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Oleuropein aglycone induces protective autophagy: molecular mechanisms and therapeutic targets in pathological models of autophagy dysfunction.

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Dottorando
Dott. (Miceli Caterina)

Tutore
Prof. (Berti Andrea)

Coordinatore
Prof. (Dello Sbarba Persio)

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This thesis reports three studies:

1) Molecular mechanisms of OLE-induced autophagy*: in vitro* and *in vivo* study.

Supervisor: Prof. Andrea Berti

Department of Biomedical, Experimental and Clinical Sciences, "Mario Serio", Florence, University of Florence (Italy).

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2) Effect of OLE on PARP1-SIRT1 interplay.

Supervisor: Prof.Andrea Berti

Department of Biomedical, Experimental and Clinical Sciences, "Mario Serio", Florence, University of Florence (Italy).

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3) OLE-enhanced autophagic flux ameliorates oxidative stress injury in cardiac cells.

Supervisor : Dr. Jeanne-Mialet Perez

INSERM, Institut des Maladies Métaboliques et Cardiovasculaires, Toulouse (France).

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2) Effect of OLE on PARP1-SIRT1 interplay.

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Abstract

Abstract

Macroautophagy, also referred to as autophagy, is an intracellular process aimed to degrade and recycle cytoplasmic components, including long-lived proteins and damaged organelles. Due to the pivotal role of autophagy in maintaining cellular proteostasis, its dysfunction is associated with a wide number of human diseases such as cancer, cardiomyopathies and neurodegenerative disorders. In these pathological conditions, autophagy is initially activated as a survival mechanism but subsequently becomes defective, leading to cell damage. Many recent studies aim to better understand why autophagy is compromised in pathological conditions. Consequently restoration of defective autophagy now appears as an important therapeutic strategy in disease contexts.

Several small molecules acting as autophagy modulators, such as plant polyphenols, or natural compounds present in fruit and vegetables, have been proposed as potential therapeutic applications. Plant polyphenols are able to regulate autophagy through different pathways. In particular, resveratrol and epigallocatechin-3 gallate (EGCG) stimulate the autophagic pathway via CamKKβ-AMPK-mTOR signalling. Polyphenols can also activate the sirtuins (SIRT), family of class III histone deacetylases, which are also involved in autophagy modulation. SIRT-induction results in many cellular outcomes and is considered responsible for the epigenetic effects of polyphenols. Oleuropein is the main polyphenol found in the olive tree and its main product, olive oil. Our previous studies have highlighted the beneficial effects of oleuropein aglycone (OLE), both in neuroblastoma cell lines (N2a) and in TgCRND8 mice, a model of Aβ deposition. In the latter, food supplementation with OLE resulted in remarkable plaque reduction and in the reduction of cognitive impairment when compared to non-OLE fed littermates. These protective effects were strongly correlated to an increased activation of autophagy in OLE fed mice.

In light of the benefits associated with the upregulation in autophagy, the aim of this thesis was to investigate the cellular and molecular effectors of OLE-induced autophagy *in vitro*, by use of cultured human neuroblastoma cells (SH-SY5Y) and *in vivo*, using our TgCRND8 mice. Our *in vitro* results showed that OLE supplementation induces a rapid release of Ca^{2+} from the endoplasmic reticulum stores which, in turn, activates CAMKKβ with subsequent phosphorylation and activation of AMPK. The interplay

between AMPK activation and mTOR inhibition shown in the OLE-fed animal model supports the idea that autophagy activation by OLE proceeds through mTOR inhibition. SIRT1 activation, another mechanism that synergizes with OLE-induced Ca^{2+}-CaMKKβ-AMPK-mTOR signalling was also found in N2a cells.

Given our findings for OLE dependent promotion of autophagy in our *in vitro* and *in vivo* neurodegeneration models, we aimed to determine whether OLE promotion of autophagy is ubiquitous and could protect against other pathological conditions displaying autophagy dysfunction. We selected an *in vitro* model of cardiomyopathy characterized by overexpression of monoamine oxidase-A (MAO-A). It is well established that catecholamine and serotonin degradation by MAO-A produces H_2O_2, which then disrupts nuclear translocation of TFEB, a master regulator of autophagy, causing autophagosome accumulation and ultimately cell death. Using this model we have shown that OLE treatment counteracts the effects of the MAO-A/H_2O_2 axis by improving mitochondrial function and decreasing cell necrosis. We demonstrate that these protective outcomes are, at least in part, related to the activation in autophagy. Indeed, increased autophagy observed in cardiac cells treated with OLE was a measure of the increase in autophagic vacuoles and autophagy-specific marker (LC3II) expression. Double immunofluorescence imaging of RFP-GFP-LC3 after 6 h of OLE treatment showed an increase of the autophagic flux; in addition, nuclear translocation of TFEB in OLE-treated cells was also observed. Together these data suggest that OLE treatment evokes transcriptional regulation of autophagy.

In conclusion, our findings demonstrate that the underlying molecular mechanism of OLE stimulated autophagy includes the activation of the Ca^{2+}-CaMKKβ-AMPK-mTOR signalling pathway. The identified molecular underpinnings of OLE treatment are indeed similar to other plant polyphenols such as resveratrol and EGCG. We show further that SIRT1-activation could synergize to maintain OLE-induced autophagy. TFEB translocation to the nucleus supports the importance of the transcriptional regulation of autophagy, findings that warrent further investigation. The results of this thesis add to the growing knowledge base of the molecular mechanisms of OLE-induced autophagy and provide strong evidence that similar to other plant polyphenols OLE can be a potential therapeutic against age-related diseases associated with autophagy dysfunction, including neurodegeneration and cardiovascular diseases.

Introduction

Mediterranean diet

1) Diet and human health

Eating is not only a physiological response to the energetic needs of the human body, yet beyond basic nutrition diet can also provide beneficial effects for health and play an essential role in the prevention of chronic diseases. In particular, as shown in several case-control and cohort studies, the consumption of fruits and vegetables is associated with reduced risk of cancer, cardiovascular diseases, stroke, Alzheimer's disease, cataracts, and other age-related diseases. On the contrary, diets comprising high intake of saturated and partially hydrogenated fats appear to increase the risk of coronary heart diseases (Liu, 2003). These findings led to the launching of the "Recommended Dietary Allowances (RDAs)" in 1943, that persist today representing nutritional guidelines for individuals and institutions. The main goal of the RDAs was to prevent chronic diseases and reduce the consumption of unhealthy foods such as saturated fats. In 1989 the National research Council (NRC) introduced new recommendations concerning food to prevent cardiovascular diseases. An increase in dietary carbohydrate, primarily in the form of complex carbohydrate, was recommended to replace dietary fats. Other recommendations include: high consumption of fruit and vegetables, maintaining protein intake at moderate levels, practising moderate and regular physical activity, limiting the consumption of alcohol and salt, and, maintaining adequate intake of minerals such as calcium and fluoride, especially during growth. The first "Food Pyramid", released by the Department of Agriculture in the United States, was a tentative to translate the nutritional knowledge to a recommended eating pattern (Willet, 1994). The basis of all these initiatives was the concept that prevention is more effective strategy than treatment of chronic diseases (Liu, 2003).

2) Mediterranean Diet as a model of healthy diet

Several epidemiological studies have shown that the Japanese and Mediterranean Diet (MeDiet) are the most healthy dietary patterns. They have many similarities and both emphasize physical activity. In both dietary patterns the key components are cereals, vegetables, along with lean meat and fish, but the MeDiet remains to this day the most recommended dietary pattern likely attributed to the consumption of olive oil.

The MeDiet promotes plant-based foods like fruits, vegetables and healthy fats such as olive oil, along with fish and poultry. The foods that have to be consumed in moderation are red meat (which has a suggested consumption of few times a month) and red wine (normally with meals; about one or two glasses per day for men and one glass per day for women). However, only if the MeDiet is associated with moderate and regular physical activity it is possible to achieve the healthy effects of this dietary pattern (Willet, 1994). Given the numerous beneficial effects of this diet, on November 17th 2010, UNESCO recognized the MeDiet as an "Intangible Cultural Heritage" of Italy, Greece, Spain and Morocco. The MeDiet was defined "a set of traditional practices, knowledge and skills passed on from generation to generation and providing a sense of belonging and continuity to the concerned communities."

2.1) History

The term MeDiet is often associated with "pizza or noodles with meat sauce" and this generates a lot of confusion. MeDiet is instead is a combined and balanced diet able to ensure mental and physical well-being through the intake and use of substances that help the body to maintain normal vital functions (Altomare et al, 2013). The MeDiet is characteristic of the Mediterranean area (mainly Greece, Italy, Spain and France), but its origin dates to about 10,000 years ago, in the "Fertile Crescent" region (Levant, Egypt and Mesopotamia), considered the cradle of civilization, and the birth and development of the first forms of agriculture. Adoption of the Fertile Crescent technique allowed for the arrival of cereals, legumes, olive oil, vegetables, fruit and wine, fish and eggs, along with sheep farming products (milk and dairy products) and meat into common diets. The MeDiet is also the result of a dynamic context that has characterized this area for a long time. Previously, the Mediterranean area was in fact a meeting place for people of different languages, cultures and religions. Different civilizations have dominated this area: Egyptian, Phoenician, Greek, Carthaginian, Roman, Arab, Byzantine, Ottoman and more. They introduced different foods, culinary practices and food traditions throughout the Mediterranean region. Therefore, MeDiet is a combination of the plethora of dietary habits of the people that lived in this region (Dernini et al, 2016).

2.2) Beneficial effects of Mediterranean Diet

"The term Mediterranean Diet" started to be associated with a model of healthy diet only in the '50s when the American Scientist Ancel Keys noticed a correlation between cardiovascular diseases and diet. The scientist observed that the poor population of South Italy was surprisingly healthier than the wealthier American relatives or others that emigrated to the United States in earlier decades. Keys hypothesized that the health state was attributed to the diets and to validate his hypothesis he started the famous "Seven Countries Study": a study conducted in seven countries (Italy, Holland, Finland, Greece, United States, Japan and Yugoslavia) to determine if lifestyle, nutrition and cardiovascular diseases would positively correlate. The study provided clear evidence that adherence to MeDiet was associated with a minor incidence of coronary heart diseases. This was mainly due to a large consumption of plant foods, such as cereals, fruit, vegetables and oil olive and to a rather moderate use of animal products (Altomare et al, 2013). This study found that all populations of the Mediterranean region, Northern Europe and the United States consumed relatively high amounts of dietary fats, but the sources of consumed fats were different. Indeed, the Northern European and US populations preferred saturated fat of animal origin, whereas Southern European diets consisted of unsaturad fats from plant origin, in particular olive oil. In accordance with Keys' findings, many other researchers have now shown associations between diet and diseases as briefly outlined below.

1998: the Lyon heart study reported that MeDiet including fatty acid supplementation (omega 3) led to a 56% reduced risk for death in patients with coronary heart disease compared to a prudent low-fat diet (Zacharias, 2012). Similar findings were obtained in trial carried out in India (Indo-Mediterranean diet Heart study) (Serra Majem et al, 2006).

2003-2004: two large European cohort studies suggested that a high degree of adherence to the MeDiet was associated with a reduction in both total and coronary mortality rates (Knoops et al, 2004; Trichopoulou et al, 2003)

2003-2013: two clinical trials were conducted in Italy (the MedDiet Project) and in France (Medi-RIVAGE Study). The most ambitious and only large-scale clinical trial (the PREDIMED Study) was conducted in Spain. The MedDiet Project was a randomized clinical trial conducted on a sample of 115 women to investigate if the

MeDiet had preventive effects against the risk of developing breast cancer. The study reported that the group that followed MeDiet showed a decrease in total estrogen levels and a decreased risk for breast cancer (Carruba et al, 2006). In particular, the intake of antioxidants, due to a large presence of fruit, vegetables and the wild greens in the diet, was proposed as a potential reason for the protective effects of MeDiet against cancer (Trichopoulou et al, 2000). The Medi RIVAGE study was conducted in a mixed-sample of 212 males and females exhibiting at least one cardiovascular risk factor and aimed to evaluate the effect of two diets (the classical MeDiet and a low-fat diet) on arteriosclerosis risk factors while implementing the biological investigation in relation to the dietary intervention (Serra Majem et al, 2006). The study showed a 9% reduction in cardiovascular disease risk with the low-fat diet and a 15% reduction with the Mediterranean-type diet. The PREDIMED Study represented the first long-term clinical trial that enrolled high-risk patients to follow a Mediterranean diet supplemented with extra-virgin olive oil (50 ml/day) or 30 grams (g) tree nuts (15g walnuts, 7.5g almonds and 7.5g hazelnuts) for primary cardiovascular disease prevention. This study was started in 2003 and concluded in 2013 with the following results: Mediterranean diet supplemented with either extra-virgin olive oil (EVOO) or nuts resulted in an absolute risk reduction of approximately 3 major cardiovascular events per 1000 person-years, for a relative risk reduction of approximately 30%, among high-risk persons who however did not exhibit cardiovascular diseases (CVD). (Estruch et al, 2013).

In addition to these clinical trials, meta-analysis of observational cohort studies by Sofi et al. (Sofi et al, 2008) showed the relationship between MeDiet and Health status, yielding new interesting data: a 20% increase in MeDiet adherence could significantly associate with a 9% reduction in overall mortality, a 10% reduction in death for CVD, a 6% reduction in cancer incidence and mortality and a 13% reduction in Alzheimer and Parkinson's diseases incidence (Serra Majem and Trichopoulou, 2016).

Beyond the protective effects against chronic diseases (CVD, cancer et others), the MeDiet has other numerous beneficial effects, which are still under investigation. These include prevention against allergic diseases and psychiatric disorders such as depression (Serra-Majem and Trichopoulou, 2016). However, despite its recognized beneficial effects the MeDiet alone is not able to induce these benefits if it is not associated with an healthy lifestyle with moderate and regular physical activity (Leitzmann et al, 2007). Altogether these studies gave rise to the notion of the "Mediterranean Diet pyramid".

2.3) Mediterranean Diet pyramid

The MeDiet provides a proper balance between fats (25-30%), proteins (10-15%) and carbohydrates (55-60%). The "Food Pyramid", designed for the first time by the U.S. Department of Agriculture, summarized the guidelines of the MeDiet, showing a balance between different nutrients, and the proportions and frequencies with which the foods should be consumed. The Food Pyramid is based on four main concepts: i) "proportionality, the right amount of foods to choose from for each group; ii) the "portion, standard quantity of food in grams, which is suggested for a balanced diet iii) "variety", importance of changing the choices within a food group; iv) "moderation", limited consumption of certain foods, such as fats or sweets. Each group includes foods that are "equivalent" on the nutritional value and are therefore interchangeable, ensuring in this way a certain variety in the nutritional choices (Figure 1).

Figure 1: Mediterranean diet pyramid (Altomare et al., 2013)

At the base of Food pyramid there are cereals followed by fruit and vegetables, legumes, olive oil, cheese and yogurt. These components should be eaten daily. For fish and eggs the MeDiet pyramid suggests a weekly consumption. Meat products are not excluded but consumption should be limited. Particularly, white meat (chicken, rabbit, turkey) should be preferred over red meat; the latter in fact should be eaten only few times a month (Altomare et al, 2013). In the top vertex of the pyramid are foods rich in sugars and unhealthy fats (sweets). Sugar, candies and beverages, such as sweetened fruit juices and soft drinks, should be consumed in small amounts and only for special

occasions. These foods can contribute to weight gain (Mozaffarian et al, 2011) and are associated with an increased occurrence of tooth decay (World Health Organization, 2003).

Foods in The Mediterranean Diet Pyramid can be divided into five main groups: grains, fruits and vegetables, milk and dairy products, meat-fish and eggs, and dressing fats (Altomare et al, 2013). A more detailed description of each group and its nutritional benefits is given below:

1) Grains. They include cereals and tubers, such as rice, pasta, bread, oats, barley, spelt, corn, potatoes. The components of this group should always be present in the diet, because they are an important source of starch, energy that our body can easily utilize. Some of these contain vitamins of the B group and different proteins, but do not contain essential amino acids such as lysine. For this reason it is advisable to associate them with legumes, an important source of lysine. Conversely, the legumes do not contain amino acids otherwise present in cereals, such as methionine. Taken together, grains and legumes compensate their mutual shortcomings by providing a good protein intake (Altomare et al, 2013).

*2) Fruits and vegetables***.** Fruits and vegetables are an important source of Vitamins (A, C), minerals (such as potassium), and sugars (in particular sucrose and fructose in the fruit). In addition this group is characterized by the presence of water (for instance up to 95% of weight in watermelon) and antioxidant substances, able to contrast oxidative stress. They are also high in dietary fibres (cellulose, hemicellulose and pectin), important for intestinal transit regulation and moderation of blood cholesterol levels. Fruits and vegetables should be taken daily and in large quantities (Altomare et al, 2013).

3) *Milk and dairy products.* The dairy group include: milk, yogurt, cheese and dairy products. Dairy products should be present in moderate amounts (two servings per day), with a preference for low-fat dairy (Bach-Faig et al, 2011). This group represents an important source of vitamins (B12 and A), proteins (the most prominent is casein), and calcium (important for maintaining bone structure and preventing diseases such as osteoporosis) (Altomare et al, 2013).

4) Meat, fish and eggs. This group provides essential amino acids for protein synthesis, vitamins (mainly of B group) and trace elements, such as zinc, iron, and copper. Within this group, lean and white meat is preferable, while red meat and sausages should be limited. In regards to eggs, the guidelines suggest no more than two or three times a week. Fish consumption is important because of the presence of polyunsaturated fatty acids, which are known to regulate haemostatic factors, protect against cardiac arrhythmias, cancer, hypertension, and maintain neural functions. Sweets should be consumed with moderation but they are not completely excluded in this diet. The foods of this group should be consumed weekly, with the exception of red meat, recommended only few times a month. Despite the limitations, this group is considered irreplaceable for the high protein content (Altomare et al, 2013; Ortega, 2006).

5) Dressing fats. Dressing fats include: vegetable fats (such as olive oil) and animal fats (butter, cream, lard). Among the two types, the former are to be preferred according to the guidelines of the MeDiet. Olive oil is located at the centre of the pyramid and the MeDiet suggests it as the principal source of dietary lipids for its high nutritional quality (especially EVOO). Dressing fats are important for the intake of fatty acids essential for the absorption of fat-soluble vitamins and the formation of cellular membranes and other components. However the consumption of dressing fats should be limited because when they are not consumed with moderation they represent an important risk factor for cardiovascular diseases, tumours and obesity (Bach-Faig et al, 2011; Altomare et al, 2013)

Daily physical activity at the base of MeDiet pyramid. The Mediterranean Diet pyramid also includes moderate and regular physical activity, which means practicing motor activity (not necessarily sport) that is not too intense (eg. walking, running, swimming or cycling etc.), for at least 30 minutes per day at least 5 days a week. Kavouras et al. studied the association between physical activity, adherence to the Mediterranean diet and total antioxidant capacity (TAC). The value of TAC considers all the antioxidants present in plasma and body fluids and is largely used in the diagnosis and treatment of different diseases such as cardiovascular, cancer, diabetes, and ageing. The study included a random sample of 1514 men and 1528 women selected from the Attica region. All participants were interviewed by trained personnel (cardiologists, dieticians, and nurses) that used questionnaires to evaluate lifestyle habits, as well as clinical aspects. The result was that adherence to MeDiet associated with a moderate and

regular physical activity increased antioxidant defences. Alternatively, physical inactivity associated with a high-fat diet, especially saturated fats, induced oxidative stress (Kavouras et al, 2011).

2.4) Extra-Virgin olive oil, a cornerstone of the Mediterranean diet.

Olive oil, is one of the hallmarks of the MeDiet. Specifically when olive oil is mentioned in the context of MeDiet, it refers to EVOO. It is a vegetable oil obtained directly from olive fruit using only mechanical extraction (without chemical treatments that can alter the oil quality), with a maximum free acidity in terms of oleic acid of 1g for 100g (Ramirez Tortosa et al, 2006). It needs to be underlined that there is a considerable difference between "olive oil" and "EVOO". The former has a very low concentration of phenolic compounds whereas the latter contains up to 1g/Kg of phenolic compounds (Cheynier et al, 2012). Several studies have shown the effectiveness of EVOO in the prevention of CVD. The proposed mechanism by which EVOO could exert beneficial effects against CVD include the improvement of lipid profile via an increase in HDL with a reduction in LDL, including its susceptibility to oxidation, an improvement in endothelial function, and an improved regulation of blood pressure. Descriptive clinical studies have suggested that EVOO is protective against age-related cognitive decline, Alzheimer's disease (AD), metabolic syndrome and diabetes. In the international EVOO conference held in Spain (2008) the beneficial effects against cancer were shown (Lopez-Miranda et al, 2010). For example it was reported by several different studies that approximately 80% of human cancer cases are associated with bad eating habits and lifestyle. However, in countries where the population followed the traditional MeDiet and where EVOO was the principal source of fat, cancer incidence was lower (Trichopoulou et al, 2000; La Vecchia, 2004; La Vecchia et al, 1995). It is proposed that EVOO could exert beneficial effects against initiation, promotion and progression of cancer through several mechanisms. Some of these may include alterations in tumour cell composition and structure of cell membranes, changes in eicosanoid biosynthesis or signalling pathways, modulation of gene expression, reduction of cellular oxidative stress and DNA damage, modulation of the immune system and/or hormonal balance in hormone-dependent cancers (Escrich et al,, 2006; Bartsch et al, 1999; Escrich et al, 2007; Menendez et al, 2006; Menendez and Lupu, 2006; Kossoy et al, 2002). The most recognized hypothesis is that these protective effects are a result of synergistic and convergent interplay of all oil

components triggering a broad range of cell responses that may play a role in cancer prevention; or, if the disease already exists, lead to a reduced biological aggressiveness (Lopez-Miranda et al, 2010).

The biological and therapeutic value of EVOO is related to its composition. It consists of two fractions: the major or saponifiable fraction and the minor or insaponifiable fraction.

1) Saponifiable fraction. The saponified fraction represents more than 98% of the total oil weight and consists mainly of triacylglycerols, diacylglycerols, monoacylglycerols, free fatty acids and phospholipids. The fatty acids present in this fraction can be divided into two groups: saturated and unsaturated. The most common monounsaturated fatty acid in olive oil is oleic acid, whereas the saturated group includes palmitic and stearic fatty acids. Linoleic acid is the predominant polyunsaturated fatty acid (Šarolic et al, 2014).

2) Insaponifiable fraction. This fraction represents 1-2% of the EVOO composition. It includes:

- hydrocarbons, classified in saturated and polyunsaturated, that represent 50-60% of insaponifiable fraction (De Leonardis t al., 1998);
- aliphatic and triterpenic alcohols, 20-35% of this fraction, that influence oil aroma;
- **polyphenols**, 18-37% of the insaponifiable fraction, which include phenolic acids, phenolic alcohols, flavonoids, secoiridoids and lignans. Polyphenols have been recognized to have the following biological effects: antioxidant, anti-inflammatory, anti-allergic, antibacterial and antiviral (Servili et al, 2002);
- tocopherols, 2-3% of the unsaponifiable fraction, known for their antioxidant properties. Among these compounds there is alpha-tocopherol, constituent of vitamin E (about 150-300 mg / kg of oil). It is lipophilic with a strong antioxidant capacity mainly toward the polyunsaturated fatty acids, which are the most likely to oxidize (Baldioli et al, 1996);
- pigments, classified in chlorophylls and carotenoids. The carotenoid pigments are responsible for the yellow tint of oil, while chlorophylls are the source of the color green. However, these compounds can undergo oxidation under certain

conditions, thus they can be degraded to uncoloured products and promote oxidation of fatty acids (Minguez-Mosquera et al, 1990).

2.5) Phenols: classification, bioavailability, biological effects

Phenolic compounds, often referred to as polyphenols, include a mixture of compounds characterized mainly by the presence of phenol structural units (at least one aromatic ring with one or more hydroxyl groups (Rodrigo et al, 2014). They are secondary metabolites in plants and have various functions, such as the protection from herbivores and from microbial infection, pollinator attraction, UV protection and others. The oil phenols are derived from fruit with some modifications. In oil phenol content varies in the range of 40 to 1000 mg/kg. Several factors can affect the quantity and quality of phenols in the oil. For instance the cultivar, olive variety, age of the trees, agronomic techniques (such as irrigation), fruit ripening, pedoclimatic conditions of production are some factors that directly impact on phenol content (Servili et al, 2002).

A brief description of the main classes of phenolic compounds contained in EVOO is given below:

Phenolic acids. There are two main series of these acids depending on the carbon skeleton: first, benzoic acids (C6-C1: 3-hydroxybenzoic, p-hydroxybenzoic, protocatechuic, gentisic, vanillic, syringic and gallic acids); and second, cinnamic acids (C6-C3: o-coumaric, p-coumaric, caffeic, ferulic and sinapic acids).

Phenolic alcohols. The most represented in EVOO are hydroxytyrosol (HT) and tyrosol (the only structural difference is the hydroxyl group in the meta position.)

Secoiridoids. They belong to the Oleaceae family. The most important are oleuropein aglycone and ligstroside aglycone. Secoiridoids are present in olive fruit as glycosides but fruit ripening, oil extraction and storage cause removal of the glucose moiety by endogenous β glucosidases and result in their conversion into aglycones.

Lignans. (+)-1-Pinoresinol, (+)-1-hydroxypinoresinol and (+)-1-acetoxypinoresinol are ubiquitous.

Flavonoids. Within this group, the most abundant are apigenin and luteolin, which originate from corresponding glucosides present in the drupe (Gomez-Romero et al, 2012).

A schematic diagram of the main polar phenolic compounds present in olive oil is reported below (Figure 2).

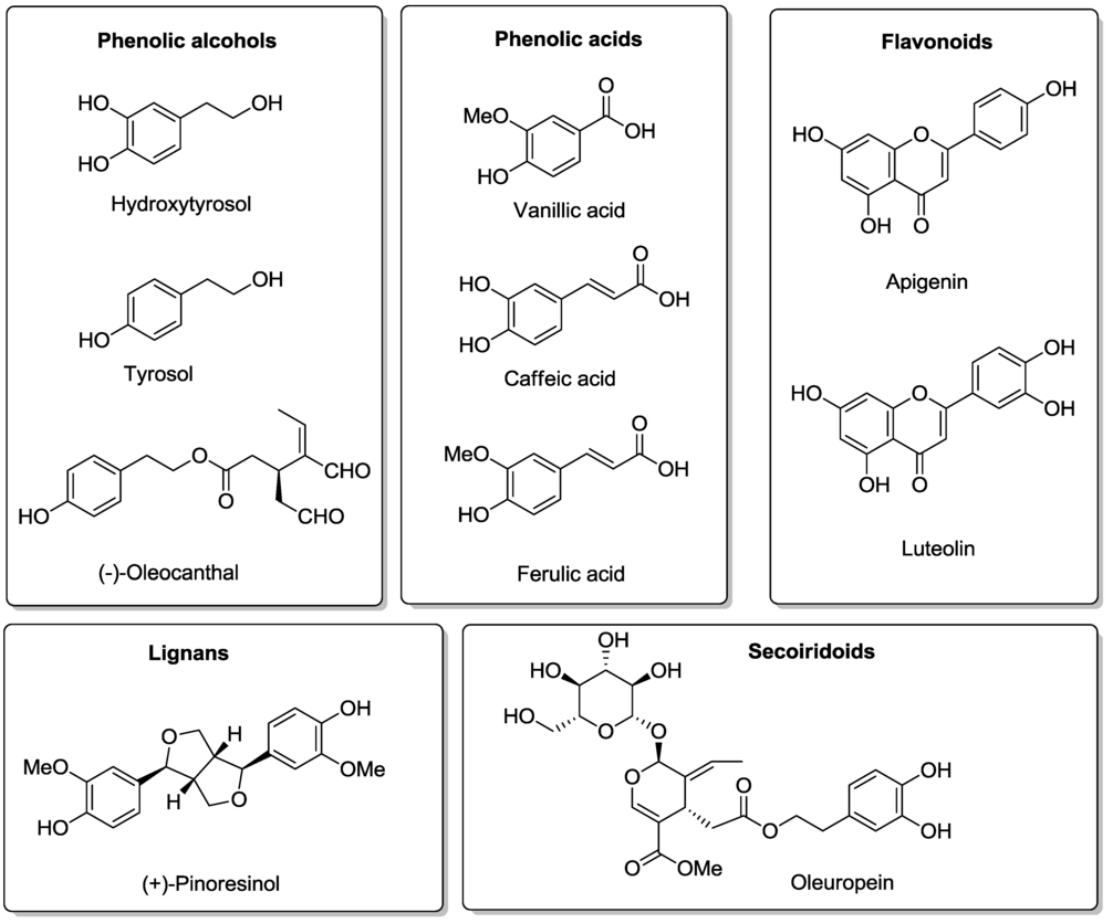

Figure 2: The different classes of polar phenolic compounds present in olive oil with molecular structures of representative examples.(Rodríguez-Morató et al, 2015)

The phenols of EVOO have a wide array of biochemical activities. Among these, the most known is their antioxidant activity. These compounds can act as antioxidants through several mechanisms, including the free radical scavenging activity, transcriptional regulation and metal chelation.

1) **Free-radical-scavenging activity.** As scavengers, they can deactivate radical species through donation of a hydrogen atom. This process depends primarily on the chemical

structure, relying on the total number and the configuration of hydroxyl groups on the molecule (Heim et al, 2002). Such antioxidant effect can be useful for all the conditions characterized by intense oxidative stress (such as cancer, hypertension, diabetes, degenerative disorders) (Rodrigo et al, 2014).

2**) Transcriptional regulation.** Polyphenols can act as transcriptional regulators. It is shown that low doses of polyphenols can stimulate the transcription of enzymes involved in antioxidant defences, such as the γ-glutamyl-cysteine-synthetase (γGCS) (Myhrstad et al, 2002), the rate-limiting enzyme in the synthesis of glutathione (Moskaug et al, 2005). The mechanism through which they exert this effect includes the activation of the Nrf2 transcription factor (Lu, 1998)

3) **Metal chelators**. The activity as metal chelators is important because some metals, such as iron, can lead to the formation of hydroxyl radicals (•OH) through the Haber–Weiss/Fenton reaction that damages DNA and other biomolecules (Huang et al, 2003).

Despite their recognized protective effects, one of the aspects that can limit their effectiveness is their bioavailability. Bioavailability can be defined as the fraction of an oral dose of either parent compound or active metabolite, from a particular preparation that reaches the systemic circulation. The bioavailability is different for every polyphenol. Evidences on bioavailability of olive oil polyphenols have been obtained measuring the concentration of the polyphenols directly in plasma and urine after ingestion of pure compounds or mixtures enriched in particular phenols of olive oil (Gomez-Romero et al, 2012). After acute ingestion the absorption of most of polyphenols in the circulatory system occurs in the small intestine (Del Rio et al, 2013). Small polar phenols as tyrosol (Tyr) and hydroxitirosol (HT) are absorbed via passive diffusion in a dose-dependent manner. If other polar but larger phenols, such as oleuropein can pass through the lipid bilayer of the epithelial cell membrane via diffusion in a similar manner is under investigation, but other mechanisms have been suggested (Vissers et al, 2002). In particular many authors support the hypothesis that polyphenolic glycosides can undergo cleavage and release aglycone either through lactase phloridzin hydrolase (LPH) present in the brush border of the small intestine epithelial cells, or through a cytosolic β–glucosidase found within the epithelial cells (CBG). For CBG-mediated hydrolysis, it is necessary that the phenolic glycosides are first transported into the epithelial cells probably through the sodium-dependent glucose

transporter (Del Rio et al, 2013). For glycosylated oleuropein, this latter hypothesis seems to be the most plausible. A similar mechanism was proposed by Hollman et al. for quercetin glycoside, another phenolic compound (Hollman et al, 1995; Hollman et al, 2000). In addition to LPH and CBG hydrolysis, some phenolic glycosides can be hydrolised by the intestinal microflora into various simple aromatic acids, an event that reduces their bioavailability. Oleuropein-glycoside, oleuropein- and ligstroside-aglycones are hydrolized into HT or Tyr and elenolic acid and then subsequently metabolized. It is not clear where this splitting occurs. They are possibly split either in the gastrointestinal tract or in the intestinal cell before absorption, or they are split in blood or liver after they are absorbed (Vissers et al, 2002). The metabolism of polyphenols occurs essentially in the small intestine and later in the liver. Their metabolic processes include methylation, sulphation, and glucuronidation. These metabolic reactions require different enzymes such as sulfotransferases (SULT), catechol-O-methyl transferases (COMT), uridine 5' diphosphates, and glucuronosyltransferases (UDPGT). The conjugation mechanisms are highly efficient and aglycones are generally absent in blood or present in very low concentrations after oral consumption. Circulating polyphenols are often conjugated metabolites that are extensively bound to albumin (Manach et al, 2004). Different studies have shown the presence of metabolites of HT, Tyr and oleuropein in human plasma and urine following oral ingestion. The metabolites are mainly glucuronate and sulphate conjugates and in some cases after glucuronation or sulphation they are also methylated at the catechol group. Other possible fates for phenols include the efflux and site-specific accumulation. The efflux can carry some metabolites back into the lumen of small intestine through the action of P-glycoprotein, a protein involved in mechanisms of multidrug resistance (MRP). The mechanism of site-specific accumulation of phenols is not known, but a number of studies suggest that some cells may induce specific mechanisms for the incorporation of polyphenols (Del Rio et al, 2013; Gomez-Romero et al, 2012).

2.6. Oleuropein aglycone, typical phenol of extra-virgin olive oil

Oleuropein is a secoiridoid typical of the Oleaceae family. The biosynthesis of oleuropein in Oleaceae is via the mevalonic acid pathway, through branching that results in the formation of oleosides. Secoiridoids are derived from these compounds (Damtoft et al, 1993).

Oleuropein was identified for the first time in olive fruit by Bourquelot and Vintilesco, but elucidation of its chemical structure was described much later (Bouerquelot et al, 1908; Panizzi et al, 1960). Oleuropein, such as demethyloleuropein and verbascoside, is present in all the constitutive parts of olive fruits such as peel, pulp and seed, but particularly in the pulp. Chemically oleuropein is an ester between HT and elenolic acid. It is important to distinguish two forms of oleuropein; glycated and aglycone (OLE). Glycated oleuropein is characteristic of olive fruit and leaves, whereas OLE is obtained from the deglycosilation operated by β–glycosidase, which is released from olive fruit during crushing (Rigacci et al, 2011) (Figure3).

