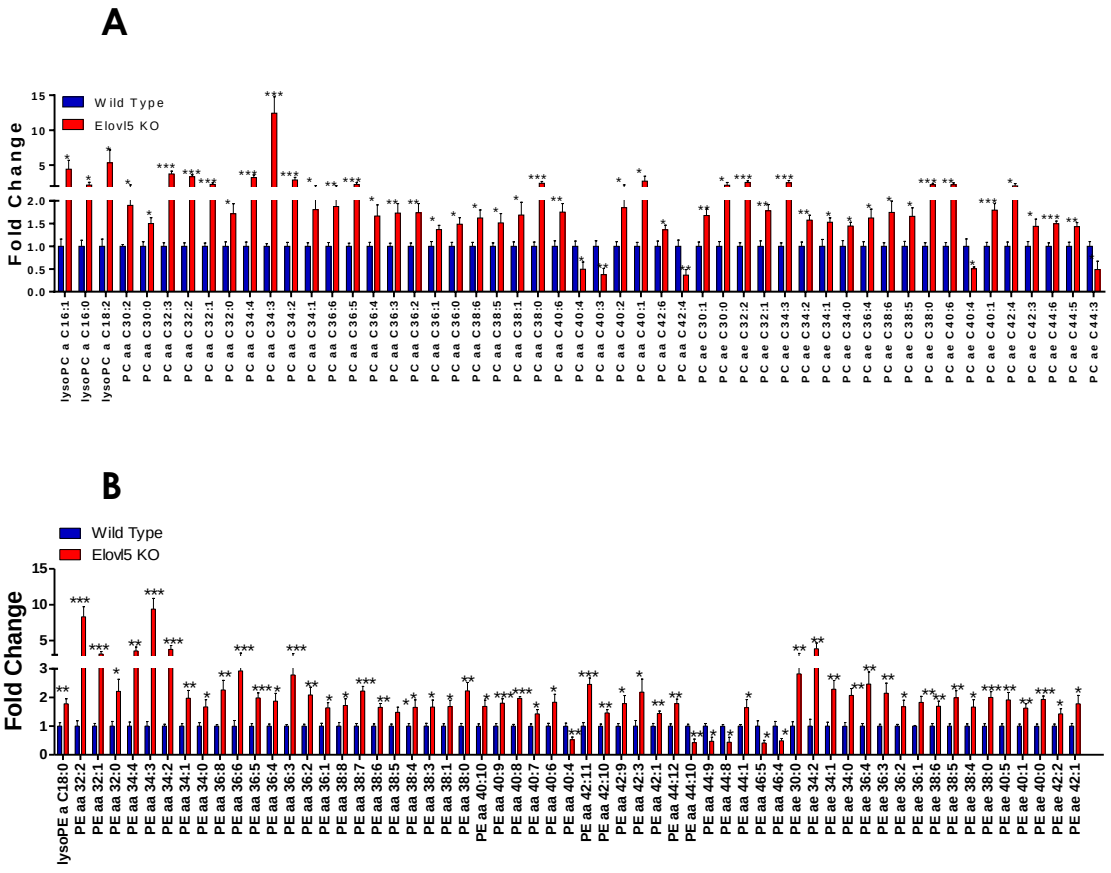


Fig.30: effect of ELOVL5 mutation on olfactory bulb (OB) phospholipids family. **A)** Volcano plot reported all different phospholipids families detected. **B)** composition of phospholipid species observed in OB of two experimental groups (% of total PLs). **C)** Total amount of different PLs families detected. Data are expressed as mean± SEM.). *p* values for two groups were determined by Unpaired Student's t-test. * $p < 0.05$, **<0.01, ***<0.001 vs Wild type.

Specifically, Figure 31A shows statistically significant difference in PCs carrying different fatty acids bound to the glycerol by two ester linkages at sn-1 and sn-2 position (di-acyl form, PCaa) and plasmanylcholines (PCae) with a vinyl ether linkage at the sn-1 position and ester linkage at the sn-2 position of the glycerol (alkyl-acyl form, ae). PCaa and PCae carrying less of 38 carbon atoms in fatty acid composition displayed an increase in ELOVL5KO mice in comparison with control mice, while PLs with a long chain fatty acid composition (>38 total carbon atoms) showed a significant decrease in ElovI5KO group when compared with wild type group (figure 31A).

