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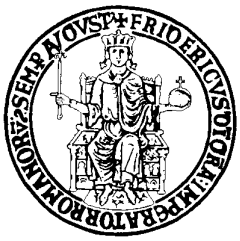

**DOCTORATE SCHOOL IN BIOLOGY**

XXXI Cycle

# *Embryonic stem cells and 3D Minibrains as model systems to study the role of oxidative stress in neurological diseases*

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# SYNOPSIS

Oxidative stress is a common issue in neurodegenerative diseases such as Parkinson´s disease (PD), Alzheimer´s disease (AD), amyotrophic lateral sclerosis (ALS), Huntington´s disease (HD), and fronto-temporal dementia (FTD). Oxidative stress occurs when the cellular redox balance is impaired leading to excessive production of reactive oxygen and nitrogen species (ROS/RNS). ROS/RNS and oxidative modified molecules accumulate in the cells over the time and contribute to cellular senescence and to the aging process. Since most neurodegenerative diseases are late-onset it is likely that oxidative stress-mediated damage accumulation occurring over the time contribute in the onset of the pathology. However, numerous evidences of oxidative stress-mediated damage have been found in developmental brain diseases, such as Rett Syndrome (RTT), in which there is not aging contribution. Rett Syndrome is an X-linked pathology mainly caused by the loss of function (LoF) mutations in the gene encoding Methyl CpG binding Protein 2 (MeCP2). Despite the studies done so far, the molecular mechanisms that link MeCP2 loss of function to the pathology outcome are still not fully understood. Oxidative stress is looked at as a possible actor involved in the disease. High level of oxidatively modified proteins and lipids and altered expression of genes involved in both oxidative stress defense and mitochondrial activity have been observed in RTT patients. Moreover, MeCP2 reactivation in mouse models not only attenuates neural dysfunction but also restores the redox homeostasis. However, the molecular pathways that link the absence of MeCP2 protein to oxidative stress state in the pathophysiology of this brain disease are still unknown.

I investigated the role of oxidative stress in Rett syndrome, taking advantage from a mouse embryonic stem (mES) cell line carrying a deletion of MeCP2 gene (MeCP2-/Y mES). In particular, I analyzed the effect of oxidative stress, induced via treatment with the enzyme Glucose oxidase (GOX) that produces $H_2O_2$ in the culture medium, on WT and MeCP2-/Y mES derived neurons. I studied the mitogen-activated protein kinase (MAPK) cellular response pathways and the apoptotic activation in mES derived neurons upon oxidative stress stimulation. I observed that MeCP2-/Y mES derived neurons resided in an active cellular response state and that the response rate increased upon treatment with GOX. Moreover, GOX treated MeCP2-/Y neurons did not die via apoptosis as shown

by the lack of activation of caspase 3 protein and the high level of the anti-apoptotic Bcl2 protein. Interestingly, MeCP2 protein level increased in GOX treated WT mES derived neurons compared to untreated condition thus giving strength to the possible role of MeCP2 protein in the regulation of cellular response against oxidative stress.

So far, studies on Rett Syndrome, as well as other brain diseases, have been carried out using mainly rodent animal models. However, it is important to take into account the differences that exist between humans and rodents. During the very last years, new strategies to model and study human brain diseases have been emerging. Relying on the use of human embryonic or induced pluripotent stem cells, it is possible to generate complex three-dimensional (3D) structures, the so-called cerebral organoids (Minibrain/MB). The use of human specific stem cells allows following the species-specific differentiation patterning and the presence of multiple human brain-like structures allows studying the interactions between several areas of the brain in an *in vitro* model system that resembles human brain development and architecture.

I was fascinated by the possibility to use MBs to analyze the role of oxidative stress in neurological disease. To this aim, I joined the lab directed by Prof. Dr. Peter Heutink at the German Center for Neurodegenerative Diseases (DZNE) in Tübingen where I experienced the methodology to generate cerebral organoids in order to evaluate their possible use to model brain developmental and neurodegenerative diseases. In particular, I sought to establish a 3D model for Parkinson's disease (PD). PD is the most common neurodegenerative disease with motor symptoms. This complex multifaceted disease has long been believed to be only environmental. However, the recent genome wide association studies (GWAS) on PD patients have identified hundreds of genetic risk factors and many of them are involved in mitochondrial function and/or oxidative stress related. Alpha-synuclein (SNCA) gene is the most common genetic risk factor associated with both familiar and sporadic forms of PD. The alpha-synuclein (α-syn) protein function is still unclear, but it is known that mutations of α-syn proteins lead to aggregates formation in dopaminergic neurons (Lewy bodies and Lewy neurites) that cause toxicity in the cells. The molecular mechanisms that lead to α-syn aggregation and toxicity are not fully defined. I aimed to establish 3D Minibrains using lentiviral-transduced induced pluripotent stem (iPS) cells overexpressing SNCA in order to study the molecular mechanisms leading to α-syn aggregation and toxicity. I reproduced a previously established 3D MB

differentiation protocol and characterized MBs growth and differentiation up to three months in culture. 3D MBs acquired a good degree of neural maturation starting from two months in culture and retained the human cell identity specification. Indeed, differentiated neurons showed a spatial patterning similar to that observed in the *in vivo* developing human brain. However, the MB generation was highly variable between and within the experiments and this highlighted the necessity to make MB generation more reproducible. I tested the effect of SMAD inhibitors, to modulate the TGF-beta and BMP signaling pathways responsible for cell differentiation. SMAD inhibitors are known to repress cell differentiation towards mesoderm and endoderm lineages and they have already been successfully used in two-dimensional (2D) iPS neural differentiation protocols. The SMAD inhibitors treatment reduced significantly the expression of early mesodermal (BRACHYURY) and endodermal (GATA4) genes and increased the expression of neural precursor genes (OTX1 and PAX6) in one-month old MBs. Nevertheless, this treatment did not significantly improve the mature neuron genes expression at any of the time points analyzed. Interestingly, mature neuron genes were less expressed in MBs overexpressing SNCA compared to WT, suggesting an impairment of differentiation in SNCA overexpressing MBs that might occur in the late stages of differentiation. Moreover, I observed that three-months old SNCA overexpressing MBs exhibited higher level of heme oxygenase 1 (HMOX1) mRNA compared to age-matched WT MBs. In line with previous studies, these results suggested the enhanced activation of nuclear factor E2-related factor 2 (Nrf2) signaling pathway in response to SNCA overexpression. Therefore, these results showed that MBs are an interesting tool to study some aspects of neurodegenerative diseases, such as the link between α-syn protein aggregation and oxidative stress response in late onset Parkinson´s disease.

The generation of 3D organoid cultures opened the way to a new human-specific model system for brain research that compensates the limitations derived from both animal models and 2D *in vitro* systems. In this perspective, MBs might be incredibly useful to model Rett Syndrome and to test possible therapeutic strategies in a complex human-specific model system.

# Chapter 1

# OXIDATIVE STRESS

## 1.1 General overview on Oxidative stress biochemistry

Oxidative stress (OS) is defined as the cytotoxic condition in which the biochemical processes leading to the production of reactive oxygen and nitrogen species (ROS/RNS) overcome those responsible for their removal, the so-called antioxidant cascade (Sayre et al., 2008). ROS production arises from the formation of the anion superoxide ($O2^-$), mainly generated by the reaction of the molecular oxygen with electrons leaked from the mitochondrial electron transport chain (Fridovich, 1978). Anion superoxide is not the most dangerous component, but the precursor of other reactive intermediates that are produced along the consecutive univalent reduction reactions of molecular oxygen to water. These intermediates are superoxide ($HO_2$), hydrogen peroxide ($H_2O_2$), and the hydroxyl radical ($HO^-$) (Imlay et al., 1988). The hydroxyl radical is considered the most reactive specie and the main instigator of oxidative stress damage (Sayre et al., 2008). This is rapidly generated by the decomposition of $H_2O_2$ to $HO^-$ in the presence of $Fe_2^+$ by the Fenton reaction. Accumulating levels of superoxide can also react with the diffusible nitric oxide (NO) and generate peroxynitrite (ONOO−) and related RNS. These RNS are capable of both oxidation and nitration chemistries, that result in a condition known as “nitrosative stress” (Alvarez and Radi, 2003).

Aerobic organisms have developed several systems to counteract the dangerous action of ROS and RNS and these include both enzymatic and non-enzymatic antioxidants. About the enzymatic antioxidant cascade, the first enzymes involved are the superoxide dismutases (SOD) that catalyze superoxide dismutation to hydrogen peroxide and oxygen (Fridovich et al., 1978). Subsequently, hydrogen peroxide is reduced by the catalase (CAT) and peroxidase (PrDx) enzymes to form $H_2O$ and $O_2$ (Evans, 1907; Fridovich et al., 1978). The non-enzymatic molecules capable of the reduction of oxidant species include the hydrophilic glutathione (GSH) and ascorbic acid (vitamin C), or α-

tocopherol (vitamin E), hydrophobic, that is concentrated in the membranes (Coyle and Puttfarcken, 1993).

In physiological conditions, ROS are produced as byproducts of normal metabolic processes, such as mitochondrial phopshorylative oxidation, and they play a regulatory role as second messengers in cellular processes via activation of various enzymatic cascades and several transcriptional factors (Droge, 2002).