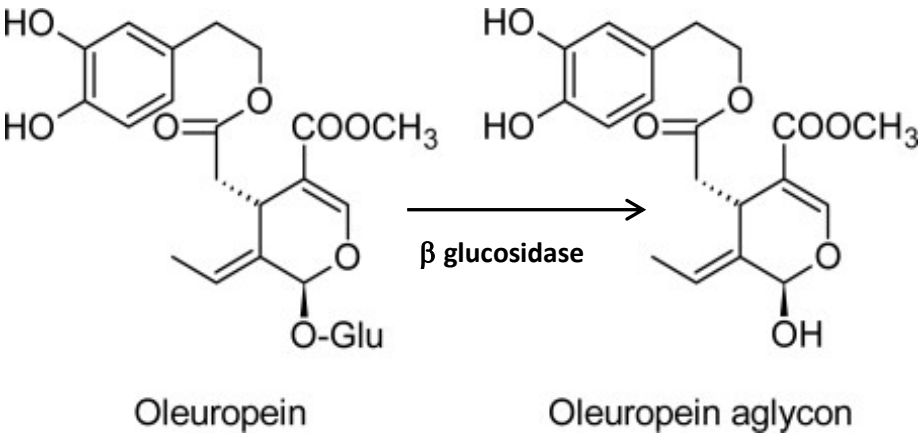

Figure 3: Structure of Oleuropein and Oleuropein aglycone (OLE)

The content of oleuropein in the fruit and OLE in the oil can be affected by several factors: drupe ripeness, cultivation techniques, and the type of oil. The drupe ripeness plays an important role for the oleuropein content as it is higher in the first stage of drupe maturation, and decreases as the fruit ripens. Irrigation also decreases the content of oleuropein in the fruit and the corresponding amount of OLE in the oil. There is also significant difference among the types of olive oil. EVOO, obtained from the first cold extraction, has a higher concentration of OLE as compared to virgin olive oil and olive oil.

Regarding the biological properties of oleuropein (glycated or aglycone), in general oleuropein shows a wide spectrum of activity in numerous contexts including an antioxidant property, cardio-protection, anti-cancer, antimicrobial, antiviral, and

inhibition of amyloid toxicity (Omar, 2002; Rigacci et al,2011). Recent studies have also highlighted its role as an activator of protective responses in cells, in particular the stimulation of autophagy (Grossi et al, 2013, Rigacci et al, 2015).

A brief description of its main beneficial properties is given below:

Antioxidant: Oleuropein is a strong antioxidant, demonstrating similar effect to ascorbic acid (vitamin C) and α tocopherol (vitamin E) (Visioli et al,1998). It causes a dose-dependent inhibition of copper sulphate-induced oxidation of low-density lipoproteins (LDL) (Omar, 2002). De la Puerta et al. (De la Puerta et al, 2001) have shown the ability of oleuropein to scavenge nitric oxide and to cause a parallel increase in the inducible nitric oxide synthase (iNOS) expression. Visioli et al. further reported a scavenging effect of oleuropein against hypochlorous acid (HOCl), oxidative substance produced in vivo by neutrophil myeloperoxidase at the site of inflammation (Visioli et al, 2002). Compared to the glycated form, OLE is more effective against oxidative stress, as its liposolubility permits faster action on penetrating the intracellular context and inhibiting radicals (Rigacci et all,2011).

Cardioprotective: CVD*s* are the result of a plethora of pathological conditions such as atherosclerosis, hypertension, hyperlipidemia. Several studies report the beneficial effects of oleuropein, and its metabolite HT against atherosclerosis. Oleuropein can act in different ways to counter atherosclerosis. Some of these mechanisms include: i) inhibition of LDL-oxidation, ii) reduction of monocytoid cell adhesion to stimulated endothelium (Carluccio et al, 2003), iii) inhibition of the stimulated expression of ICAM1 and E-selectin, and, iv) downregulation of inflammatory genes, in particular those encoding for IL-6 and IL-8 cytochines, over-expressed in atherosclerosis (Rigacci et al, 2016). Thrombin-induced platelet aggregation is a pathological condition closely related to CVD. Oleuropein, in particular OLE, can act against platelet aggregation through inhibition of cAMP phosphodiesterase, which leads to an increase in cAMP concentration. As an anti-hypertensive agent, OLE inhibits both angiotensin converting enzyme (ACE) and neprilysin (NEP). The role of ACE enzyme is the conversion of angiotensin I to angiotensin II (AngII). Ang II constricts blood vessels, producing high blood pressure. On the other hand, NEP is an enzyme highly expressed in the kidney and lung tissues that degrades signalling peptides such as encephalins, substance P, endothelin and atrial natriuretic peptide. *In vitro* studies have shown the effectiveness of

OLE (extracted by Ligustrum vulgare) against both ACE and NEP with IC50 20μM for ACE and 35 μM for NEP. Notably, under the same conditions oleuropein glycate was not shown to be effective. Moreover, hyperlipidemia is a risk factor for cardiovascular diseases. Oleuropein, both glycated and aglycone, is effective on lipid metabolism, lowering LDL plasma levels and total cholesterol, and increasing high-density lipoproteins (HDL) levels (Rigacci et al, 2011).

***Anticancer*:** Some studies showed anti-cancer properties of oleuropein and OLE**.** Corona et al. have shown that olive oil extract (where OLE was the most abundant phenol) was able to block cell cycle in G2/M phase in colon adenocarcinoma cells (Caco2). This G2/M block was mediated by the ability of OLE and other polyphenols present in the extract to inhibit p38 and CREB phosphorylation, which then led to a downstream reduction in COX-2 expression (Corona et al, 2007). Barbaro et al. reported anti-proliferative and proapoptotic effects of oleuropein glycate in breast adenocarcinoma, colorectal adenocarcinoma, glioblastoma, lung carcinoma, prostate cancer, melanoma, renal cell adenocarcinoma (Barbaro et al, 2014). A dose-dependent antiproliferative effect of OLE was reported in breast cancer (Mendenez et al, 2008). In this study, HER2 oncogene, overexpressed in human breast cancer, was proposed as specific target of OLE (Mendenez et al, 2008). Beneficial effects against cancer can also depend on antioxidant (protection of cells against oxidative stress characteristic of oncogenesis) and anti-angiogenic effects (prevention of tumor progression). Tumours release vascular endothelial growth factor (VEGF), a factor that promotes the formation of new vessels resulting in angiogenesis. In the absence of VEGF, the vessels cannot develop and the tumour is no longer able to obtain necessary nutrients or oxygen for growth. VEGF is therefore an important target for cancer therapy. In addition to VEGF, other factors such as metalloproteinases are closely linked to growth, invasion, metastasis and angiogenesis of cancer. Kimura et al. have shown inhibition of VEGF, as well as the metalloproteinases MMP-2, MMP-9 and MMP-13 by OLE in a model of chronic UVB-induced skin damage and carcinogenesis (Barbaro et al, 2014; Kimura et al, 2009).

Antimicrobial: Oleuropein has strong antimicrobial activity against Gram negative and Gram positive bacteria and mycoplasma. The mechanism of antimicrobial activity is not completely understood, even if some studies suggest that the catecholic portion plays an important role. There are three proposed mechanisms: i) damaging of the bacterial

membrane and/or disrupting cell peptidoglycans, ii) interference with the synthesis of amino acids necessary for the growth of microrganism, and, iii) stimulation of the immune system that leads to phagocytosis of microbes. (Omar, 2010). Saija and Uccella (Saija et al, 2011) reported a greater efficacy of OLE compared to the glycated form. The glycoside group in fact could reduce the ability of the phenol to penetrate the cell membrane and get to the target site (Omar, 2010).

Antiviral: Oleuropein has antiviral activities against herpes mononucleosis, hepatitis virus, rotavirus, bovine rhinovirus, canine parvovirus, feline leukemia virus, respiratory syncytial virus and para-influenza type 3 virus (Omar, 2010). The protective effect against the human immunodeficiency virus was demonstrated; an action likely mediated via the inhibition of HIV 1 integrase activity (Lee-Huang, 2007).

Inhibition of amyloid toxicity and neuroprotection: Amyloidosis are a group of diseases related to proteins or their fragments, which lose their native soluble form and are transformed into insoluble aggregates with consequent formation of amyloid plaques. This process is called "amyloidogenesis". Amyloid plaques have been found in different pathological conditions such as Parkinson's (PD) and Alzheimer's diseases (AD) and diabetes (Stefani and Dobson, 2003). In AD, the amyloid plaques are characterized by extracellular deposits of β-amyloid peptide and intracellular aggregates of tau protein. It is generally accepted that the oligomeric species formed during the amyloid formation are the more cytotoxic as compared to the mature fibrils present in amyloid plaques (Chiti et al, 2006). Recent studies also suggest that mature fibrils could fragment and release oligomers, acting as storage of toxic species (Xue et al, 2009). Increasing evidence reported that OLE was able to interfere with the *in vitro* aggregation of hIAPP (a peptide that aggregates in type II diabetes), Aβ and tau neutralizing the toxicity of the oligomers. These studies showed that if amylin or Aβ were aggregated in presence of OLE, the result was the production of less toxic molecular species (Rigacci et al, 2010; Rigacci et al, 2011; Daccache et al, 2011).

Regarding the peptide Aβ, NMR and Electrospray Ionization Mass Spectrometry (ESI-MS) demonstrated non-covalent interaction between OLE and the peptide. OLE forms a complex with Aβ 1-40 in a 1:1 stoichiometry, with a preference for the apolar region of Aβ (Rigacci et al,2011). A recent study of Leri et al. further reported a protective effect of OLE in a model of systemic amyloidosis, transthyretin-amyloidosis (TTR-

amyloidosis). TTR-amyloidosis is characterized by deposits of insoluble fibrils in the heart, peripheral nerves and other organs. OLE can interfere with TTR-aggregates, similarly to the effect showed on amylin, Aβ and Tau plaques. In the heart TTR-fibrils can bind the glycosaminoglycan GM1 of cardiomyocyte inducing cell damage and consequent cytotoxicity. In the presence of OLE these aggregates were unable to interact with GM1. The reported data suggested that OLE induced a remodelling of the supramolecular structure of the growing aggregates, reducing TTR aggregates-GM1 interaction, with consequent loss of cytotoxicity (Leri et al, 2016).

Although all these results confirmed the ability of OLE to interfere with amyloid aggregation and propose the use of OLE as preventive therapy, the protective effect of OLE in the presence of already formed toxic plaques is also known. Various mechanisms are under investigation to explain this effect. *In vivo* studies on TgCRND8 mice, a model of Aβ deposition, fed with OLE for 8 weeks showed improvement in cognitive performance that correlated with improved synaptic function, reduced size and compactness of Aß plaques, reduced inflammatory response and strong autophagy activation in specific brain areas (Casamenti et al, 2015). These *in vivo* results were further confirmed by studies in a transgenic C.elegans model, displaying Aβ aggregates in muscles that were diminished in worms fed with OLE (Diomede et al, 2013).

Autophagy activation: Autophagy is a cellular pathway involved in degradation of altered proteins and organelles. Several pathological conditions such as neurodegenerative diseases, infectious diseases, and cancer are characterized by dysfunction in autophagy. Restoring defective autophagy in these conditions has been shown to be a promising therapeutic strategy. Grossi and colleagues highlighted the effect of OLE as autophagy activator *in vitro* and *in vivo* while investigating its protective effect in neurodegenerative disorders (Grossi et al, 2013). In TgCRND8 mice, OLE administration (50 mg OLE /kg of diet for 8 weeks) induced autophagic response, promoting autophagosome-lysosome fusion, the last step of the autophagy machinery, which leads to the clearance of misfolded proteins (Grossi et al, 2013). In light of these findings, the aim of this thesis was to discriminate the cellular and molecular mechanisms underlying OLE-induced autophagy (Rigacci et al, 2015).

Autophagy

1. Degradation and recycling of cellular components.

In eukaryotic cells two main systems aimed at degrading cellular waste include the ubiquitin-proteasome system (UPS) and the lysosome based degradation. The UPS is an ATP-dependent system with high affinity for ubiquitinated substrates, which are primarily short-lived proteins (Mizushima et al, 2011). The lysosomal system is a less selective process consisting of single-membrane vesicles that contain hydrolytic enzymes such as lipases, nucleotidases, glycosidases and proteases. These enzymes are particularly effective at the acidic pH of the lysosomal lumen. Endo– and ectoproteases convert proteins into smaller peptides and free aminoacids that are released into the cytosol, or recycled to synthesize the novo proteins (Vilchez et al, 2014). The lysosomal system is involved in the degradation of both extracellular and intracellular material. Extracellular constituents can be delivered to lysosomes by endocytosis or phagocytosis, whereas cytosolic components are delivered to lysosomes by autophagy (Mizushima et al, 2011).

1.1. Ubiquitin-proteasome system

The UPS involves the ubiquitin (a polypeptide of 76 amino acids, which serves as a marker for degradation), and a multienzyme complex, the proteasome. UPS degrades preferentially short-lived nuclear and cytosolic proteins (Ciechanover, 2006), yet it was also reported to have a role in the degradation of misfolded proteins in the endoplasmic reticulum. UPS mediated degradation comprises two essential steps: first, the covalent binding of molecules of ubiquitin to the protein substrate; and second, the degradation of the ubiquitinated protein by the proteasome.

Three different enzymes are involved in the ubiquitin-substrate conjugation: E1 (ubiquitin-activating enzyme), that hydrolyses ATP and forms a thioester-bond with ubiquitin; E2 (ubiquitin-conjugating enzyme) that receives ubiquitin activated by E1 and forms a similar thioester-bond with ubiquitin, and finally with E3 (ubiquitin ligase) that binds both E2 and a substrate thereby promoting substrate degradation through ubiquitin. Ubiquitination occurs several times, forming a chain of polyubiquitin, before

the protein is degraded by the proteasome and the ubiquitin molecules are recycled (Rubinsztein, 2006).

1.2 Autophagy system

Autophagy, which literally means "self-eating", represents a non-selective degradation system, regulating the turnover of several cellular constituents, included damaged organelles, soluble or aggregated proteins amongst others (Rubinsztein, 2006). The interest in autophagy was instigated when genetic studies in yeasts identified a series of autophagy-related (ATG) genes (Klionsky et al., 2003). Several of the autophagy specific genes are highly conserved emphasizing the importance of this process in all organisms (Nakatogawa et al., 2009). Three defined types of autophagy, which include macro-autophagy, micro-autophagy, and chaperone-mediated autophagy, are known. They differ in how the material to be degraded is transported into the lysosomal lumen; in the type of material to be degraded; and, in the cellular regulation (Glick et al, 2010) (Figure 4). In general the term autophagy is classically referring to macroautophagy.

A brief description of the main types of autophagy is given below:

Macroautophagy. The macroautophagy pathway was first described by Christian De Duve in 1963 (De Duve, 1963). Macroautophagy is considered the main type of autophagy and has been more extensively studied than microautophagy and chaperone-mediated autophagy. It is important in physiological conditions, but is also activated by stress and nutrient starvation. This process includes three different steps: i) phagophore formation, ii) closure of phagophore and formation of an "autophagosome", and, iii) autophagosome-lysosome fusion.

In the first step the phagophore or pre-autophagosome, a double membrane structure, sequesters cytoplasm constituents, including mutated proteins and damaged organelles. Despite that this step is well characterized the origin of the autophagic sequestering cisterna remains unknown. Two hypotheses have been formulated regarding the origin of the phagophore. One line of thought is *de novo* synthesis or utilization of pre-existing cytoplasmic membranes (endoplasmic reticulum, mitochondrial membrane, plasma membrane and Golgi cisterns) (Vilchez et al, 2014). In the second step, the phagophore is sealed and forms an autophagosome. The autophagosomes then fuse with lysosomes or alternatively with endosomes, forming an amphisome, before fusing with lysosomes.

Amphisomes can be ultrastructurally recognized as organelles containing both cytoplasmatic components and endocytic markers. They are delimited by a single or double membrane with an acidic interior compartment allowing for autophagocytosed cytoplasm to become denaturated. Amphisomes are distinguished from autophagosomes by endocitic markers, but the distinction between amphisomes and lysosomes is more problematic since the endosomal fusion can bring some lysosomal enzymes inside the amphisome. Once autophagosomes or amphisomes fuse with lysosomes, the sequestered material is degraded or recycled (Vilchez et al. 2014; Mizushima et al, 2011).

Microautophagy. Microautophagy consists of the incorporation and degradation of entire cytosolic regions by lysosome itself, without the involvement of autophagosomes (Glick et al, 2010). This type of autophagy is important for the degradation of proteins as well as entire organelles. In yeasts, a specific form of microautophagy, the "micropexophagy", was described. It is involved in the selective degradation of peroxisomes (Klionsky et al, 2007). Microautophagy is active in basal conditions, but unresponsive to stimuli such as nutrient deprivation, glucagon or amino acids, which are shown to activate other types of autophagy (Cuervo, 2004).

Chaperone mediated autophagy (CMA). In chaperone-mediated autophagy (CMA), chaperone proteins (such as Hsc-70, heat-shock protein 70) and co-chaperones recognize the proteins containing a particular pentapeptide consensus motif (KERFQ) to form a complex. Targeted proteins associated with chaperones are then translocated across the lysosomal membrane where they are recognized by lysosome-associated membrane protein 2 (LAMP-2A), resulting in their unfolding and degradation (Glick et al, 2010). This type of autophagy is very selective and is especially activated in stressful conditions, such as prolonged nutrient deprivation or exposure to toxic compounds (Cuervo, 1998; Dice, 2000). Both macroautophagy and CMA can act during nutrient starvation. Macroautophagy represents the first response aimed to degrade indiscriminately cytosolic components to obtain energy. However if the starvation is prolonged then to prevent the cell from 'eating itself', the activity of macroautophagy begins to decline and the CMA becomes the new supplier of amino acids for the synthesis of new proteins, allowing selective degradation of substrates. Interestingly, recent studies reported CMA has tissue and cell-specific effects. In fact, starvation activated preferentially CMA in the liver, the heart, and the kidney (Cuervo, 2004;

Wing et al, 1991), and among the cells there were differences in the induction of CMA. Although the CMA induced by starvation is less present in the brain, cultured astrocytes showed high levels of CMA after serum deprivation (Martin et al, 2002)

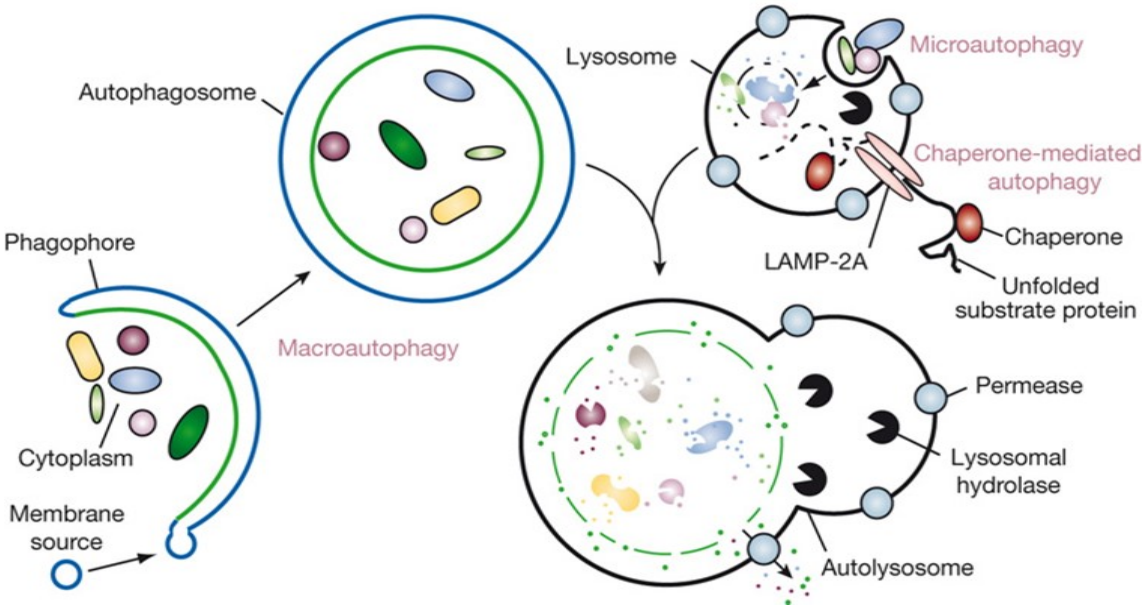

Figure 4 Types of autophagy. **Microautophagy:** sequestering of cytosolic constituents by lysosomes through invaginations in their membrane. **Macroautophagy.** Macroautophagy requires three steps: i) cytosolic substrates are sequestered within a unique double-membrane vesicle called "pre-autophagosome" or "phagophore" ii) closure of phagophore forms an"autophagosome". iii) fusion of autophagosome-endosome (not shown) or autophagosome-lysosome and formation of the "autolysosome". The material is degradated into the autolysosome and the recycled macromolecules are released back into the cytosol. **Chaperone mediated autophagy**: proteins marked with the KFERQ motif are recognized and complexed by chaperone proteins (such as Hsp70) The complex substrate-chaperone moves toward lysosomes and the proteins are translocated by Lamp2 into the lumen for degradation *(Mizushima et al, 2008).*

2. Molecular mechanisms of macroautophagy

The discovery of the genes that regulate autophagy in the yeast Saccharomyces cerevisiae has permitted a step forward for the understanding of the molecular mechanisms of autophagy. The process of macroautophagy is under control of four main protein systems: 1) ULK complex 2) Class III phosphatidylinositol 3-kinase 3) Ubiquitin-like conjugation systems (Atg5–Atg12/ Atg8- PE) 4) SNAREs and other fusion proteins (Figure 4)

2.1 ULK complex.

The ULK complex is required for the early stages of autophagy. It includes ULK1, Atg13 and Atg 17. ULK1, known as **Atg1** in yeasts, interacts with autophagy-proteins such as Atg13, Atg17, Atg29 and Atg31 in a nutrient status- and TOR kinase activity dependent manner. In particular, the interaction with **Atg13-Atg17** is essential for Atg1 activity and autophagy induction. When there is availability of nutrients, the kinase mTOR phosphorylates and deactivates Atg13, resulting in the repression of autophagy. Under starvation periods mTOR is inactive, Atg13 phosphorylation is suppressed and Atg13 is activated. Activated Atg13 induces the recruitment of Atg1, Atg17 and other Atg proteins to the pre-autophagosome structure (PAS) (Jung et al, 2010).

2.2. Class III Phosphoinositide 3-kinase

The role of class III phosphoinositide 3-kinase (PI3K-III), also called "Vps34" (vesicular protein sorting 34), and its binding with Beclin-1 (Atg6) in phagophore formation and autophagy is not entirely known in mammalian systems. PI3K-III is unique amongst PI3-kinases in only using phosphatidylinositol (PI) as substrate to generate phosphatidyl inositol triphosphate (PI3P), which is essential for phagophore elongation and recruitment of other Atg proteins. The PI3K-**Beclin1** interaction promotes the catalytic activity of PI3K (Glick et al, 2010). Beclin1, and PI3K, can interact with other proteins. Beclin1 protein contains three prominent domains: "BH3 domain", "CCD domain" and "ECD" domain**.** The BH3 domain promotes the interaction with Bcl-2, an antiapoptotic protein, and several studies reported that this interaction had an autophagy-inhibitory effect. Through the CCD domain Beclin1 can interact with UVRAG, Atg14L (also named Barkor) and RUBICON. PI3K binds Beclin1 at its ECD (McKnight et Zhenyu , 2013). A study of Russell et al. reported that

ULK1 activated Beclin1 through phosphorylation. ULK1 is recruited to the PI3K-Beclin-1 complex via binding to ATG14L. After recruitment, it can phosphorylate Beclin-1, activate PI3K and promote PI3P production at the nascent autophagosome (Russell et al, 2013).

2.3. Ubiquitin-like conjugation systems.

Two convergent-systems are required for the second stage of autophagy: maturation of phagophore and autophagosome formation. These systems recall some features of the ubiquitination of proteins in the ubiquitin-proteasome system.

The first conjugation system (Atg5-Atg12). It involves the Atg proteins 5, 7, 10, 12. Atg7 and Atg10 are similar to the E1 and E2 enzymes in the ubiquitin pathway. The final complex formed consists of Atg5–Atg12 non-covalently associated with Atg16 (Meijer et al, 2004)

The second conjugation system (Atg8-PE). It is characterized by the conjugation of the protein LC3 (Atg8) with the phospholipid phosphatidylethanolamine (PE). The interaction LC3-PE is dependent on Atg7 and Atg3 activity. **LC3** (19 kDa) is a full-length cytosolic protein that is proteolytically cleaved by Atg4 to generate **LC3B-I** upon the induction of autophagy**.** The carboxyterminal glycine of LC3B-I exposed by Atg-4 mediated cleavage is then activated by Atg7, an E1-like protein and LC3B-I activated is subsequently transferred to Atg3, an E2-like protein. At this point phosphatidylethanolamine (PE) can bind activated LC3B-I generating the processed **LC3B-II** (17Kda). Recruitment of LC3B-II into the phagophore is dependent on Atg5–Atg12, and LC3BII is found on both the internal and external surfaces of the autophagosome. It is considered one of the main markers of autophagy induction. LC3B-II, acts as a 'receptor' at the phagophore and autophagosome membranes where it interacts with scaffolding proteins such as p62 in addition to several other molecules (eg protein aggregates, damaged mitochondria) to promote their selective uptake and degradation. **p62/SQSTM1** is a protein involved in both UPS and autophagy degradation systems. It promotes the turnover of poly-ubiquitinated protein aggregates, to which it is linked through the UBA domain. The binding site for LC3 is called LIR domain and is formed by 8 aminoacids. The LC3-p62 link encourages the recruitment to autophagic vacuoles of damaged organelles or mutates proteins. P62 is an essential protein, and its mutation is linked to Paget's disease, a pathology characterized by the

abnormal turnover of bone (Glick et al, 2010). NBR1 is a protein similar to p62, and could compensate a possible loss of p62 and as the latter has UBA and LIR domains and is degraded by autophagolysosomes. However, NBR1 contains a FW domain, a four tryptophan residues domain, not present in p62. The FW domain plays an important role in activation, control and differentiation of T cells and in inflammatory processes. The loss of this domain induces alterations in p62 expression and hyperactivation of the p38 MAPK (Johansen et al, 2011).

2.4. SNAREs and membrane-fusion .

The last step of autophagy requires the fusion between the autophagosome and lysosome.

Microtubules and actin filaments have been implicated in the transport of autophagosomes towards the lysosomes, and the movements along the microtubules are driven by kinesin and dynein . While microtubules are formed by dimers of α and β tubulins, the actin filaments are comprised of polymerized actin monomers. Rab GTPase is an important player ìn the trafficking of autophagosomes, connecting autophagosomes or endosomes to microtubule proteins through the interaction with the protein RILP (Rab-interacting lysosomal protein). Rab-GTPase is also involved in the recruitment of the protein FYCO1 (FYVE and coiled-coil domain containing 1) on the autophagosome membrane. FYCO1 in turn connects kinesins to autophagosomes. FYCO1 also contains an LC3-interacting motif promoting LC3-BII recruitment to autophagosomes (Ganley et al, 2013). With regards to the autophagosome-lysosome fusion, a recent study demonstred that HDAC6 (histone deacetylase 6) recruiting cortactin, a protein responsible of actin polymerization, could be involved in the mechanism of fusion and degradation of protein aggregates (Lee et al, 2010).

In addition during this step, Rab GTPases, membrane-tethering complexes and SNAREs proteins are recruited. Rab proteins, localized on both membranes, bind tethering complexes (HOPS complex, TECPR1 complex) that act as bridges approaching the compartments involved in the fusion. The tethering complex in turn activates SNAREs proteins (Vam3, Vam7, Vti1 e Ykt6), that drive the fusion of lipid bilayers between autophagosome and lysosome. SNAREs localized on the opposing membrane interact with each other forming complexes essential for their activity.

Lysosomal-associated membrane protein-2 (LAMP-2) is a component of the lysosomal membrane, also involved in the autophagosome-lysosome fusion. Given its pivotal role, mutations of LAMP-2 are associated with accumulation of autophagosomes and Danon disease, a disorder characterized by skeletal myopathy and cardiomyopathy, as well as mental retardation (Ganley et al, 2013).

Finally the cargo is degraded by the lysosomal enzymes cathepsins D, B and L. The cathepsins are a class of proteases able to degrade modified proteins. Many cathepsins involved in autophagy machinery are characterized by the presence of cysteine residues in their structures. **Catepsin D** is often studied as a late marker of autophagy (Eskelinen et al, 2005).

As described above, the autophagy pathway requires different steps and several drugs have been reported as activators or inhibitors acting on specific steps of the autophagy machinery (Figure 5). Among these, drugs such as BCL-2 homology 3 (BH3) mimetics disrupt the inhibitory interaction between Beclin 1 and Bcl-2/Bcl-X(L), thus activating autophagy. In contrast, inhibition of Beclin 1 protein by Spautin-1 or PI3K by 3-methyladenine (3-MA) can inhibit autophagy. Some drugs can inhibit the later stages of autophagy. These include chloroquine (CQ), which inhibits the acidification inside the lysosome; bafilomycin A1, which inhibits the vacuolar ATPase (V-ATPase) located in the lysosomal membrane; and, pepstatin A or cystatin B that inhibit the lysosomal proteases.

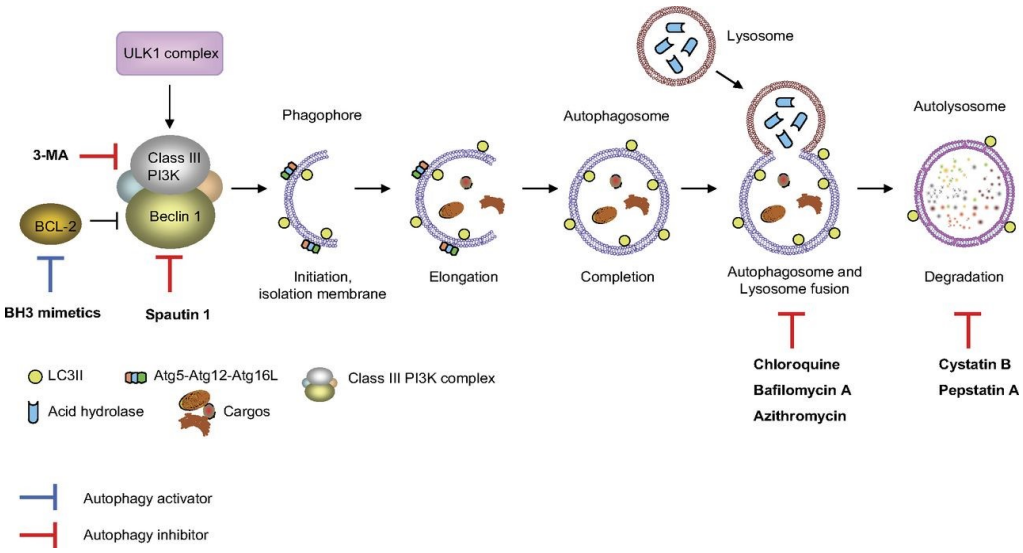

Figure 5: Molecular mechanisms of macroautophagy. Macroautophagy starts with the formation of a double-membrane structure "phagophore" of undefined origin. The protein complexes involved in this

step are represented by ULK1 and Class III phosphoinositide 3-kinase (PI3K-III). Atg5-Atg12-Atg16L complex and LC3-II phosphatidylethanolamine (PE) conjugate promote the phagophore elongation and substrate sequestering. The closure of phagophore forms autophagosome. In this step LC3B-II is found on both the internal and external surfaces of the autophagosomes. SNAREs proteins mediate the fusion autophasgosomes-lysosomes and the cargos are degraded by lysosomal hydrolases. Some drugs can interfere at different levels with autophagic machinery, activating or inhibiting macroautophagy *(Nakahira and Choi, 2013)*

3. Signalling pathways of autophagy

Autophagy is a well-regulated process. A variety of extracellular (starvation, hormones or pharmacological treatments) and intracellular stimuli (accumulation of misfolded proteins, pathogens invasion) can modulate the machinery controlling autophagy (Yang et al, 2005). In most cases these stimuli, although different from each other, converge to a common target upstream of the molecular machinery, the kinase **mTOR**, which is involved in the formation of the autophagosomes (Meijer et al, 2004).

mTOR is a target of rapamycin, a macrolide-antibiotic with antifungal and immunosuppressive properties. Rapamycin, inhibiting mTOR, has an autophagy activating effect. In mammalian cells mTOR interacts with regulatory proteins to form two different complexes: mTORC1 and mTORC2 (Alayev et al, 2014). mTORC1 is particularly sensitive to the antibiotic rapamycin, whereas mTORC2 is sensitive to rapamycin and rapalogs (analogues of rapamycin) only after prolonged exposure. The best characterized substrates of mTORC1 kinase activity are eukaryotic initiation factor 4E-binding proteins 1, 2 and 3 (4E-BP1,2 and 3) and the p70 S6 kinases (S6K1 and S6K2). mTORC1 phosphorylates p70-S6K on at least two residues, promoting its efficiency in stimulating protein synthesis through the activation of the ribosomal S6 protein and other components of transcription apparatus. Phosphorylation of 4E-BP1 on four Ser/Thr residues induces the dissociation of 4E-BP1 from eIF4E (Eukaryotic translation initiation factor 4E). This event causes the repression of 4E-BP1 and the activation of eIF4E, ultimately promoting eIF4E mediated growth and cell proliferation.

mTORC2 can target the serine / threonine Akt, phosphorylating it on serine S473. The phosphorylation of this residue leads to full activation of Akt and Akt-mediated inhibition of autophagy (Dunlop et al, 2009).

Although the mTOR-dependent autophagy is predominant, mTOR-independent autophagy is also present. Lithium, carbamazepine and valproate are so-called "mood-stabilizing medications" used in the treatment of mental disorders and can induce autophagy independently from mTOR signalling (Rubinsztein et al, 2011).

3.1 mTOR-dependent autophagy

The serine/threonine kinase mTOR is an important regulator of autophagy, acting as a sensor for *amino acids* and *ATP levels* and integrating *hormonal stimuli* via the class I PI-3K/PKB pathway (Dennis et al., 2001; Marygold et al, 2002; Rohde et al, 2001) (Figure 6).

Aminoacids. Mortimore and Schworer (1977) showed for the first time that amino acids, the final products of autophagic degradation, had an inhibitory effect on autophagy in rat liver cells. The inhibitory effects were observed on the formation of autophagosome, a step regulated by mTOR (Mortimore et al, 1994; Seglen and Bohley, 1992). Other studies showed the interference of amino acids in different steps of autophagy. For example, studies on isolated rat hepatocytes showed that high concentrations of leucine together with glutamine or histidine were particularly effective in inhibiting autophagic sequestration of cytosolic substrates. Asparagine, if used at high concentrations, interfered with the fusion between autophagosomes and lysosomes. An effect on the lysosome activity was also reported for leucine. The latter increased the lysosomal pH at high concentrations; presumably through direct inhibition of the lysosomal proton pump (Codogno et al, 2003). The link between mTOR and amino acids in the control of autophagy was showed in rat hepatocytes, with the discovery that the inhibitory effect of amino acids on autophagy could be reverted by rapamycin (Blommaart et al, 1995).