In figure 31B, statistically significant differences of PEs carrying different fatty acids bound to the glycerol by two ester linkages at sn-1 and sn-2 position (di-acyl form) and plasmenylethanolamines, (PEae) with a vinyl ether linkage at the sn-1 position and ester linkage at the sn-2 position of the glycerol (alkyl-acyl form, ae) are reported. Specifically, we found that PEaa and PEae carrying less of 38 carbon atoms in fatty acid composition highlighted an increase in ELOVL5KO mice in comparison with group mice, while phospholipids with a long chain fatty acid composition (> 38 total carbon atoms) showed a significant decrease in ELOVL5KO group when compared with wild type group.



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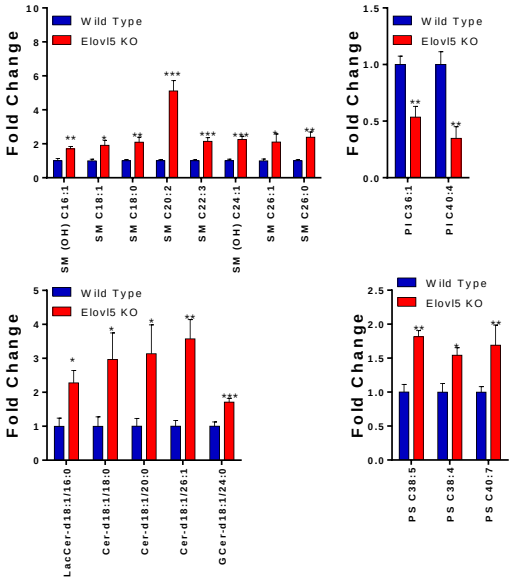

Fig.31: effect of ELOVL5 mutation in olfactory bulb (OB) of different species altered. **A)** bar graph denoting fold change (in log2 base) of the statistically different levels of PCs between the experimental groups (n=6 for group). **B)** bar graph denoting fold change (in log2 base) of the statistically different levels of PEs between the experimental groups (n=6 for group). **C)** bar graph denoting fold change (in log2 base) of the statistically different levels of SM, PI, ceramides and PS between the experimental groups (n=6 for group). Data are expressed as mean± SEM.). *p* values for two groups were determined by Unpaired Student's t-test. * $p < 0.05$, **<0.01, ***<0.001 vs Wild type.

Finally, in figure 31C are reported other PLs that showed statistically significant difference in ELOVL5KO group when compared with wild type animals. Specifically, we found that SM, LacCer, Gcer, PS highlighted an increase in ELOVL5KO mice in comparison with WT group ans two phospholipids belong to phosphatidylinositolo (PI) family showed a decrease in ELOVL5KO group when compared with wild type animals. These two PLs are PI carrying 36 carbon atoms and 1 double bound and PI carrying 40 carbon atoms and 4 double bound.

6.3.6 PHOSPHOLIPIDS CHANGING IN DIFFERENT TISSUES

Phospholipids analysis highlighted alterations of different species in all tissues considered. As reported in figure 32, Olfactory Bulb analysis showed more changes (green) in different species of PLs compared to other tissue.

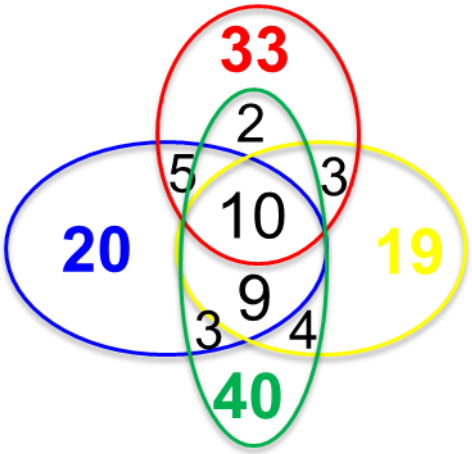

Fig.32: Diagram showing the phospholipids change in plasma (red), cerebellum (yellow), sciatic nerve (blue) and olfactory bulb (green).

In addition, figure 32 shows that in all tissue in all tissues considered, 10 PLs significantly changed in ELOVL5KO group in comparison with control animals. A significant decrease in the PC aa C:40:4 and PE aa C:40:4 were detected (Figure 33).