However, ROS and RNS are highly reactive molecules and in oxidative stress conditions they can react and damage unconditionally proteins, nucleic acids and lipids (Fig. 1.1; Frijhoff et al., 2015). Proteins and amino acids are the most affected molecules by the action of ROS and RNS and the damage can occur both at backbone and side chain sites. Oxidation of protein results in cross-linking, backbone fragmentation and side-chain cleavage (Stadtman, 2006). Reaction of ROS and RNS with amino acids, especially the sulfur-containing cysteine (Cys) and methionine (Met), leads to the formation of carbon-centered or nitrogen-centered radicals, hydroperoxide and carbonyl groups and, in minor amounts, of nitrosated products (Alvarez and Radi, 2003; Davies, 2005). Oxidation of Met to methionine-sulfoxide (MetO) is rescued by the action of specific methionine-sulfoxide reductases that promote the thioredoxin-dependent reduction of MetO back to Met (Stadtman, 2006). Thus, this cyclic oxido-reduction of Met serves as a buffering mechanism to increase the resistance to oxidative damage. Oxidation of nucleic acids results in strand breaks and base modifications (Imlay et al., 1988; Aruoma at al., 1989; Honda et al., 2005; Nunomura et al., 2012). RNA molecules rather than DNA are more susceptible to oxidative stress because of their single-stranded structure and the absence of protective histones (Nunomura et al., 2012). The hallmark of RNA oxidation is the formation, iron-dependent, of 8-hydroxyguanosine (8-OHG) (Nunomura et al., 2012). ROS can also promote lipid peroxidation that, beside the cellular membrane disruption, generates other toxic products such as aldehydes, which include malonic dialdehyde (MDA) and 4-hydroxyalkenals, in particular 4-hydroxynonenal (4-HNE) and 4-hydroxyhexenal (Comporti, 1989; Esterbauer et al., 1991). All of these are highly reactive aldehydes that can react and modify thiol groups and amino acids and exert mutagenic and toxic effects in cells and tissues (Esterbauer et al., 1991).

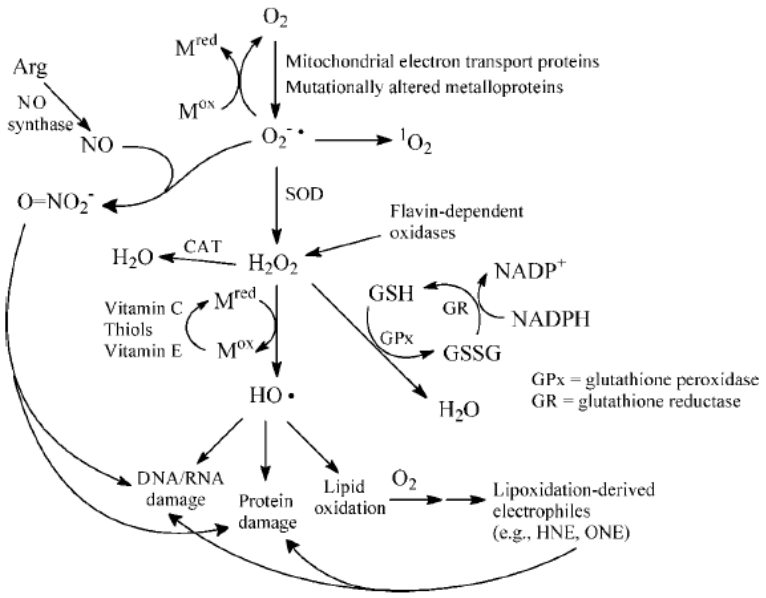


Fig. 1.1 **Schematic representation of the ways of production of ROS and RNS and main effect on biological macromolecules** (source from Sayre et al., 2008)**.**

The amounts of ROS and, in turn, of the oxidatively modified macromolecules increase with age in various tissues due to metabolic or pathophysiologic changes and environmental stress exposure (Zhang et al., 2015). Moreover, it has been hypothesized that such accumulation of oxidative damage might be a former cause of aging and cellular senescence and of wide variety of diseases such as cancer, arthritis, autoimmune disorders, cardiovascular and neurological diseases (Harman, 1956; Harman, 1981; Rebrin et al., 2003; Stadtman, 2006)

## 1.2 Cellular response to oxidative stress

### *1.2.1 Cellular response to oxidative stress: Activated molecular pathways*

The treatment with oxidant agents stimulates the activation of mitogen activated protein kinase (MAPK) superfamily in the cells in a dose-dependent manner (Usatyuk et al., 2003). MAPKs constitute a signal transduction pathway well-known to exert a major role in regulating cell response to a multitude of

external stimuli. Three main families belong to MAPK superfamily: the extracellular signal-regulated protein kinases (Erk 1/2s), the c-Jun N-terminal kinases or stress-activated protein kinases (JNKs) and p38 kinase family (Cowan et al., 2003). MAPKs are the last actors of the signal transduction cascades that activated, via phosphorylation, in response to specific class of external stimuli. Erk 1/2 proteins are activated by mitogens and growth factors, while JNK and p38 proteins are mainly active upon inflammatory cytokines and various physical and chemical stressors stimulation (Raingeaud et al., 1995; Hibi et al., 1993; Sluss et al., 1994; Usatyuk et al., 2003). The stimulus specific activation of MAPKs culminates with the phosphorylation, and hence the activation of the target proteins, mainly nuclear protein such as transcription factors responsible for gene expression regulation (Fig. 1.2; Cowan et al., 2003). As the stimulation of MAPKs differs among each other also the cellular responses are different. Erk1/2 downstream effectors are mainly involved in cell proliferation, differentiation and survival, while JNK and p38 MAPKs targets regulate inflammatory response, and osmoregulation respectively and both regulate apoptosis (Cowan et al., 2003).

Important to note, all the MAPKs have been associated with regulation of autophagy (Sridharan et al., 2011). While Erk 1/2 and JNK MAPKs have been positively associated with induction of autophagy, p38 contribution is controversial, although its activation seems to inhibit autophagy (Sridharan et al., 2011; Zhang et al., 2015).

Autophagy is usually referred to as a survival mechanism activated in either starvation or stress conditions even though has also been suggested as another cell death pathway (Klionsky et al., 2000; Li et al., 2017). Cells activate autophagy to counteract nutrient deprivation via self-component organelles digestion to recycle the basal molecules needed to sustain very essential metabolic pathways. In case of prolonged starvation, active autophagy can trigger apoptosis or necrosis, thus contributing to cell death. Another study suggested that the Erk 1/2-mediated autophagic activation might be one of the causes of neurodegeneration in an MPP+-induced *in vitro* model for PD (Chu et al., 2007). Defective autophagy has been reported in RTT derived primary skin fibroblasts (Sbardella et al., 2017). In particular, the authors showed the inability of RTT fibroblasts to activate autophagy upon starvation (Sbardella et al., 2017). Although, autophagy might contribute differently in fibroblasts than in post-mitotic neural cells (Tsujimoto and Shimizu, 2005). Post-mitotic neurons,

indeed, lose their proliferation potential. Therefore, autophagy might be extremely important as a survival pathway in post-mitotic neurons upon stress and injuries.

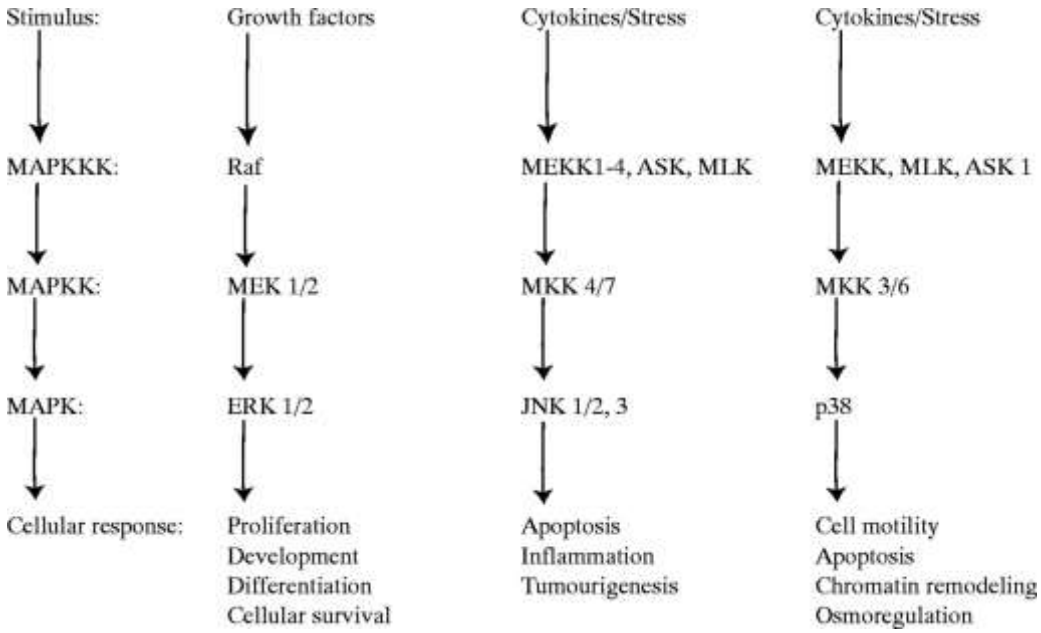


Fig. 1.2 **MAPKs activation pathways** (source from Cowan et al., 2003)**.**

However, Erk 1/2 is also known to promote cell survival by regulation of the Bcl2 family.

Moreover, p38 and AMPK have been reported to regulate Nrf2/ARE-mediated response to oxidative stress induced by oxidants such as 4-HNE (Zimmermann et al., 2015; Ma et al., 2015; Wu et al., 2015).