Although it is generally accepted that amino acids activate mTOR, how the amino acids signal their presence to the cell is not clear. Initially it was thought that amino acids bound a plasma membrane localized receptor and this signal was transmitted inside the cell. According to studies, amino acid starvation is sensed by the degree of amino acid charging of tRNA. In fact, it was observed in yeasts, that free tRNA, which did not bind amino acids, prevented the phosphorylation of p70S6K protein mediated by mTOR. Despite these controversial findings for amino acid signalling, the fact that the inhibition of the transport of amino acids markedly inhibited the phosphorylation of p70S6K protein system demonstrated that the amino acids exerted their effects upon

entering the cell (Van Sluijters et al, 2000). Along the same lines, insulin was shown to have an inhibitory effect on autophagy signalling, independent from amino acid signalling. In fact the administration of amino acids, leucine in particular, resulted in phosphorylation of ribosomal protein S6K and 4E-BP1 in the absence of insulin or other growth factors. Both are targets of mTOR. Furthermore amino acids and insulin can also act synergistically on mTOR signalling to inhibit autophagy. According to this, leucine can activate glutamate dehydrogenase, that promotes insulin production in β-cells and the insulin signalling (Yang et al, 2005).

ATP/ADP levels. Autophagy is also regulated by ATP levels. A decrease in cellular ATP levels is sensed by the AMP-dependent protein kinase (AMPK). The activation of AMPK occurs through phosphorylation by LKB1 in response to an increase in the AMP/ATP ratio and by calmodulin-dependent protein kinase kinase-beta (CaMKKβ, activated by cytosolic calcium) (Cai et al, 2012). Activated AMPK phosphorylates TSC1/2 complex, which inhibits mTOR activity through Rheb (Inoki et al, 2003). When mTORC1 is inactive, dephosphorylation of ULK1/2 activates its kinase activity, phosphorylating Atg13, FIP200 and ULK1/2 itself and promoting autophagy (Jung et al., 2010). Other studies have reported a direct effect of pAMPK on ULK1 phosphorylation thereby affecting the initial step of autophagy (Cai et al, 2012).

Hormonal stimuli. The hormones insulin and glucagone exert an opposite effect on autophagy. As previously reported insulin appears to have an inhibitory effect on the autophagic pathway, whereas glucagone has stimulatory effects.

In response to excess of nutrients, insulin is produced. Insulin binds a specific receptor on plasma membrane, IR receptor. The binding hormone-receptor induces autophosphorylation that in turn phosphorylates the insulin receptor substrate IRS that activates PI3K (Fulop et al, 2003). Activated PI3K phosphorylates the lipid phosphatidylinositol present on the internal surface of the cell membrane, producing PtdIns (3,4,5)P3 which in turn activate protein kinaseB (AKT/PKB) and other enzymes. This is followed by the activation of mTOR and autophagy inhibition. The phosphatase PTEN and the hormone glucagone are among the known signals that induce autophagy . PtdIns(3,4,5)P3 can also be hydrolysed by phosphatase PTEN resulting in a stimulatory effect on autophagy. Glucagone, released in nutrient starvation, activates instead autophagy through PI3K inhibition. (Meijer et al, 2004; Yang et al, 2005).

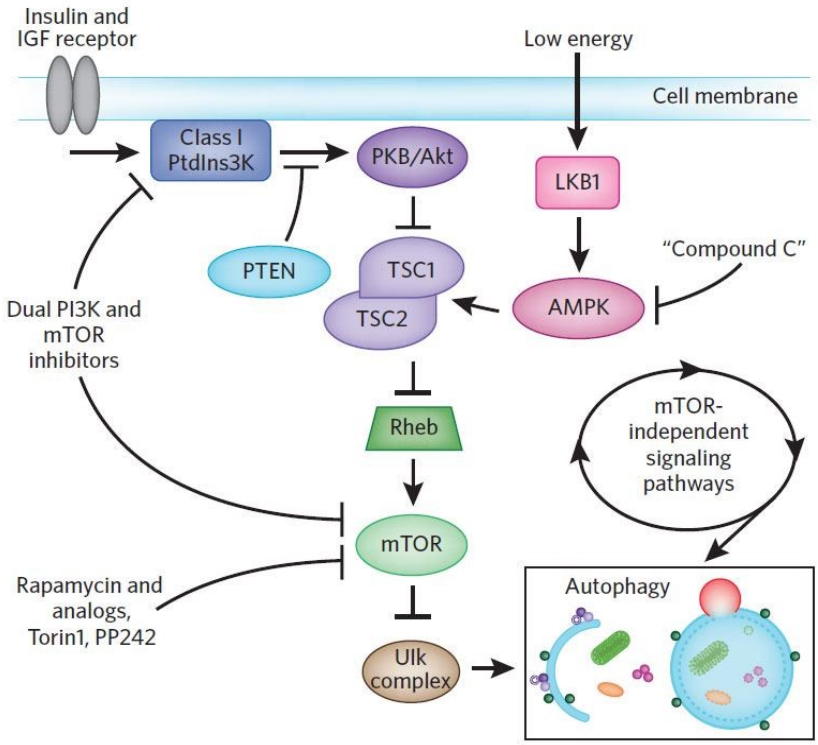

Figure 6. Schematic representation of the mTOR signalling pathway. In mTOR dependent signalling, autophagy is inhibited by the serine/threonine kinase mTOR. Different signals such as amino acids, insulin and ATP converge to the kinase mTOR. mTOR is inhibited by rapamycin (and its analogs), Torin1 and PP242, which induce autophagy. AMPK activation also causes inhibition of mTOR signalling, resulting in autophagy stimulation. Dual PI3K and mTOR inhibitors induce robust autophagy activation compared to single mTOR inhibitor (Fleming et al, 2011).

3.1.1.CamKKβ-AMPK/mTOR signalling in autophagy modulation by caloric restriction and polyphenols.

Several studies reported that the reduction in food and/or calorie intake without malnutrition may be associated with the prolongation of life- and health span. Despite the beneficial effects of caloric restriction (CR), CR regime for long time is hard to sustain. Recently it was demonstrated that polyphenols, compounds with recognized properties against a wide number of human pathologies (from neurodegenerative to cardiovascular diseases, cancer and diabetes, age-related diseases), showed other new mechanisms of action. Among these, their ability to raise the autophagic response was involved in the protection against neurodegeneration, liver and vascular diseases (Kim

et al, 2013; Zou et al, 2014). Polyphenols, in particular EVOO secoiridoids, are referred to as mimetics of CR. The discovery that natural compounds, easily assimilated from fruit and vegetables, could have the same effects of CR has immediately aroused great interest. Plant polyphenols induce CR-like effects in muscle, brain, fat tissue and the kidneys through different signalling pathways. Most of the effects seem to be mediated by CamKKβ-AMPK/mTOR signalling (Rigacci et al, 2015; Rigacci et al, 2016) (Figure 7). In this signalling pathway calcium seems to be the main regulator of autophagy. Calcium ions are the major intracellular second messengers propagating signals throughout cellular compartments. Calcium is involved in contraction, cellular death, regulation of gene transcription etc. Inside the cells, Ca^{2+} is distributed in different compartments: cytosol, mithocondria, endoplasmic reticulum (ER) and nucleus (Ghislat et al, 2012). The ER represents the main site of deposit of intracellular Ca^{2+} (Yang et al, 2005). Ca^{2+} is released from intracellular stores or fluxed inside the cells from extracellular space through calcium channels localized on plasma membranes, and stimulates CamKKβ. In turn this activates AMPK phosphorylating its threonine residue (Thr172) on catalytic α-subunit. Finally, AMPK inhibits mTOR, inducing autophagy (Carling et al, 2008; Kania et al, 2015). Among the polyphenols, evidence for autophagy modulation by CamKKβ-AMPK/mTOR was obtained for EGCG and resveratrol. In the case of EGCG, the increase in Ca^{2+} cytosolic was related to a release from ER because treatment with CPA, a blocker of ER Ca^{2+}-ATPase, reduced cytosolic Ca^{2+} load observed after EGCG treatment. Furthermore, although CaMKKβ is essentialy involved in AMPK activation in calcium-dependent manner, it was shown that CaMKKβ can autonomously activate other substrates, including CaMKI and CaMKIV, without Ca^{2+}/CaM binding. Resveratrol, the characteristic polyphenol in red wine, activates CaMKKβ/AMPK through the inhibition of cAMP-degrading phosphodiesterase which leads to the activation of phospholipase C and ryanodine receptor to increase intracellular calcium levels.(Kim et al, 2013). The involvement of CamKKβ in resveratrol-mediated autophagy was demonstred using STO-609 (Camkkβ inhibitor). STO609 decreased the phosphorylation of AMPK and its substrate Acetyl-CoA carboxylase (ACC) after treatment with resveratrol, indicating that resveratrol stimulated the phosphorylation of AMPK and ACC in a CamKKβ-dependent manner (Park et al, 2012). The signalling of CamKKβ-AMPK/mTOR reported for EGCG and resveratrol could correspond to a common molecular mechanism shared by

polyphenols in the induction of autophagy. This could explain, at least in part, the healthy effects of these substances against ageing, neurodegeneration, cancer, diabetes and other diseases with autophagy dysfunction.

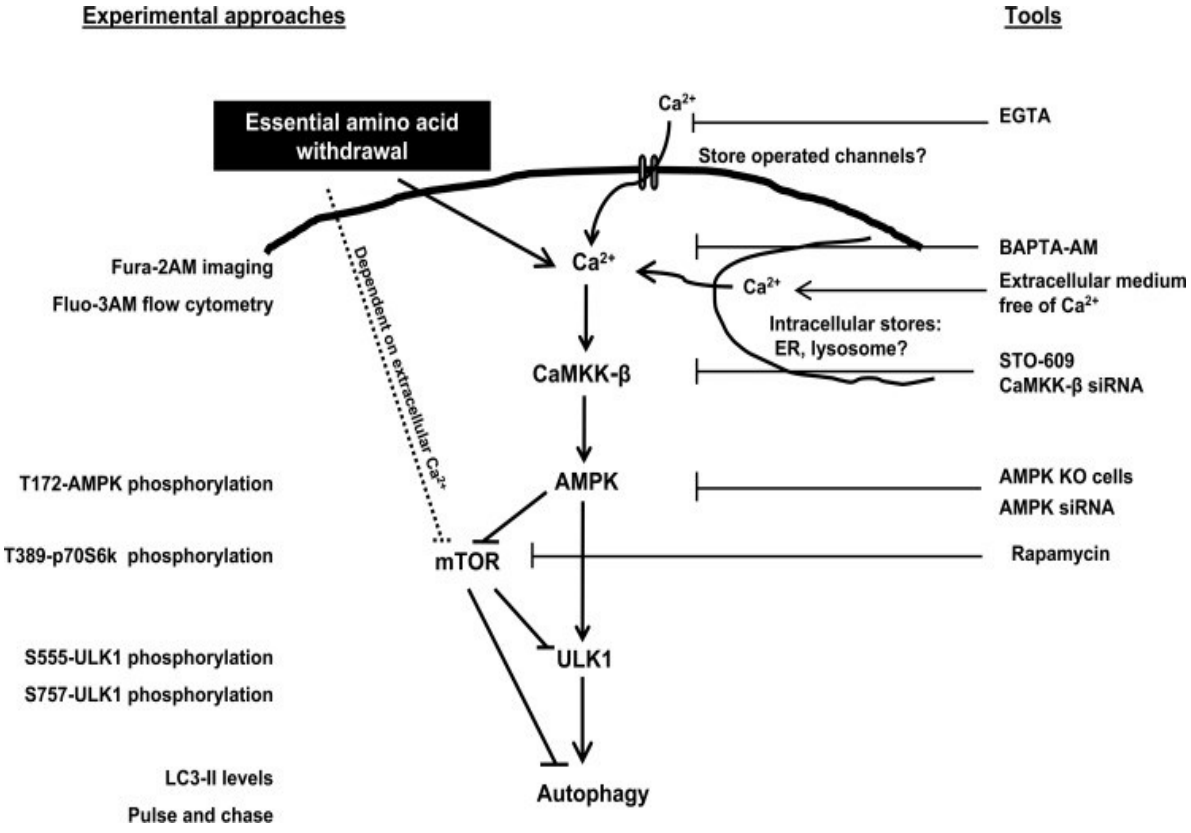

Figure 7. Ca^{2+}-dependent signalling pathway from amino acid starvation and polyphenols. Starvation induces autophagy through CamKKβ-AMPK/mTOR . The same pathway was proposed for polyphenols, mimetics of CR. Methods/experimental approaches and assays/tools used for monitoring CamKKβ-AMPK/mTOR are shown next to the signalling cascade in the figure (Ghislat et al, 2012).

3.1.2 Autophagy modulation by Sirtuins.

One of the main effectors of CR are Sirtuins, a family of NAD^+-dependent protein deacetylases involved in the regulation of several physiopathological processes, such as inflammation, cellular senescence/ apoptosis/proliferation, metabolism, and cell cycle (Chung et al, 2010). In contrast to other deacetylases proteins, the sirtuins are the only effectors that require NAD^+ for their activity. NAD^+ is an important co-factor for the electron transport chain, but it is also involved in various enzymatic reactions. There are two different ways to obtain NAD^+ inside the cells: *de novo* production or via a salvage pathway. In this latter case, nicotinamide (NAM) is first converted to nicotinamide

mononucleotide (NMN) by the enzyme nicotinamide phosphoribosyltransferase (NAMPT), and then to NAD^+ by nicotinamide mononucleotide adenylyltransferase (NMNAT). For this reason the activation of the enzymes such as NAMPT or NMNAT involved in NAD^+ salvage pathway plays an important role in regulating sirtuin activity (Chung et al, 2010). Mammalian cells contain seven sirtuins (SIRT1-7) that are structurally similar, all of them showing a binding-domain for NAD and a catalytic domain, but they differ in their N-and C-terminal domains. Other differences reside in the cellular localization, the specificity for substrates, and enzymatic activity. With regards to their cellular localization, SIRT1 can shuttle between the nucleus and the cytosol, SIRT2 is mainly cytosolic, SIRT3-SIRT4-SIRT5 localize to the mitochondria, and, SIRT6/7 are located in the nucleus (Chung et al, 2014). The deacetylation mediated by Sirtuins occurs in two steps: i) cleavage of NAD^+ by sirtuins and nicotinamide (NAM) production, and ii) transfer of the acetyl group from the substrate to the ADP-ribose moiety of NAD to generate O-acetyl-ADP ribose and the deacetylated substrate (Nakagawa, T., 2011). Sirtuin-activity can be inhibited by the reaction product, NAM (Figure 8). Polyphenols, inducing CR-like effects, are the most known activators of Sirtuins (Jayasena et al, 2013).

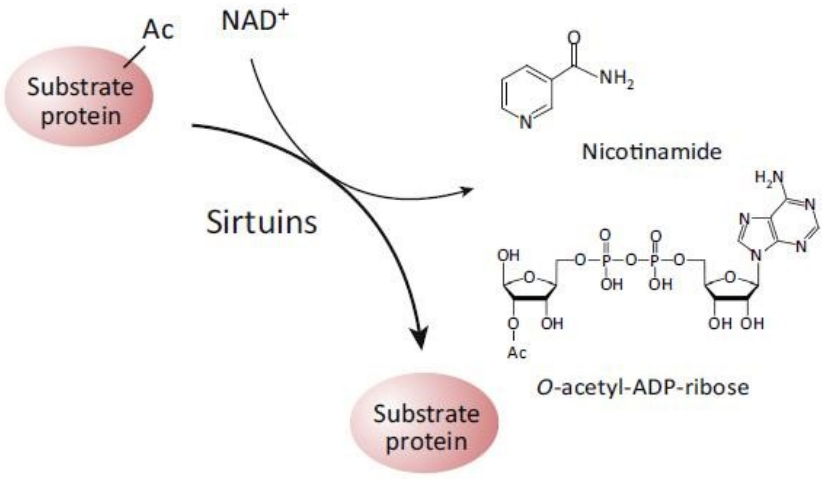

Figure 8 . Mechanism of action of Sirtuins.(*Fasting, circadian biology, and epigenetics*, 2014)

SIRT1 is the most studied among the sirtuins. The main targets of SIRT1 are nuclear factor (NF)-κB, Forkhead box class O (FOXO) 3, p53, peroxisome proliferator-

activated receptor (PPAR)-γ, PPAR-γ coactivator 1α (PGC-1α), and endothelial nitric oxide synthase (eNOS). SIRT1 also plays an important role in autophagy activating LKB1/AMPK signalling. Some authors reported the ability of polyphenols (in particular resveratrol) to activate AMPK in *in vitro* and *in vivo* models through a SIRT1 and LKB1 dependent signalling (Hou et al, 2008). Following this model , SIRT1 coud directly activate AMPK. Other studies suggested different mechanisms involved in SIRT-AMPK interconnection. Among these, two independent groups reported that AMPK could function as a SIRT1 activator (Figure 9). First, Fulco et colleagues observed that glucose restriction induced a decrease of ATP, AMPK activation and inhibited the differentiation of C2C12 skeletal muscle myoblasts. They obtained the same effects using the AMPK activator, 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside AICAR. In both situations, an increase in NAD^+ levels was observed. Conversely, myoblasts derived from SIRT1+/− mice and cells transduced with shRNAi for SIRT1 were resistant to AICAR effect on muscle differentiation, suggesting that it was a SIRT1 mediated effect (Fulco et al, 2008). The second group headed by Canto and Auwerx demonstrated that AMPK activation increased PGC-1α-mediated gene expression in a SIRT1-dependent manner. They reported that different AMPK activators such as AICAR and metformin increased NAD^+ levels and the NAD+/NADH ratio and that this resulted in SIRT1 activation as evidenced by the deacetylation and activation of PGC-1α, substrate of Sirtuin1(Canto et al, 2009).

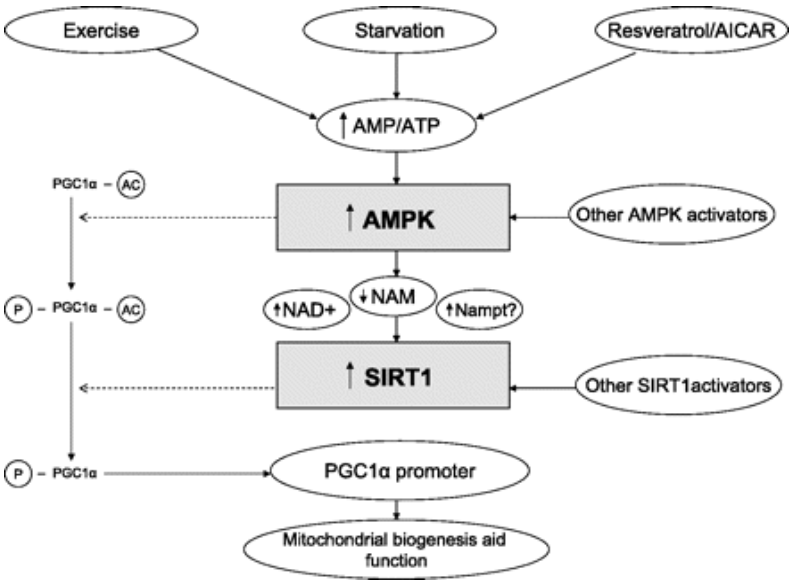

Figure 9: Proposed mechanisms for SIRT1 activation AMPK dependent. AMPK activates SIRT1 that in turn promotes deacetylation of PGC-1α. PGC-1 deacetylated stimulates mitochondrial biogenesis

and function. This schema considers activation of AMPK and phosphorylation of PGC-1α as early events. SIRT1 activation and PGC-1α deacetylation are depicted as later events. The phosphorylation of PGC-1α by AMPK makes it more susceptible to deacetylation by SIRT1 (Ruderman et al, 2010).

Despite the close relationship between AMPK and SIRT1 in autophagy modulation, SIRT1 is also able to induce autophagy in a AMPK-independent fashion. Kume et al. (Kume et al, 2010) demonstrated that CR increased SIRT1 expression in the kidneys of old mice. They observed that SIRT1 could attenuate hypoxia-associated mitochondrial and renal damage in old mice by stimulating the FoxO3-induced expression of Bnip3, a potent inducer of autophagy. Hariharan et al. (Hariharan et al, 2010) revealed that SIRT1 deacetylated FoxO1 which subsequently triggered autophagy by increasing the expression Rab7, a small GTP-binding protein that mediated autophagosome-lysosome fusion. In addition to the FoxO-mediated signalling, SIRT1 can induce autophagy through other mechanisms. Among these the direct deacetylation of autophagy (ATG) proteins, e.g. Atg5, Atg7 and Atg8 was reported. SIRT1 forms a complex with Atg proteins, deacetylating them in an NAD dependent fashion and promoting therefore autophagosome formation (Lee et al, 2008; Salminen and Kaarniranta, 2012)

3.1.3 Crosstalk PARP1-SIRT1

SIRT1, using NAD+ as substrate, is involved in the control of genomic stability, DNA repair and transcriptional regulation (Kolthur-Seetharam et al, 2006). In addition to SIRT1, poly(ADP-ribose) polymerase-1 (PARP1) requires NAD+ for its activity. The main biochemical activity catalysed by PARP1 is the Poly(ADP-ribosyl)ation (PARylation) of proteins, which consists of the transfer of negatively charged poly(ADP-ribose) (PAR) polymers on specific proteins. The main targets of PARP1 include PARP itself, core histones proteins, the linker histone H1, and many other transcription-related factors (Kim et al, 2005; Canto et al, 2013). Under basal conditions, the essential function of PARP-1 is to detect and repair DNA damage activating pro-survival mechanisms. However, cells with severely damaged DNA have excessive PARP-1 activity resulting in high NAD+ consumption (depleting ATP pools). If unchecked, this activity inevitably leads to prolonged ATP depletion and cell necrosis (Chaitanya et al, 2010). It has been reported that exposure to N-methyl-N0-nitro-N-nitrosoguanidine (MNNG), an alkylating agent commonly used as chemotherapeutic

drug, activates PARP-1 (Zhou et al, 2013). PARP1 activation following MNNG induced DNA damage is rapid and intense and leads at first to pro-survival autophagy through AMPK-mTOR signalling pathway, but upon prolonged exposure to MNNG NAD + depletion occurs, leading to overcome the beneficial effect of AMPK activation and the cells die by necrosis or apoptosis . PARP-1 over-activation may trigger apoptotic or necrotic responses, depending largely on cell type, functional signaling pathways and stimulus. In HELA cells PARP-1 activation MNNG induced resulted in an AIF-dependent cell death preceded by complete loss of phosphorylated ERK1/2. In HEK293 cells treated with the same alkylating agent, necrotic response has been observed (Éthier et al, 2012).

PARP and SIRT proteins are thought to operate as essential components of the balance that determines cell survival and death in response to stress. Sirtuins promote cell survival through autophagic response whereas poly(ADP-ribose) polymerases (PARPs) can act both as survival and death inducing factor (Kolthur-Seetharam et al, 2006). The use of the same substrate suggest a functional cross-talk between the two protein families. Indeed, PARP1 has been shown to enhance the transcription activity of NF-KB, whereas SIRT1 is an inhibitor of NF-KB. SIRT1 can also inhibit p53 activation or block the release of apoptosis- inducing factor (AIF), events that occur in the hyper-activation of PARP1. How the two proteins influence each other is under investigation. It was proposed that hyper-activation of PARP1 reduced intracellular levels of NAD+ and increased levels of nicotinamide (NAM). These PARP1-induced events could lead to a downregulation of SIRT1. In contrast, the activation of SIRT1 by genotoxic stress or resveratrol treatment reduced PARP activity (Chung et al, 2014). SIRT1 has been proposed as a direct inhibitor of PARP1, through its deacetylation activity. PARP1 acetylation enhances its activity. Therefore, deacetylation of PARP1 induced by SIRT1 could lead to its enzymatic deactivation (Figure 10). SIRT1 can also inhibit the PARP1 gene promoter, leading to repressed synthesis of the PARP1 protein (Rajamohan et al., 2009).

In a most general context, the existence of complex interplay between pro-survival mechanisms (autophagy), cell death (necrosis or apoptosis) SIRT1 and PARP1 activity can be exploited for therapeutic purposes. In the central nervous system (CNS), PARP overactivation is involved in several neurological diseases including cerebral ischemia, AD, multiple sclerosis, PD, traumatic brain injury, and others (Chaitanya et al, 2010). In

cardiovascular field, ROS-PARP pathway is reported to be involved in cardiac and endothelial dysfunction associated with various forms of cardiovascular injury and heart failure (HF) (Pacher and Szabó, 2007). In all these conditions PARP1 appears as an attractive therapeutic target for chemical inhibiton. PARP1 inhibition has been shown to be effective, on the one hand, in the treatment of cancer in combination with alkylating agents by suppressing the mechanisms of DNA repair and thus triggering apoptosis in cancer cells (Beneke et al, 2004). Recently, in addition to classical PARP1-inhibitors, small molecules modulating SIRT1 activitity have aroused great interest as potential therapeutic agents for treatment of PARP1-dependent diseases.(Chung et al, 2014). In this scenario, polyphenols as Sirtuin-modulators are effective through a dual mechanism: 1) activation of pro-survival mechanism by induction of autophagy 2) interference with the PARP1-mediated cascade of events leading both to apoptotic or necrotic cell death or inflammatory response through NF-kb signalling. These findings highlight new mechanisms of protection by polyphenols with possible implications in pathologies characterized by high oxidative stress and DNA damage (Luccarini et al, 2016)

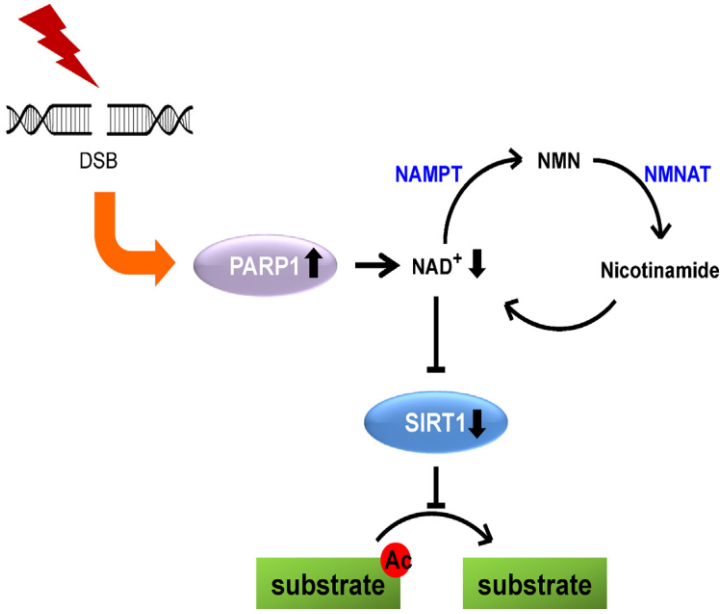

Figure 10 Antagonistic crosstalk between SIRT1 and PARP1 in response to DNA damage. DNA strand break activates PARP1, enzyme that use NAD^+ as substrate. If the DNA-damage is intense, PARP1 is hyper-activated, leading to the depletion of NAD^+ and deactivation of SIRT1. On the contrary, SIRT1 inhibits PARP1 activity by deacetylation (Chung et al, 2014).

3.2 mTOR independent autophagy

Apart from the regulation of autophagy by mTOR, various mTOR independent pathways have been proposed in autophagy modulation. These include: 1) the inositol signalling pathway, 2) the $Ca^{2+/}$calpain pathway, and 3) the cAMP/Epac/Ins (1,4,5) P3 pathway (Figure 11)

1) *Inositol signalling pathway*: Inositol (Ins) and inositol 1,4,5-trisphosphate (IP3) are negative regulators of autophagy via an mTOR-independent pathway. In particular an increase of intracellular Ins or IP3 interfere with autophagosome synthesis. Mood-stabilizing drugs, such as lithium, carbamazepine and valproate induce autophagy through this pathway. They inhibit inositol monophosphatase (IMPase) therefore reducing free inositol and IP3. Valproic acid acts to inhibit myo-inositol-1-phosphate synthase, resulting in the reduction in inositol biosynthesis. (Fleming et al, 2011)

2) *$Ca^{2+/}$calpain pathway.* Ca^{2+} can act as a positive or a negative regulator of autophagy (for autophagy calcium mediated see section 3.1.1). In the Ca^{2+}/calpain pathway, the increase in cytosolic Ca^{2+} can negatively modulate autophagy by activating calpains, Ca^{2+} -dependent cysteine proteases that interfere with autophagy machinery. Inhibition of calpains with calpastatin and calpeptin increases autophagic flux independently of mTORC1. Opposingly, overexpression of calpains or activation of calpains by Ca^{2+} channel openers inhibits autophagosome synthesis (Sarkar et al,2013).

3) *cAMP/Epac/Ins(1,4,5)P3 pathway*. Autophagy can also be regulated by intracellular levels of cAMP produced by adenylate cyclase (AC). High levels of cAMP inhibit autophagy, whereas pharmacological inhibition of AC activity induces autophagic flux independently of mTORC1. The main targets of cAMP are Epac (a protein directly activated by cAMP) and PKA (protein kinase A). The cAMP-EPAC signalling can exist independently of PKA since it was demonstrated that EPAC activators that did not have any effects on PKA inhibited autophagy. EPAC activates RAP2B, a small G protein of the RAS family, that in turn activates PLCε. Activation of PLCε produces IP3 that can directly inhibit the autophagy machinery. Alternatively, IP3 can bind an IP3 receptor on endoplasmic reticulum and induce release of calcium into the cytosol. Activation of calpains by Ca^{2+} inhibits autophagy as previously reported. This implies that the cAMP/Epac pathway is closely related to the inositol signalling and Ca^{2+} /calpain pathways in mTOR-independent autophagy (Sarkar et al, 2013) .

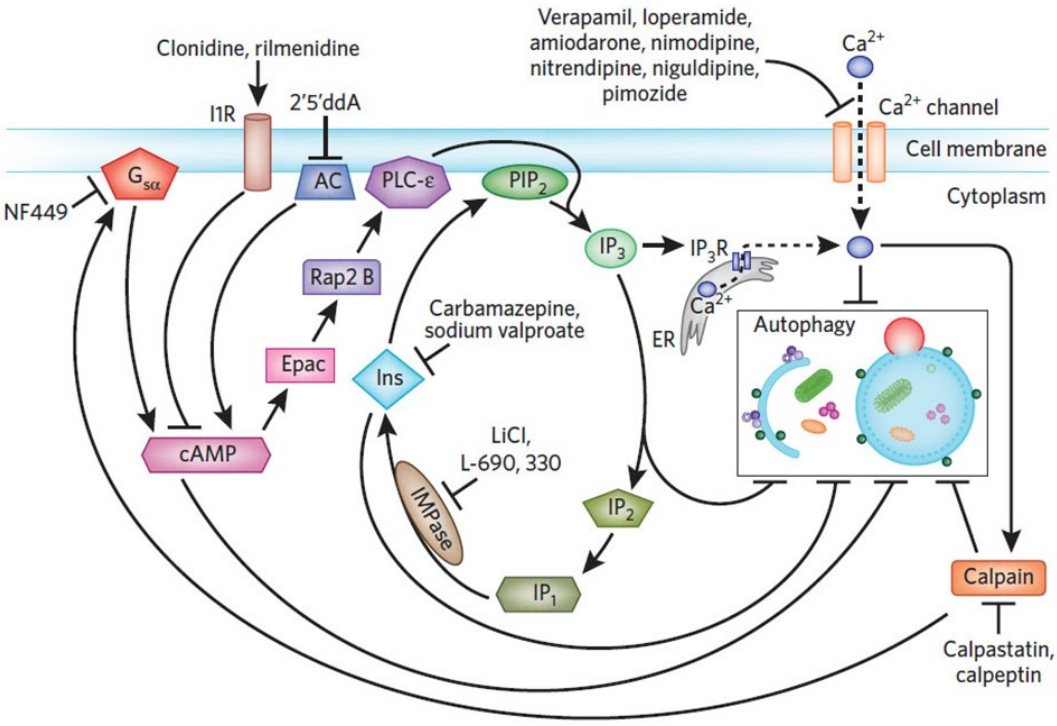

Figure 11 : mTOR independent signalling pathway. mTOR independent autophagy includes: 1) Inositol signalling pathway 2) $Ca^{2+/}$calpain pathway 3) cAMP/Epac/Ins(1,4,5)P3 pathway. Inhibition of various components of these pathways results in the activation of autophagy (Fleming et al,2011)

3.3 TFEB regulation: mTOR dependent and mTOR independent autophagy

Lysosomes are the main organelles involved in the degradation and recycling of intracellular components. Maintaining functional lysosomes is essential for the cells, but the exact mechanisms of the regulated lysosomal function and homeostasis are unknown. Roczniak-Ferguson and colleagues identified an interaction between mTOR and the TFEB transcription factor on the lysosomal surface that allowed for mTOR to transduce signals related to the lysosomal status to TFEB. TFEB in response can translocate to the nucleus and induce the expression of autophagy genes and other genes involved in lysosomal biogenesis (Roczniak-Ferguson et al, 2012). To transduce the signal related to the lysosomal amino acid content mTOR interacts with the vacuolar ATPase (Zoncu et al, 2011). V-ATPase are multisubunit complexes, present in all eukaryotic cells, formed by two domains: the Vo domain, responsible for proton translocation and the V1 domain that obtains energy by ATP hydrolysis.

V-ATPases are found within the membranes of many organelles, such as endosomes, lysosomes, and secretory vesicles, where they play a variety of roles crucial for the function of these organelles. V-ATPase are also recognized target of TFEB (Forgac, 2007).

In conditions of nutrient availability the interaction between amino acids and v-ATPase regulates Rag guanosine triphosphates (GTPase), which in turn activates mTORC1 inducing its translocation on lysosomal surface (Sancak, 2008; Zoncu, 2011). TFEB is recruited to the lysosomes by mTORC1 that phosphorylates it on serin 211, maintaining it inactive into the cytoplasm (Roczniak-Ferguson et al, 2012). Recently other proteins, called "YWHA 14-13-3 proteins", have been considered, with mTORC1, the main responsible of the retention of TFEB in the cytosol. Furthermore the phosphorylation induced by mTOR is required to the interaction of TFEB with 14-13-3 proteins (Roczniak-Ferguson et al, 2012).

Under conditions of starvation, translocation of TFEB to the nucleus was observed. Similar effects were obtained with the blockade of the lysosomal function using cloroquine, that raises lysosomal ph and bafilomycin, an inhibitor of the vacuolar H+ pump. Since mTORC1 phosphorylated TFEB and maintained it inactive into the cytoplasm, it was supposed that the inhibition of mTOR, as a consequence of Rag GTP ase deactivation, could dephosphorylate TFEB that translocated to the nucleus (Figure 12). To test this hypothesis different mTOR inhibitors were used. The first inhibitor tested was the rapamycin, the most known inhibitor of mTOR. Contrary to expectations, the rapamycin had minimal effects on TFEB translocation in *in vitro* models. A possible explanation is that rapamycin is mTOR inhibitor highly dependent on cell type and substrate (Benjamin et al, 2011; Thoreen et al, 2009). Nuclear translocation of TFEB was instead observed with torin, a recently developed ATP – competitive inhibitor that inhibits mTOR and blocks mTORC activity towards all substrates (Thoreen et al, 2009). In particular, the effect of TFEB translocation and nuclear accumulation was significant after 30 minutes of mTOR inhibition and was maximal after 1h of treatment of HELA cells with 2 μM of torin1(Roczniak-Ferguson et al, 2012). These studies confirmed the involvement of mTOR in regulation of transcriptional factor TFEB. The discovery that lysosomal mTORC1 regulates TFEB and its nuclear translocation has shown an intriguing lysosomal-to-nucleus signalling mechanism.