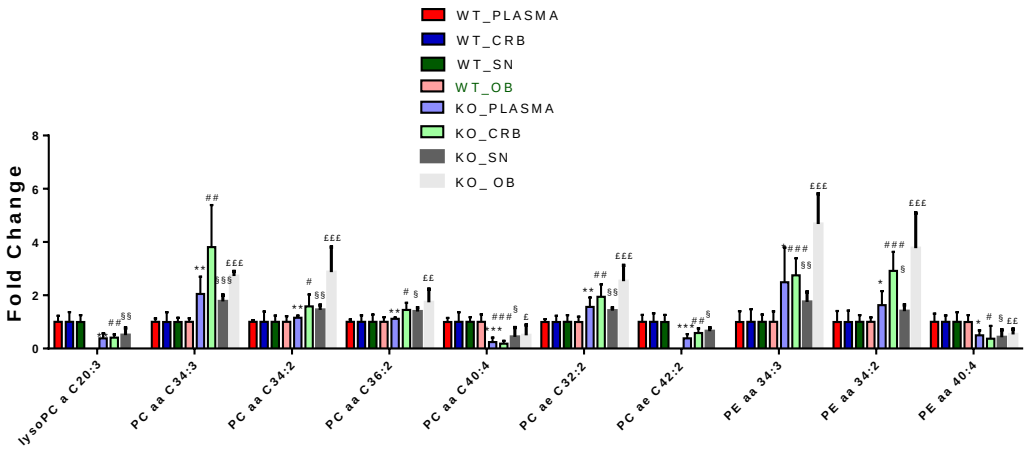

Fig.33: bar graph denoting fold change (in log2 base) of the statistically different levels of common phospholipids in all tissues considered between the experimental group (n=6 for group). Data are expressed as mean± SEM.). *p* values for two group comparisons were determined by Unpaired Student's t-test. PLASMA: * *p*< 0.05, **<0.01, ***<0.001 vs Wild type. CRB # <0.05, ##<0.01, ###<0.001 vs WT. SN § <0.05, §§<0.01, §§§<0.001 vs WT. OB £ <0.05, ££<0.01, £££<0.001 vs WT

6.4 DISCUSSION

Spinocerebellar Ataxia 38 (SCA38) is a new rare form of Ataxia caused by single missense mutations in the Very Long Chain Fatty Acid Elongase 5 Gene, (ELOVL5) described for the first time by Di Gregorio Di Gregorio et al., 2014). The ELOVL5 protein belongs to a family of enzymes localized in the endoplasmic reticulum involved in elongation of very long-chain (>16 C) fatty acids, are. The main clinical findings in this disease are ataxia, hyposmia and cerebellar atrophy. Mice in which ELOVL5 has been knocked out represent a model of the loss of function of SCA38. In agreement with this aspect mice with a complete deletion of ELOVL5 are generated. These animal show the main symptoms observed in SCA38 patients, motor deficits at the beam balance test and hyposmia (Di Gregorio et al., 2014; Borroni et al., 2016, Hoxha et al., 2017).

Hoxha et al. (2017) described as ELOVL5KO mice displayed a marked deficit in the beam balance test. On the contrary, the rotarod test did not reveal any significant difference between two experimental groups. A phenotype similar to that of ELOVL5KO mice was described by Larivière et al.(2015) in a murine model of autosomal recessive spastic ataxia of Charlevoix-Saguenay. They were able to detect the first signs of ataxia with the balance beam test but not with the rotarod test. On the other hand, other researchers have found an impaired performance of SCA3 mice in the rotarod test despite the absence of deficits in the balance beam test (Switonski et al., 2015). These different data suggest that the motor tests might have a different sensitivity in revealing motor impairment, depending on the severity of ataxia and of the peculiar features of each ataxic syndrome.

The motor impairment observed in ELOVL5KO mice is reminiscent of the initial stages of ataxia, especially in slowly progressing forms

observed in spinocerebellar ataxia (SCA) patients, in which instability can be revealed by tandem gait while regular walking is not affected (Bodranghien et al., 2016).

ELOVL5 encodes an elongase enzyme involved in the synthesis of long-chain polyunsaturated fatty acids (lc-PUFA) and is highly expressed in murine and human Purkinje cells (Di Gregorio et al., 2014). They are inhibitory neurons that regulate the complex and coordinated movements, preventing an abrupt movement. In humans, Purkinje cells can be damaged by a variety of causes: toxic exposure, for example alcohol or lithium; autoimmune diseases; genetic mutations that cause spinal-cerebellar ataxias. Given the specific expression of ELOVL5 in Purkinje cells a reduction in the fatty acid synthesis could damage cerebellar function.