The Nrf2 signaling pathway is maybe the most important in defense against oxidative stress (Zhang et al., 2015) and Nrf2 is thought to be the master regulator of redox homeostasis in the cells. Nrf2 is a transcription factor that binds to the antioxidant response element (ARE), also referred to as electrophile response elements (EpRE) (Moi et al., 1994) and regulates the expression of antioxidant genes. Under physiological condition, Nrf2 is sequestered in the cytoplasm by interaction with the Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1, (Keap1), also referred to as inhibitor of Nrf2 (INrf2). Through the Kelch-domain, Keap1 protein sequester Nrf2 in the cytoplasm where it is constantly degraded by the ubiquitin proteasome machinery. Upon stimulation by oxidants or electrophiles, Keap1 loses its

capacity to bind Nrf2 that is released and able to translocate into the nucleus where it heterodimerizes with other proteins, such as Mafs, c-Jun, and ATF4, and binds to the ARE in the DNA (Zhang et al., 2015).

The binding of Nrf2 to ARE controls the expression of hundreds of antioxidant genes including γ-glutamylcysteine synthetase (GCS; Moinova et al., 1998), heme oxygenase 1 (HMOX-1), peroxidoredoxin (Ishii et al., 2000) superoxide dismutase family proteins, catalase, glucose-6-phosphate dehydrogenase (G6PDH) and NAD(P)H:quinoneoxidodreductase (NQO-1). Beside the oxidants and electrophiles, also phosphorylation might play a role in regulation of Nrf2/ARE-mediated antioxidant response. Protein kinase C (PKC)-mediated phosphorylation at Ser 40 of Nrf2 has been shown to be important for its release from Keap1, even though is dispensable for Nrf2 stabilization and nuclear translocation (Huang et al., 2002; Bloom and Jaiswal, 2003). Noteworthy, the cytotoxic effect of oxidants follows a dose-dependent mechanism. For example, at physiological level, 4-HNE stimulates cell survival through activation of Nrf2-ARE, NFkB and MAPK pathways and promotion of cell proliferation and autophagy. Higher 4-HNE concentration, instead, induces proliferation arrest and induction of apoptosis. The threshold that distinguishes between physiological or cytotoxic condition depend on the cell type, DNA repair capacity or cellular metabolic circumstances (Ayala et al., 2014).

### *1.2.2 Cellular response to oxidative stress: Programmed cell death*

*Apoptotic cell death*

Since oxidative stress is a matter of equilibrium, both ROS and oxidatively modified macromolecules play a dual role in the cells. At low physiological, or under-toxic, levels ROS can stimulate a pro survival response. As the ROS concentration and the cellular damage increase the cellular response can switch from a pro-survival to a cell death program such as apoptosis or necrosis (Elmore, 2007). Apoptosis refers to a tightly-regulated energy-dependent mechanism of cell death that involves various molecular pathways. Apoptosis occurs as a normal process during development and aging and cellular turnover in tissues as well as a defense mechanism against toxins or stressors and during immune response (Elmore, 2007). Apoptotic cells are characterized by shrinkage and

exhibit several biochemical modifications such as protein cross-linking and cleavage, DNA fragmentation, membrane loosing and phosphatidylserine (PS) exposure. PS exposure to the outside face of membrane cells is recognized by phagocytic cells that get rid of dead cells and cell debris.

There are three main apoptotic pathways: the extrinsic or death receptor pathway, the intrinsic or mitochondrial pathway and the perforin-granzyme dependent pathway (Fig. 1.3; Elmore, 2007). All the three pathways involve the group of cysteine proteases, known as caspases, which activate upon stimulation via autocatalytic cleavage.

The extrinsic and perforin/granzyme pathways are mediated by cytotoxic T lymphocytes (CTL) to kill specifically virus-infected or transformed cells. The extrinsic pathway is initiated by activation of death-receptors such as Fas receptor (FasR) or tumor necrosis factor (TNF) receptor and as consequence lead to the formation of the death-inducing signaling complex (DISC). This, in turn, stimulates the auto-catalytic activity of procaspase 8. Active caspase 8 triggers the execution phase via activation of procaspase 3.

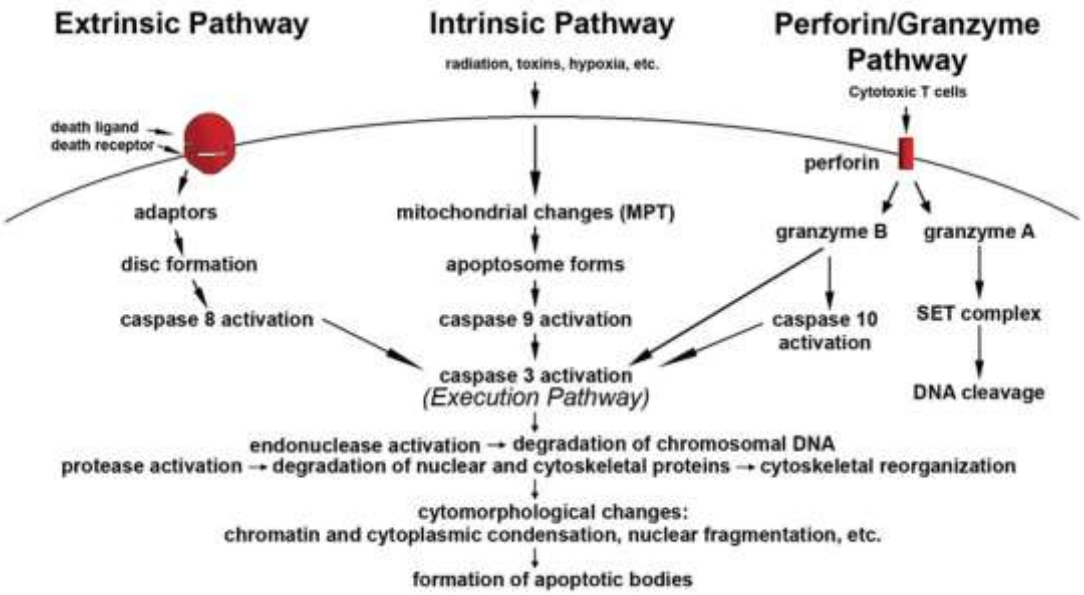


Fig. 1.3 **Representation of the three apoptotic pathways.** The three apoptotic pathways are activated upon different stimulations but converge in the activation of the same execution pathway (source from Elmore, 2007).

Perforins, a class of pore forming proteins, and granzymes, a class of serine proteases, are secreted by CTL via exocytosis and subsequently uptaken by the

target cells. Granzymes A and B are the main responsible for apoptotic induction and, in particular, they induce cell death via caspase-independent and caspase-dependent mechanisms respectively. Granzyme B activates by cleavage procaspase 10 which, in turn, activates procaspase 3, or else directly activates procaspase 3. Granzyme A, instead, disrupts by cleavage the complex responsible for DNA protection and repair (SET complex) and induces DNA degradation via DNase activation (Elmore, 2007).

The intrinsic pathway activated by a wide range of stimuli that include starvation, radiations, toxins and oxidative stress and it is usually triggered by mitochondria that act as sensors of cellular health. All of these stimuli result in change of mitochondrial membrane permeability, opening of the mitochondrial permeability transition (MPT) pore, and release of cytochrome c and Smac/DIABLO protein groups from the intermembrane space into the cytosol. These, in turn, trigger the activation of procaspase 3 (Elmore, 2007). The intrinsic pathway is regulated by the members of the Bcl2 family of proteins (Cory and Adams, 2002) which include both pro-apoptotic (Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, Blk, Puma and Noxa) and anti-apoptotic (Bcl2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG) proteins. These proteins are mainly involved in the regulation of cytochrome c release from the mitochondria. Bcl2 inhibits cytochrome c release in the cytoplasmic space via inhibition of Bax. Upon stimulation, Puma and Noxa localize to the mitochondria and interact with anti-apoptotic Bcl2. Inhibition of Bcl2 allows the release of cytochrome c and, in turn, the activation of caspases 9 and 3 (Oda et al., 2000).

Both intrinsic and extrinsic apoptotic pathways culminate in the execution pathway with the activation of procaspase 3. Activated cleaved caspase 3 triggers proteases which, in turn, cleave their respective substrates such as cytoskeleton or DNA repair proteins (i.e. PARP) and nucleases responsible for DNA fragmentation thus leading to cellular degradation.

Noxious agents might also lead to necrotic death. In contrast to apoptosis, necrosis does not involve tight-regulated molecular pathways and is usually accompanied by inflammation.

*Autophagic cell death*

Whether or not autophagy is a cell death mechanism is still under debate since it is unclear if the activated autophagy observed in dying cells is the cause of cell death either the last effort of a survival mechanism (Tsujimoto and

Shimiyu, 2005; Liu and Levine, 2015). Autophagy is activated by starvation, as well as hypoxic or toxic conditions and high temperature, to counteract nutrient deprivation and to get rid of damaged organelles and cellular components. The molecular machinery is regulated by the autophagy-related gene (Atg) protein family, identified in yeasts, and the autophagic process has been conserved during evolution (Fig. 1.4). Autophagy starts with the formation of a double membrane structure (vesicle nucleation) promoted by phosphatidylinositol 3 (PI3) kinase class 3 complex, which include Atg6. The double membrane elongates to form a vesicle, the so-called autophagosome, which include the cytoplasmic material to digest. Autophagosome elongation and completion are carried out by two ubiquitin-like conjugation systems: the Atg12 and LC3 conjugation systems.