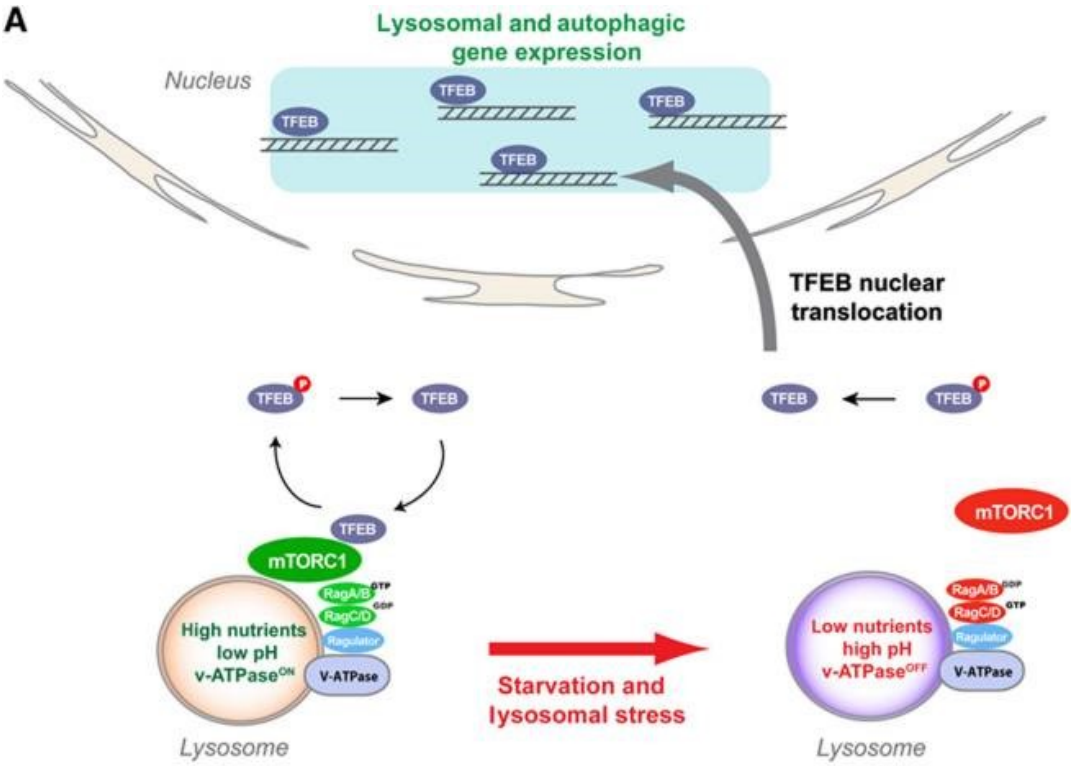

Figure 12 A lysosome to nucleus signalling modulated by mTOR and TFEB. (Left) When nutrients are available, the complex formed by vATP ase- Rag and Ragulator is activated and it, in turn, recruits and activates mTORC1 on lysosomal surface. mTORC1 phosphorylates the TFEB transcriptional factor, maintaining it in the cytoplasm and preventing its translocation to the nucleus. (Right). In starvation, lysosomal stress or v-ATP ase inhibition, Rag proteins are inactive and mTORC, dissociated from the complex, is detached from the lysosome and inactivated. TFEB is no more phosphorylated and translocates to the nucleus, where it activates genes involved in autophagy and lysosomal biogenesis (Settembre et al, 2012)

In addition, recently a new lysosomal-to-nucleus signalling mechanism that involves calcineurin and TFEB was highlighted. The lysosomes are among the calcium storage sites in the cell. In starvation or other stress conditions, the calcium channels localized on lysosomal membranes release the calcium into the cytoplasm and, in particular, the channel mucolipin 1 (MCOLN1) seems to be the main channel involved. Lysosomal Ca^{2+} released by MCOLN1 can activate a calcium dependent phosphatase calcineurin, that binds and dephosphorylates TFEB, promoting its nuclear translocation. Medina and colleagues reported that starvation could regulate TFEB translocation using both the mTOR and the calcineurin pathway. As previously described, in normal feeding

conditions, mTORC1 phosphorylates TFEB preventing its nuclear translocation, while in starvation, mTORC1 dissociates from the lysosomal surface and its activity is inhibited. At the same time, in starvation lysosomal calcium released by MCOLN1 can activate calcineurin that dephosphorylates TFEB. This leads to both a decreased TFEB phosphorylation, through mTORC1 inhibition, and an induction of TFEB dephosphorylation, through calcineurin activation. The two pathways sinergize to maintain TFEB dephosphorylated, which translocates to the nucleus to induce the expression of lysosomal and autophagic genes (Figure. 13) (Medina et al, 2015).

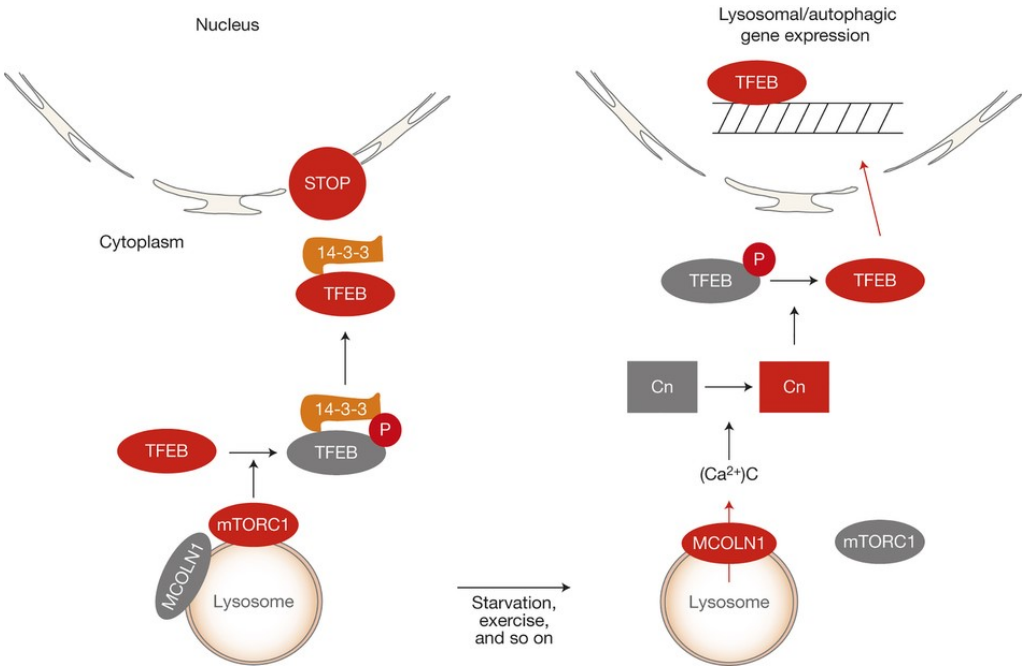

Fig 13 Ca^{2+} mediated regulation of TFEB. Under nutrient-rich conditions, TFEB is phosphorylated by mTORC1 on a lysosomal surface, and sequestered and complexed with 14-13-3 proteins. During starvation, mTOR is inhibited and this leads to a decreased TFEB phosphorylation. In addition in starvation, Ca^{2+} released from the lysosome through MCOLN1 channel activates dephosphatase calcium dependent calcineurin. Calcineurin activated dephosphorylates TFEB that translocates to the nucleus (Medina et al, 2015)

4 Selective autophagy

Autophagy has been considered for a long time as a non selective mechanism of intracellular degradation. Recently however, selective forms of autophagy have been identified. In selective autophagy redundant or damaged organelles are recognized and degradated. How the cells can recognize specifically these structures is under investigation. Recent studies have allowed to better elucidate these new mechanisms of intracellular degradation. Identified forms of selective autophagy include: pexophagy (elimination of peroxisomes), mitophagy (mitochondria degradation), reticulophagy (degradation of ER membranes), ribophagy (degradation of mature ribosomes), autophagy of glycogen (specific glycogen degradation) (Meijer et al, 2009).

Pexophagy "Pexophagy" or selective peroxisomes degradation was initially observed in yeasts when the cells were switched to culture media where the peroxisomes are no longer necessary for the growth. Two peroxisomal membrane proteins, Pex3 and Pex14 (Pex, peroxisomal import protein), were identified for peroxisomes recognition in yeasts. In mammalian cells it is still not clear how the cells specifically recognize peroxisomes that are to be degraded. Thus far the 69 Kda peroxisomal membrane protein was proposed as recognition target. (Meijer et al, 2009)

Mitophagy. Mitophagy involved the selective degradation of mitochondria. Mitophagy was identified in Saccaromyces Cerevisiae when the cells were switched from a medium containing lactate, where the mitochondria are required for the oxidation, to a medium where the mitochondria are no longer necessary and are therefore degraded. In mammalian cells, the signal used for recognition of damaged or non-functional mitochondria could be the pro-apoptotic mitochondrial associated protein BNIP3, which induces a loss of mitochondrial potential membrane. Mitochondria undergo to several cycles of fusion and fission, but only the products of mitochondrial fission with a low membrane potential are involved in mitophagy. The exact mechanism for BNIP3 recognition of defective mitochondria is unknown, but ROS production or protein aggregates on mitochondrial membrane can be involved in the signalling. BNIP3, recognized the substrates then induces a loss of mitochondrial membrane potential and targets them to the autophagy pathway disrupting the complex Bcl2-beclin1 or activating Rheb. In addition, the low membrane potential can reduce ATP concentration and induce AMPK activation. Another protein involved in targeting damaged

mitochondria is the ubiquitine ligase Parkin, a protein frequently mutated in PD. Parkin acts independently from fission mitochondrial, as its recruitment precedes the mitochondrial fission. In basal condition Parkin is free in the cytosol, but when the mitochondrial oxidative phosphorylation is uncoupled, it moves onto the mitochondrial outer membrane, where the defective mitochondria are sequestered and subsequently fragmented and degraded (Meijer et al, 2009).

Reticulophagy The accumulation of misfolded proteins in ER causes stress that induces the selective degradation of ER membranes. The proteins that recognize the "ER stress" are Ire1 (inositol-requiring enzyme-1), JNK, eIF2α kinase and PERK (PKR-like ER kinase) (Meijer et al, 2009).

Ribophagy. Degradation of RNA is a well documented process. Only recent data showed that mature ribosomes can be degraded by a process called "ribophagy". Both ribosomal subunits are involved in the degradation, but only the subunit 60S requires ubiquitin protease Ubp3/Bre5 for its turnover. The signal that modulates the turnover of ribosomal 40S subunit is unknown (Meijer et al, 2009).

Autophagy of glycogen. Autophagy of glycogen has been reported in newborn mammals. Once the supply of maternal nutrients is interrupted and prior to the start of breastfeeding, autophagy of glycogen is induced by glucagone and adrenaline in liver, heart and muscle to cope the energy deprivation (Kotoulas et al. 2006; Schiaffino et al, 2008). Once incorporated in the autophagolysosomes, glycogen is degraded by α glucosidase to release glucose. In basal conditions glycogen is converted in glucose-6-phosphate in the cytosol and then dephosphorylated by glucose-6-phosphatase. The activity of the glucose-6-phosphatase is very low at birth, furthermore the autophagy of glycogen is an essential mechanism to mantain glucose homeostasis (Meijer et al, 2009).

5. Autophagy: cell survival or cell death?

Although autophagy is generally considered a cellular protective mechanism that regulates a number of physiologic roles; excessive autophagy results in cell death. As a mechanism of cell death, autophagy in this regard is defined as " type II programmed cell death" (II PCD) and presents morphological characteristics that distinguish it from apoptosis, "type I programmed cell death" (I PCD). Apoptosis is characterized by

condensation of chromatin, DNA fragmentation and breakdown of the cell into apoptotis bodies that are subsequently degraded by lysosomes of phagocytic cells. Conversely, in II PCD cellular degradation is operated by lysosomes of the same dying cells (Shintani et al., 2004). Autophagy can lead to cell death inducing subsequently apoptosis or represents a type of cell death that occurs as consequence of cell inability to survive under conditions with the degradation of large amount of cytoplasmic constituents. Recent studies described autophagic cell death in the context of tumor cells treated with chemotherapeutic drugs such as tamoxifen. The inhibition of the first step of autophagy with 3-methyl adenine led to the inhibition of tamoxifen- induced autophagic cell death (Cheng et al, 2013). The crosstalk between autophagy and apoptosis is complex and is still under investigation. Some signalling molecules such as Bcl2, BH3-proteins, ATG5, p62 represent a link between autophagy and apoptosis. Anti-apoptotic factors such as Bcl2 block autophagy inhibiting Beclin1, whereas proaptotic factors, such as BH3 proteins, have the opposite effect disrupting the inhibitory effect of Bcl2 on Beclin1 thereby activating autophagy. While Atg5 is involved in the first step of autophagy, in some conditions it can undergo caspase mediated cleavage thus generating pro-apoptotic fragments that lead to the mithocondrial death pathway (Mizushima et al, 2008). Protein p62 is involved in selective autophagic degradation of many proteins and damaged mitochondria, but can also interact with different apoptotic and survival proteins including caspase-8, TRAF6 (which modulates NF-kB survival pathways) and ERK. As such, p62 promotes activation of caspase 8 , subsequently triggering apoptosis. This pathway is inversely regulated as caspase-8 can also cleave p62 in response to death receptor activation. In addition, caspase-8 can be degraded by autophagy, most likely via a p62 mediated mechanism. Though it is generally recognized that autophagy and apoptosis are interconnected, further studies are needed to better understand how these two pathways are related. Among the proteins involved in both mechanisms, p62 and Beclin1 are likely critical molecular players that act as upstream and downstream effectors of pro and anti-apoptotic molecules. Understanding the exact role of these proteins in autophagy and apoptosis pathways can be useful for autophagy modulation in cancer treatment and other diseases (Gump. et al, 2011).

6. Autophagy and diseases

Autophagy can play a protective or deleterious role in many pathological conditions, depending on the context. Defective autophagy can contribute to the pathogenesis of several diseases, such as infection and inflammatory diseases, lysosomal storage disorders, cancer, obesity, cardiovascular and, neurodegenerative diseases (Figure14).

A brief describe of autophagy role in human diseases is given below.

Autophagy and cancer. The role of autophagy in cancer is controversial and is now thought that controversial findings are in fact a representation of the different stages of cancer development. In the initial stages, autophagy is protective counteracting DNA damage and suppressing growth of pre-cancerous cells. In the advanced stages, when the tumor is consolidated and the cancer cells have to face unfavorable conditions (nutrient deprivation or hypoxic condition), autophagy can facilitate the survival of malignant cells providing them nutrients and energy through degradation of cytoplasmic constituents. Interference of autophagy with cancer chemotheraphy was reported. These studies show that the mechanism that reinforces the chemoresistance is through the ability of autophagy to promote survival of cancer cells because it facilitates degradation of their damaged macromolecules (Cheng et al, 2013).

Autophagy and obesity. Autophagy plays also a role in lipid metabolism and insulin signalling. Defective autophagy was reported by Yang et al. (Yang et al, 2010) in hepatocytes in both genetic and dietary model of obesity. In particular Atg7 appears to play a role in insulin signalling, since its suppression leads to defective insulin signalling and endoplasmic reticulum stress.

Autophagy and cardiovascular diseases. In cardiovascular field, autophagy at low levels is normally activated and its downregulation can lead to CVD. Under stress conditions autophagy is up-regulated and represents a strategy to ensure the survival of cardiomyocytes. Despite the protective role of this process in basal and moderate stress conditions, excessive levels of autophagy can lead to myocardial cell death (Cheng et al, 2013)

Autophagy and neurodegenerative disorders. Neurodegenerative diseases such as AD, PD, amyotrophic lateral sclerosis, prion diseases, Huntington's disease (HD) and spinocerebellar ataxias are characterized by dysfunction in autophagy. Data has

indicated that common characteristics in all these diseases include an accumulation of autophagic vacuoles containing misfolded proteins. In AD the misfolded proteins are the peptide Aβ and tau, in HD (and other polyglutamine disorders) the mutant proteins present polyglutamine-rich extensions, and in PD the protein involved is α sinuclein. In most of these conditions, autophagy is initially stimulated to remove misfolded proteins, but the excessive load of material to degrade leads to autophagy dysregulation with accumulation of autophagosomes (Cheng et al, 2013). Several studies report that pharmacological stimulation of autophagy could be a promising strategy in the treatment of neurodegenerative disorders, resulting in the clearance of protein aggregates and reduction of neurodegeneration-related symptoms (Choi et al., 2013).

Autophagy and lisosomal storage disorders. Defective autophagy is also the basis of "lysosomal storage disorders". They are characterized by genetic mutations that perturb lysosomal function. Defective lysosomes are no longer able to fuse with autophagosomes, leading to an accumulation of toxic substrates (Lieberman et al, 2012).

Autophagy and immunitary system. The role of autophagy in infection and inflammatory diseases is a fairly new avenue of research. In these conditions autophagy has a dual role. It can be protective, for example in targeting intracellular bacteria and viruses to autophagosomes for subsequent degradation by lysosomes (xenophagy). However, it can be deleterious because it can provide microrganisms nutrients for growth and substances beneficial to their metabolic pathways. Many pathogens have develop strategies to escape targeting to autophagy and degradation. Some bacteria or virus use autophagy for their replication, and others can even block the membrane trafficking required by the cell to target these invaders to lysosomes for degradation (Cheng et. al, 2013).

Since the autophagy can positively or negatively modulate many human diseases, pharmacological approaches to activate or inhibit it are required. Among the autophagy modulators, rapamycin (and analogs), torin1, threalose and carbamazepine are used to activate autophagy, whereas CQ, hydroxycloroquine, 3-methyladenin can be used as inhibitors (Jiang et al, 2014). Furthermore despite the great interest in the use of autophagy modulators as pharmacotherapeutic agents some questions need to be addressed to ensure efficient progress in this emerging field.

First, although the knowledge regarding this process at the molecular, biochemical, and cellular levels is considerably increased in this last decade, the precise role of autophagy in individual diseases is not fully characterized especially for clinical use. Second, since in each of these pathologies not all the patients respond to the treatment in the same manner, predictive studies appear necessary before application of a treatment strategy. Third, most of the autophagy modulators are not specific impacting also other cellular processes. The identification of more selective modulators can maximize therapeutic potential. Finally, since the autophagy modulators should be used complementary to traditional therapy, the possible drug-drug interactions must be considered before designing therapeutic autophagic modulators. Only solving these fundamental questions, autophagy modulators can come into clinical practice, ensuring benefits for human health (Cheng et al, 2013).

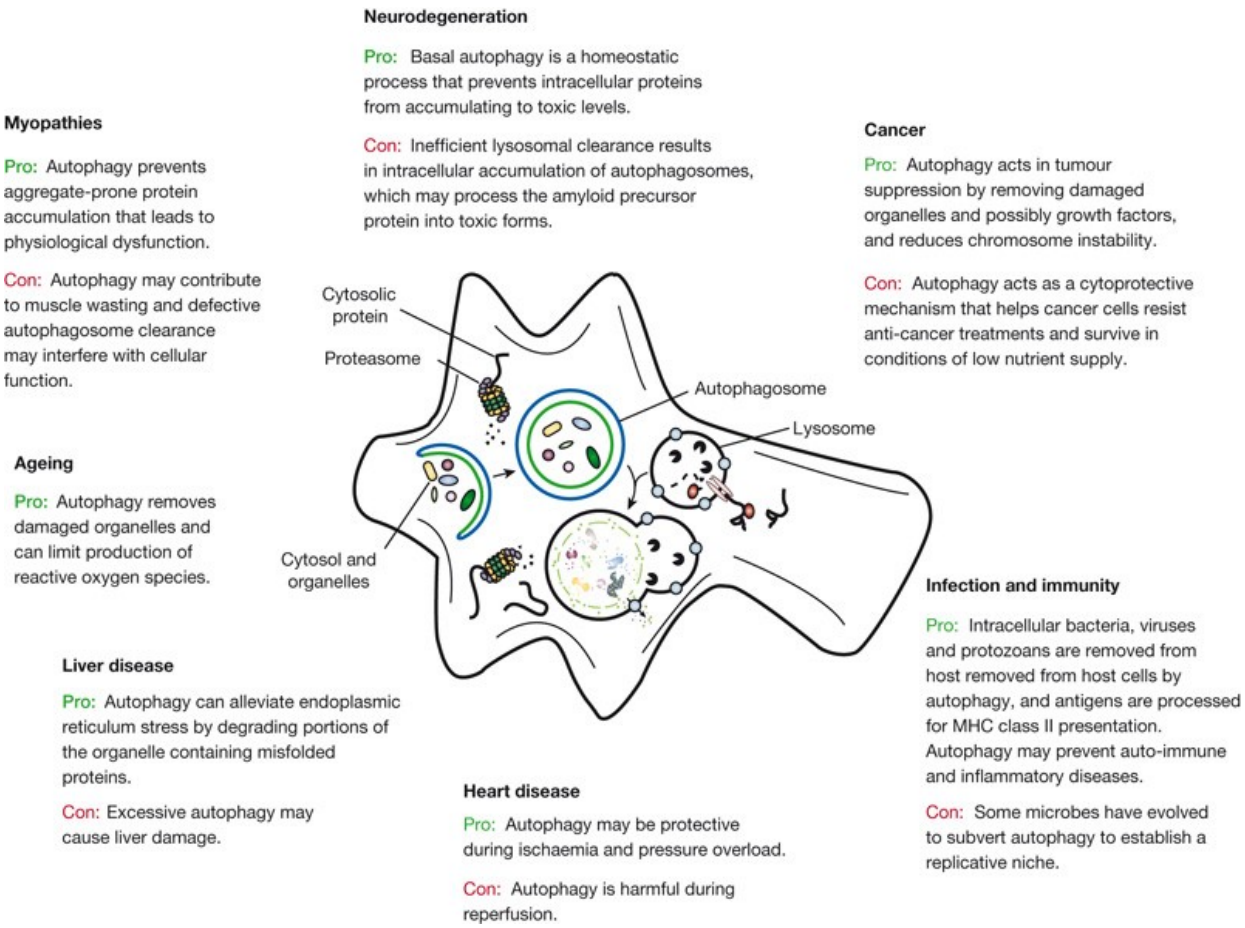

Figure 14) Role of autophagy in human diseases. In basal conditions autophagy is considered protective for cell homeostasis. Due to its crucial role, dysregulation in autophagy is associated to several

pathological conditions The type and progression of diseases have to be considered to determine whether autophagy inhibition or stimulation may be beneficial (Mizushima et al, 2008).

6.1 Autophagy and Alzheimer's disease.

Alzheimer's disease (AD) is considered the most common form of dementia. It was identified for the first time in 1907 by psychiatrist and neuropathologist Alois Alzheimer in a 51 year-old-woman that showed deteriorating memory with psychiatric disturbances four years prior to her death (Nixon, 2007, Allosop, 2000). Post-mortem histological analysis of the brain highlighted the presence of unusual fibrils in the neurons (neurofibrillary tangles) and focal lesions within the cerebral cortex (senile plaques) (Allosop, 2000). AD was distinguished from the classic forms of dementia for the simultaneous presence of neurofibrillary tangles, senile plaques and dementia in subjects not necessarily over 65 years. In 1923 the scientist Divry (Divry, 1923) identified the presence of amyloid in the senil plaques, subsequently called "amyloid plaques". Amyloid plaques were originally thought to be starch-like in nature, but later it was discovered that they were formed by a variety of proteins that lost their natural conformation forming oligomeric intermediates and subsequently insoluble fibrils (fibrillogenesis). (Allosop, 2000). At the present the general consensus is that the oligomeric intermediates formed during amyloid aggregation represent the most toxic species. In AD the peptide involved in amyloid plaques is the Aβ peptide that is generated by cleavage of amyloid precursor protein (APP). APP can be turned over in two different pathways: non-amyloidogenic pathway implicating α secretase, and, the amyloidogenic pathway regulated by the β and γ secretases. Protein cleavage by α secretase forms a soluble N terminal fragment (sAPPα) that is released from the cells and C terminal fragment that remains associated to the membrane (CTF). The amyloidogenic pathway provides instead that APP is internalized into the endosomes and degraded in a more distal site at extracellular domain by β secretase, that releases a soluble APP fragment (sAPP β) and a C terminal fragment (βCTF) containing Aβ peptide. Aβ is subsequently produced by CTF through an intramembrane cleavage operated by the multi enzymatic complex of γ secretase (Nixon, 2007). The catalytic presenilin (PS1 or PS2) protein is part of this complex. Mutations in APP or in PS (presenilin) 1 or 2 have been recognized as the causes that lead to the amyloidogenic

pathway. Regarding its localization, most researchers agree that Aβ is produced essentially inside the cells as result of endocytic and secretory pathways (Orr and Oddo, 2013) and then is released in the extracellular compartment in soluble or aggregate form, where it exerts its toxicity in different ways (interaction with surface receptors, membrane lipid bilayer damage or intracellular toxicity after re-internalization into the endosomal-lysosomal compartments). In contrast, McGowan et colleagues reported cognitive deficit in AD models with intracellular elevated levels of Aβ but in absence of extracellular amyloid deposition, suggesting that intracellular Aβ can be neurotoxic (McGowan et al., 2005). Intracellular neurofibrillary tangles are instead characterized by the microtubule-associated protein tau. This protein plays an important role in microtubule stabilization but in some pathological conditions it is abnormally hyperphosphorylated and aggregated (Son et al, 2012). Tau-positive aggregates are detected not only in AD, but are observed in other neurodegenerative disorders (Orr and Oddo, 2013).

Autophagy has been extensively studied in AD, since Aβ aggregates and tau inclusions seem to be a consequence of defective autophagy. In neurons autophagy plays a role in both health and disease. Neurons in basal conditions require autophagy to remove long-lived proteins and organelles, but autophagosomes are rarely detected in these conditions. The scarcity of autophagic vacuoles (AVs) in healthy neurons may be related to the high efficiency of autophagy that quickly leads to autophagic flux and clearance of autophagosomes. Autophagy is also activated by misfolded proteins and aggregates, but defective autophagy is observed in AD, and this is thought to be related to the excess of protein burden that has to be degraded. The exact mechanism that leads to autophagy dysfunction in AD is unknown even if several hypotheses exist (Figure 15). Many authors have reported altered autophagy at the initiation phases of AD. These hypotheses are confirmed by an observed deficit in Beclin 1 in AD brains. Caspase 3-mediated cleavage of Beclin-1 occurs in AD and this event could lead to defective autophagy with Aβ accumulation. Conversely, other studies reported an up-regulation of autophagy in AD, due both ROS-induced PI3 kinase III activation and transcriptional up-regulation of positive regulators of autophagy (Liang et al, 2014). In addition to possible defects in earlier step of autophagy there is emerging evidence suggesting defective autophagosome transport or a failure in lysosomal clearance. The presence of autophagosomes containing Aβ peptide in the dystrophic dendrites and tau inclusions in

the perikarya of affected neurons confirmed the hypothesis for a deficit in the maturation of autophagosomes or in retrograde transport towards the neuronal cell body, where the lysosomes are present (Orr et al, 2016, Sarkar, 2013). Other studies have further showed the presence of both autophagosomes and autolysosomes in dystrophic neurites of AD patients, suggesting that autophagosomes can fuse with lysosomes but substrate degradation in the autolysosomes is defective. These findings point to the disruption of substrate proteolysis within autolysosomes as the principal mechanism underlying autophagic dysfunction in AD. This possibility is supported by the recent discovery that presinilin (PS1) plays a role in the defective proteolysis of autophagic substrates in patients with AD. PS1 is essential for the targeting to lysosomes the v-ATPase, complex responsible for lysosomal acidification and protease activation. Genetic mutations of PS1 are present in familial forms of AD and this could explain the defective proteolysis that leads in turn to vacuole accumulation and impaired autophagic substrate turnover in AD (Son et al, 2012; Liang et al, 2014).

Altogether these studies suggest autophagic dysfunction in the pathogenesis of AD, although the exact mechanisms in terms of where and when is not entirely understood. Manipulating neuronal autophagy appears as an intriguing therapeutic measure for AD. The kinase mTOR is a critical negative regulator of autophagy as previously reported and the treatment with rapamycin, in transgenic model of AD, has shown to reduce the accumulation of Aβ and tau aggregates. Induction of autophagy by administration of a lentiviral vector expressing Beclin-1 reduced the accumulation of Aβ and tau aggregates in APP transgenic mice. These findings suggest that pharmacological modulation of autophagy, inhibiting mTOR signalling or activating Beclin1 can have potential benefits in AD.

However before moving to clinical practice some considerations are necessary. First,, the increased autophagy does not necessarily leads to an increase in autophagic flux. If autophagy is induced but the lysosomal functionality is not restored the activation of autophagy may synergize the deleterious accumulation of intermediate AVs, worsening further Aβ accumulation. In this case the induction of autophagy should not be used before the autophagic efficiency is restored. Second, the stage of the disease has to be considered. In fact in the early stages of AD it is proposed that autophagy inducers can ameliorate Aβ accumulation, whereas at advanced stages the combination therapy based

on autophagic inductors with agents that promote the completion of autophagic degradation might be a promising intervention strategy. Third the optimal level and the duration of therapy based on autophagy modulation must be established for both early and advances stages of AD as overactivation of autophagy can be as deleterious as defective autophagy. Further studies are needed to clarify the exact mechanism of autophagy dysfunction in AD before proceeding with targeted therapeutic interventions based on autophagy modulation (Liang et al, 2014).

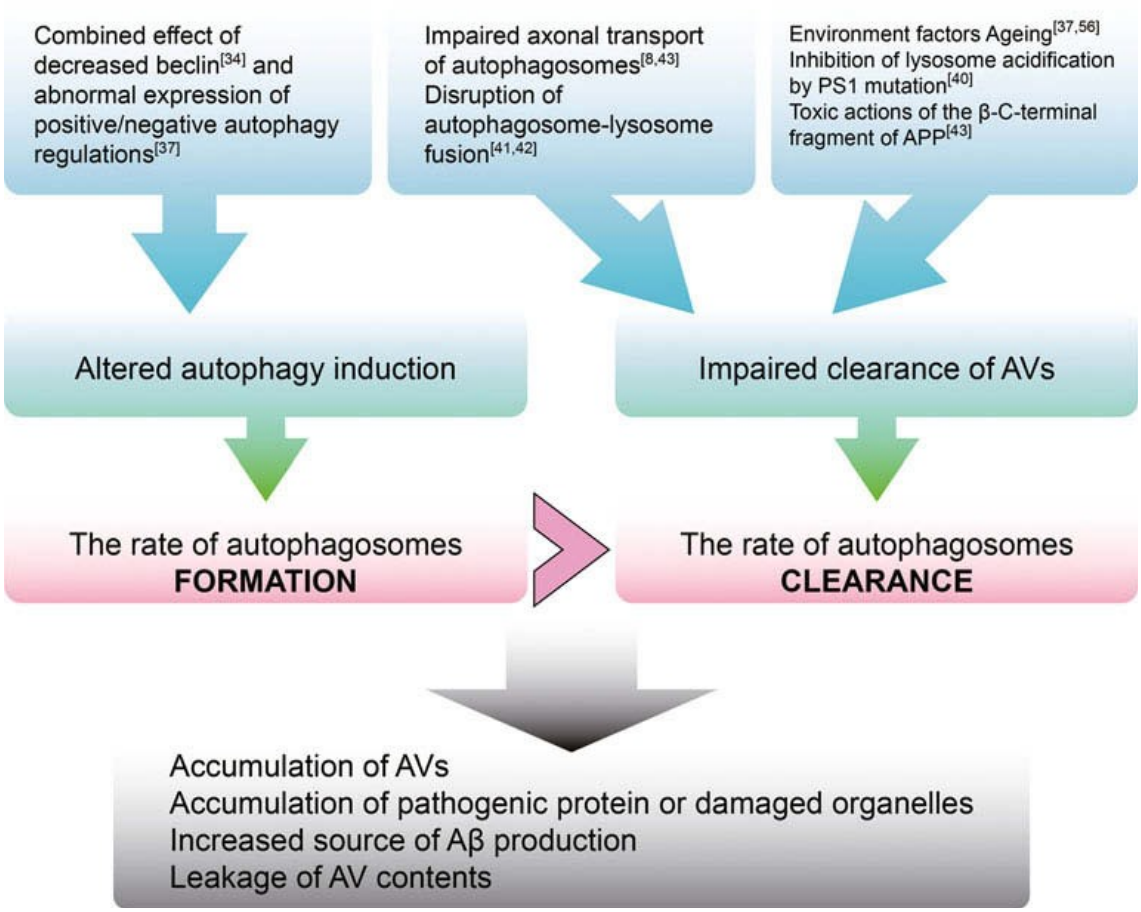

Figure 15 Model of autophagic dysfunction in AD. Defective autophagy in AD leads to accumulation of amyloid aggregates. The exact mechanisms of autophagy failure are unknown, but three essential steps could be compromised: 1) induction 2) axonal transport of autophagosomes to lysosomes and autophagosome-autolysosome fusion 3) clearance of substrates by lysosomes (Liang et al, 2014)

6.2 Autophagy and cardiovascular diseases.

In cardiovascular field, autophagy can act in either beneficial or detrimental ways, depending on the context. Several studies support the essential role of autophagy in the heart under basal conditions. For example, rapid ablation of Atg5, protein required for autophagy, in adult hearts resulted in left ventricular (LV) dilatation and contractile dysfunction, accompanied by increased levels in ubiquinated proteins. Furthermore, Atg5 deficient mice showed alteration in mithocondrial size and damage to the intramithocondrial structure (Nakai et al, 2007). Mutation in the gene coding for LAMP2, protein essential for autophagosome-lysosome fusion, is reported to be associated to Danon's cardiomyopathy, pathology characterized by intracytoplasmic vacuoles containing autophagic material and glycogen in skeletal and cardiac muscle cells (Nishino et al, 2000). Despite the essential role of autophagy in mantaining cellular homeostasis, upregulation of autophagy was reported in several conditions associated to CVD such as ischemia/reperfusion, hypertension, cardiomyopathy and HF. In **ischemia-reperfusion**, autophagy is initially considered a survival mechanism, that subsequently becomes dysfunctional and deleterious. During the initial phase of ischemia, many factors can contribute to autophagy activation. Among them depletion of ATP, low oxygen availability and oxidative stress play a pivotal role. ATP depletion in myocardial ischemia increases the ratio AMP/ATP with subsequently activation of AMPK by both LKB1 and CamKKβ. AMPK in turn activates autophagy through mTOR or eukaryotic elongation factor-2 phosphorylation (eEF2) inhibition. Low oxygen availability and oxidative stress activate hypoxia inducible factor 1 α (HIF 1 α). HIF-1 α can protect ischemic heart through different pathways, among which one is autophagy. HIF-1 α activates Bnip3 that can induce mitophagy. Mitophagy activated by HIF-1a-Bnip3 mechanism decreases the size of mitochondria thereby preventing alterations in oxidative phosphorylation as well as mitochondrial damage and ultimately cellular death (Nishida et al, 2009). Glycogene synthase kinase 3 β (GSK-3 β) is also involved in the modulation of autophagy during ischemia-reperfusion. During the ischemic phase, activation of GSK-3 β was associated with activation of autophagy, whereas in reperfusion phase the deactivation of GSK-3 β inhibited autophagy (Mei et al, 2015).