Lipidomic studies in this mice model were performed to characterize the phenotype. These studies have revealed alterations in total fatty acid composition and particularly phospholipids of different species have changed in ELOVL5KO animal when compared with wild type group. Lipidomic analysis in purkinje cells isolated from ELOVL5KO animal has indicated alterations in the FAs synthesis; we have found a trend to decrease in the main metabolites product by the action of ELOVL5 enzyme such as AA 20:4 and DHA 22:6. Different species of phospholipids founding significantly different in ELOVL5KO group when compared with wild type. Particularly, we have found that phosphatidylethanolamine with long chain polyunsaturated fatty acid composition (PE aa 42:10) significantly decreased in ELOVL5KO animal; these phospholipids could have in its FAs composition DHA and AA. These founding suggest that mutations within elovl5 gene induces an alteration in the synthesis of long chain-polyunsaturated fatty acids in ELOVL5 knockout mice.

Total fatty acids profile and phospholipids were evaluated in in plasma, cerebellum, sciatic nerve and olfactory bulb ELOVL5KO and

wild type mice at 15 months of age, where animals display Motor Deficits and Cerebellar Atrophy (Hoxha et al., 2017).

Total fatty acids analysis in cerebellum and sciatic nerve didn't show any difference between two experimental groups. we do not observe the same pattern obtained in cells because the characterization of the fenotype was carried out in cerebellum (due to its involvement in hyposmia) and not in cerebellar cortex, which Purkinje cells are mainly located.

In plasma is evident the same trend observed in serum analysis of SCA38 patients (Di Gregorio et al.,2014) Specifically, Arachidonic Acid (AA, 20:4, ω6), Eicosapentaenoic Acid (EPA, 20:5, ω3) and Docosahexaenoic Acid (DHA, 22:6, ω3) levels were significantly decreased in ELOVL5KO group with respect to wild type group. Data obtained from plasma analysis could suggest alterations in FAs synthesis in liver, where mainly produced.

Phospholipids analysis have indicated many changes in ELOVL5KO mice levels when compared with wild type group in all tissues considered. Specifically, we have observed two trends depending from fatty acids composition for cerebellum and sciatic nerve and olfactory bulb analysis. PLs with a total of 38 carbon atoms significantly decreased in ELOVL5KO group in comparison with wild type group; in contrast, phospholipids carrying long-chain polyunsaturated fatty acids composition (>38 carbon atoms) showed a significant decrease in the levels in ELOVL5KO group with the respect to control.

Lipid metabolism plays an important role in the development and normal functionality of the CNS, given that this organ has the highest lipid content after adipose tissue. Altered levels of lipid molecules, such as fatty acids, are demonstrated in the neuronal system of individuals affected by neurodegenerative diseases, mental disorders, stroke, and trauma (Adibhatla et al., 2007).

A variety of neurological diseases, particularly hereditary spastic paraplegias (Tesson, C et al.,2012; Boukhris, A et al.,2012; Martin, E et al.,2013) and related diseases (Morgan, N.V et al.,2006) also have altered lipid metabolism in human cells, which confirms the importance of lipid homeostasis in brain membranes.

Data obtained in this study, have indicated alterations in lipids pattern; In particularly these results have highlighted as PLs composition is affected in ELOV5KO mice model, suggesting that PLs could have an important role in molecular mechanism underlying SCA38 considering their ability to modulate the eicosanoid pathway (Burri et al 2012; Hishikawa et al 2017; Murray et al 2015).

Olfactory Bulb analysis showed more changes in different species of PLs compared to other tissue suggesting probably a cross talk between alteration in lipidomic pattern with hyposmia observed in patients (Borroni et al.,2016) and in olfactory test performed in mice model (Hoxha et al.,2017).

However, different phospholipids showed significantly changes in all of tissues considered between two experimental groups.

In all tissues considered, 10 PLs significantly changed in ELOV5KO group in comparison with control animals. A significant decrease in the PC aa C:40:4 and PE aa C:40:4 were detected; these two phospholipids probably contain arachidonic acid (20:4) and arachic acid (20:0) confirming that arachidonic acid could have a crucial role in the pathology.

Due to their amphipathic properties, naturally occurring PLs either from plant or animal origin, generally contain an unsaturated fatty acid in the sn-2 position, such as oleic acid, linoleic acid, a-linolenic acid, arachidonic acid (pro-inflammatory molecule usually from animal origin) or eicosapentaenoic acid (anti-inflammatory molecule usually from marine origin), whereas the sn-1 position predominantly carries a saturated fatty acid (SFA), such as stearic

acid or palmitic acid (Zhang et al., 2012).

The correct ratio of saturated to unsaturated fatty acids in the phospholipid membrane is essential to sustain the membrane characteristics, since the fatty acid composition and degree of saturation directly affects the fluidity of the cell membrane.