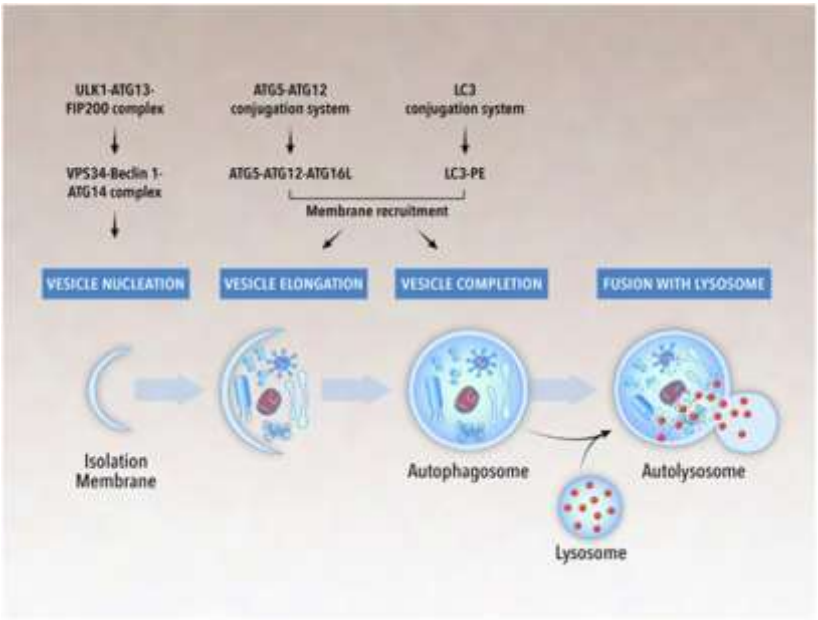


Fig. 1.4 **Representation of the autophagic pathway** (source from Liu and Levine, 2015)

Finally, the autophagosome fuses with lysosome to form the autophagolysosome. The lytic enzymes contained in the autophagolysosome are responsible for the degradation of the sequestered cytoplasmic material (Fig. 1.4; Liu and Levine, 2015).

Autophagic cell death has been proposed after the observation that the inhibition of autophagosome formation suppresses cell death induced by some specific cytotoxic agents in apoptotic deficient mouse and human cellular

systems (Shimizu et al., 2004; Yu et al., 2004). Moreover, the observation that autophagic cell death is triggered by Bcl2 family members in this model systems, let the authors hypothesize that mitochondria might be involved in regulation of autophagic cell death (Shimizu et al., 2004). The balance between pro-survival autophagy and autophagic cell death might rely on the regulation of Atg protein genes (Shimizu et al., 2004). Moreover, JNK activation has been suggested as another possible mediator that triggers autophagic cell death (Yu et al., 2004). However, the involvement of Atg members as well as JNK in other cell death mechanisms, such as apoptosis, makes challenging the statement of the existence of autophagic cell death, which remains controversial.

### *1.3 Oxidative stress and neurodegeneration*

The central nervous system (CNS) is the most susceptible organ to oxidative stress given the relatively low levels of antioxidants (i.e. GSH) and the high content of oxidable molecules such as catecholamines and polyunsaturated fatty acids (PUFA; Sayre et al., 2008). The brain alone consumes approximately 20% of the total energy supply of the body, including oxygen.

The bioenergetics of neural cells mostly relies on pentose phosphate pathway (PPP) for glucose metabolism (Herrero-Mendez et al., 2009; Rodriguez-Rodriguez et al., 2013). This represents an important route for neuronal cells to counteract the susceptibility to oxidative stress. Indeed, PPP is essential to sustain glutathione buffering via production of reduced NADPH during the first rate-limiting reaction catalyzed by Glucose-6-Phosphate Dehydrogenase (G6PD). Beside the redox balancing, PPP in neurons is also essential to reduce the mitochondrial mediated apoptotic cell death. Indeed, the increase in glycolytic pathway has been linked to increased cell death in neurons (Herrero-Mendez et al., 2008). This process can be prevented by the overexpression of G6PD in primary neurons (Herrero-Mendez et al., 2008; Vaughn and Deshmukh, 2008). Nevertheless, other forms of stress can injure the survival of neural cells and concur to oxidative stress.

Formerly in the 1970s has been introduced the concept of “excitotoxicity” claiming that high doses of excitatory neurotransmitters such as glutamate (Glu), aspartate (Asp) and cysteine (Cys) might be involved in neuronal degeneration (Meldrum, 1993). Glu has been shown to exert a toxic effect via overstimulation

of N-methyl-D-aspartate (NMDA)-type glutamate receptors (NMDARs) and subsequent increase in calcium ions ($Ca^{+}$) concentration in the cytoplasm (Ferreira et al., 1996; Bai et al., 2013). Increased $Ca^{+}$ influx through the activation of Glu-gated anion channels seems to be an important source of ROS, such as NO or hydrogen peroxide, in neuronal cells (Coyle and Puttfarcken, 1993) which, in turn, trigger apoptotic cell death (Goldshmit et al., 2001; Pugazhenthi et al., 2003; Vincent and Maiese, 1999). In addition, NO plays a role as a second messenger for inflammatory response by activating the microglia in the brain. Microglia are the resident immune system of the brain which originate from the extraembryonic endoderm and migrate in the central nervous system during embryonic development. Activated microglia are responsible for removal of apoptotic cells in the brain and for stimulation of the active immune response upon infection, trauma, molecules or protein aggregates-induced toxicity. Indeed, activated microglia promote the activation of astrocytes and peripheral immune system via production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and free radicals such as NO and superoxide (Sankarapandi et al., 1998).

The involvement of NO in both neuroinflammatory response and oxidative stress suggest a possible interplay that might underlie the pathogenesis of neurodegenerative diseases such as Parkinson´s disease (PD), Alzheimer´s disease (AD), and amyotrophic lateral sclerosis (ALS), in which both of them are common features.

Parkinson's Disease (PD) is the most common neurodegenerative disease with motor symptoms that occurs usually around 55 years age. Most of the cases are identified as sporadic (idiopathic PD) late-onset, while only few cases have a familiar transmission and are characterized by an early-onset. The hallmarks of the pathology are resting tremors, rigidity, postural instability and other non-motor symptoms such as pain, depression, constipation, sleep disturbances and urogenital problems. The molecular hallmark of PD is the formation of insoluble aggregates of alpha-synuclein (α-syn) protein in the neurons, the so-called Lewy bodies or Lewy neurites. Moreover, PD is characterized by the loss of dopaminergic neurons in the Substantia nigra *pars compacta*. PD, as well as, other neurodegenerative diseases, is also characterized by mitochondrial and lysosomal dysfunction, proteasomal impairment and oxidative stress. Reported evidences of oxidative damage in PD include accumulation of protein carbonyls and 4-HNE protein adducts in PD brains, decreased levels of antioxidant, such as

GSH, ascorbate and vitamin E in brain and plasma from PD patients, increased mitochondrial ROS in PD fibroblasts and increased expression of HMOX2 and mitoSOX in differentiated neurons from patient-derived iPS cells (Giordano et al., 2014). The etiology of the disease is complex, because genetic alteration, environmental factors and aging concur with the outcome of the pathology. Genome wide association studies (GWAS) have identified several generic risk factors that might be implicated in the outcome. These include the 16 "PARK" loci, including the PTEN-induced kinase 1 (PINK1 or PARK6), Parkin (PARK2), SNCA (PARK1/4), LRRK2 (PARK8), e DJ-1 (PARK7) (Simón-Sánchez et al., 2009). Interestingly, all of these genes are responsible for mitochondrial dysfunctions in familial PD (Subramaniam et al., 2013). The first indication of the link between mutations in alpha-synuclein (SNCA) gene and PD dates to 1997 (Polymeropoulos et al., 1997; Maroteaux et al., 1988). Mutations in SNCA gene lead to protein misfolding and/or overexpression and are usually associated with increased α-syn protein aggregation and toxicity. However, the α-syn protein function and the molecular mechanisms that lead to α-syn aggregation and toxicity in dopaminergic neurons are still unclear. Noteworthy, dopaminergic neurons are even more susceptible to oxidative stress due to the products of dopamine oxidation that represent another source of oxidative stress.

Even though oxidative stress is extremely common in neurodegenerative disease where aging is an important feature, it seems to play an important role also in other brain diseases not age-related, such as Rett Syndrome. However, whether oxidative stress is a mere consequence of degenerative processes initiated by some other factors, for example genetic, or whether oxidative stress is an early event that contributes integrally to the etiology of brain diseases is still an open question

# Chapter 2

# UNREVEALING OXIDATIVE STRESS IN RETT SYNDROME

## 2.1 Introduction

Rett Syndrome (RTT, OMIM #312750) is a neurological disease affecting almost exclusively girls with an incidence of 1:10.000 live births (Lyst and Bird, 2015).

The pathology is characterized by a former, apparently normal, post-natal development followed by a development arrest around 6 to 18 months of age. At this stage the clinical symptoms arise, and these include loss of acquired motor and cognitive skills, including speech, and the insurgence of stereotypical movements, breathing disturbances, microcephaly, seizure and mental retardation. Most of the cases (from 85 to 94%) are also characterized by a strong growth delay that might be one of the first indications of the pathology.