During reperfusion other factors play a role in the modulation of autophagic response. Among them are the overexpression of Beclin1, deficiency in Lamp2, oxidative stress and ER stress (Nishida et al, 2009). Reperfusion was associated to overexpression of Beclin1 in the heart as well as in other tissues including the brain and kidney, but its persistent activation during this phase resulted in excessive degradation and cell death (Schiattarella et al, 2016). In addition to Beclin1 overexpression, reduced levels of LAMP-2 were observed in this phase (Lavandero et al, 2016). ROS levels increased in riperfusion, following a mechanism called "ROS-induced ROS release" that involves the mitochondria. Mitochondria damaged produce ROS that in turn damage proteins and organelles and provoke lipid peroxidation in mitochondria themselves, thereby promoting autophagy. ER stress is induced by accumulation of misfolded proteins, among which polyglutamine repeat (poliQ) 72 aggregates. ER activate the RNA-dependent protein kinase-like ER kinase (PERK)/eukaryotic initiation factor 2 α (eIF2α) signaling pathway, that in turn stimulates autophagy to remove protein aggregates. Autophagy plays a different role in ischemia and in reperfusion, therefore it is difficult establish if autophagy is protective or deleterious in the stress-response related to this condition. The severity and duration of ischemia and consequent tissue damage during reperfusion could determine the outcome of autophagy. Other studies suggest that the specific pathway activating autophagy can decide the final effect. According to this hypothesis, AMPK-induced autophagy seems to be protective, conversely autophagy induced by overexpression of Beclin1 can have deleterious effects (Nishida et al, 2009) (Figure 16)

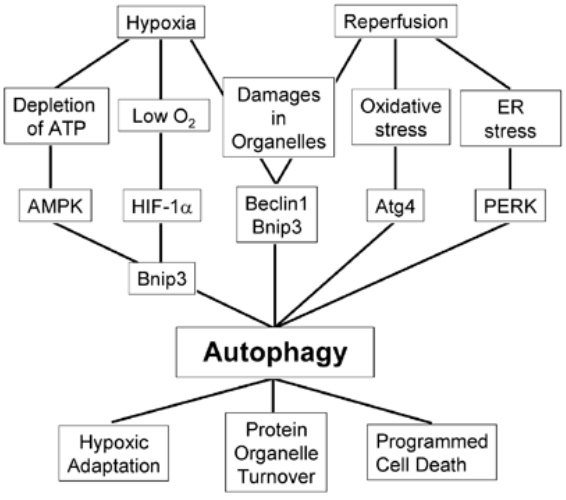

Figure 16 Autophagy during stress induced by ischemia-reperfusion (Nishida et al, 2009)

Hypertension is classified as primary and secondary hypertension. Primary hypertension represents 90–95% of cases and may develop as a result of environmental or genetic causes. Secondary hypertension, 5–10% of cases, has instead multiple etiologies, including renal, vascular, and endocrine causes. In response to hypertension, the heart exhibits hypertrofic growth. In this condition autophagy is upregulated as adaptative mechanism. However, excessive degradation of intracellular components by autophagy may lead to cell death and contribute to hypertension-related heart disease. According to this, autophagy inhibitors decreased neurogenic hypertension in spontaneously hypertensive rats, suggesting autophagy could be a potential therapeutic target in neurogenic hypertension (Chao et al, 2013). In another study, the inhibition of autophagy using CQ, antimalarial and antirheumatoid drug, was reported as protective in the prevention of monocrotaline-induced pulmonary hypertension. Take together these studies suggest that autophagy is maladaptative in pulmonary and neurogenic hypertension and its inhibition could be a new therapeutic strategy to treat these conditions (Mei et al, 2015). In contrast, McMullen and colleagues reported that the activation of autophagy with rapamicyn regressed cardiac hypertrophy induced by pressure overload (McMullen et al, 2004) .

Cardiomyopathy is classified in hypertrofic, dilatative and restrictive. In dilatative cardiomyopathy, several studies show the potential benefits related to the modulation of autophagy. LAM A/C gene (LMNA) mutation leads to dilatative cardiomyopathy with enlargement of left ventricle and altered systolic function. Defective autophagy was observed in this model of cardiomyopathy as consequence of upregulated AKT/mTOR signalling. Drugs inhibiting mTOR restored autophagic flux and improved cardiac function in this context. Desmin-related cardiomyopathy (DRC), another model of dilatative cardiomyopathy, is characterized by accumulation of misfolded proteins related to a mutation in alphaβ cristalline gene. In DRC ablation of Beclin1 accelerated HF development, suggesting a protective effect of autophagy stimulation in cardiomyopathy. Recently the role of autophagy in diabetic cardiomyopathy was also explored. Diabetes is associated with impaired autophagy and cardiomyocyte apoptosis. Accord to this, metformin, AMPK activator, restored autophagic function and protect against cardiac apoptosis, preventing cardiomyopathy. The above studies suggest that defective autophagy contributes to cardiomyopathy development; therefore,

pharmacologic interventions to augment autophagy might ameliorate cardiomyopathies (Mei et al., 2015).

All the pathological conditions above described can ultimately lead to heart failure (**HF).** This is a multifactorial clinical syndrome characterized by adverse ventricular remodeling which involves changes in the balance between cardiomyocyte protein synthesis and degradation. In this context, autophagy may be protective antagonizing ventricular hypertrophy by increasing protein degradation and decreasing tissue mass. (Mei et al, 2015). HF is molecularly characterized by accumulation of damaged mitochondria and harmful proteins, probably consequence of autophagic failure in ageing. Damaged mitochondria release reactive oxygen species (ROS) that are deleterious for lipids, proteins and DNA. Alteration of mitochondrial functionality decrease ATP levels, leading to AMPK activation. In this context autophagy is upregulated by both AMPK and ROS signalling. Upregulation of autophagy is considered as an adaptive response that protects cardiomyocytes. It is also possible that high ROS levels and severely damaged mitochondria induce excessive autophagy, transforming it from adaptive mechanism to autophagic cell death (Rothermel et al, 2008).

Recent studies have been investigated the link between ROS and autophagy in HF (Santin et al, 2016). Among the potential sources of oxidative stress in HF, mitochondrial enzyme monoamine oxidase-A (MAO-A) has been recently characterized. MAO–A is overexpressed in HF and ageing and this correlates with mitochondrial dysfunction and cardiac damage (Santin et al, 2016; Villenueve et al, 2013). Santin et al. reported that MAO-A mediated degradation of catecholamine and serotonin resulted in the production of H_2O_2, which could promote the onset and progression of cardiac diseases. H_2O_2 generation by MAO-A blocked autophagic flux with accumulation of p62 and LC3II, leading to mitochondrial fission and cell necrosis . In addition MAO activation induced an accumulation of catepsin D and Lamp1 (proteins essential for the lysosomal acidification and the completion of autophagosome-lysosome fusion) and lack of nuclear translocation of TFEB, master regulator of autophagy. Interestingly TFEB overexpression prevented mitochondrial fission and cell death in HF associated to MAO-A-overexpression. In conclusion the authors reported the possibility to restore lysosomal function and the autophagy

machinery as potential therapeutic target to prevent age-associated cardiac diseases (Santin et al, 2016) (Figure 17)

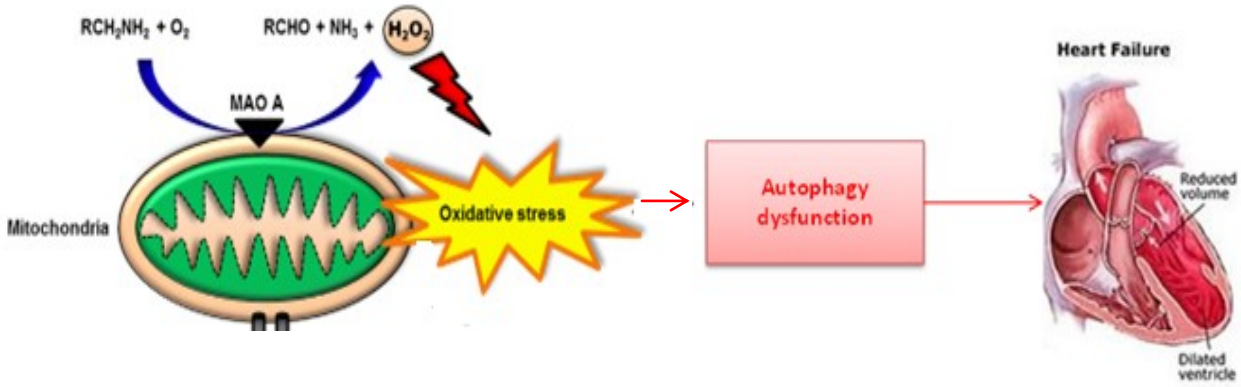

Figure 17: Proposed mechanism for autophagy dysfunction in HF. MAO-dependent H_2O_2 production negatively impacts on the elimination of damaged mithocondria through autophagosome-lysosome pathway, leading to cardiomyocyte death and HF.

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Aim of the study.

Aim of the study.

As previously reported, increasing evidence points to natural polyphenols, in particular those characterizing the so-called "Mediterranean Diet", as protective tools in a wide number of human pathologies, including neurodegenerative and cardiovascular diseases, cancer and diabetes. Most of their beneficial effects can be explained by their ability to induce "autophagy", acting as mimetic of CR. In light of this evidence, the aim of this thesis is to investigate the effects of OLE, the main phenol found in EVOO, focusing in particular on autophagy activation and therapeutic effects arising from autophagy modulation .

This thesis has essentialy three goals:

1) To describe the molecular and cellular mechanisms of autophagy induction by OLE in *vitro* and in *vivo*.

Autophagy activation can result from different pathways, some of which are Ca^{2+}-dependent such as Ca^{2+}-calpains-Gsα, cAMP/Epac/Ins(1,4,5)P3 and Ca^{2+}-CaMKKβ-AMPK-mTOR pathways. The latter appears to be the signalling pathway activated by resveratrol and EGCG in autophagy modulation. To date, the mechanism of autophagy induction showed by resveratrol and EGCG could not be generalized to other plant polyphenols. So, the first aim of this study was to investigate if OLE-induced autophagy proceeded through a similar signalling pathway using human SH-SY5Y neuroblastoma cell line and animal models of Aβ deposition (TgCRND8 mice).

2) To study the effect of OLE on SIRT1-PARP1 interplay.

The activation of SIRT is another mechanism by which polyphenols induce a CR-like effect. SIRT are a family of class III histone deacetylases that use NAD+ for their activity. In addition to SIRT1, other enzymes such as PARP1 require NAD+ as substrate. Since PARP1 overactivatiοn was reported to inhibit SIRT1 activity, is possible that the two proteins can counterbalance each other's activity controlling in this manner the cell survival/cell death equilibrium. In light of these evidence, another aim of this study was to investigate the effect of OLE on SIRT1-PARP1 interplay using murine N2A neuroblastoma cell line.

3) To evaluate the protective effects of OLE against pathological models of autophagy dysfunction

Following the results obtained with neurodegeneration models, we wondered whether OLE could protect against other pathological conditions displaying autophagic dysfunction. To this end, we selected an *in vitro* model of cardiomyopathy characterized by MAO-A overexpression, in which autophagy was defective. MAO-A is responsible for the oxidative deamination of catecholamines and serotonin in the heart and during this reaction produces H_2O_2, the corresponding aldehyde and ammonia. The generation of H_2O_2 disrupts nuclear translocation of TFEB, a master regulator of autophagy, causing autophagosome accumulation and ultimately cell death. So the last aim of this study was to evaluate if OLE-induced autophagy could be protective against MAO/ H_2O_2 axis and if TFEB translocation to the nucleus could be involved in OLE-mediated signalling pathway.

Materials and Methods

Molecular mechanisms of OLE-induced autophagy: in *vitro* and in *vivo* study.

In vitro study

For *in vitro* studies, two different immortalised cell lines were used: human neuroblastoma SH-SY5Y cells and and rat insulinoma RIN-5F cells. OLE was obtained by oleuropein glycate through enzymatic deglycosilation. To study the molecular mechanisms of OLE-induced autophagy $Ca^{2+/}$CaMKKβ/AMPK/mTOR autophagy signalling pathway was checked. The cytosolic levels of free Ca^{2+} after OLE-treatment were measured by confocal microscopy using the Fluo-3-acetoxymethyl ester (Fluo-3AM) probe. Autophagy induction was investigated using Cyto-ID Autophagy detection Kit (detection of autophagic vacuoles) and by western blot analysis through protein expression of beclin1, Lc3 II, pAMPK involved in autophagy signalling. For Cyto-ID and western blot analyses, STO-609 (a CaMKKβ inhibitor), EGTA (an impermeant Ca^{2+} chelator), compound C (CC, an AMPK inhibitor), or cyclopiazonic acid (CPA, an ER Ca^{2+}-ATPase blocker) were also added before OLE exposure.

1.1 Immortalised cell lines.

.SY-SY5Y and RIN-5F were both from American Type Culture Collection (ATCC). SY-SY5Y were cultured in 1:1 mixture of DMEM and HAM medium, supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich, Steinheim, Germany), 3.0 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. RIN-5F were cultured in RPMI medium supplemented with 10% FCS, 2.25 g/L glucose, 10 mM HEPES, 1.0 mM, sodium pyruvate, glutamine and antibiotics. The cells were maintained in a humidified incubator at 37°C with 5% CO2.

1.2. Oleuropein deglycosylation

Oleuropein glycated was from Extrasynthase, Genay Cedex, France. OLE was obtained by oleuropein deglycosylation according to Konno et al. (Konno et al, 1999), with minor modifications. Briefly, oleuropein glycated was solubilized at a 10 mM concentration in 0.1 M sodium phosphate buffer pH 7.0, and incubated with 9.0 I.U./mL of β glycosidase overnight at room temperature in the dark. The reaction mixture was centrifuged at 10,000g for 15 minutes to precipitate the aglycone. The complete

oleuropein deglycosylation was confirmed by assaying the glucose released in the supernatant with the Glucose (HK) Assay Kit (Sigma-Aldrich). The glucose determination is based on two enzymatic reactions:

1) Glucose + ATP $\xrightarrow{\text{Hexokinase}}$ Glucose-6P +ADP

2) Glucose 6-Phosphate + NAD $\xrightarrow{\text{G6PD}}$ 6- Phosphogluconate + NADH

In the first reaction the hexokinase catalyses the phosphorylation of glucose into glucose-6-phosphate. Glucose-6-phosphate (G6P) is then oxidized in the second reaction to 6-phospho-gluconate in the presence of oxidized nicotinamide adenine dinucleotide (NAD) through the glucose-6-phosphate dehydrogenase (G6PDH) enzyme. During this reaction, an equimolar amount of NAD is reduced to NADH. The increase in absorbance at 340 nm of NADH is directly proportional to glucose concentration, which is equimolar to OLE. The determination of the amount of OLE allowed its solubilisation in DMSO at a final concentration of 50mM. This stock solution was kept in the dark at -20°C until further use. OLE working solution was obtained by diluting the stock solution into buffer (for experiments *in vitro)* or seed oil (supplements for mice) .

1.3 Analysis of autophagic vacuoles

Analysis of autophagic vacuoles was performed using the Cyto-ID® Autophagy Detection Kit (Enzo Life Sciences). This assay uses a 488 nm excitable green fluorescent reagent that selectively labels autophagic vacuoles in living cells. SHSY-5Y and RIN-5F were plated onto poly-l-lysine-coated glass coverslips in 24-well plates for 24h at density of 200.000 cells/well, and treated without or with 50 µM OLE for different time periods. In some experiments cell were pre-treated for 30 minutes with 13,3µM STO-609 (a CaMKKβ inhibitor), 4mM EGTA (an impermeant Ca^{2+} chelator), 10 µM compound C (CC, an AMPK inhibitor), or for 1h with 40 µM cyclopiazonic acid (CPA, an ER Ca^{2+}-ATPase blocker) before OLE exposure. After treatment, the cells were washed twice with 100 µl of PBS and then with 100 µl of 1 × Assay-Buffer provided with Detection Kit supplemented with 10% FCS. The cells were incubated for 30 min at 37 °C with 100 µl of Dual detection reagent (prepared by diluting Cyto-ID Green Detection Reagent 330 times in a mixture of 1× Assay Buffer plus FCS), protected from light, and washed again with the previous solution (Assay buffer plus

FCS). The coverslips were placed onto microscope slides and the fluorescent-labelled cells were analysed. Confocal Leica TCS SP5 scanning microscope (Mannheim, Germany) equipped with an argon laser source for fluorescence measurements at 488 nm and a Leica Plan Apo 63 × oil immersion objective was used. A series of optical sections (1024 × 1024 pixels) was taken through the cell depth for each examined sample and projected as a single composite image by superimposition. The confocal microscope was set at optimal acquisition conditions (e.g., pinhole diameters, detector gain and laser powers), which were maintained constant for all analysis. Phase-contrast images were also acquired using the DIC channel. To quantify the green fluorescent signal, 10-22 cells were analysed for each condition in three different experiments using ImageJ software (NIH, Bethesda, MD)

1.4. Cell treatment and western blotting

OLE-treated cells were also analysed by western blotting to evaluate the expression of specific autophagy markers and the signalling involved in the induction of autophagy. After the treatment, SH-SY5Y and RIN-5F were lysed in Laemmli buffer (60 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10 mM EDTA, 10% (w/v) glycerol). Before immunoblotting, the protein concentration was determined with a BCA detection kit (Pierce, USA) and adjusted to equal concentrations across samples. The protein lysates were boiled for 5 minutes with β-mercaptoethanol and bromophenol blue, clarified at 10000 × g for 10 min, run on SDS-PAGE, and transferred to PVDF membranes (Amersham Bioscience, UK). After blocking with 5.0% (w/v) BSA in 0.1% (v/v) PBS-Tween-20, membranes were incubated overnight at 4 °C with specific primary antibodies: rabbit polyclonal anti-beclin1 antibody (1:2000, Abcam), rabbit polyclonal anti-LC3II A/B antibody (1:1000, Cell Signaling), rabbit monoclonal anti-phospho-AMPKα (Thr172), anti-AMPKα antibodies (1:1000, Cell Signaling). Mouse monoclonal anti-β−actin antibody (1:1000, Santa Cruz Biotechnology Inc.) was used as loading control. Following an overnight incubation the membranes were washed in PBS-Tween-20, and incubated for 1 h with specific secondary antibodies as appropriate (1:10000, goat anti-rabbit antibody and goat anti-mouse antibody, Molecular Probes, Life Technologies); the immunoreactive bands were detected with the Immobilon Western Chemiluminescent HRP substrate (Millipore) or SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific), and quantified by densitometric

analysis using the ChemiDoc system and the Quantity One software (Bio- Rad Laboratories, Italy).

1.5. Measurement of intracellular Ca^{2+} levels

The intracellular calcium levels were measured using the Fluo-3-acetoxymethyl ester (Fluo-3AM) probe (Molecular Probes). Fluo-3AM excitation is compatible with a 488 nm and the intensity of emitted fluorescence is largely increases upon Ca^{2+} binding. Semi-confluent SH-SY5Y cells cultured on poly-l-lysine-coated glass coverslips were treated with 50 μM OLE for different time periods, washed with PBS, and incubated with 10 μM Fluo-3AM and Pluronic F-127 at a 1:1 (v/v) ratio for 30min at 37 °C. The cells were then washed and fixed into 2.0 % buffered paraformaldehyde for 15 min. Cell fluorescence was analyzed by confocal microscopy using a Leica Plan 7 Apo X63 oil immersion objective. The punctate green dots were quantified using ImageJ software (NIH, Bethesda, MD).

In vivo study

For ***in vivo*** study**,** TgCRND8 mice, a model of amyloid β-peptide (Aβ) deposition (Chitsi et al, 2001) were used. TgCRND8 mice treated with OLE for 8 weeks (50 mg/kg of diet) were sacrificed by cervical dislocation and the brains were rapidly removed and divided sagittally. For protein analysis, cortical and hippocampal samples from one hemibrain were immediately sectioned, snapfrozen and stored at -80. The other hemibrain was postfixed in phosphate-buffered 4.0% paraformaldehyde, pH 7.4, at 4°C for 48 h, rinsed in PBS and paraffin embedded for immunohistochemistry and staining. To explore the involvement of mTOR and AMPK in OLE-triggered autophagy immunoistochemical and western blot analyses were used.

1.6 Animals and experimental treatment

All animal experimentation was performed according to the ECC (DL 116/92, Directive 86/609/EEC) and National guidelines for animal care. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Italian Ministry of Health (Permit Number: 283/2012-B).

The TgCRND8 mice encode a double mutant form of amyloid precursor protein 695 (KM670/671NL1V717F) driven by the *PRNP* (prion) promoter; the mutations used were originally identified in familial Alzheimer's disease. Transgenic hemizygous TgCRND8 (tg) mice with a (C57)/(C57/C3H) genetic background and non-tg hybrid (C57)/(C57/C3H) wild-type (wt) , control littermate male, and female mice, were used. Three month old TgCRND8 mice exhibited Aβ amyloid deposits in the cortex and hippocampus, with denser plaques at 6-7 months. At 7 months intracellular aggregates of hyperphosporylated tau were also observed in their brains. These neuropathologic manifestations were accompanied by learning deficits. TgCRND8 mice usually die at around 12 months of age.

TgCRND8 and wild type (wt) of 4 and 10 months of age at the beginning of treatment were used, n = 6 group/genotype, equally divided for sex. The mice were treated for 8 weeks with modified low-fat AIN-76A diet (10 g/day per mouse) composed of 50.0% sucrose, 5.0% fat, 20.0% casein, 15.0% corn starch, 5.0% powdered cellulose, 3.5% AIN-76 mineral mix, 1.0% AIN-76A vitamin mix, 0.3% DL-methionine and 0.2% choline bitartrate supplemented with OLE (50 mg/kg of diet) or not (untreated mice).

1.7 Immunohistochemistry

The immunohistochemical analyses were performed on 5.0 μm coronal paraffin-embedded sections of the brain, obtained by microtome sectioning (Leica Microsystem, UK). Brain cross-sections were incubated for 30 min in xylene at RT to allow the removal of the paraffin and rehydrated using decreasing ethanol concentrations solutions up to distilled water. The cross-sections were then incubated overnight at 4° with rabbit anti-phospho-mTOR (S2448) polyclonal antibody (Abcam, Cambridge, UK) used at 1:100 dilution in 0.1 M PBS (pH 7.4) with Triton X-100 (0.3%) and BSA (5 mg/ml). The cross-sections were then incubated for 1 h with the secondary antibody at 1:1000 dilution in 0.1 M PBS plus BSA (1 mg/ml), and the immunostaining was visualized using the avidin-biotin system (Vectastain: Vector Laboratories, Burlingame, CA) and 3,39-diaminobenzidine plus Nickel (DAB Kit: Vector Laboratories, Burlingame, CA) as chromogen.

1.8 Western blotting

For western blotting, TgCRND8 and wt tissue brains were homogenized in cold RIPA buffer composed of.

- 2x lysis buffer (50 mM Tris-HCl ph 7,5; 50 mM NaCl; 10 mM EGTA; 5mM EDTA)
- Phosphatase inhibitors (2 mM NaPP, 4mM PNFF, 1mM Na3VO4)
- Protease inhibitors (1mM PMSF, 20 μg/ml leupeptin, 30μγ/ml aprotinin)

Before immunoblotting, protein concentration was determined and forty μg of proteins were separated by SDS-PAGE for electrophoresis. The separated proteins were transferred onto 0.45 mm nitrocellulose/PVDF membrane (Hybond-C, Amersham Life Science) and incubated overnight at 4° with specific primary antibody: rabbit anti-phospho-p70 S6 kinase (Thr389), anti-phospho-AMPK (Thr172) and anti-AMPK (Cell signalling, MA, USA) ; anti-actin antibody (SIGMA-ALDRICH, MO, USA) diluted 1:4000 was used as loading control. Following the overnight incubation, the membranes were washed and then incubated with HRP-conjugated secondary Ab for 1h. The immunocomplexes were visualized using enhanced chemiluminescence (ECL, Pierce USA) and acquired using ImageQuant 350 system. Band densitometric analysis was performed using Image Quant TL software (GE, Healthcare UK).

1.9. Statistical analysis

One-way ANOVA, plus Bonferroni's post-hoc, was used to analyse Western blotting experiments in *vitro and in vivo*. Statistical analyses were carried out with GraphPad Prism and the statistical significance was defined as $p < 0.05$.

2)Effect of OLE on PARP1-SIRT1 interplay

In *vitro* study

For *in vitro* study murine neuroblastoma N2a cells were used. To study the effect of OLE on PARP1-SIRT1 interplay, the cells were treated with the alkylating agent MNNG, PARP1 activator, in presence or in absence of OLE and the levels of PARylated proteins were analysed by western blot. Since PARP1 use NAD+ as substrate for its catalytic activity, NAD+ levels were measured by enzymatic reaction in cells exposed to MNNG in presence or in absence of OLE. OLE-treated cells were also analysed for SIRT1 protein expression by western blot. SIRT1 is involved in autophagy signalling and similarly PARP1 induces autophagy upon moderate DNA damage. On this basis, N2a cells exposed to MNNG in presence or in absence of OLE were analysed for Beclin1 protein expression, autophagy marker, by western blot analysis.

2.1 Cell cultures

Murine neuroblastoma N2a cells (European Collection of Authenticated Cell Cultures, ECACC) were plated in Eagle′s minimum essential medium (MEM) supplemented with non-essential amino acids, 10% FCS (Sigma-Aldrich, Steinheim, Germany), antibiotics and glutamine and maintained in a humidified, 5% CO2, 37°C incubator.

2.2. Cell treatment and western blotting

To induce protein poly-ADP-ribosylation, N2a cells were incubated with 100 μM of the PARP1 activator methylnitronitrosoguanidine (MNNG) for 15 min or pretreated with 100 μM OLE or with the PARP inhibitors 6(5H)-phenanthridinone (PHE, 30μM) or N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-(N,N-dimethylamino) acetamide hydrochloride (PJ-34, 20 μM) for 24 h before MNNG exposure. After the treatment the cells were lysed with Laemmli buffer (60mM Tris-HCl, ph 6,8; 2% (w/v) SDS, 10 mM EDTA, 10% (w/v) glycerol) and protein concentration was determined with a BCA detection kit (Pierce, USA). The samples were added with β-mercaptoethanol and bromophenol blue, boiled for 5 min, clarified at 10000 × g for 10 min, run on 8% SDS-PAGE and transferred to PVDF membranes (Amersham Bioscience, UK). After blocking with 5.0% (w/v) BSA in 0.1% (v/v) PBS-Tween-20, the membranes were incubated

overnight at 4 °C with anti-Poly (ADP-ribose) (PAR) 1:1000 to detect the expression of parylated proteins. In another experiment N2a treated with 100 μM OLE for 24h were also analysed for SIRT1 protein expression. Rabbit anti-SIRT1 (Proteintech) was used for the analysis. The day after the membranes were incubated for 1.0 h with specific secondary antibodies (1:10000, goat anti-rabbit antibody and goat anti-mouse antibody, Molecular Probes, Life Technologies); the immunoreactive bands were detected with the Immobilon Western Chemiluminescent HRP and quantified by densitometric analysis using a ChemiDoc system and the Quantity One software (Bio- Rad Laboratories, Italy). The bands were normalized to β actin, used as loading control.

2.3. Measurement of nicotinamide adenine dinucleotide (NAD+)

Since PARP1 activation requires NAD+ as substrate, N2a cells treated with 100 μM MNNG for 90 min or with 100 μM OLE for 24h before MNNG (90 min) were analysed for NAD+ levels. NAD levels were measured by means of an enzymatic cycling procedure according to Buonvicino D. et al. (Buonvicino D et al, 2013). Briefly, cells grown in a 48 well plate were killed with 50 μl HClO41N and then neutralized with an equal volume of KOH 1N. After the addition of 50 μl of bicine 50 mM, 100 μl of the cell extract was mixed with an equal volume of the bicine buffer containing 23 μl/ml ethanol, 0.17 mg/ml MTT, 0.57 mg/ml phenazine ethosulfate and 20 μg alcohol dehydrogenase. Mixture was kept at room temperature for 10 minutes and then absorbance a 550 nm was measured.

2.4 Data analysis

Statistical analysis was performed using One way ANOVA followed by Bonferroni post-hoc test and the significance was defined $p < 0.05$. Data are reported as mean ± standard error of the mean (S.E.M)

3)OLE-enhanced autophagic flux ameliorates oxidative stress injury in cardiac cells.

In *vitro* study

For *in vitro* study, naonatal rat cardiomyocytes were used. Autophagy induction was investigated using Cyto-ID Autophagy detection Kit (detection of autophagic vacuoles) and western blot analysis (expression of Lc3 II, autophagy marker). Monitoring autophagic flux was performed using the tandem RFP-GFP-LC3 construct (immunofluorescence analysis) and the LC3 turnover assay in presence or absence of CQ (western blot analysis). TFEB translocation was analyzed by immunfluorescence using anti-TFEB antibody.

3.1 Primary cultures of cardiomyocytes.

Neonatal rat cardiomyocytes were isolated from hearts of Sprague-Dawley Neonatal rats (1–2 days). The rats were euthanized by decapitation, hearts excised and atria were removed. The ventricles isolated were washed with a solution of ADS buffer (NaCl 1,16 M, HEPES 200 mM , NaH_2PO_4 10 mM, Glucose 55 mM, KCl 3,4 mM , MgSO4 8,3 mM) and then cut into small pieces with a scalpel. The washing solution was aspirated and the ventricles transferred in falcon were then incubated for 20 minutes at 37° with an enzymatic solution containing 0,04% collagenase (Roche Applied Science) and 0,05% pancreatine (Sigma Aldrich) diluted in ADS buffer. At this point two phases were observed: precipitate and supernatant.

The following steps can be summarized as follows:

1) The pellet was incubated again with the previous enzymatic solution (collagenase+pancreatine) at 37° for 20 minutes.

2) The supernatant was recovered and incubated in 1 ml of newborn calf serum (NCS, Gibco) and then centrifuged at 250g for 6 min. After centrifugation the supernatant was removed and the pellet was resuspended in 2 ml of NCS and then incubated at 37 ° C (the latter represents the cellular suspension).

These steps (1,2) were repeated 4 to 5 times until complete digestion of the ventricles. All cell suspensions were then pooled and centrifuged again at 250g for 6 min to

remove the NCS and the pellet was resuspended in ADS 1x. To specifically isolate cardiomyocytes, a Percoll gradient was used. The Percoll, consisting of silica gel, was diluted to obtain a denser Percoll solution (Bottom:Percoll (58%)) and a less dense (Top:Percoll (41%)). The cell suspension was placed on the gradient and then centrifuged for 30 min at 950g. After 30 min, each cell type was separated on the gradient by different density (Figure 18). After removing fibroblasts by aspiration, the cardiomyocytes were recovered. The fraction of cardiomyocytes was resuspended in ADS and then centrifuged for 6 minutes at 250g to remove the Percoll. The pellet, containing the cardiomyocytes, was then resuspended in culture medium (Plating medium) composed by 68% DMEM + Glutamax (Gibco), 17% Medium 199 (Gibco) supplemented with 10% horse serum (Invitrogen), 5% NCS (Invitrogen) and 1% antibiotics (penicillin / streptomycin). Cardiomyocytes were finally seeded into wells previously coated with 0.1% gelatin (Sigma). The day after the isolation the medium was changed and replaced with complete Medium (HAM F12, 10% FBS, 10%Horse Serum, 1% penicillin/streptomycin). All procedures for neonatal rat ventricular myocytes (NRVMs) isolation were performed in accordance with the Guide for the care and use of laboratory animals.

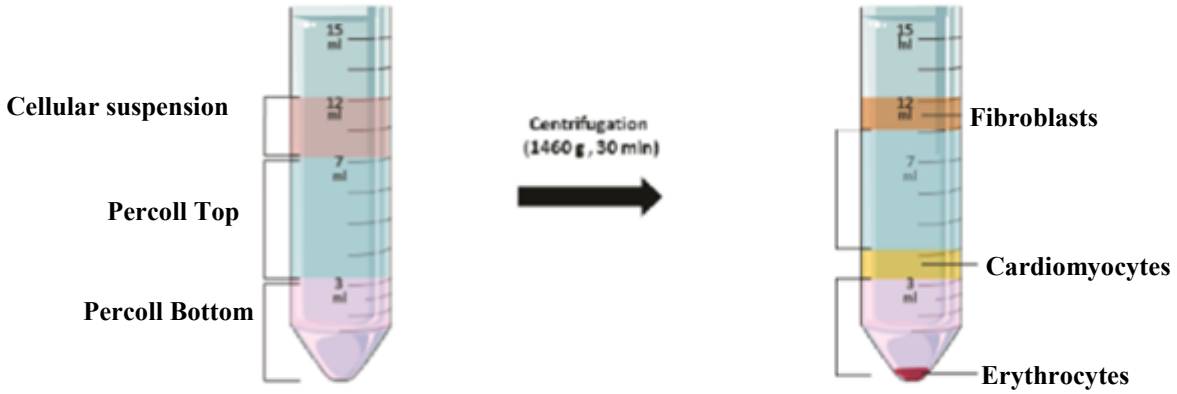


Figure 18 Isolation of neonatal rat cardyomyocites on gradients of Percoll. The Percoll, consisting of silica gel, is diluted to obtain a denser Percoll solution (Bottom : Percoll (58%)) and a less dense (Top: Percoll (41%)). These two solutions are deposited one on the other to obtain a density gradient. The difference in density of different cell types separates them into three rings (erythrocytes at the bottom, cardiomyocytes in the middle, fibroblasts on the top)

3.2 Cytotoxicity assay

MTT, LDH and DCFDA were used to assess the potential OLE toxicicy at the concentrations used for the experiments

3.2a MTT test

MTT is a cell viability test using 3-(4,5,dimethylthiazol-2-yl)-2,5 dipheniltetrazolium-bromide (MTT) , a compound yellowish in solution. The MTT substrate is prepared in a physiologically balanced solution, added to cells in culture, usually at a final concentration 0.5mg/ml, and incubated for 1 to 4 h. Mitocondrial dehydrogenases of viable cells cleave the tetrazolium ring converting MTT into purple colored formazan cristals which are insoluble in aqueous solution (Figure 19). The formazan crystals are then solubilized in DMSO and the resulting purple solution is measured spectrophotometrically. The quantity of formazan (presumably directly proportional to the number of viable cells) is measured by recording changes in absorbance at 570 nm. The exact mechanism responsible of MTT reduction into formazan is not well understood, but probably NADH or similar reducing molecules that transfer electrons to MTT are involved (Marshall et al, 1995). Neonatal rat cardiomyocytes were seeded into 96-well plates at a density of 50.000 cells/well. The day after the medium was replaced with fresh complete medium (HAM F12, 10% FBS,10%Horse Serum,1% penicillin/streptomycin) and the cells were treated with OLE 50μM and 100 μM for 6h. At the end of the exposition the culture medium was removed and the cells were incubated with 200μl of MTT solution (0,5mg/ml) for 3h at 37°. After the incubation, MTT solution was aspirated and 200μl DMSO was added to solubilize the formazan crystals precipitated. The absorbance of the blue formazan was read at 570 nm using spectrophotometric microplate reader.