Even though the main function of PLs is to support the formation and biofunctionality of cell membranes.

For example, PLs also contain bound PUFAs to be released on demand as precursors of prostaglandins and other eicosanoids (Tessaro et al., 2015), while other PLs and their metabolites are a source of secondary messengers in cell signalling (i.e., diacylglycerols, phosphoinositides, etc.) and carry out essential functions within organelles such as the mitochondria (Mejia et al.,2016; Choy et al.,2017).

7.MASS SPECTROMETRY: NEW TOOL IN PATHOLOGY RESEARCH

Mass spectrometric (MS) techniques are applied in various areas of medical diagnostics. Mass spectrometry provides unique advantages for the analysis of clinical specimens, and these capabilities have been critical to the advancement of diagnostic medicine. (Norris et al., 2013). Cells, whether bacterial, fungal or mammalian, are able to cover different metabolic pathways capable of producing an assortment of structurally and functionally distinct lipid species. The molecular mechanisms that are under this structural diversity remain poorly understood. In part, this is due to the lack of adequate analytical techniques capable of measuring the structural details of lipid species in a direct, comprehensive and quantitative manner. Today, increased understanding of the molecular basis of disease is revolutionizing the practice and delivery of health care. Analytical measurement of molecular markers has been a challenge of diagnostic medicine for many years and technologies for clinical measurements have significantly improved, adding increased throughput and sensitivity. These capabilities allow for the further development and validation of molecular markers for clinical use, provide important insights into the mechanism of disease that augment observations of tissue morphology. MS plays an important role due to the many advantages such as high sensitivity, wide dynamic range, molecular specificity, and the flexibility to address many varied analytes on a single platform. The impact of MS on diagnostic medicine is unequivocally significant. In the last decade years, test for biomarkers of endocrine function and drugs of abuse in body fluids (for example serum, plasma, urine, etc.) are among those most commonly used in clinical laboratories. Today, liquid chromatography mass spectrometry (LC-MS) is the platform most commonly used for diagnostics. LC-MS applied to targeted molecular species is the strategy most widely adopted by clinical laboratories for carrying out routine testing. The tecnique allows the

comprehensive characterization and quantification of molecular glycerophospholipid species, molecular diacylglycerol species, molecular sphingolipid species including ceramides, glycosphingolipids and inositol containing sphingolipids, and sterol lipids including cholesterol.

However, LC-MS can be used to analyze samples in solution, making it difficult to adapt to the analysis of tissue sections including the lack of information that is necessary to relate the molecular measurements to specific populations of cells in the tissue. To this aim analysis matrix assisted laser-desorption/ ionization imaging mass spectrometry (MALDI IMS) provide a substantial throughput advantage over chromatography-based approaches.

MALDI IMS allows the localization of the analytes in the tissue as well as histology-directed experiments because the tissue pathology is used to guide the mass spectrometry analysis.

Because change in lipid metabolism represent the major factors in the development of cancer. (Rompp et al., 2013), imaging of lipids of cell in the tissues could provide unique inside information about the state of the disease as well as its perspectives for pathology research and practice (Sparvero et al., 2010).

Rujoi et al have mapped PLs composition of mammalian eyes using MALDI-TOF.MS. Specifically, feasibility of direct in situ analysis of two relevant PLs classes, phosphatidylcholines (PCs) and sphingomyelins (SMs) were demonstrated (Rujoi et al., 2004).

An interesting study was conducted by Kurabe et al to explore lipid metabolism of 34 human colorectal cancer tissues to identify a cancer specific phospholipid signature.

They have identified a news biomarker, PC (16:0/16:1), in colorectal cancer using imaging mass spectrometry. Specifically, elevated levels of PC (16:0/16:1) expression were observed in the advanced

stage of CRC. In addition, imaging mass spectrometry allowed to localize PC (16:0/16:1) in the cancer region (Kurabe et al., 2013).

In conclusion, it has been shown that histology-directed mass spectrometry measurements provide unique insight into clinical questions. The in situ analysis of PLs by MALDI-TOF MS offers a rapid and sensitive tool for the mapping of PLs in biological tissues.

Histology images are routinely used by the pathologist to diagnose disease leading MALDI IMS to revolutionize the practice of anatomical pathology in the coming decade. The implementation of molecular information into molecular pathway databases or the integration of multidimensional and complex MALDI Imaging data into systems medicine tools makes MALDI Imaging a valuable tool for a deeper understanding of the complexity of tissue and disease (Aichler et al., 2015).

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