RTT is a monogenic disorder mostly caused by *de novo* sporadic loss of function (LoF) mutations in the X-linked gene encoding for Methyl CpG binding Protein 2 (MeCP2; Amir et al., 1999). Thus, the pathology has a negative dominant inheritance in female and, given to the X chromosome localization, it is early lethal in hemizygous males.

MeCP2 gene encodes for two protein isoforms highly expressed, even though not exclusively, in post mitotic neurons throughout the brain where are supposed to be responsible for normal neuronal function and structural maintenance (Lyst and Bird, 2015). Indeed, RTT patients show a reduction in neuronal cell size, neuritic length and dendritic spines density.

MeCP2 binds to DNA at the cytosine site in metilated CpG rich domains, where it regulates gene expression and epigenetic modifications. MeCP2 binds to methylated DNA mainly through the methyl-binding domain (MBD) even though other DNA binding motifs have been describing (Lyst and Bird, 2015). DNA methylation is a known signature for gene silencing and MeCP2 has been proposed to regulate gene expression via recruitment to DNA of co-repressors

and histone deacetylates leading to inhibition of transcription (Nan et al., 1998; Jones et al., 1998). Nevertheless, microarray studies have shown the downregulation of several genes in MeCP2 null mice supporting its role as transcriptional activator (Chahrour et al., 2008). Moreover, Chahrour and collaborators (2008) have shown MeCP2 interaction with the transcriptional activator CREB on active promoters. One of the main targets of MeCP2 is the Brain Derived Neurotrophic Factor (Bdnf), which plays an essential role in neuronal survival and plasticity (Filosa et al., 2015).

Beside its role in gene expression regulation, MeCP2 is also responsible for epigenetic modification in the nervous system. MeCP2 has been shown to regulate chromatin structure, in particular the structural organization of chromocenters and, in turn, in the heterochromatinic architecture in neural cells (Bertulat et al., 2012). Moreover, MeCP2 plays a role in alternative splicing (Maunakea et al., 2013), miRNA processing (Cheng et al., 2014) and perhaps it regulates transposition in neurons (Muotri et al., 2010).

Despite many studies have been done to understand the function of MeCP2, are still unknown the molecular mechanisms that link MeCP2 mutations to the pathology outcome. Especially, it is still unknown the role of MeCP2 in brain development and physiology (Guy et al., 2011). Most of the mutations causing the pathology onset are missense mutations occurring in the DNA binding domains that, affecting the MeCP2 binding to DNA, might influence co-repressor recruitment and heterochromatin structure, ultimately influencing normal neuronal functions (Lyst and Bird, 2015). Important to note, the re-expression of MeCP2 protein rescues the phenotype in RTT mouse models.

During the last decade has emerged that RTT patients have high levels of circulating oxidative stress markers (De Felice et al., 2009; Pecorelli et al., 2011; Leoncini et al., 2011). De Felice et al. (2014) confirmed redox alterations in two RTT mouse models (Mecp2-null e Mecp2-Loss of Function), showing reduced antioxidant enzyme activity and increased levels of oxidative stress markers in brain tissues. Noteworthy, oxidative stress seems to precede the pathology outcome in mouse models of RTT, even though is still unclear the role of oxidative stress in disease progression (De Felice et al., 2014).

Several *in vivo* and *in vitro* models are currently available for studying RTT. Over the last decades, stem cells have been emerging as a powerful model system for several studies.

Mouse embryonic stem (mES) cells are derived from the inner cell mass (ICM) of a mouse embryo at the blastocyst stage. mES cells can be maintained undifferentiated in culture for several passages without losing their differentiation potential and are able to differentiate, upon induction, into various cell lineages. Several protocols have been established to differentiate stem cells in several cell types derived from all the three embryonic germ layers.

Moreover, it is possible to genetically engineer stem cells in order to obtain specific mutant isogenic cell lines. Upon injection into a mouse blastocyst, genetically engineered stem cells integrate within the ICM, thus generating a chimeric mouse. If the engineered cells colonize germinal tissues of the chimeric mouse it is possible to generate, through chimeric mice inbreeding, transgenic animals carrying the desired mutation.

Beside the generation of transgenic animals, it is possible to study the role of the gene of interest both in stem cells or stem cell differentiated cell lines, in a simplified *in vitro* model system. For example, the protocol described by Fico et al. (2008) is an optimized one for differentiation of mESC into neural and glial cells in monolayer, without co-culture, embryoid bodies (EBs) formation, or intermediary steps of cell dissociation and replating. The protocol relies on the use of the Knock-out serum replacement (KSR)-supplemented medium and is able to generate the four different neuronal subtypes (γ-aminobutyric acid (GABA)-ergic, dopaminergic, serotonergic, and motor neurons) after 13 days in culture.

The aim of this work was to investigate about the correlation between oxidative stress and Rett syndrome and, in particular on the molecular mechanisms that might be involved.

Taking advantage of the mES cell line carrying the null mutation for MeCP2 (MeCP2-/Y mES cells; Bertulat et al., 2012), I aimed to identify the molecular pathways activated upon oxidative stress stimulation in mES derived neurons and to understand whether or not the protein MeCP2 was involved in the protection against oxidative stress in neurons. The interest was also to analyze whether oxidative stress influenced genes expression as well as functional and morphological alterations in Wild type (WT) and MeCP2-/Y mES derived neurons in a similar way to what observed in RTT patients.

## 2.2. MATERIALS AND METHODS

### *2.2.1 mES cells culture*

WT and MeCP2-/Y mES cell lines were kindly provided by the Maurizio D'Esposito lab (IGB-CNR, Naples).

Both, WT and MeCP2-/Y were cultured feeder-free on gelatin coated six well plates and maintained undifferentiated in LIF containing medium (mES Medium). Medium change was performed every day and cells were passaged when reach a confluency of c.a. 70-80%, usually every two days as follows.

*mES cells passaging protocol*

At least 1 hour before passaging coat 6-well plate using 1 ml of gelatin solution per well and incubate at room temperature for 1 hour.
Warm a sufficient volume of mES medium at room temperature.
Aspirate the medium from the well and wash twice with 1 ml of PBS1X. Add 1 ml 0.05% trypsin solution and incubate at room temperature for 10 min. In the meantime, aspirate gelatin solution from the well and let it dry.
Neutralize trypsin solution with addition of 1ml medium and dissociate cells by pipetting up and down several times in order to obtain a single cell suspension.
Transfer the cell suspension at the desired dilution in gelatin coated well containing 2 ml of medium.
Move the plate back and forth to distribute cell suspension and place the plate in the incubator.

*mES cell freezing protocol*

One confluent well can be passaged in three vials.
Aspirate medium from the well and wash twice with 1 ml of PBS1X. Add 1 ml 0.05% trypsin solution and incubate at room temperature for 10 min.
Neutralize trypsin solution with addition of 1ml medium and dissociate cells by pipetting up and down several times in order to obtain a single cell suspension.
Transfer cell suspension in 15 ml tube and centrifuge at 1000 RPM for 5 min. Aspirate the supernatant and resuspend cells in 3 ml of freezing medium (10% DMSO in FBS). Transfer 1 ml cell suspension in labelled cryovials and place promptly in dry ice. Move and store the frozen vials in the liquid Nitrogen tank.

*mES cells thawing protocol*

At least 1 hour before passaging coat a 6-well plate using 1 ml of gelatin solution per well. Incubate at room temperature for 1 hour.
Warm a sufficient volume of mES medium at room temperature.
Thaw the cells by adding 1 ml of warm medium to the frozen pellet. And gently transfer the volume in a 15 ml tube containing 8 ml of medium.
Centrifuge at 1000 RPM for 5 min at room temperature.
Aspirate the supernatant and gently re-suspend the cells in 2 ml of medium.
Aspirate the gelatin solution from the well and plate the 2 ml cells suspension.
Move the plate back and forth to distribute cell suspension and place it in the incubator.
Change the medium the next day.

### *2.2.2 Neural differentiation*

Neural differentiation has been performed using the protocol established by Fico at al., (2008).
Briefly, the cells were washed with PBS1X and dissociated in trypsin solution to obtain a single-cell suspension. The cells were seeded in gelatin coated six-well plates in neuronal differentiation medium (KO Medium). Respectively 1000 cell/$cm^2$ and 1500 cell/$cm^2$ for WT and MeCP2-/Y mES cells were seeded (according to Bertulat et al., 2012).
The culture medium was replaced every other day until day 4 and afterwards every day until day 13.

### *2.2.3 Oxidative burden*

At day 13 of differentiation cells were exposed to oxidative stress by treatment with Glucose Oxidase (GOX) an enzyme that produces $H_2O_2$ in the plate via oxidation of the glucose contained in the medium.
GOX was diluted to different final concentrations (10, 25, 50, 75 and 100 mU/ml) in KO Medium.
The culture medium was replaced with GOX containing medium and incubated for 30 min at 37°C. The culture medium was then replaced with fresh medium

and cells were harvested at different time points, respectively 1, 3, 5, and 8 hours after the pulse with GOX.