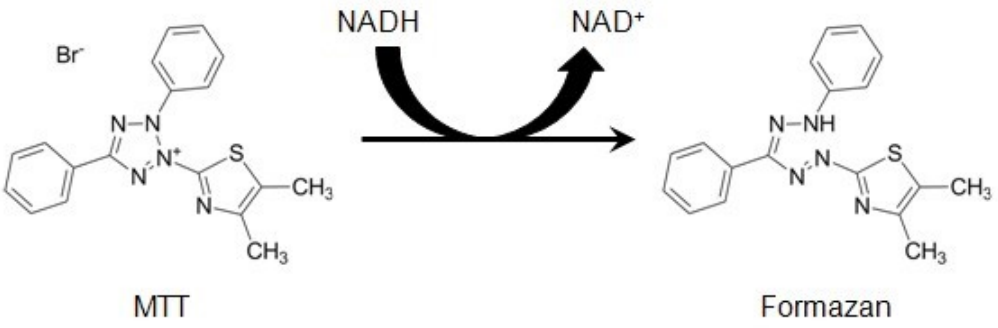


Figure 19: Conversion of MTT in colored formazan product (Riss, T.L. et al, 2013)

3.2b DCFDA assay

ROS generation in neonatal rat cardiomyocytes was evaluated using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA). H2DCFDA is a chemically reduced form of fluorescein used as an indicator for reactive oxygen species (ROS) in cells. In presence of ROS, the cleavage of the acetate groups by intracellular esterases and oxidation converts the non-fluorescent H2DCFDA into the highly fluorescent 2',7'-dichlorofluorescein (DCF). Oxidation of this probe can be monitored by increase in fluorescence. Neonatal rat cardiomyocytes were seeded at a density of 50.000 cells/well in 96-wells dark plate and growth for 24h. The day after the cells were treated with OLE 50µM and 100µM for 6h before the incubation with DCFDA (7,5µM) in complete medium for 30-40 minutes at 37° in dark. After the incubation, the medium was replaced with HBSS and the fluorescence value at 535 nm was detected by Fluoroscan Ascen FL (Thermo-Fisher).

3.2c LDH test

Lactate Dehydrogenase (LDH) assay is a colorimetric method to measure quantitatively the release of LDH into the media from damaged cells as a biomarker of cellular cytotoxicity and cytolysis. Extracellular LDH present was measured using LDH cytotoxicity Assay kit according to the manufacturer's instructions, based on a coupled enzymatic reaction. First, LDH catalyzes the conversion of lactate to pyruvate via

reduction of NAD+ to NADH. Second, diaphorase uses NADH to reduce a tetrazolium salt (INT) to a red formazan product that can be measured at 490 nm. Therefore, the level of formazan formation is directly proportional to the amount of released LDH in the medium, which is indicative of cytotoxicity (Figure 20)

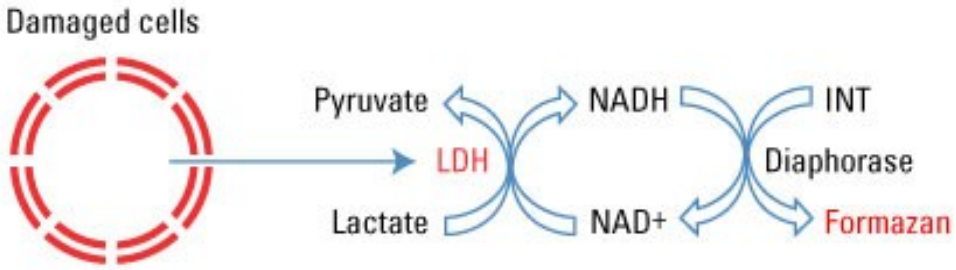


Figure 20: Mechanism of LDH cytotoxicity assay

Neonatal rat cardiomyocytes were seeded at a density of 200.000 cells/well in 24-well plates and growth for 24h. The day after the Plating medium was replaced with complete medium and the cells were treated with OLE 50µM and 100 µM for 6h. A complete medium without cells was used as a control to determine LDH activity present in serum used for medium supplementation. At the end of treatment 50 µl of supernatant of each sample was transferred into a 96 well-plates and mixed with 50µl reaction mixture prepared dissolving the lyophilized substrate mix present in the kit with ultrapure water and combining it with Assay buffer; the resulting solution was protected from light until use. After 30 minute room temperature incubation with reaction mixture , the reaction was stopped by adding 50 µl Stop solution. Absorbance at 490nm and 680 nm was measured using a plate-reading spectrophotometer. The 680 nm absorbance value (background) was subtracted from the 490nm absorbance to determine LDH activity (Figure 21).

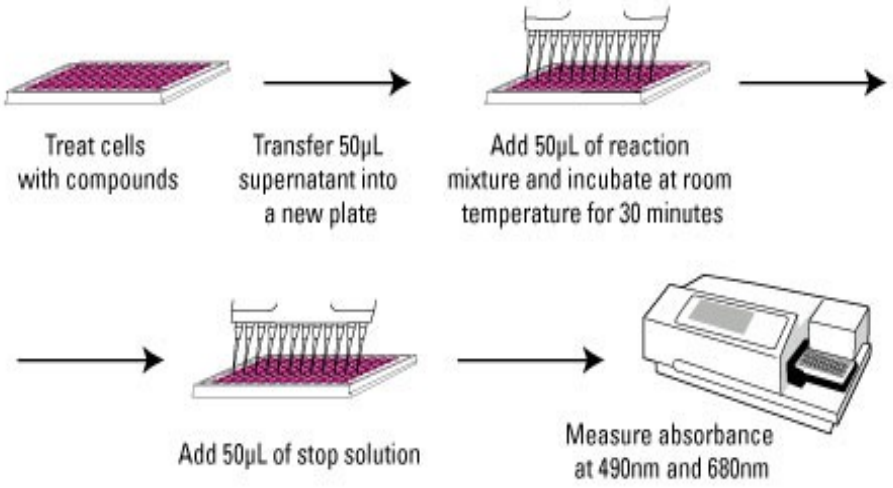


Figure 21: Procedure for LDH cytotoxicity Assay using The Thermo Scientific Pierce LDH Cytotoxicity Assay Kit

3.3. Autophagic vacuoles detection in neonatal rat cardiomyocytes

For autophagic vacuoles analysis a Cyto-ID autophagy Detection Kit was used (see 1.3 Methods). Cardiomyocytes were seeded in 96 well plate black at a density of 50.000 cells/well. The day after the cells were treated with OLE 100µM for 1h and 3h in complete fresh medium. Control cells were treated with DMSO, solvent used to solubilize OLE. After the treatment the medium was removed and the cells were washed twice with 100 µl Assay buffer 1x supplemented with FBS5%. The cells were then incubated with dual color detection solution (100 µl/well) for 30 min at dark, prepared by adding for every 1ml of 1x Assay buffer (supplemented with 5% FBS) 2µl of Cyto-ID Green Detection Reagent and 1 µl of Hoechst 33342 Nuclear staining. After two washing steps with 100µl Assay buffer, the cells were analysed by fluorescence microscopy. The increase in Cyto-ID green dye fluorescence was used as index of autophagy induction.

3.4 Cardiomyocytes treatment for western blotting

In parallel with autophagic vacuoles staining, western blot analysis was performed to analyse autophagy-specific markers (LC3II) expression. The cells were treated with OLE 100 µM for 1h and 3h in complete fresh medium. Control cells we treated with the

same concentration of DMSO used to solubilize OLE. In some experiments the cells were also treated with CQ (lysosomal inhibitor) and with CQ plus OLE to check OLE induced efficient autophagic flux. After the treatment the cells were lysed in RIPA buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) and equal quantity of proteins was resolved by SDS-PAGE and transferred to a PVDF membrane (Trans-blot turbo transfer system, Bio-Rad). After blocking with 5.0% (w/v) BSA in 0.1% (v/v) PBS-Tween-20, the membranes were incubated overnight with anti-Lc3 B antibody (Cell signalling Technology). After washing steps and incubation for 1h with 1:10000 goat anti-rabbit antibody the immunoreactive bands were detected by Chemiluminescence with a Bio-Rad Chemidoc XRS$^+$ camera. Relative densities were quantified using the ImageLab 4.0 software (Bio-Rad). β actin was used as loading control.

3.5 Monitoring autophagic flux by the tandem RFP-GFP-LC3 construct.

Plasmid transfection with RFP-GFP-LC3 is used to monitor the various stages of autophagy (through LC3B protein localization) in OLE-treated cells. This assay is based on the pH difference between autolysosome (acid) and the autophagosome (neutral) through pH sensitivity difference of GFP (green fluorescent protein) and RFP (red fluorescent protein). GFP fluorescence is quenched at acidic pH, making difficult to trace the delivery of GFP-LC3 to lysosomes, in contrast the RFP fluorescence is more stable at acidic compartment and RFP-LC3 can easily be detected in autolysosomes. So autophagosomes are labelled with yellow puncta (GFP and RFP signal), autolysosomes with red puncta (RFP signal). If autophagic flux is increased both yellow and red puncta are increased, on the contrary the only autophagosomes formation or the block of autophagosomes maturation into autolysosomes are detected as yellow puncta. (Mizushima et al, 2010). To vehicle RFP-GFP-LC3 into the cells, Lipofectamine 2000 reagent (Life technologies) was used. Neonatal rat cardiomyocytes were seeded at a density of 200.000 cells/well in four-well Labtek chamber slides and growth for 24h. The day after plasmid transfection with RFP-GFP-LC3 was performed. The ratio lipofectamine/plasmid used was 1μg plasmid/2μl lipofectamine. First plasmid and lipofectamine were separately diluted in Optimem-glutamax, then they were gentle mixed together and the solution containing the complex was incubated for 10 minutes at

room temperature. The medium was removed and the cells were incubated with transfection solution (200µl/well) for 2h. The solution was then removed and complete medium was added. After 48h the cells were treated with OLE 100µM for increasing times (1h, 3h, 6h). Control cells were treated with DMSO at the same concentration used to solubilize OLE. At the end of the treatment, the cells were washed two times with PBS, fixed 5 minutes in paraformaldehyde 4%, washed again and analysed for autophagic flux using fluorescence microscopy.

3.6 Transfection of cardiomyocytes with MAO-A adenovirus.

Neonatal rat cardiomyocytes were seeded into 96-well plates at a density of 50.000 cells/well for MTT test or into 24-well plates at a density of 200000 cells/well for LDH test . The day after adenoviral infection with replication-deficient adenoviral vector expressing MAO-A MOI-1(MOI1: multiplicity of infection or number of virus particles for cardiomyocytes) was performed. Infections were made at a certain MOI based on this definition, e.g $1*10^6$ bav (banna virus)/$1*10^6$ cell. The adenovirus was diluted in complete medium and distributed into the wells. After 2h the wells were replaced with new fresh medium. The day after the cells were treated with the MAO-substrate Tyramine (500µM) for 6h to induce oxidative stress or pretreated with 100 µM OLE for 1h before tyramine treatment. At the end of treatment MTT and LDH test were performed as previously reported in 3.2a and 3.2c Methods.

3.7 Analysis of TFEB translocation by immunofluorescence

TFEB translocation in OLE-treated cells was analyzed by immunfluorescence using anti-TFEB antibody. Neonatal rat cardiomyocytes were seeded at a density of 200000 cells/well in four-well Labtek chamber slides. The day after the cells were treated with 100 µM OLE for 30 minutes in complete medium. After washing once in PBS, the cells were fixed in paraformaldehyde 4% (5 minutes), washed again and incubated with TBS-0,2% -Triton for 10 minutes at room temperature (500µl/well). The cells were then blocked with TBS-3% BSA 1h at room temperature and incubated overnight with anti-TFEB antibody (Bethyl Laboratories), diluted 1:400 in blocking solution. The immunoreaction was revealed using Alexafluor 546 goat antirabbit (diluited 1:1000). After washing steps finally the slides were mounted with cover-slip with Mounting

Medium containing DAPI and analysed for TFEB translocation to the nucleus using fluorescence microscopy.

3.8 Statistical analysis

Results are expressed as mean± standard SEM. Experimental groups were compared using one-way ANOVA and Tukey post-hoc test was used. A value of $p < 0,05$ was considered significant.

Results

1) Molecular mechanisms of OLE-induced autophagy: *in vitro* and *in vivo* study.

Previous studies performed in our laboratory have highlighted the benefits of OLE in both murine neuroblastoma N2a cells and in TgCRND8 mice (Grossi et al, 2013). In the latter, food supplementation with OLE countered Aβ toxicity, leading to a remarkable reduction of plaque load and improved cognitive performance. These protective effects were mainly related to the activation of a strong autophagy response (Grossi et al, 2013; Luccarini et al, 2015).

In this work, we decided to explore more in depth the molecular and cellular mechanisms of OLE-induced autophagy, both *in vitro* and *in vivo*, using cultured human neuroblastoma cells SH-SY5Y and TgCRND8 mice.

1.1. OLE induces a biphasic increase in AMPK phosphorylation in SH-SY5Y cells.

To investigate at which level OLE activated the autophagic cascade, we took benefit of what was known regarding plant polyphenol induction of autophagy. Indeed, increasing evidences suggest that polyphenols, such as resveratrol and EGCG, induce autophagy by increasing intracellular Ca^{2+} with subsequent activation of AMPK by CamKKβ (Figure 22). Therefore, our aim was to assess if the molecular and cellular mechanisms of OLE-induced autophagy was similar to that reported for other polyphenols. Towards this goal we exposed SH-SY5Y to OLE 50μM for 24h, condition under which we previously showed the activation of autophagy by OLE in N2A cells (Grossi et al, 2013), and then we checked for Beclin1 (early marker of autophagy induction) protein expression and AMPK phosphorylation by western-blotting analysis. After 24h of OLE treatment, we observed an increase in Beclin1 protein expression but no increase in AMPK phosphorylation (Figure 22 panel A). To evaluate a potential early and transient OLE-mediated AMPK activation, we exposed SH-SY5Y for 4h to OLE treatment, and we checked autophagy induction at this time using Cyto-ID detection kit. As shown in Fig. 22 panel B, the number of autophagic vacuoles, detected as green fluorescent puncta, was increased in OLE treated cells even at this short-time of treatment. At this point we decided to analyse the level of AMPK phosphorylation within a rapid time frame kinetic after OLE treatment. Western blot analysis showed a

biphasic increase in AMPK phosphorylation under these conditions, with two peaks after 10 minutes and 4h of OLE-treatment, suggesting that AMPK was involved early in OLE-autophagy induction (Figure 22 panel C)

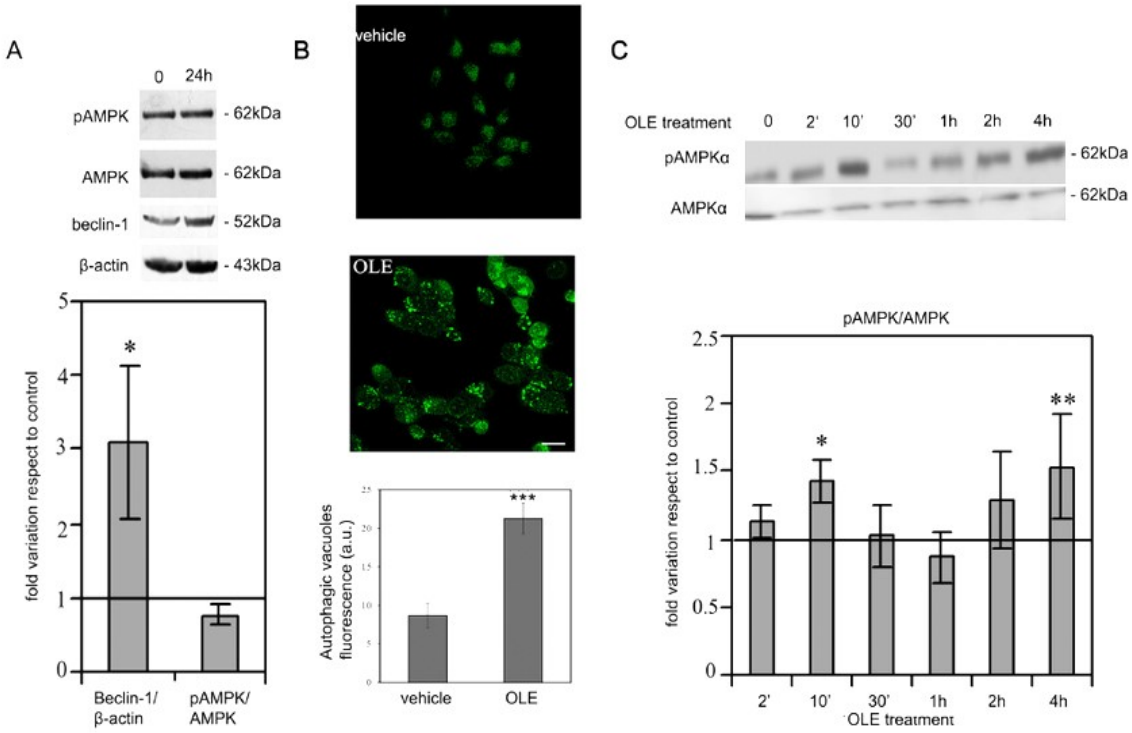


Figure 22: OLE induces autophagy mediated by a biphasic increase in AMPK phosphorylation after short time treatment times. (**A.)** (Upper panel) SH-SY5Y were treated with OLE 50μM for 24h, then Beclin1 expression and AMPK phosphorylation were analysed by western blotting. β actin was used to normalize Beclin1 expression. (Lower panel) Quantitative analysis. (**B)** SH-SY5Y treated with OLE 50μM for 4h showed an increase in autophagic vacuoles (green fluorescence). Autophagic vacuoles staining was performed using Cyto-ID autophagy Detection Kit. (Lower panel) Quantitative data.(**C)** (Upper panel) OLE induces a significant increase of AMPK phosphorylation at 10 minutes and 4h of treatment, as shown by time-course analysis of OLE-treated cells for increasing time periods. (Lower panel) Quantitative data. The data are represented as Mean ± S.E.M. The asterisk (*) indicate a statistical significance difference with respect to vehicle-treated cells * P < 0,05 **P< 0,01 ***P< 0,001. n:3. Scale bar: 25 μm

1.2 OLE induces a biphasic increase in the intracellular Ca^{2+} levels.

Two upstream kinases have been identified as responsible of AMPK phosphorylation: the serin/threonin protein kinase LKB1 and the $Ca^{2+/}$calmodulin dependent protein kinase kinase β, CamKKβ. While LKB1 is activated by an increase in AMP:ATP ratio in the cells, the activation of AMPK by Camkkβ is only dependent on intracellular increase of Ca^{2+} (Ma et al, 2012). Since other plant polyphenols such as resveratrol and EGCG are known as CamKKβ activators (Kim et al, 2013; Zhou et al, 2014), we investigated if Camkkβ was also involved in our conditions. Considering that CamKKβ is dependent on free Ca^{2+}, the first step was to investigate the levels of cytosolic Ca^{2+} in OLE treated cells. Using the calcium-sensitive fluorescent probe Fluo-3AM, we observed an early increase in cytosolic Ca^{2+} that followed a biphasic kinetic: a first peak after 5-10 minutes of OLE treatment followed by a second peak between 1-2h; 5h after treatment, the levels of Ca^{2+} were similar to baseline. The time points at which we observed the two peaks of Ca^{2+} increase seemed immediately precede the increase in AMPK phosphorylation, suggesting a temporal correlation between increase in cytosolic Ca^{2+} and AMPK phosphorylation in OLE-treated cells (Figure 23)

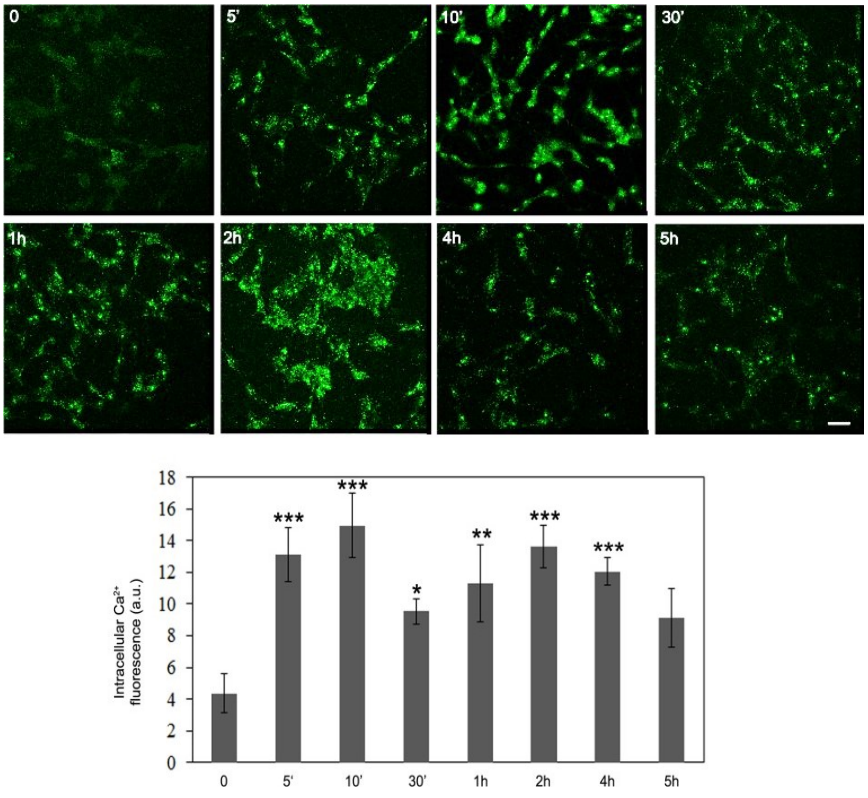


Figure 23) OLE induces a biphasic increase in Ca^{2+} intracellular during short-treatments. SH-SY5Y were treated with OLE 50 μM for increasing time periods. Ca^{2+} levels were detected using Fluo-

3AM. Asterisks (*) indicate significant statistical differences with respect to time zero *P < 0,05 **P< 0,01 ***P< 0,001 n:3. Scale bar: 25 μm.

1.3. OLE induces release of Ca^{2+} from endoplasmic reticulum (ER) after short treatment times.

We next decided to focus on the early peak of Ca^{2+} increase and to check whether it came from extracellular medium or it was released from intracellular storage. To this end, cells were incubated for 30 minutes with a cell impermeant Ca^{2+} chelator (4.0 mM EGTA) or with an inhibitor of the endoplasmic reticulum Ca^{2+} pump (40 μM CPA), which depletes Ca^{2+} from ER storage, before OLE treatment for 10 minutes. We found that only the pre-treatment with CPA was able to abolish the increase of Ca^{2+} induced by OLE; in contrast, EGTA did not interfere with Ca^{2+} release. These data strongly suggest that OLE induced a release of Ca^{2+} from ER after short time of treatment. These first experiments supported our hypothesis confirming that in OLE-treated cells AMPK phosphorylation paralleled Ca^{2+} release from the ER, suggesting an involvement of CamKKβ in the autophagic cascade (Figure 24).

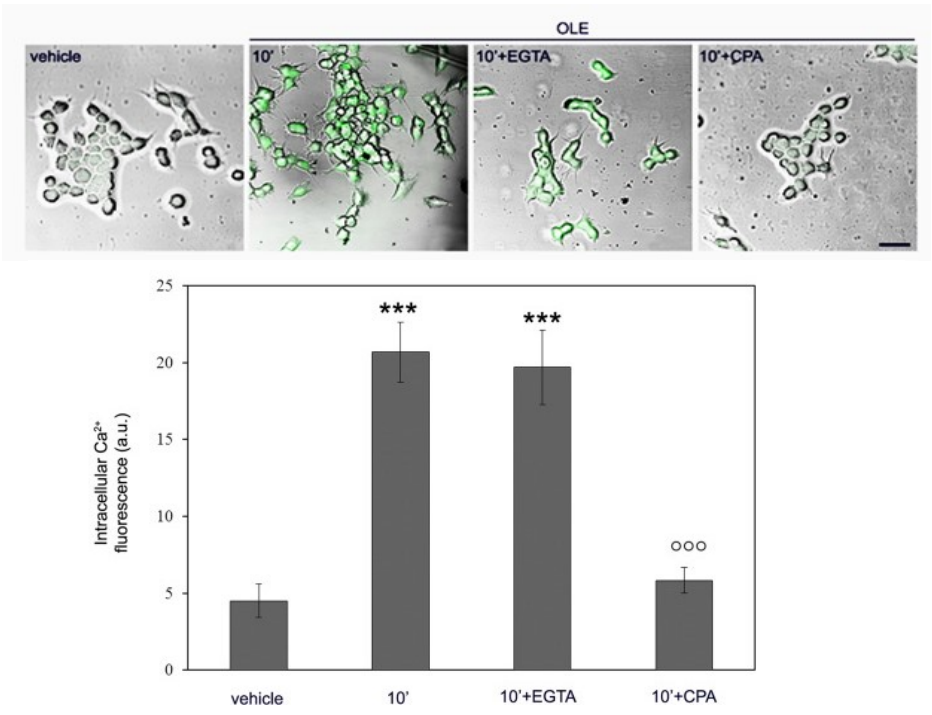


Figure 24) OLE induces a release of Ca^{2+} from intracellular stores after 10 minutes of cell stimulation.SH-SY5Y were pretreated with CPA or EGTA before OLE (50μM) stimulation for 10

minutes. Cytosolic calcium levels were detected using Fluo-3AM probe and the fluorescent signal was superimposed to phase-contrast images of the cells. The asterisk (*) indicate a statistical significance difference with respect to vehicle-treated cells, circles (°) indicate a statistical significance difference with respect to OLE-treated cells ***P< 0,001; °°° P< 0,001 n:3. Scale bar: 25 μm

1.3 CamKKβ plays a pivotal role in OLE-autophagy induction

To investigate the involvement of CamKKβ in OLE-triggered AMPK activation, SH-SY5Y were pre-treated with STO-609 (CamKKβ inhibitor), before OLE treatment for 10 minutes and 4h, at which points the level of AMPK phosphorylation was analysed by western blotting. As shown in Figure 25, CamKKβ inhibition abolished the induction of AMPK phosphorylation compared to OLE-treated cells. This result suggested a pivotal role of CamKKβ in OLE-autophagy induction. These first experiments showed that OLE induced a rapid release of Ca^{2+} from ER after 10 minutes of treatment, which subsequently led to CamKKβ activation and AMPK phosphorylation.

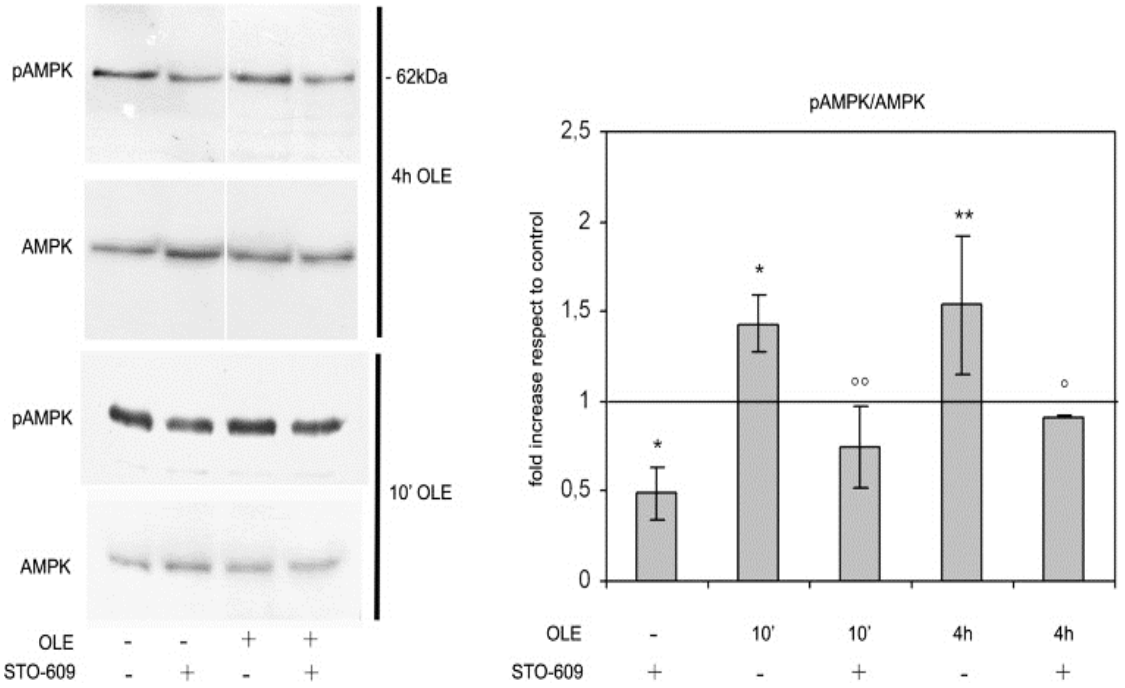


Figure 25): OLE-induced AMPK phosphorylation is dependent from CaMKKβ signalling. (Left). SH-SY5Y cells were incubated with STO-609 (Camkkβ inhibitor), before OLE treatment for 10 minutes and 4h and then the levels of AMPK phosphorylation were analyzed by immunoblotting. (Right) Quantitative analysis. The asterisks indicate a statistically significant difference respect to vehicle treated

cells, the circles indicate statistically significant differences with respect to OLE-treated/STO-609-untreated cells. *$P < 0.05$, ** $P < 0.01$, ° $P < 0.05$, °°$P < 0.01$

1.4 Short time cell treatment with OLE efficiently triggers autophagy

At this point, we aimed to determine whether a short-time treatment with OLE was sufficient to trigger autophagy and, if so, whether Ca^{2+} increase and CaMKKβ activation could also be involved. Toward this end, cells were pre-treated with 4.0 mM EGTA, 40 μM CPA or 13.3 μM STO-609 for 30 min and then incubated with OLE for 10 min. The cells were then washed with PBS and incubated for 5h in complete fresh medium, after which we analysed autophagic vacuoles. Under these conditions we still observed an increase in autophagic vacuoles in OLE treated cells, suggesting that a short time exposure to OLE is sufficient to stimulate the induction of autophagy. In addition cells pre-treated with CPA and STO-609 before OLE-treatment did not exhibit autophagic vacuoles confirming that release of Ca^{2+} from ER and CaMKKβ activation were essential for OLE-mediated induction of autophagy. On the contrary and as expected, the pre-treatment with EGTA did not blocked the induction of autophagy, suggesting a minor role of extracellular calcium in this signalling (Figure 26). However, since these experiments were focused on short time of OLE treatment, it is not possible to exclude a role of extracellular calcium at longer time after OLE treatment.

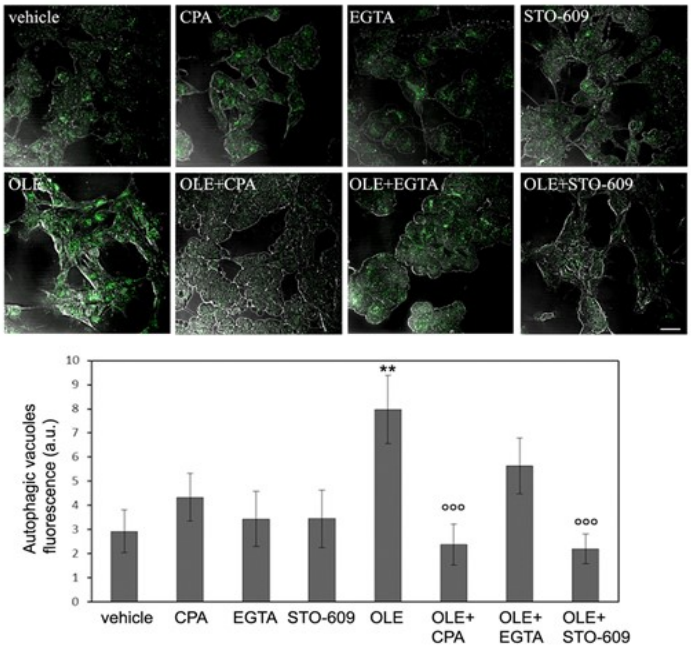


Figure 26) Short time of OLE-treatment efficiently induces autophagic cascade. (Upper panel) SH-SY5Y cells were incubated with STO-609, CPA or EGTA before treatment with OLE for 10 minutes.

Then the cells were washed and incubated for 5 h in complete fresh medium. At the end of the treatment autophagy induction was measured with Cyto-ID autophagy detection kit**.** Scale bar: 30 μm. (Lower panel) Quantitative data. The asterisks indicate astatistically significant difference respect to vehicle treated cells, the circles indicate statistically significant differences with respect to OLE-treated **P < 0.01, °°° P < 0.001 n = 3

These results were confirmed by analysing the time course of Beclin-1 expression after OLE treatment, which showed an increase after 30 minutes of treatment. (Figure 27). Considering the short time of OLE treatment, we reckoned that the increase in Beclin-1 expression did not result from an increased synthesis but rather could result from the dissociation of the complex Beclin1-Bcl2 in the cytoplasm. Once dissociated Beclin1 interacts with Vps34 and the Beclin-1/Vps34 complex is essential for both autophagosome biogenesis and maturation. A second and higher increase in Beclin1 protein expression was observed after 24h of treatment and was probably related to neo-synthesis (Figure 22 panel A).