### *2.2.4. Fixation and Immunostaining*

At day 13 of differentiation cells were fixed for immunostaining as follows.
Aspirate the medium and wash once with 1 ml D-PBS1X.
Fix the cells in 4% paraformaldehyde in PBS1X (4% PFA) for 15 min at room temperature.
Remove the fixation solution and wash three times with 1 ml D-PBS 1X for15 min at room temperature.
Perform cell permeabilization and aspecific blocking by incubating 10% normal goat serum (DakoCytomation, Glostrup, Denmark)/0,1 % triton X-100 in PBS 1X for 15 min RT.
Dilute primary monoclonal anti-βIII-Tubulin antibody (1:400; Sigma-Aldrich) in 10% NGS (Dako)/PBS and incubate overnight at 4°C.
Wash three times in PBS for 5 min at room temperature.
Dilute secondary antibody in 10% NGS (Dako)/PBS and incubate 1 h at room temperature, protect from light.
Wash three times in PBS for 5 min at room temperature.
Counterstain nuclei with DAPI (250 ng/mL, Sigma-Aldrich) for 2-3 min, at room temperature, protect from light.

Images were acquired using epifluorescent inverted microscope (Leica-Microsystem) and processed with FiJi.

### *2.2.5. Western Blotting*

*Protein isolation*

Total cell lysates were prepared from differentiated mES cells treated with GOX at different time points, 1h, 3h, 5h, and 8h, after treatment in 100-500 µl Tergitol-type NP-40 lysis buffer:

- Tris-HCl pH 8 20 mM
- NP40 0.1 %
- Glycerol 10 %

- NaCl 138 mM
- EDTA pH 8 10 mM
- $H_2O$ MilliQ

supplemented with:

- 0.5 mM DTT;
- 0.5 mM PMSF;
- 2 mM benzamidine;
- 20 μg/μl aprotinin;
- 4 μg/μl pepstatin;
- 10 μM leupeptine;
- 10 mM NaF;
- 1 mM $Na_3VO_4$;
- 25 mM β-glicerophosphate (Sigma-Aldrich)

From 100 to 500 μl of lysis buffer was added to each sample according to the pellet size and incubated on ice for 25 min, vortexing briefly every 5 min.
After centrifugation at 14000 RPM for 30 min at 4°C, the supernatants were transferred in a new 1.5 ml tube and the pellet were discarded.
Protein concentrations were determined with Bradford assay by measuring the absorbance at 595 nm with the spectrophotometer and relative comparison to BSA standard curve.
Protein lysates were stored at -80°C.

*SDS-PAGE and blotting on PVDF*

Total cell extracts were normalized to 10 μg and separated on either 10% or 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were then transferred on PVDF membrane (Millipore) at 100 Volt for 1 hour at 4°C.
Aspecific sites were blocked with 5% nonfat milk solution for 1 hour at room temperature and membranes were incubated with primary antibody overnight at 4°C. Membranes were washed three times for 10 min at room temperature with washing solution and incubated with secondary antibody for 1 hour at room temperature.
The immune complexes were detected by the ECL detection system according to the manufacturer's protocol (Amersham Bioscience).

List of Antibodies

| Antigen | Host species | Cat. n | Supplier | Dilution |
|---|---|---|---|---|
| *P-Erk1/2* | rabbit | #4376 | Cell signaling | 1:1000 |
| *Erk1/2* | rabbit | #4695 | Cell signaling | 1:1000 |
| *P-JNK* | rabbit | #9251 | Cell signaling | 1:1000 |
| *JNK* | rabbit | #9252 | Cell signaling | 1:1000 |
| *P-p38* | mouse | sc-166182 | Santa Cruz Biotechnology | 1:1000 |
| *P38* | rabbit | #9212 | Cell signaling | 1:1000 |
| *Bcl-2* | rabbit | #3498 | Cell signaling | 1:1000 |
| *PARP* | rabbit | #9542 | Cell signaling | 1:300 |
| *Cleaved Caspase-3* | rabbit | #9661 | Cell signaling | 1:1000 |
| *MeCP2* | rabbit | M9317 | Sigma Aldrich | 1:2000 |
| *Actin* | rabbit | A2066 | Sigma Aldrich | 1:2500 |
| *Anti-rabbit* | goat | AP307P | Sigma Aldrich | 1:10000 |
| *Anti-mouse* | goat | 31431 | Thermo Fisher Scientific | 1:10000 |

### *2.2.6. Media and Solutions*

**mES cell medium (mES Medium)**

| | |
|---|---|
| Dulbecco's Modified Eagle Medium (DMEM) | 500 ml |
| Fetal bovine serum 15% | 90 ml |
| Glutamine 2 mM | 6 ml |
| Sodium pyruvate 1 mM | 6 ml |
| β-mercaptoethanol 1 mM | 1.2 ml |
| LIF 1000 U/ml | |

**Neuronal differentiation medium (KO Medium)**

| | |
|---|---|
| Knockout Dulbecco's minimal essential medium | 500 ml |
| Knockout Serum Replacement (KSR) (Invitrogen) 15% | 90 ml |
| Glutamine 2 mM | 6 ml |
| Penicillin/ streptomycin 100 U/ml | 6 ml |
| β -mercaptoethanol 0.1 mM | 1.2 ml |

| **PBS1X** | **final vol. 5 L** |
|---|---|
| $Na_2HPO_4$ | 5.75 g |
| NaCl | 40 g |
| KCl | 1 g |
| $KH_2PO_4$ | 1 g |
| $H_2O$ MilliQ | Up to 5 L |

| **Trypsin solution** | **final vol. 1 L** |
|---|---|
| NaCl | 8 g |
| KCl | 0.4 g |
| Glucose | 1 g |
| $NaHCO_3$ | 0.59 g |
| EDTA | 0.29 g |
| Trypsin | 0.5 g |
| $H_2O$ MilliQ | Up to 1 L |

**Acrylamide gel preparation:**

| **Lower gel** | **final vol. 10 ml** | |
|---|---|---|
| | **12.5%** | **10%** |
| Tris-HCl pH 8.8 1.5 M | 2.5 ml | 2.5 ml |
| Acrylamide 30% (SIGMA) | 4.17 ml | 3.3 ml |
| $H_2O$ MilliQ | 3.12 ml | 4 ml |
| SDS 10% | 100 µl | 100 µl |
| APS 10% | 100 µl | 100 µl |
| Temed | 10 µl | 10 µl |

| **Upper gel 5%** | **final vol. 5 ml** |
|---|---|
| Tris-HCl pH 6.8 1 M | 630 µl |
| Acrylamide 30% (SIGMA) | 830 µl |
| $H_2O$ MilliQ | 3.435 ml |
| SDS 10% | 50 µl |
| APS 10% | 50 µl |
| Temed | 5 µl |

| **Laemly buffer 4X** | **STOCK** | **final vol. 10 ml** |
|---|---|---|
| Tris-HCl pH 6.8 0.25 M | 1 M | 2.5 ml |
| SDS 8% | | 800 mg |
| β -mercaptoethanol 20% | | 2 ml |
| Glycerol 40% | 100% | 4 ml |
| bhromo phenol-blue 0.04% | 1% | 400 µl |
| $H_2O$ MilliQ | | Up to 10 ml |
| Stored a 4°C | | |

| **Running Buffer 1X** | **STOCK** | **final vol. 1 L** |
|---|---|---|
| Tris-Gly 1X | 10X | 100 ml |
| SDS 0.1% | 20% | 5 ml |
| $H_2O$ MilliQ | | up to 1 L |

| **Transferring Buffer 10X** | **STOCK** | **final vol. 1 L** |
|---|---|---|
| Tris-Gly 1X | 10X | 100 ml |
| MetOH 10% | 100% | 100 ml |
| $H_2O$ MilliQ | | 800 ml |

| **Blocking Solution** | **STOCK** | **final vol. 50 ml** |
|---|---|---|
| TBS pH 8 1X | 10X | 5 ml |
| Tween20 0.1% | 100% | 50 µl |
| Nonfat Milk 5% | | 2.5 g |
| $H_2O$ MilliQ | | Up to 50 ml |

| **Washing Solution** | **STOCK** | **final vol. 500 ml** |
|---|---|---|
| TBS pH 8 1X | 10X | 50 ml |
| Tween20 0.1% | 100% | 500 µl |
| $H_2O$ MilliQ | | Up to 500 ml |

| **TBS10X pH 8** | **STOCK** | **final vol. 500 ml** |
|---|---|---|
| Tris-HCl pH 8 100 mM | 2 M | 25 ml |
| NaCl 1.5 M | 5 M | 150 ml |
| $H_2O$ MilliQ | | Up to 500 ml |

| **Stripping buffer** | **STOCK** | **Final vol. 50 ml** |
|---|---|---|
| Tris-HCl pH 6.7 62.5 mM | 1 M | 6.25 ml |
| SDS 2% | 20% | 5 ml |
| $H_2O$ MilliQ | | Up to 50 ml |
| Supplement with β-mercaptoethanol | 100 mM | (357.5 µl per 50 ml) |

## 2.3 RESULTS

### *2.3.1 Neural differentiation of WT and MeCP2-/Y mES cell lines.*

I used the single-step monolayer differentiation protocol mentioned above (Fico et al., 2008) to generate neurons from both WT and MeCP2-/Y mES cell lines (Bertulat et al., 2012) and I confirmed the efficient differentiation of both cell lines via immunostaining for the pan-neuronal marker βIII-Tubulin (Fig. 2.1).

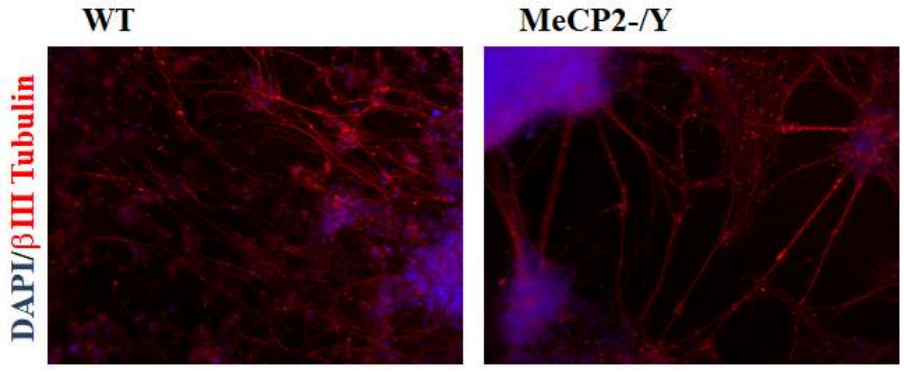


Fig. 2.1 **Immunostaining for the neural marker βIII-Tubulin in WT and MeCP2-/Y mES derived neurons at day 13 of differentiation.**

### *2.3.2 MAPK activation upon oxidative stress stimulation of WT and MeCP2-/Y mES cells derived neurons.*

Starting from the published data showing increased oxidative stress in RTT patients and RTT mouse models I analyzed whether MeCP2 was modulated by

oxidative stress. To this aim I tested the effect of oxidative burden on WT and MeCP2-/Y mES differentiated neurons. Neurons at day 13 of differentiation were treated with Glucose Oxidase (GOX), an enzyme that oxidizing glucose produces $H_2O_2$. I analyzed the effect of different concentrations of GOX on MeCP2 expression.

As shown in Fig. 2.2, MeCP2 expression directly increased depending on $H_2O_2$ concentration. This result encouraged me to analyze if the MAPK pathways were differentially activated in MeCP2-/Y neurons after an oxidative burden. The Western blot analysis showed that the level of the active form of Erk 1/2 (P-Erk 1/2) increased in both WT and MeCP2-/Y derived neurons in a dose-dependent manner (Fig. 2.2), although Erk 1/2 was already phosphorylated in untreated MeCP2-/Y neurons. GOX treatment induced the activation of P-p38 in both cell lines even though the level of P-p38 was lower in MeCP2-/Y mES derived neurons compared to the WT counterparts.

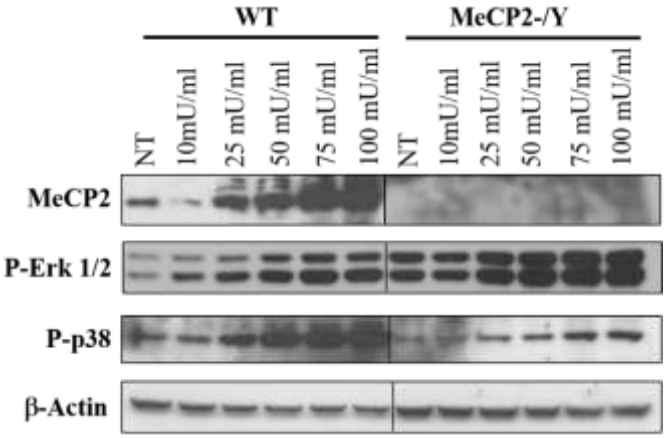


Fig. 2.2 **Dose-dependent MAPKs activation in WT and MeCP2-/Y mES derived neurons 1 hour after GOX treatment. β-Actin was used as loading control.**

To analyze if the observed MAPKs activation was time dependent, WT and MeCP2-/Y neurons were treated with 50 mU/ml GOX. This concentration was chosen since, as previously observed, it was sufficient to strongly activate the MAPKs. Moreover, neurons detachment was observed at higher GOX concentration (data not shown). I analyzed the MAPKs activation at four different time points after GOX treatment (1h, 3h, 5 h and 8h). Western blot analysis confirmed the differences in MAPKs activation. Indeed, P-Erk 1/2 activation was strongly activated during the time both in WT and MeCP2-/Y

treated neurons, and the maximum activation was observed 1 hour after treatment (Fig. 2.3).

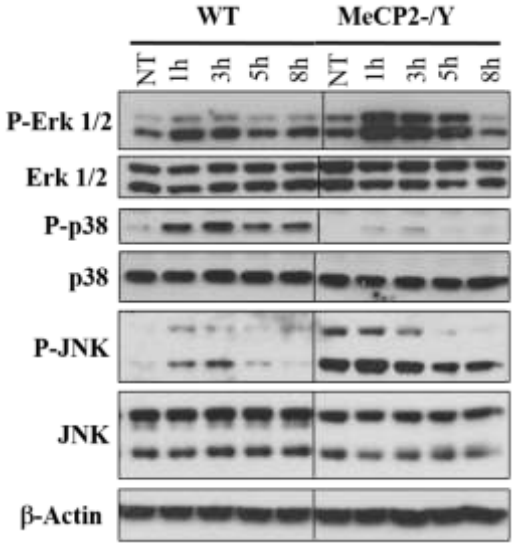


Fig. 2.3 **Time-course MAPKs activation in WT and MeCP2-/Y mES derived neurons after 50 mU/ml GOX treatment. β-Actin was used as loading control.**

P-p38 resulted almost not activated in MeCP2-/Y neurons, while in WT neurons its activation reached the maximum 3 hours after treatment and persisted until 8 hours. Finally, the analysis of JNK activation (P-JNK) showed that this pathway was already active in MeCP2-/Y untreated neurons and its activation decreased over the time after treatment. Instead, JNK activation reached the maximum 3 hours after treatment in WT neurons (Fig. 2.3).

### *2.3.3 Apoptotic response upon oxidative stress stimulation of WT and MeCP2-/Y mES cells derived neurons.*

As described above, MAPKs can induce either apoptotic or survival response after oxidative stress injury. To distinguish the role of MAPKs activation in these cellular systems, I analyzed if GOX treatment induced apoptosis in WT and MeCP2-/Y neurons. The Western blot analysis for apoptotic markers, 8 hours after treatment, showed that oxidative stress induced apoptosis only in WT neurons in a dose-dependent manner. Indeed, I observed a dose-dependent

cleavage of PARP and Caspase 3 only in WT neurons (Fig. 2.4). On the contrary, the anti-apoptotic marker Bcl2 was highly expressed in MeCP2-/Y neurons.

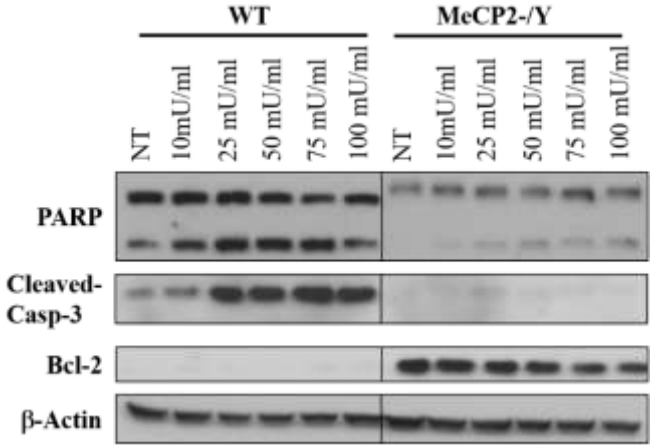


Fig. 2.4 **Dose-dependent apoptotic pathway activation in WT and MeCP2-/Y mES derived neurons 8 hours after GOX treatment. β-Actin was used as loading control.**

## 2.4 CONCLUSIONS

The first indication of oxidative stress in Rett Syndrome dates to 1987 when reduced level of the antioxidants GSH and vitamin E where detected in a single RTT patient (Sofic et al., 1987). Over the time, mounting evidences suggested the hypothesis that oxidative stress might play an important role in RTT pathophysiology. High levels of lipid peroxidation products, such as isoprostanes (IsoP) and 4-HNE, and level of oxidized proteins have been observed in plasma of RTT patients and both in plasma and brain of RTT animal models (De Felice et al., 2014). Regarding the possible role of oxidative stress in the RTT etiology, oxidative stress-mediated CpG modification has been reported to inhibits MeCP2 binding to DNA (Valinluck et al., 2004)

This work was made with the interest to investigate the possible role of MeCP2 protein in oxidative stress response. I took advantage of a mES cell line mutant for MeCP2 and the possibility to easily differentiate mES in neuronal cell to analyze MeCP2 function specifically in neurons. I differentiated both WT and MeCP2-/Y mES cell lines into neuronal cells to investigate the role of MeCP2 in oxidative stress-mediated cellular response.

As expected, oxidative treatment of WT and MeCP2-/Y neurons activated the MAPK pathway. Activation of MAPK pathways following oxidative stress has been described to induce different outputs. Indeed, according to the intensity and persistence of the stimulus, they can either increase the survival potential of the cells by inducing the transcription of several antioxidant enzymes or determine cell death.

Although my data do not permit to determine which MAPK is essential to induce apoptosis in WT cells, the activation of ERK and JNK in the untreated MeCP2-/Y neurons suggested a possible role of these MAPK in the induction and maintenance of the anti-apoptotic protein Bcl2 during oxidative stress.

The usage of specific inhibitors will allow to discriminate the precise role of the analyzed MAPK pathways.

The data presented also suggest a block of the apoptotic pathway induced by oxidative stress in MeCP2-/Y neurons. In particular, MeCP2 might act as a modulator of the expression of anti-apoptotic proteins, such as Bcl2.