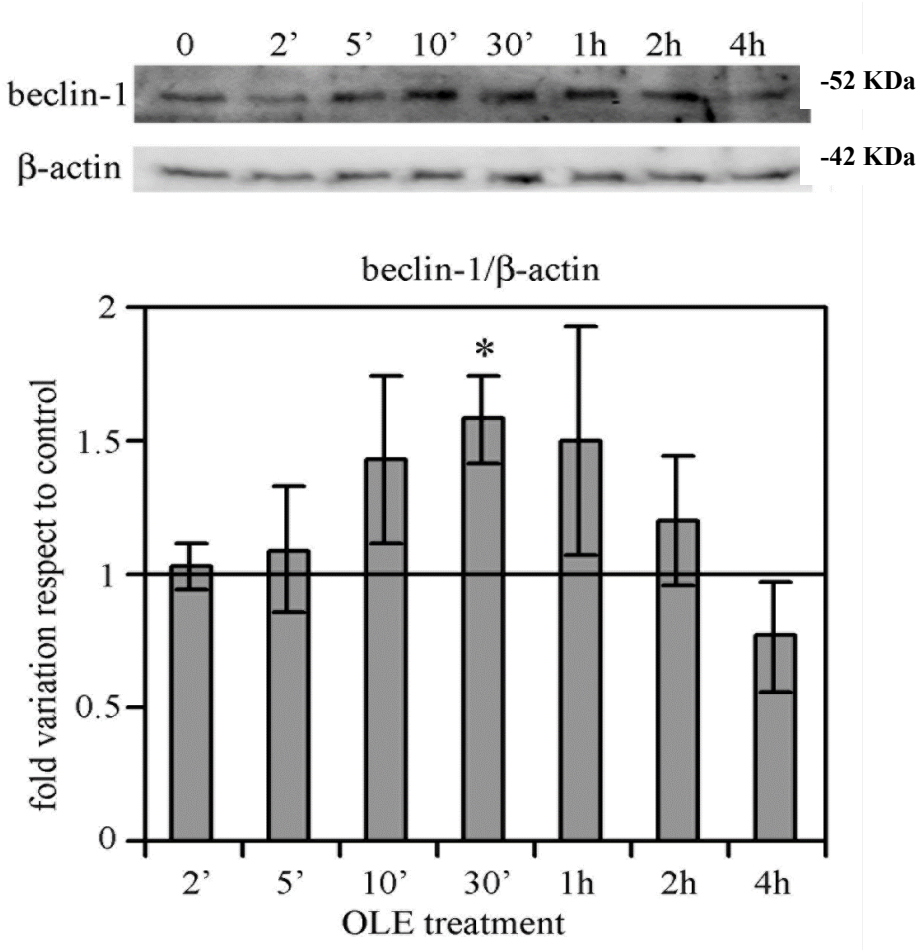


Figure 27). Time course of Beclin1 protein expression after short time of OLE treatment. (Upper panel) Time course of Beclin-1 expression during short time treatments with OLE. β-actin was used to

normalize Beclin-expression. (Lower panel) Quantitative analysis. The asterisks (*) indicate statistically significant differences with respect to vehicle treated cells. * P < 0,05

1.5. AMPK activation is required for autophagy induction by OLE

To confirm the pivotal role of AMPK in OLE-induced autophagy, we pre-treated the cells with compound C (CC), the only known AMPK-specific inhibitor, before OLE treatment. Unexpectedly, autophagy was still induced under these conditions, although at lesser extent. (Figure 28). This unexpected observation could in part be explained by the fact that compound C is an AMPK-independent autophagy activator *per se* in this cell line as well as in other cancers cells (Vucicevic et al, 2011; Liu et al, 2014).

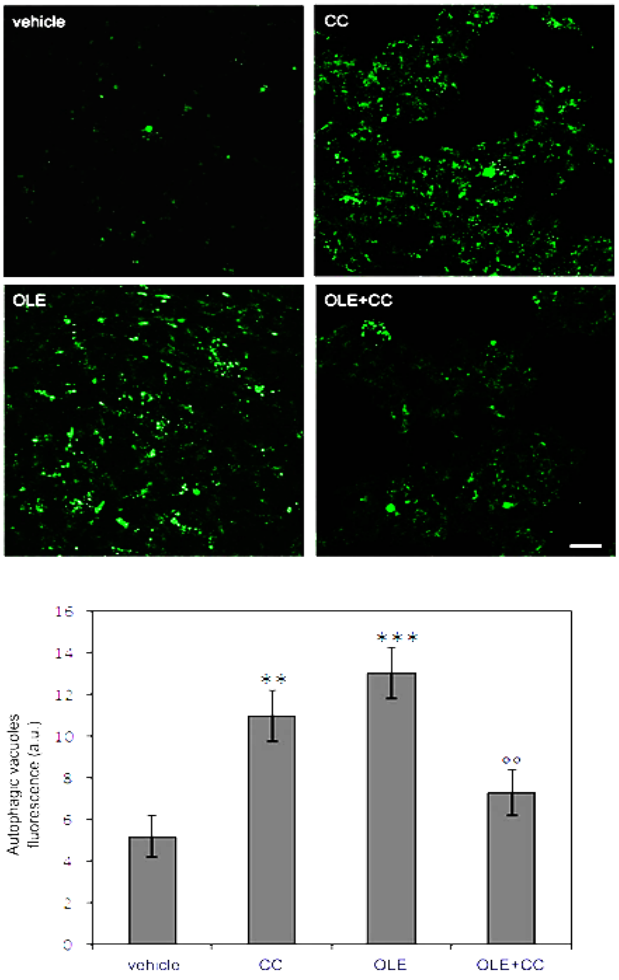


Figure 28 The compound C, AMPK inhibitor, induces autophagy *per se* in SH-SY5Y and partially inhibits OLE-induced autophagy. (Upper panel) SH-SY5Y were treated with OLE 50 µM in the

presence or in the absence of compound C. Autophagic vacuoles were labelled with Cyto-ID Autophagy detection kit. Scale bar 25 μm. (Lower panel) Statistical analysis. The asterisks (*) indicate significant difference compared to vehicle-treated cells, the circles indicate a statistical significant difference compared to OLE-treated cells **P < 0.01, ***P < 0.001, °°P < 0.01 n :3

Therefore we concluded that SH-SY5Y was not the ideal model to study the role of AMPK in OLE-induced autophagy and we looked for another CC-insensitive cell line. After screening different cell lines, we identified the rat pancreatic insulinoma RIN-5Fcells, in which CC does not activate autophagy on its own. In this cell line, OLE induced autophagy similarly to what observed in SH-SY5Y. However and contrary to what we observed in SH-SY5Y, compound C blocked OLE-induced autophagy (Figure 29A). These results were also confirmed by western blot analysis of LC3II, another marker of autophagy (Figure 29B).

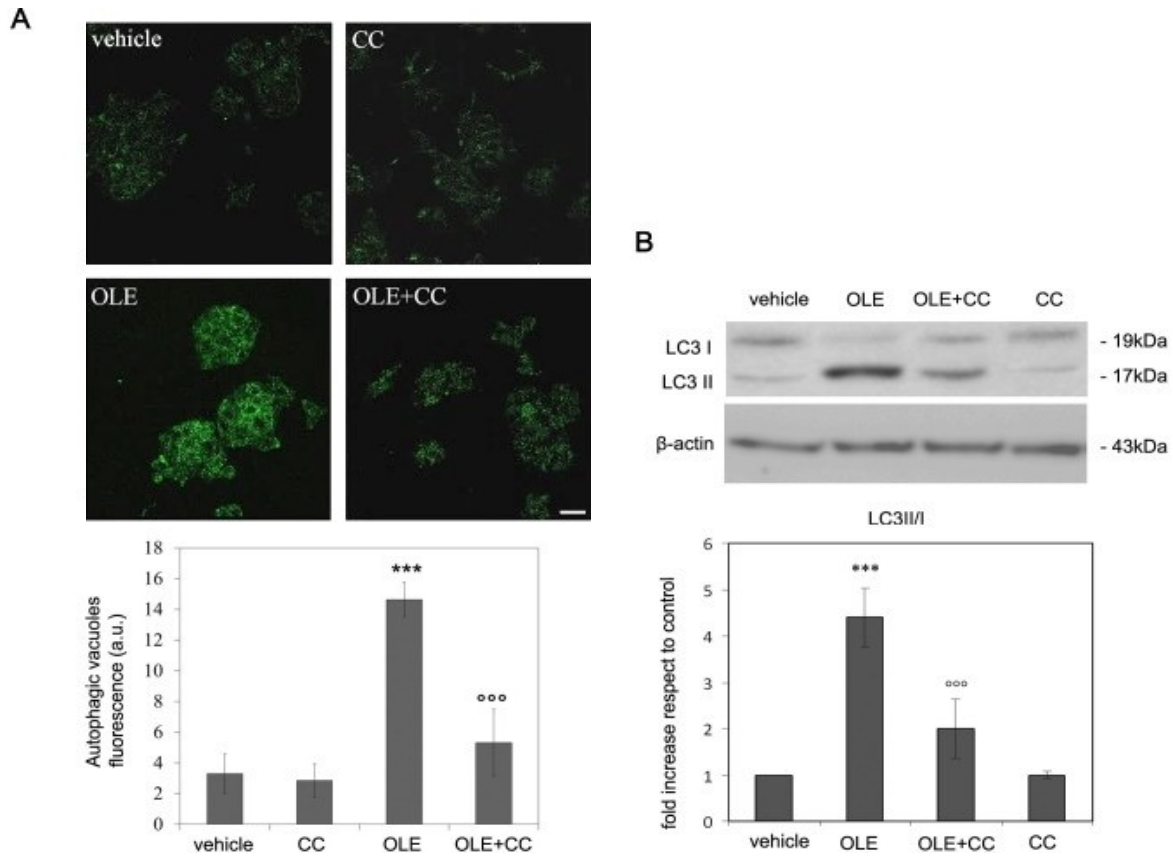


Figure 29) CC inhibits OLE-induced autophagy in RIN-5F cells. RIN-5F cells were incubated with compound C (CC) before OLE treatment for 4h. **A** (Upper panel) Autophagic vacuoles were detected with Cyto ID Autophagy Detection Kit. (Lower panel) Statistical analysis. Scale bar 25 μm. **B.** (Upper panel) RIN-5F treated with OLE plus CC were also analysed for LC3 protein expression by immunoblot.

(Lower panel) Quantitative data. The asterisks (*) indicate significant difference compared to vehicle-treated cells, the circles indicate a statistical significant difference compared to OLE-treated cells ***P < 0.001; °°P < 0.01 n :3

Taken together these experiments showed that the activation of AMPK played an essential role in OLE-induced autophagy, supporting the hypothesis that OLE activated autophagy through the Ca^{2+}-CaMKKβ-AMPK pathway.

1.6 OLE inhibits mTOR through AMPK activation in TgCRND8 mice.

To confirm our *in vitro* results, we decided to investigate the involvement of mTOR and AMPK in OLE-induced autophagy in the cortical tissue of 6/12 month-old TgCRND8 mice fed with OLE for 8 weeks (50 mg/Kg diet). Since no age-related differences were observed between 6 month- and 12 month-old animals, the two groups were pooled for this analysis to increase statistical power. Immunohistochemical analyses showed that the levels of both phospho-mTOR (Figure 30A) and the phosphorylated mTOR substrate p70-S6K (Figure 30B) were markedly increased in the cortex of TgCRND8 untreated mice compared to controls. In marked contrast, the levels of both phospho-mTOR and phospho-p70-S6K were reduced to the level of control when TgCRND8 mice received OLE diet. Therefore, these data support the hypothesis that autophagy activation by OLE proceeds through mTOR inhibition. We next wondered if mTOR inhibition occurred in parallel to AMPK activation. To this end, we checked in these same conditions the levels of AMPK phosphorylation. AMPK phosporylation was reduced in TgCRND8 while increased in TgCRND8-treated animals. (Figure 30C). Taken together these results indicate that in the cortex of OLE-fed TgCRND8 autophagy activation proceeds through AMPK activation and mTOR inhibition. These results confirmed those obtainend *in vitro* in SH-SY5Y and matched those previously reported in N2a cells (Grossi et al, 2013), confirming similar mechanisms of OLE-induced autophagy activation both *in vitro* and *in vivo.*

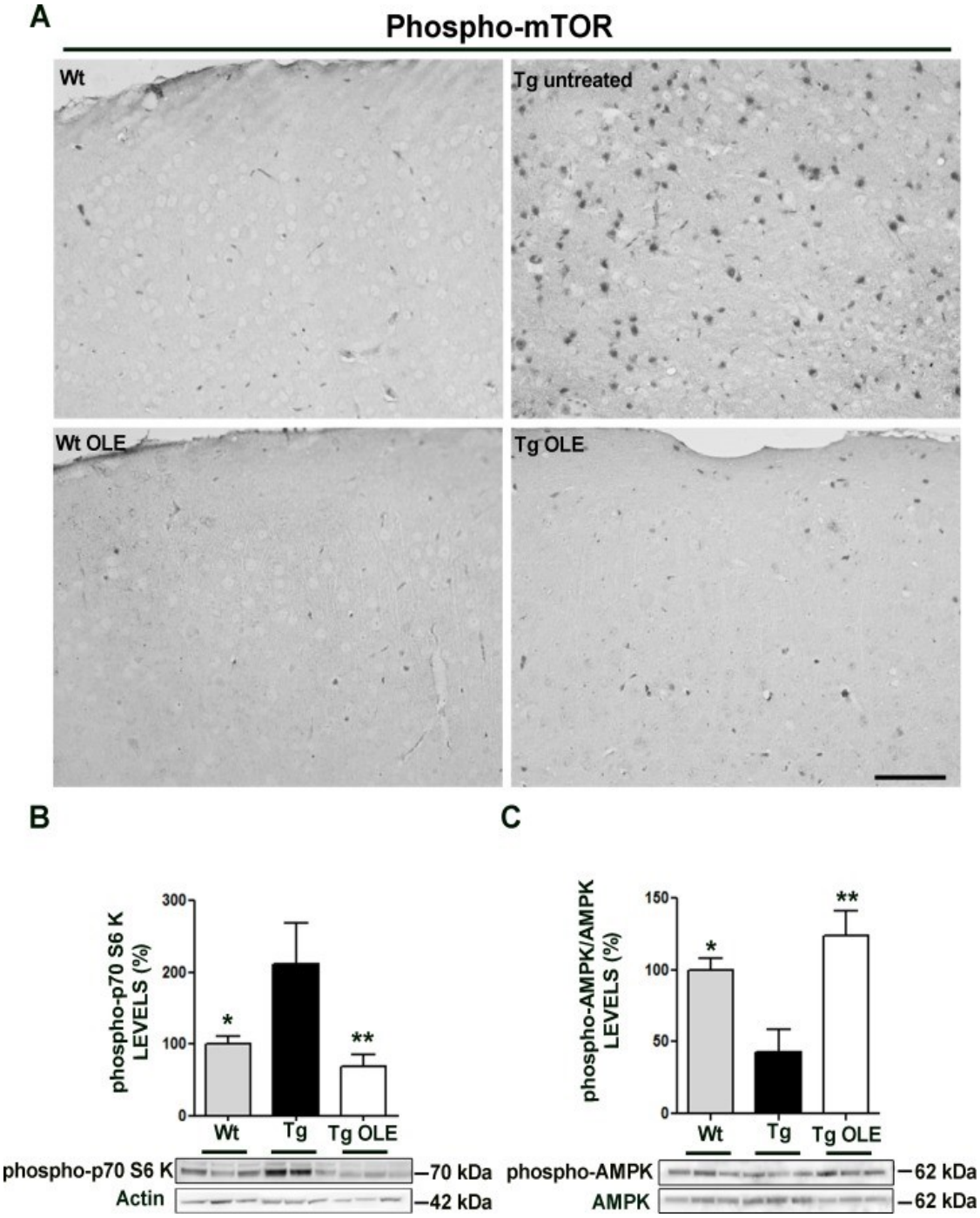


Figure 30) OLE induces autophagy through dual AMPK activation and mTOR inhibition in TgCRND8 mice. **A** Immunohistochemical analysis shows phospho-mTOR immunoreactivity in the cortex of TgCRND8 mice. In OLE-fed Tg mice the levels of phospho-mTOR were reduced compared to untreated Tg and appeared similar to wt mice. Scale bar: 50μm . **(B,C)** Immunoblot analysis shows increased phospho-p70S6K **(B)** and decreased AMPK phosporylated **(C)** in TgCRND8 mice, whose levels were restored after OLE treatment The asterisks (*) indicate a statistically significant difference with respect to untreated Tg mice. *P<0,05 ** P<0,01 (n: 5-6 mice/group)

2) Effect of OLE on PARP1-SIRT1 interplay.

Polyphenols are known activators of Sirtuins, class III Histone DeACetylase (HDAC), which require NAD+ for their activity. In addition to SIRT1, PARP1 requires NAD+ as a substrate, therefore interplay between these two proteins is suggested. The main effect of PARP1 activity is formation of PAR polymer. Several studies reported an increase in PARP1 activity and PAR accumulation in AD patients, which was associated with neuronal death. (Love et al, 1999; Abeti et al, 2012). In contrast, deletion or inhibition of PARP1 gene improved neuronal survival (Abeti et al, 2012) In light of these evidence and the potential benefits related to modulation of the SIRT1-PARP1 crosstalk, we decided to investigate the potential role of OLE as a SIRT1 activator and the relationship between PARP1-SIRT1 using murine N2a neuroblastoma cell line.

2.1 OLE inhibits MNNG-induced PARP1 activation and attenuates NAD+ depletion in N2a cells.

To assess the role of OLE as PARP1-inhibitor, N2a neuroblastoma cells pre-treated or not with 100μM OLE for 24h were exposed for 15 min to 100 μM PARP1-activator MNNG and the levels of PARylated proteins were analysed by immunoblotting. N2a cells were also pre-treated with two PARP1 inhibitors (PHE, 30μM; PJ-34, 20μM) for 24h before MNNG exposition, along with the OLE pre-treatment. As expected, MNNG induced an increase in PARylated proteins compared to untreated control cells. In contrast, PARP1 activation was abolished when the cells were pre-treated with OLE 100μM, PHE or Pj34. It is worth mentioning that neither OLE, PHE nor PJ-34 alone affected PARylation. (Figure 31 A). Since PARP1 requires NAD+ for its activity, NAD+ levels were measured in N2a exposed to MNNG (100μM) for 90 minutes. We observed under these conditions a 27% reduction of NAD+ compared to the levels measured in control cells. This reduction was slightly attenuated when cells were pre-treated with OLE, PHE or Pj-34. Take together these first results suggested that OLE could act as PARP1 inhibitor, similarly to PHE or Pj34 in cultured neuroblastoma cells (Figure 31B).

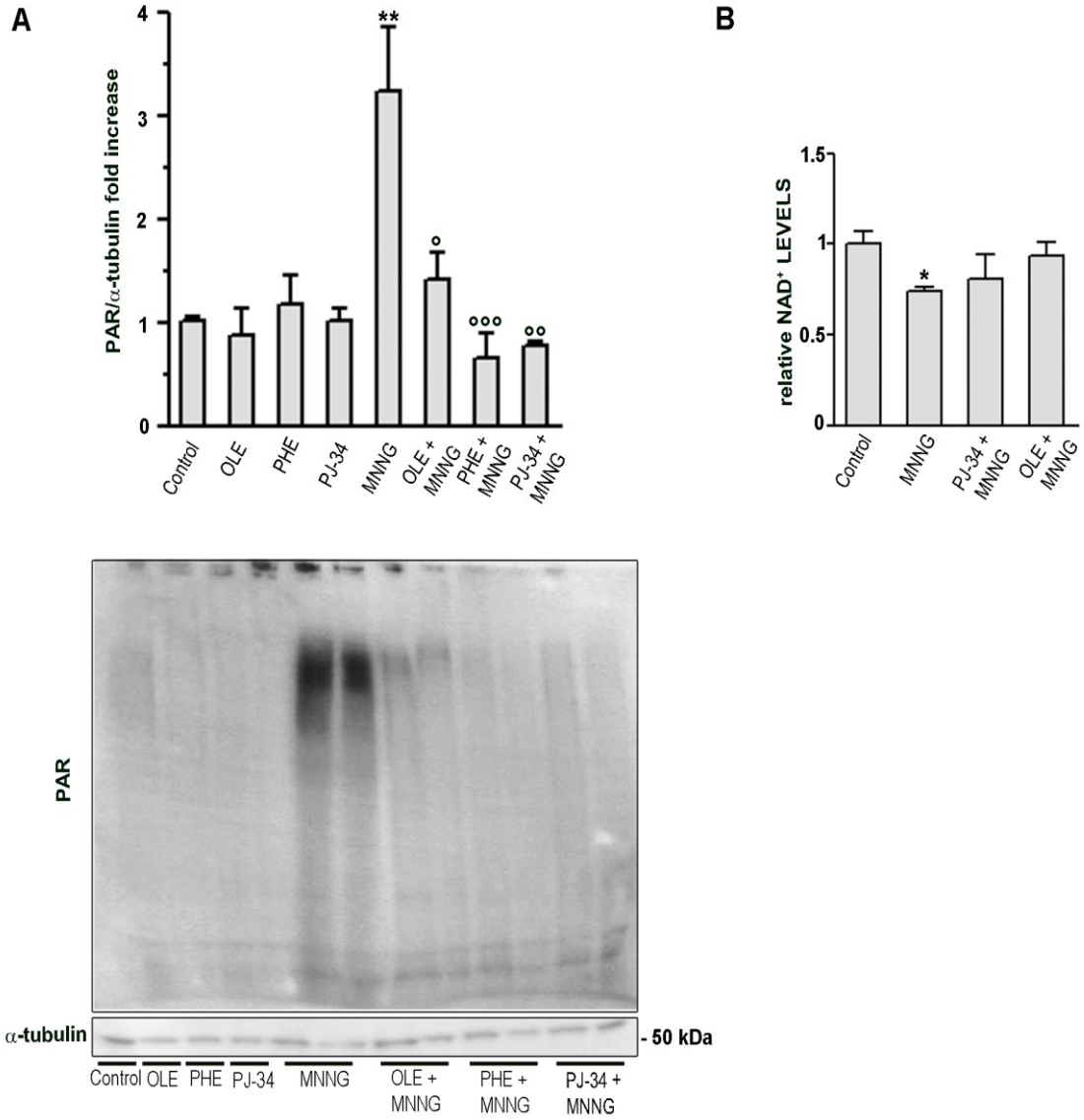


Figure 31. OLE inhibits PARP1 activation and attenuates NAD+ levels in cells exposed to MNNG. **A** (Lower panel) N2a cells were treated with MNNG (PARP1 activator) and PARylated were detected by immunoblotting. Pretreatment with OLE or with known PARP1-inhibitors (PHE or PJ-34) inhibited PARylation MNNG-induced. (Upper panel) Quantitative data. **B.** MNNG treatment for 90 minutes reduced NAD+ levels. OLE, PHE and PJ-34 pretreatment attenuated slightly NAD+ depletion MNNG induced. The asteriks (*) indicate significant difference compared to control cells, the circles(°) indicate a statistical significant difference compared to MNNG treated cells * P<0,05 ; ° P<0,05 °° P<0,01 °°° P<0,001.

2.2.OLE increased Sirtuin1 and Beclin1 expression in N2a cells

Polyphenols are shown to stimulate autophagy through activation of Sirtuins (Chung et al, 2010). Therefore, we decided to investigate the effect of OLE on SIRT1 modulation and the involvement of SIRT1 in OLE-induced autophagy. As expected, we observed a strong increase in SIRT1 protein expression in OLE-treated cells compared to control cells (Figure 32A). In addition, besides its role in cell death, PARP1, can also induce cell survival via autophagy stimulation in the presence of low levels of DNA damage (Kim et al, 2005). In light of these evidence, we treated N2a cells for 15 min with 100 μM MNNG or with 100 μM OLE (for 24H) and MNNG (15 min), and checked the effect on Beclin1, protein involved in the first steps of autophagy pathway. We found that Beclin1 expression level increased after 15 min of MNNG exposure, but its levels were further increased in OLE plus MNNG (Figure 32 B). Several studies support the hypothesis that MNNG and OLE induce autophagy through AMPK signaling pathway (Rigacci and Miceli, 2015; Zhou et al, 2013). Moreover, since OLE increases SIRT1 levels and autophagy is further increased in cells exposed to MNNG plus OLE, our results highlight SIRT1 activation as another mechanism of autophagy stimulation by OLE in addition to the Ca^{2+}-CaMKKβ-AMPK-mTOR axis previously reported (Rigacci and Miceli, 2015). .

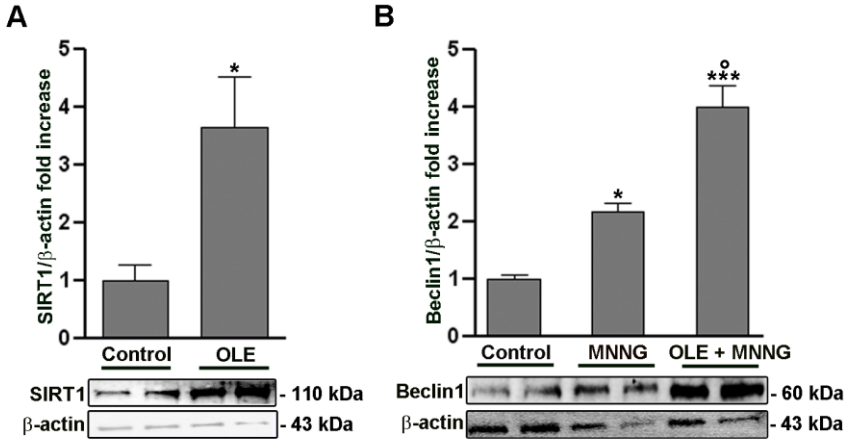


Figure 32) OLE increases SIRT1 and Beclin1 protein expression in N2a cells A. N2a cells were treated with 100μM OLE for 24 h, then SIRT1 expression was analysed by immunoblotting. **B** Beclin1 protein levels in N2a cells were detected after exposition to 100 μM MNNG (15 min) or after OLE treatment (24 h) plus MNNG (15 min). (A)*P<0.05 OLE treated cells vs control cells; (B) * P<0.05 MNNG treated cells vs control cells; *** P<0.001 OLE + MNNG-treated cells vs control cells; ° P<0.01 OLE + MNNG-treated cells vs MNNG-treated cells.

3)OLE-enhanced autophagic flux ameliorates oxidative stress injury in cardiac cells

Following these results obtained with neuroblastoma cell lines and *in vivo* models of AD, we wondered whether OLE could activate autophagy in another cell lines and protect against other pathological conditions associated with autophagy dysfunction. Towards this end, we used neonatal rat cardiomyocytes and selected an *in vitro* model of cardiomyopathy characterized by overexpression of monoamine oxidase-A (MAO-A).

3.1 OLE is not toxic in neonatal rat cardiomyocytes

Before studying a potential autophagy activation in neonatal rat cardiomyocytes we determined that OLE was not toxic in this cellular model.To this end, we exposed neonatal rat cardiomyocytes to

50 μM or 100 μM OLE for 6h and measured cell toxicity using different tests: MTT (mitochondrial functionality), LDH (cell death) and DCFDA (ROS detection). No difference was found in these assays between control and OLE-treated cells , suggesting that OLE did not induce toxicity in this range of concentrations (Figure 33).

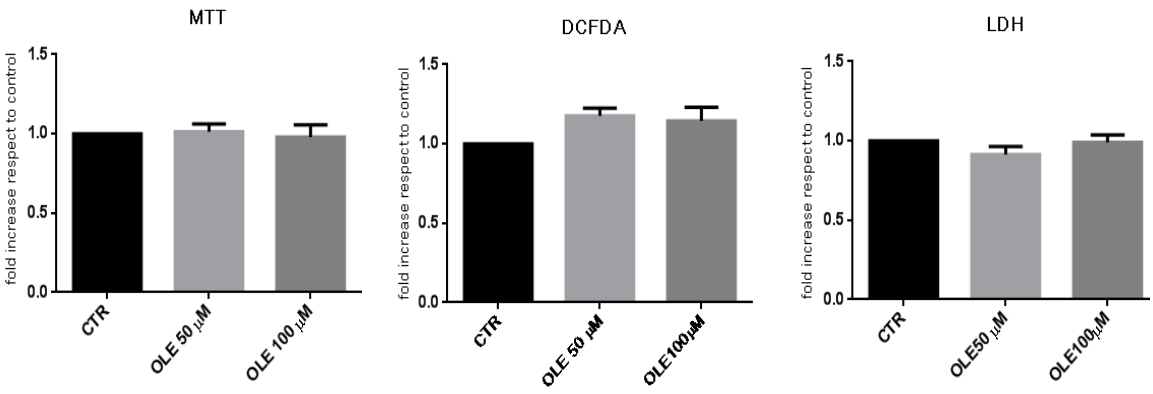


Figure 33) OLE is not-toxic in neonatal rat cardiomyocytes. The cells were treated with OLE 50μM and 100μM and no significant differences were found between control and OLE-treated cells in MTT (mitochondrial functionality), DCFDA (ROS detection) and LDH test (cell death).

3.2 OLE induces autophagy in neonatal rat cardiomyocytes

Since OLE-stimulated autophagy occurred in short and early time frame in neuronal models, we wondered if in cardiomyocytes OLE could act in a similar manner. Neonatal rat cardiomyocytes were exposed to 100 µM OLE for 1h and 3h and autophagic vacuoles were detected using Cyto-ID Detection Kit. We observed that autophagy was induced after 1h of OLE treatment and persisted at 3h (Figure 34). These results were confirmed by western-blot analysis that showed in OLE-treated cells an increase of autophagy-marker LC3II in the same temporal fashion (Figure 35). Taken together, these data suggest that OLE induced autophagy in cardiomyocytes after short-time of treatment.

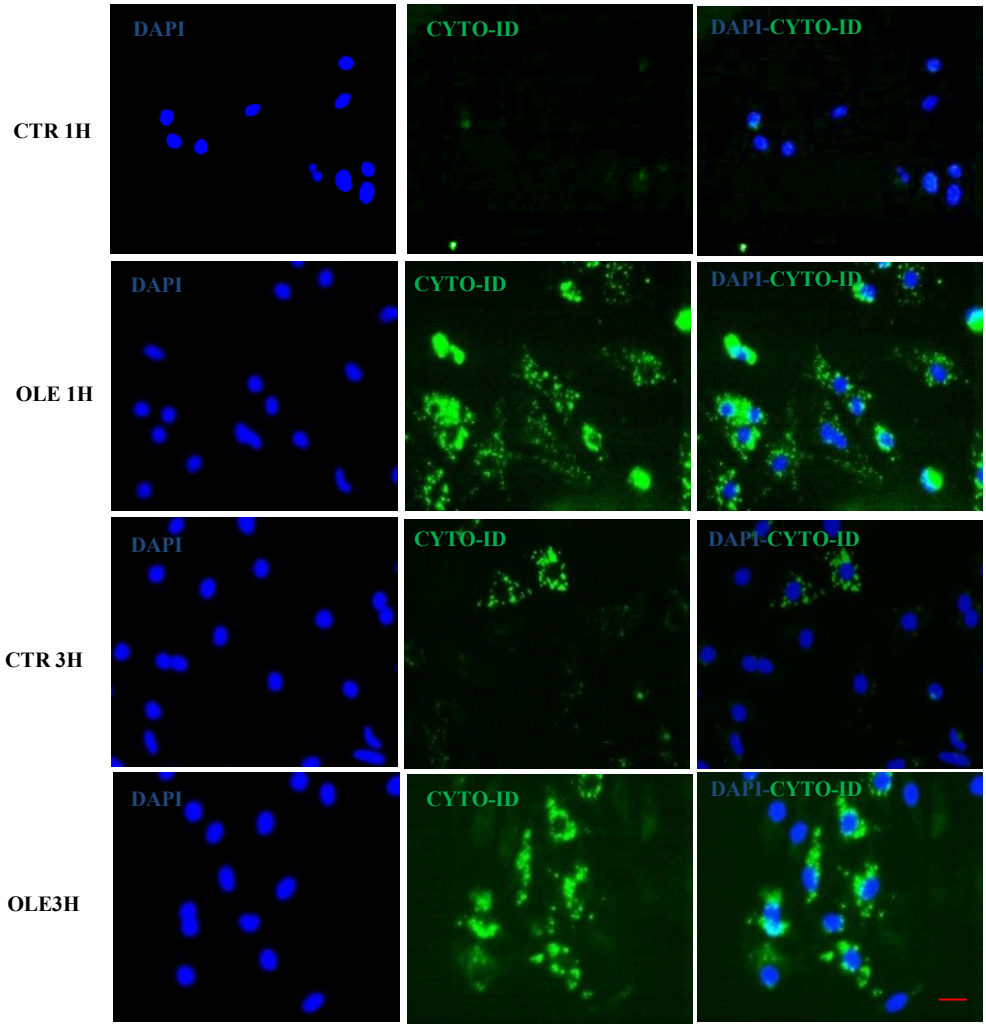
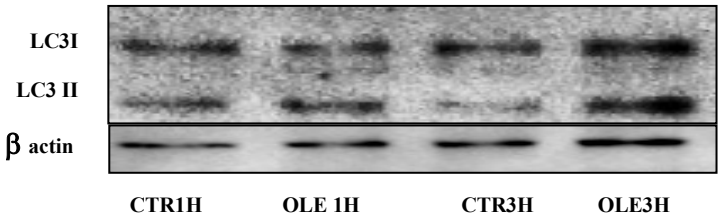


Figure 34) Detection of autophagic vacuoles in cardiomyocytes treated with OLE. Neonatal rat cardiomyocytes were cultured with DMSO (control cells) or OLE 100µM for 1 and 3h followed by staining with green detection reagent that labels autophagic vacuoles. Nuclei were counter stained with

Hoechst 33342. Note that autophagy could slightly increase also in control cells with increasing time for nutrient shortage. Scale bar 20μm. N exp 3



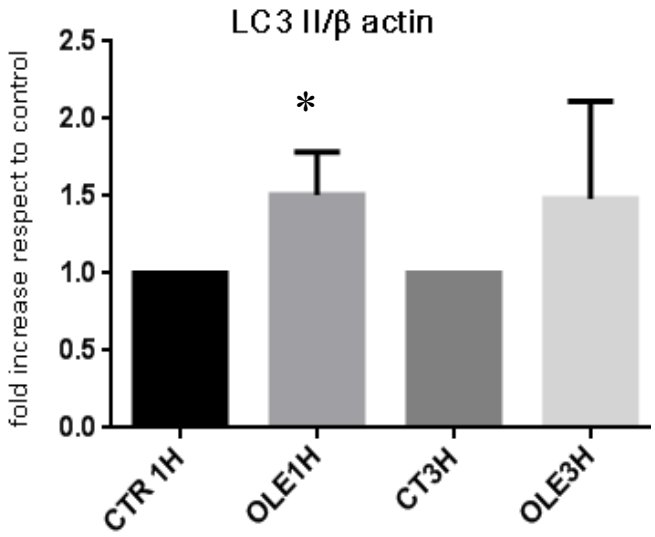
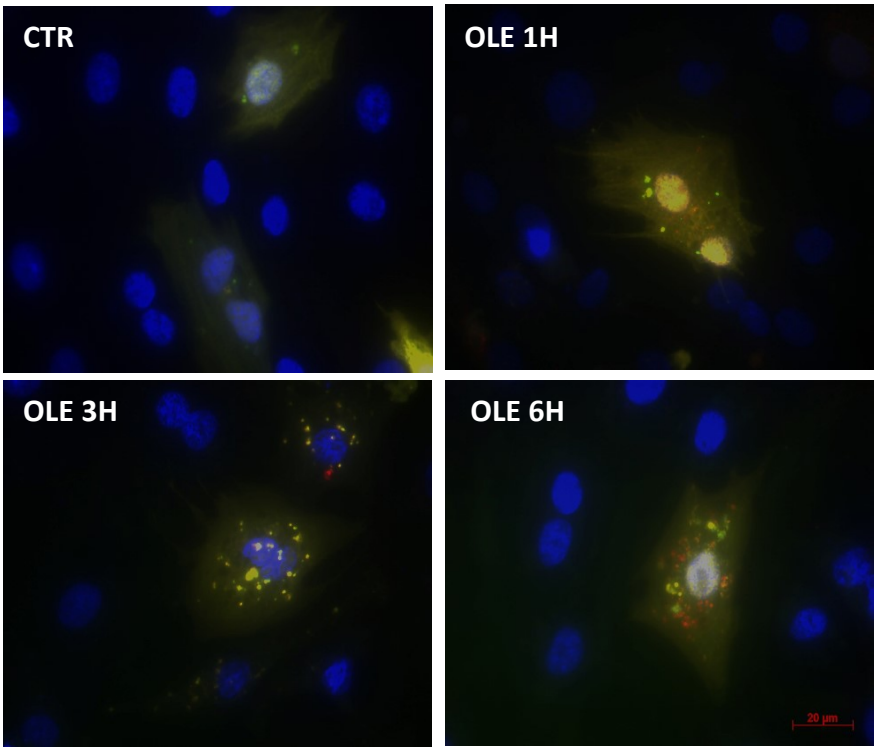


Figure 35.OLE induces an increase in LC3II protein expression in neonatal rat cardiomyocytes. Cardiomyocytes were treated with OLE 100μM for 1 and 3h and LC3II expression was analysed by immunoblot. *P<0.05 OLE treated cells vs control cells

3.3. OLE-stimulation enhances autophagic flux in neonatal rat cardiomyocytes.

Since the accumulation of autophagosomes is not always indicative of complete autophagy activation but can also represent a block in autophagosome maturation, we decided to perform an "autophagic flux" assay to distinguish between these two possibilities. "Autophagic flux "is a measure of degradative completion of autophagy that requires the autophagosome-lysosome fusion and the consequent substrate degradation. To this assay, we used the Tandem Sensor RFP-GFP-LC3B to specifically label autophagosomes and autolysosomes in OLE-treated cells. Autophagosomes appear as yellow puncta (GFP+RFP) whereas autolysosomes are only labelled in red due to the quenching of the GFP fluorescence at acidic pH. Cardiomyocytes transfected with the Tandem sensor mRFP-GFP-LC3 were treated with OLE 100 μM for 1, 3 and 6h , the autophagic flux was analysed. Dual-immunofluorescence analysis showed a slight increase in autophagosomes (yellow puncta) at 1H of OLE treatment, which peaked at 3H. Of note, the number of yellow puncta decreased after 6H of OLE, while at the same time the number of autolysosomes increased (red puncta).These results indicated that autophagosomes are processed to lysosomes and that the autophagic flux is enhanced in the presence of OLE (Figure 36).



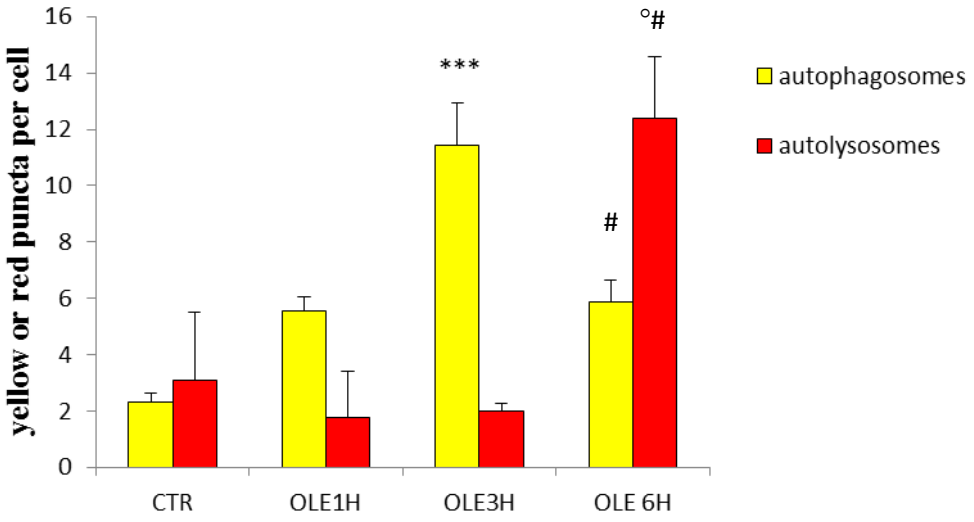


Figure 36 OLE enhances autophagic flux in neonatal rat cardiomyocytes. (Upper panel). Cells transfected with RFP-GFP-LC3 show a slight increase in autophagosomes (yellow puncta) after 1h of OLE treatment and a stronger increase at 3H. The number of yellow puncta decreased after 6H of OLE, while at the same time the number of autolysosomes increased (autophagic flux). (Lower panel) Quantification of yellow puncta (autophagosomes) and red puncta (autolysosomes) for each condition is displayed on histogram. * significativity autophagosomes in treated cells vs autophagosomes in vehicle-cells; ° significativity autolysosomes in treated cells vs autolysosomes in vehicle-cells; # significativity autophagosomes OLE3h vs autophagosomes OLE6h , # significativity autolysosomes OLE6h vs autolysosomes OLE3h ***P< 0,001; °P<0,05; #P<0,05

For LC3 turnover assays, the cells were treated for 1h with 10 μM chloroquine, (CQ), which inhibits the lysosomal acidification, or with CQ plus OLE. In the presence of CQ, the degradation of LC3-II was blocked, resulting in the accumulation of LC3-II. In cells treated with both CQ and OLE, we found an increased level of LC3II compared to CQ alone. The differences in the amount of LC3-II between OLE+ CQ and CQ alone represented an increased formation of autophagosomes due to OLE (i.e., autophagic flux) (Figure 37)

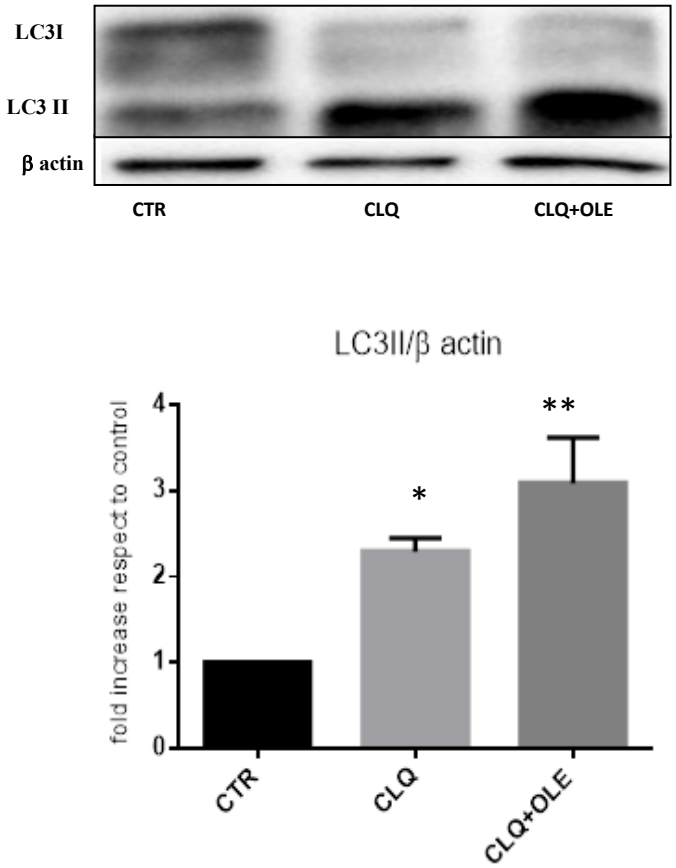


Figure 37 OLE synergizes with chloroquine to incease LC3II protein expression. Neaonatal rat cardiomyocytes were treated with 10μM CQ for 1h and LC3II protein expression was analysed. CQ, inhibiting lysosomal acidification, induced a block of LC3II degradation, resulting in LC3II accumulation. The combination of CQ (10μM) plus OLE (100 μM) increased LC3II protein expression compared to CQ alone, index of autophagic flux in OLE treated cells.* significativity vs control cells. * P<0.05 , **P<0,01

3.4. OLE is protective against autophagy dysfunction induced by MAO/H_2O_2 axis

Once verified that OLE induced the autophagic flux in neonatal rat cardiomyocytes, we aimed to identify if this effect could be protective in a model of cardiac stress characterized by autophagy dysfunction. To this end, we selected an *in vitro* model of cardiomyopathy represented by overexpression of monoamine oxidase-A (MAO-A). Cardiomyocytes transduced with Ad-MAO and stimulated for 6h with tyramine (500 μM), MAO-A substrate, exhibited an increase in oxidative stress, which in turn led to autophagy dysfunction as reported by Santin and collaborators (Santin et al, 2016). In order to evaluate the potential effect of OLE on cell survival in this model, we performed LDH (cell death) and MTT test (measurement of mitochondrial functionality). In Ad-MAO-A-transduced cardiomyocytes stimulated with tyramine, we found an alteration of mitochondrial function and an extracellular release of lactate dehydrogenase (LDH). As a negative control, we showed that non-transduced cardiomyocytes did not show necrosis activation or alteration in mitochondrial functionality in the presence of tyramine. Interestingly, in Ad-MAO-A-transduced-cardiomyocytes pretreatment with dose curve of OLE resulted in a dose-dependent inhibition of mitochondrial alteration (MTT) and cardiomyocyte necrosis (LDH) induced by tyramine (Figure 38)

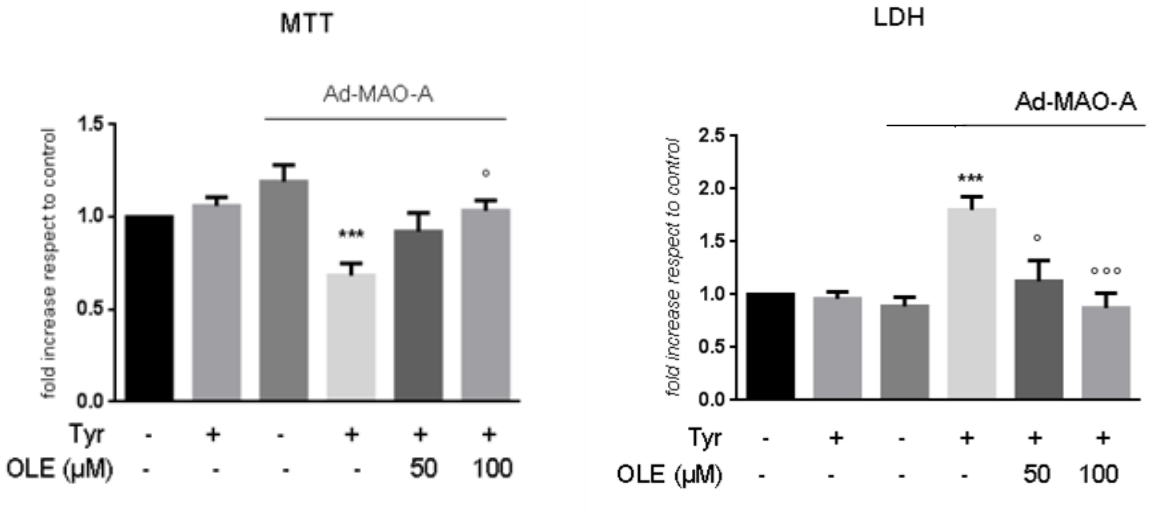


Figure 38) OLE is protective against autophagy dysfunction induced by MAO/H_2O_2 axis. Neonatal rat cardiomyocytes were transduced with Ad-MAO-A and stimulated with tyramine (MAO substrate) for 6h. OLE-pretreatment counteracts the effects of MAO-A/H_2O_2 axis improving mithocondrial functionality (Left panel) and decreasing cell necrosis (Right panel) . * vs MAO / ° vs MAO+Tyr ;
***P < 0.001, ° P<0.05 , °°° P < 0.001

3.5 OLE treatment induces nuclear tranlocation of TFEB, master regulator of autophagy.

In the light of these beneficial effects of OLE on the MAO-A/H_2O_2 axis, we sought to identify the pathway involved in OLE-induced protection. Since it was recently reported that MAO activation blocked the nuclear translocation of transcriptional factor-EB (TFEB), a master regulator of autophagy genes and lysosomal biogenesis (Santin et al, 2016) we questioned whether OLE could induce TFEB translocation and restore a proper autophagy through this pathway. To this end,, cardiomyocytes were treated for 30 minutes with 100μM OLE and cytosolic and nuclear TFEB localization were assessed by immunofluorescence. In untreated control cells, TFEB was mainly localized into the cytoplasm, where the complex with mTOR maintains it phosphorylated and inactive . Interestingly, in cells treated with OLE for 30 minutes, TFEB translocated to the nucleus (red puncta), suggesting an indirect transcriptional regulation of autophagy by OLE in this context. (Figure39).

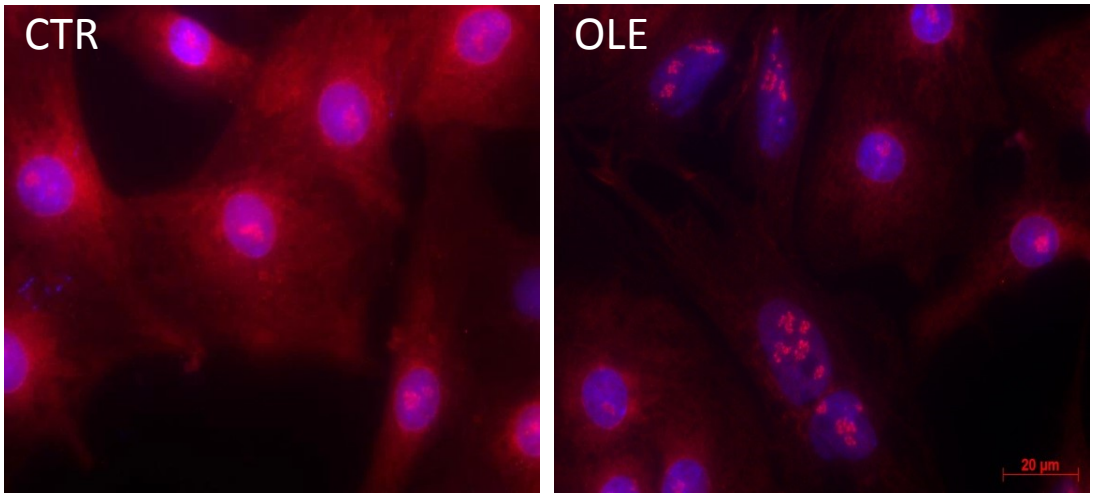


Figure 39) Proposed mechanism of action for OLE-induced autophagy. MAO activation leads to accumulation of TFEB into the cytoplasm in form inactive (not shown) Immunofluorescence analysis using anti-TFEB antibody showed translocation of TFEB to the nucleus after 30 minutes of OLE treatment (red signal) . Scale bar 20 µm. n exp 3

Discussion

Discussion

Growing evidence point to plant polyphenols as promising molecules for the prevention and treatment of several pathological conditions (Del Rio et al, 2013). The use of natural compounds to provide medicinal or health benefits is part of the so-called "nutraceutical" approach, a therapeutic strategy focusing on food molecules that are candidates to enter in clinical trials as such or following structural modification to have higher selectivity towards biological targets. Among these molecules, natural phenols present in EVOO, green tea, red wine, red berries have aroused great interest (Rigacci et al, 2014). The main feature of these natural phenols is their recognized antioxidant capacities. The antioxidant activity of polyphenols could protect against conditions in which oxidative stress is involved such as cancer, hypertension, neurodegenerative disorders, or diabetes. However their antioxidant capacity itself cannot explain the notable effects of these molecules against human diseases (Del Rio et al, 2013).

The discovery that polyphenols such as resveratrol, EGCG and curcumin could induce autophagy, acting as mimetics of CR represented a step forward in our understanding regarding the health effects of these promising molecules (Rigacci et al, 2016). Autophagy is a highly regulated intracellular process that promotes recycling of abnormal protein aggregates and damaged organelles into bioenergetic and biosynthetic materials to maintain homeostasis. Thus, dysregulation of autophagy is associated with the abnormal accumulation of aggregate proteins and defective organelles, cellular dysfunction that altogether lead to the onset of several pathological diseases, such as neurodegenerative and cardiovascular diseases, type II diabetes, or cancer (Ntsapi et al, 2016). The induction of autophagy can involve different pathways, some of which are Ca^{2+}-dependent such as Ca^{2+}-calpains-Gsα, cAMP/Epac/Ins(1,4,5)P3 and Ca^{2+}-CaMKKβ (LKB1)-AMPK-mTOR pathways. The latter appears to be the signalling pathway activated by resveratrol and EGCG in autophagy modulation. These molecules appear to induce the autophagic pathway by increasing cytosolic Ca^{2+} levels and activating AMPK by the CaMKKβ. In turn, activated AMPK promotes autophagy by ULK1 activation (Li et al, 2014) and mTORC1 inhibition. To date, the mechanism of autophagy induction showed by resveratrol and EGCG could not be generalized to other plant polyphenols, but recent studies aimed at understanding if this mechanism of action is shared by other polyphenols.

Towards this end our research group recently studied the effect of OLE, a typical phenol from extra virgin olive oil, as an activator of autophagy in murine neuroblastoma N2a cells, focusing on the molecular mechanisms involved. The research highlighted that OLE induced autophagy through mTOR inhibition (as showed by the reduced phosphorylation of p70-S6K, mTOR substrate) and did not trigger cell death in the range of used concentrations. Since OLE appeared to have a role as a protective molecule, we decided to investigate its beneficial effects *in vivo* using pathological models of autophagy dysfunction. To this end, we selected an in *vivo* model of Alzheimer's disease, TgCRND8 mice, which showed autophagy dysfunction as one of the main features. In this model, OLE supplementation with the food resulted in an improvement in performance in behavioural and cognitive tests, a decrease in Aβ load (decreased level, reduction in plaque number and size), all of which were in agreement with the *in vitro* anti-aggregative effect of OLE reported in previous studies (Rigacci et al,2011). In addition a strong autophagic response concomitantly with a recovery of the lysosomal system, severely compromised in AD, was observed in the cortex of OLE-treated Tg mice (Grossi et al, 2013).

Following these encouraging results, this thesis sought to investigate the molecular and cellular mechanisms involved in OLE-induced autophagy using human neuroblastoma SH-SY5Y cells and TgCRND8 mice.

The results of this thesis showed that OLE induced autophagy in cultured neuroblastoma cells through the $Ca^{2+/}$CaMKKβ/AMPK/mTOR pathway by a mechanism similar to that reported for other polyphenols. In particular we showed that the increase of Ca^{2+} levels, observed after few minutes of OLE treatment, came from the intracellular storage rather than from external medium. This suggested that OLE could penetrate the plasma membrane without damaging it and induce the opening of Ca^{2+} channels present in the endoplasmic reticulum (ER), producing the increase in intracellular Ca^{2+}. In turn, the increase in cytosolic Ca^{2+} activated CamKKβ which precedes AMPK phosphorylation. The link between AMPK and mTOR in OLE-induced autophagy was supported by our previous studies on N2a neuroblastoma cells where we reported a time-dependent decrease in mTOR phosphorylation after OLE-treatment. Interestingly in that study, we reported a significant decrease of p70-S6K phosphorylation at 3-6 h of OLE treatment, and at a similar time, in this study, we found the highest peak of AMPK phosphorylation (4h of treatment). Taken together, these

results supported the hypothesis that autophagy activation by OLE proceeds through AMPK/mTOR signalling pathway (Grossi et al, 2013, Rigacci, 2015). We further observed that the activation of autophagy by OLE following an AMPK-dependent signalling last only last few hours; a potential beneficial therapeutic effect would therefore require continuous intake.

The activation of Sirtuins is another mechanism by which polyphenols induce a CR-like effect. Sirtuins are NAD+ dependent type-3 deacetylases involved in the regulation of metabolism and lifespan in several organisms. The activation of sirtuins, in particular SIRT1, results in up-regulation of protective biological functions, including autophagy (Rigacci et al, 2014). We investigated the effect of OLE on SIRT1 protein expression in N2a cells and we found a strong increase in SIRT1 levels after 24h of OLE treatment. Since several reports supported a model in which SIRT1 and AMPK concordantly function to ensure an appropriate cellular response to stress, starvation or calorie restriction (Fulco et al, 2008), we speculated that also in our model SIRT1 could play a role, synergizing with the Ca^{2+}-Camkkβ-AMPK-mTOR pathway in OLE-autophagy modulation .

Among SIRT1 main targets we focused on the poly (ADP ribose) polymerase (PARP1), a protein that requires NAD+ for its catalytic activity. PARP1 is essentially involved in the maintaining of DNA integrity and its increased activity results in addition of poly (ADP) ribose polymers in response to a variety of cellular stress. Although PARP1 plays a role in cell survival , it can be also implicated in cell death processes since excessive DNA damage leads to an extensive PARP1 activation followed by dramatic alterations in the levels of NAD+ and ATP. (Pillai et al, 2005, Rajamohan et al, 2009). PARP1 and SIRT1 use the same substrate and are able to modulate many common pathways, therefore crosstalk between these two proteins is suggested (Zhang, 2003). Several authors reported that PARP1 over activation suppressed the activity of SIRT1 by depleting cellular NAD levels (Pillai et al, 2005). In contrast, SIRT1 inhibited the PARP1-mediated release of apoptosis-inducing factor (AIF) from mitochondria, thus suggesting that these two proteins might counterbalance each other's activity (Kolthur-Seetharam et al, 2006).

In light of these findings we sought to investigate the effect of OLE on PARP1-SIRT1 interplay. Exposure of N2a neuroblastoma cells for 15 minutes to the alkylant agent MNNG led to PARP1 activation and induced PAR polymer formation. This response

was abolished by a 24h pre-treatment with OLE and was similar to the effect of known PARP inhibitors (PJ-34, Phe). In addition, we analysed the levels of NAD+ in N2a cells exposed to MNNG alone, MNNG plus Pj-34 or MNNG plus OLE, and we observed a significant decrease of NAD+ levels (27%) after MNNG exposure for 90 minutes. NAD+ levels were partially maintained by 24h pre-treatment with PJ-34 (-20%) or OLE (-7%). At this point we aimed to determine whether OLE could behave as PARP1 inhibitor, similarly to PJ-34, or other mechanisms could explain its effects on PARylation. Since MNNG-exposed cells treated with OLE resulted in a reduce tendency to restore NAD+ levels we hypothesized that OLE was not a direct PARP1 inhibitor, but its effect could be due to the extended use of NAD+ by OLE-induced SIRT1 activation as previously reported. We concluded that the reduction of PARP1 activation in OLE-treated cells could be an indirect effect of the increased SIRT1 expression induced by OLE. In fact, by competing for the same substrate, SIRT1-activation can directly abolish PARP1 activity (Luccarini et al, 2016). In addition other mechanisms could explain how SIRT1 inhibits PARP1 activity. It is known that PARP1 is acetylated under stress conditions and that this enhances its enzymatic activity. At the same time SIRT1 is a strong deacetylase and therefore could deacetylate and inhibit the enzymatic activity of PARP1. A direct effect of SIRT1 on the inhibition of PARP1 gene promoter was also reported (Abeti et al, 2012). NAD+ levels dictate PARP1-SIRT1 interaction, and the activation of a protein rather than the other depends on the stress severity. Under mild stress conditions, SIRT1 activation can suppress PARP1 activity at both the transcriptional and post-translation levels, and at the same time induce autophagic response that promoted cell survival. By contrast, under severe stress conditions, excessive NAD+ depletion inhibits the activity of SIRT1, along with a hyperacetylation of PARP1, resulting in cell death (Rajamohan et al, 2009).

Since it was reported that autophagy may be cytoprotective in response to DNA damaging agents, we investigated the expression of Beclin1, an early autophagosome marker, in MNNG-treated cells and in cells treated with MNNG plus OLE. We found that MNNG exposure for 15 minutes increased Beclin1 expression as survival strategy adapted by the cells to respond to mild stress condition. Interestingly we found that Beclin1 protein expression was markedly increased compared to MNNG alone in cells pre-treated for 24h with OLE prior to MNNG exposure. Modulation of autophagy by both MNNG and OLE is reported to proceed through AMPK activation (Chiu et al, 2011; Rigacci-Miceli et al, 2015; Zhou et al, 2013). However, since OLE increased

SIRT1 protein expression and Beclin1 is markedly increased in cells exposed to MNNG plus OLE, our results suggested that SIRT1 activation could represent another mechanism of OLE-autophagy stimulation in addition to the previously reported Ca^{2+}-AMPK-mTOR pathway (Rigacci-Miceli et al, 2015, Luccarini et al, 2016).

PARP1 activation and accumulation of PAR-polymers are one of the main features of AD. Some authors showed that Aβ activates the protein poly(ADP-ribosyl) polymerase-1 (PARP-1) specifically in astrocytes, indirectly leading to neuronal cell death (Abeti et al, 2012). Other studies contradicted these results, showing that in AD brains, the cells that contained the most poly (ADP-ribose) polymers were neurons, while only a few were astrocytes. Despite controversial findings for the exact localization of PAR-polymers the authors agreed that the oxidative stress to DNA increased in the brains of patients with AD, leading to PARP1 activation and enhanced production of PAR-polymers. These findings suggested that pharmacological interventions aimed at direct or indirect inhitibition of PARP activity may be of benefit in such a disease (Love et al, 1999). In light of these findings, the inhibition of PARP1 activation as an indirect effect of SIRT1-activation by OLE could provide a new mechanism of neuroprotection by OLE with possible implications in AD.

These results supported and extended our previously reported data obtained in TgCRND8 confirming that OLE is a promising molecule with pleiotropic mechanisms of action, ranging from SIRT1-PARP1 modulation, anti-amyloidogenic effect (Rigacci et al, 2011) and AMPK activation, that could synergize providing protection in AD (Luccarini et al, 2016). The benefit of OLE-based therapy can concomitantly activate numerous beneficial mechanisms that result in a better and global intervention on the disease.

Following the results obtained with neurodegeneration models, we aimed to identify whether OLE also could protect against other pathological conditions displaying autophagic dysfunction. To this end, we selected an *in vitro* model of cardiomyopathy in which the monoamine oxidase-A (MAO-A) was overexpressed. MAO-A has recently emerged as a potent generator of hydrogen peroxide (H_2O_2) in the heart, and was proposed to be involved in promoting the onset and the progression of several cardiovascular diseases (Anderson et al, 2014; Kaludercic et al, 2014; Kaludercic et al. 2011; Pena-Silva et al, 2009). MAO is a FAD-dependent enzyme present as two-isoforms and is localized at the outer mitochondrial membrane. The two isoforms,

MAO-A and B, differ in terms of their tissue distribution and substrate specificity. MAO-A is responsible for the oxidative deamination of catecholamines and serotonin in the heart and during this reaction produces H_2O_2, the corresponding aldehyde and ammonia. Recently Villeneueve et al. (Villeneueve et al, 2013) reported that MAO-A overactivation in the heart was associated with significant mitochondrial dysfunction and cardiac damage, leading as final consequence to HF. At present it is not really understood the reason for the accumulation of dysfunctional mitochondria in situations in which MAO-A activity is increased.

Santin et al. suggested for the first time a link between MAO-H_2O_2 axis and lysosomal dysfunction that could explain the accumulation of damaged mithocondria in cardiovascular pathologies. The authors reported that MAO-A/ H_2O_2 axis negatively affected the elimination and recycling of mitochondria through the autophagosome-lysosome pathway, resulting in cardiomyocyte death and ultimately HF. In addition to autophagic flux blockade and impairment in lysosomal functionality they observed a lack of nuclear translocation of TFEB, a master transcriptional regulator of autophagy and lysosomal genes, in conditions of enhanced MAO-activity. Interestingly, TFEB overexpression counteracted the deleterious effects of MAO-A/ H_2O_2 axis by decreasing autophagosome accumulation and cell necrosis. In light of these findings, activators of TFEB could be a promising therapeutic strategy in pathological conditions associated to overactivation of MAO-A (Santin et al, 2016)

The effect we observed in neuron and AD model prompted us to test the effect of OLE on MAO-A/H_2O_2 axis, in particular whether OLE-induced autophagy could be protective in this condition. For this study we used isolated cardiomyocytes from the hearts of neonatal rats. Following the results obtained in neuroblastoma cell lines, where OLE induced autophagy at early times, cardiomyocytes were initially exposed to OLE 100 μM for short term treatment (1h and 3h) and autophagy induction was measured by western blot (expression of Lc3II, autophagy marker) or fluorescence microscopy (analysis of autophagic vacuoles). In these conditions we showed an increase in autophagic vacuoles and increased expression of LC3II, as early as 1h post-treatment. These effects were still present at 3h. However, since both the induction of autophagic flux and the inhibition of lysosomal degradation can result in LC3B-II increase and autophagosomes accumulation, in order to properly interpret the results obtained with OLE, we used different assays to measure autophagic flux that is indicative of the completion of the autophagy pathway (fusion autophagosome-lysosome and lysosomal

degradation). The first assay used for this purpose was to monitore of LC3 turnover in presence of CQ, lysosomotropic reagent that inhibits the lysosomal acidification. CQ provokes the blockade of LC3-II degradation, resulting in its accumulation . Therefore the difference in the levels of LC3-II between samples in the presence and absence of CQ represents the amount of LC3 that is delivered to lysosomes for degradation (autophagic flux). (Mizushima et al, 2010). Our results indeed showed that CQ induced an increase in the levels of LC3B-II, but this increase was even more evident in cells treated with OLE (100μM) plus CQ (10 μM), indicating a strong autophagic flux. In addition autophagic flux was also measured using the tandem RFP-GFP-LC3, a construct that distinguish autophagosomes labelled with yellow puncta (RFP and GFP signal) from autolysosomes labelled with red puncta (RFP signal). Dual fluorescence imaging of RFP-GFP-LC3 showed an increase of autophagosomes formation after 1h of OLE treatment that appeared further increased at 3h of stimulation. At longer time of treatment (6h) OLE induced autophagic flux as demonstrated by the simultaneous presence of autophagosomes and autolysosomes.

Once demonstrated the effect of OLE as inducer of autophagic flux in cardiac cells, we evaluated its effectiveness against the defective mechanism of autophagy caused by the overexpression of the MAO-A. To this end the cells were transfected with MAO-A-adenovirus (Ad-MAOA) and stimulated for 6h with tyramine, MAO-A substrate, to induce oxidative stress and block of autophagic flux, as previously reported by Santin and co-workers (Santin et al, 2016). Our results showed altered mitochondrial functionality and cell necrosis in Ad-MAOA cardiomyocytes stimulated with tyramine. In contrast, pretreatment with OLE, before tyramine stimulation, resulted in improved mitochondrial function and reduced cell death.

As previously reported MAO-A activation was also associated to lack of TFEB translocation in the nucleus, event that reduced its transcriptional activity. Thus, we wondered whether OLE could act as activator of TFEB translocation, similarly to Torin1, recognized inductor of TFEB translocation to the nucleus. TFEB translocation can be regulated in mTOR dependent or mTOR independent pathway. Under availability of nutrients TFEB is phosporylated on serine 211 in a mTOR-dependent manner. Once phosporylated TFEB is sequestered in the cytoplasm in its inactive form complexed with 14-13-3 proteins, which prevents its translocation to the nucleus. On the contrary, when mTOR is inactive TFEB is dephosphorylated and can accumulate in the nucleus, promoting the expression of autophagy genes and lysosomal biogenesis

(Roczniak-Ferguson et al, 2012). A recent paper of Medina et co-workers (Medina et al, 2015) reported another mechanism of TFEB translocation that involves calcineurin, a protein phosphatase, shown to be activated by lysosomal Ca^{2+}. This mechanism is considered independent from mTOR signalling. In this model the lysosomal calcium release activates calcineurin that in turn dephosphorylates TFEB and promotes its nuclear translocation (Medina et al, 2015). Interestingly our results showed TFEB translocation in the nucleus after 30 minutes OLE-treatment suggesting a transcriptional regulation of autophagy by OLE. At the present the mechanism by which OLE induces TFEB translocation is unknown but our previous results reporting mTOR inhibition in OLE-treated cells suggested that in this context mTOR could play a role. In addition OLE induced an increase of Ca^{2+} after short term of stimulation and the calcium has been reported as calcineurin activator. In light of this evidence the Ca^{2+} - CamKKβ–AMPK-mTOR signalling could synergize with calcineurin in maintaining de-phosporylated yet active TFEB, an event that promotes its nuclear translocation and its transcriptional activity (Rigacci, Miceli et al. 2015).

Our preliminary results on TFEB translocation were also supported by a recent findings for TFEB translocation and enhanced lysosomal function after treatment with curcumin, a hydrophobic polyphenol that induced autophagy through Akt-mTOR pathway (Zhang et al, 2016). Herein the authors reported two distinct mechanisms by which curcumin induced TFEB activation i) direct binding with TFEB that leads to increased transcriptional activity and ii) TFEB translocation as a consequence of mTOR suppression. Using a curcumin-probe the authors showed a direct binding TFEB-curcumin, suggesting TFEB as a new molecular target of curcumin. In addition, the paper reported a significant decrease in the interaction of TFEB with 14-3-3 proteins, indicating the reduced phosphorylation level of TFEB in curcumin treated cells. These findings suggested the existence of both mechanisms, TFEB binding and mTOR inhibition, in curcumin modulation of autophagy. Finally cells treated with curcumin were also analysed by real-time PCR, to determine mRNA level changes in the known TFEB target genes, such as Lamp1, Atp6v1a, Uvrag and Atg9b.Upregulation of TFEB-target genes was observed in these conditions, supporting the notion that the activation of the lysosomal function by curcumin occurs at least in part through TFEB signalling pathway.

Beyond the beneficial effects shown by OLE in *in vitro* and *in vivo* models , a factor that must be considered, before thinking to therapeutic applications, is the bioavailability. For most plant polyphenols a problem associated with their use could be their reduced bioavailability coming from incomplete intestinal absorption and rapid biotransformation favouring urinary excretion. Following ingestion, polyphenols have to cross the barrier represented by enterocytes and, once into the blood stream, they undergo substantial biotransformations, including methylation, glucuronidation, sulphation and thiol conjugation These transformations can potentially alter their chemical properties, promote their excretion or confer them new biological activities. Necessary processes include the distribution to target tissue and if the site of action is the brain, the polyphenols must cross the relatively impermeable blood brain barrier. In addition to this, the polyphenols can be extensive metabolized by colonic microflora. Together all these factors can affect polyphenols bioavailability (Rigacci et al, 2016) .

Regarding OLE, studies on bioavailability have been encouraging. Recent pieces of evidence in rats (Serra et al, 2012; Bazoti et al, 2010) and humans (Markopoulous et al, 2009) clearly showed that OLE was absorbed in discrete amounts by the intestine and subsequently distributed through the blood stream to different organs and tissues, including the brain, in native form or processed into metabolically active derivatives. Accordingly, a recent paper reports that the main OLE metabolite, HT, was found in the brain of TgCRND8 mice fed with OLE for 8 weeks, suggesting that OLE metabolites, including HT, can directly cross the blood–brain barrier or are generated from OLE *in situ* once it has crossed the blood-brain-barrier (Rigacci et al, 2016).

Conclusions

Conclusions

In summary, our results provide evidence that:

1) OLE induces autophagy through the Ca^{2+}-CaMKKβ-AMPK-mTOR signalling pathway, similarly to other plant polyphenols such as resveratrol and EGCG.

2) OLE promotes SIRT1 activation, which could synergize with the above mentioned pathway to maintain OLE-induced autophagy.

3) OLE induces TFEB translocation to the nucleus, supporting the importance of transcriptional regulation in autophagy.

4) OLE is effective in *in vitro* and *in vivo* models displaying autophagy dysfunction.

In conclusion, we believe that the protective effects showed by OLE in different experimental models and the encouraging data regarding its bioavailability, convincingly support the possibility that dietary supplementation with OLE may prevent or delay the occurrence of several pathological conditions characterized by autophagy dysfunction, in particular neurodegenerative and cardiovascular diseases.

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