It is important to note that neurodegeneration has not been observed in RTT patients. My data suggest that in absence of MeCP2, oxidative stress induced damage in neuronal cells, as previously described in mice, although the cells cannot activate the apoptotic pathway due to the overexpression of Bcl2.

During the last years, a new model system has been emerging as a very promising tool to model brain development and disease: three dimensional (3D) cerebral organoids (Pașca, 2018). This model system has already been used to model several brain diseases including Rett Syndrome (Mellios et al., 2018) and suggest that this technology has the potential to unveil aspects of brain functioning that are still hidden so far.

# Chapter 3

# CEREBRAL ORGANOID: A NEW MODEL FOR BRAIN DISEASES

## 3.1 INTRODUCTION

In recent years, new protocols have been established to generate three-dimensional (3D) cultures that mimic the physiology and the developmental process of an actual human organ (Pașca, 2018). In particular, a special effort has been made on generating brain specific 3D cultures because our knowledge of human brain development is largely derived from studies in animal models, particularly rodents (Renner et al., 2017).

Albeit, numerous are the differences between the rodent and human brain structure and development. For example, the rodent cerebral cortex is smooth, while human cortex is convoluted due to the big surface expansion during evolution. The main source of interneurons in human are the ganglionic eminences (GE) of the ventral telencephalon and the ventricular zone/subventricular zone (VZ/SVZ) of the dorsal telencephalon, while in rodents the interneurons are generated only in the GE.

The primate SVZ contains a much larger population of neural progenitors compared to rodent brain. In addition, the rodent brain misses some regions such as the Broca and Wernicke areas or the prefrontal granular cortex.

These are only few of the several differences between the rodent and primate brain.

Some limitations of mouse models have been overcome by the introduction of human pluripotent stem cells generated via cellular reprogramming. The expression of the “magic quartet” of transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) in differentiated somatic cells induces their conversion into pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006). iPS cells can be propagated in culture in an undifferentiated state as well as induced to differentiate *in vitro* into all the three germ layers derivatives, including neural cells. The use of human iPS cells-derived neurons, thus, allows studying differentiation patterning and

molecular pathways involved in both health and disease, in a human-specific model. Although numerous differentiation protocols have been established to generate a variety of neural cell types from pluripotent stem cells, two-dimensional (2D) cultures do not allow to fully recapitulate the complex architecture and functioning of the developing human brain.

Instead, 3D cultures generated from human stem cells, offer an unprecedented opportunity to model and study human brain development and diseases (Clevers, 2016; Di Lullo and Kriegstein, 2017).

By relying on the self-aggregation and self-organization capacities of pluripotent stem cells, it is possible to generate a complex structure that mimics the cytoarchitecture of an actual human brain. These so-called cerebral organoids contain several neuronal cell types that organize in brain-like structure including forebrain, midbrain, retina, choroid plexus as well as ventricular and subventricular zones.

Cerebral organoids contain also organized germinal zones and forebrain-organizing centers that are generated according to the *in vivo* spatial and temporal patterning. Moreover, both radial and tangential migrations of cortical neuron populations are observed in cerebral organoids (Renner at al., 2017).

Several studies have shown that differentiating cerebral organoids display an expression profile and epigenetic signature that correlate with fetal neocortex during human *in vivo* development (Camp et al., 2015; Luo et al., 2016; Quadrato et al., 2017).

Cerebral organoids can be grown for extended periods of time leading to the formation of structurally-defined synapses and dendritic spines that, together with the acquisition of firing potential, indicate that organoids can reach a high rate of maturation (Quadrato et al., 2017).

It is also possible to generate brain region-specific organoids, such as forebrain, midbrain and retinal organoids or cortical spheroids by addition of external factors that drive induction of unique tissue identities (Eiraku et al, 2008; Chambers et al, 2009; Kadoshima et al, 2013; Mariani et al, 2015; Pasca et al, 2015; Jo et al., 2016; Qian et al, 2016). Of course, these brain region-specific organoids lack the interactions between different regions constituting the brain (Renner et al., 2017)

Cerebral organoids have been used to model neocortex congenital diseases such as microcephaly and macrocephaly (Lancaster et al., 2013) as well as to model autism spectrum disorders (ASD; Forsberg et al., 2018), including Rett

Syndrome (Mellios et al., 2018), or Zika Virus pathology (Qian et al., 2016; Watanabe et al., 2017). Therefore, these studies suggest the possibility to study the contribution of environmental factors, such as drug exposure, viral infection, hypoxia or oxidative stress, and epigenetic alterations during early brain development. Epigenetic factors might play important role in the neurobiology of autism by regulating processes, such as cell proliferation, differentiation and survival, as well as neurite outgrowth, thereby influencing prenatal brain development (Forsberg et al., 2018).

Thus, cerebral organoids are well suited to study developmental diseases, but it is more challenging to model neurodegenerative disease such as PD, AD, ALS and HD. These pathologies are characterized by a late-onset. Idiopathic PD, for example, occurs typically around 50-60 years old age, while cerebral organoids, given their relatively poor maturation, better resemble early brain development.

Nevertheless, the use of cerebral organoids as model system for AD has already been reported. Cerebral organoids generated from AD patients-derived iPS cells recapitulate disease neuropathological phenotypes such as amyloid aggregation and tau protein hyperphosphorylation (Raja et al., 2016).

Beside the difficulty to model an adult brain, there are other limitations in the use of cerebral organoid as model system for brain studies. The structure that arises from 3D differentiation is not fully comparable to the architecture of an actual human brain and lack of rostro-caudal and dorso-ventral axes. Moreover, cortical lamination in cerebral organoid is incomplete and there is lack of vascularization in innate immune system. Lack of vascularization and microglia are major limitations that current research aims to overcome. The resident microglia, indeed, beside their role as immune system players, are directly involved in neural differentiation and maturation in the developing brain and vascularization is essential to support the oxygen and nutrient supply in the most inner part of the 3D culture. Its absence makes the long term culturing of cerebral organoids challenging. So far, vascularized organoids have been obtained via organoid engraftment in mice brains (Mansour et al., 2018).

Even though 3D cultures do not allow the full reconstruction of an actual functional brain, they recapitulate more faithfully the process of the human-specific brain development than mouse models and 2D culture systems.

The aim of this study was to first characterize the growth and differentiation of cerebral organoids (hereafter referred to as Minibrains, (MBs)) derived from a Wild type (WT) NAS6 iPS cell line and test strategies to make MBs generation

more stable and reproducible. Secondly, to use MB as model system for neurodegenerative diseases, especially PD. For this aim, I used a SNCA overexpressing iPS cell line to generate MB carrying one of the main genetic risk factors for PD in order to investigate the mechanisms that lead to α-syn protein aggregation and toxicity.

Since oxidative stress is a key phenomenon in PD pathology, I assessed whether SNCA overexpressing MBs show signs of oxidative stress. I sought to verify if MBs could recapitulate this key characteristic of PD thus allowing their use to study the mechanisms underlying the interplay between α-syn protein accumulation and oxidative stress.

## 3.2 MATERIALS AND METHODS

### *3.2.1 Human iPS cell culture*

*hiPS cell lines*

The NAS6 iPS cell line used in this study were obtained from the lab of Tilo Kunath (Devine et al., 2011). SNCA-overexpressing NAS6 iPS cell line has been generated by Dr. Salvador Rodriguez-Nieto from Peter Heutink´s lab via lentiviral transduction with SNCA-RFP virus (Fig. 3.8). mCherry-tagged SNCA gene was cloned under the constitutive EF1 promoter. Positive transduced cells were selected with blasticidine (10 µg/ml; InvivoGen) two days after transduction.

*hiPS cell culture*

WT NAS6 and SNCA overexpressing-NAS6 human iPS cell lines were maintained and propagated in feeder-free conditions in E8 flex medium supplemented with E8 flex supplement. Cells grew as colonies on Matrigel™ coated 6-well plates and were passaged as aggregates when they reach a confluency of c.a. 70/80%, usually every 4-7 days as follows.

At least 1 hour before passaging Matrigel™ dilute (according to the manufacturer) in cold DMEM/F12 medium to coat a 6-well plate using 1ml of diluted Matrigel™ per well and incubate at 37°C for 1 hour.

**Passaging with Gentle cell Dissociation Reagent**

Gentle cell Dissociation Reagent (GDR) is an enzyme-free reagent suitable for the dissociation of both human embryonic stem (ES) cells or human induced pluripotent stem (iPS) cells into cell aggregates for routine passaging or into a single-cell suspension.

Warm a sufficient volume of medium at room temperature. Do not warm medium in the water bath.

Use the microscope to visualize regions of differentiation and mark those on the bottom of the plate.

Remove regions of differentiation by aspiration.

Aspirate medium from the well and add 1 ml of GDR. Incubate at room temperature for 6-7 min.

Aspirate the GDR and add 1 ml of E8 flex complete medium. Detach the colonies by pipetting two-three times and transfer in a 15 ml tube containing a volume of E8 flex medium necessary for the appropriate dilution (usually 1:10).

Pipette once the suspension to break up the colonies in order to obtain a suspension of c.a. 100 μm aggregates.

Aspirate the Matrigel™ and plate the cell aggregates at the desired dilution. Move the plate back and forth to distribute cell aggregates and place the plate in the incubator.

Media change is required the day after passaging and every other day.

*hiPS cell freezing protocol*

One confluent well can be passaged in two vials.

Pre-treat the cells with 2 mM of thiazovidin for 1 hour at 37°C.

Label cryovials.

Aspirate medium from the well and add 1 ml GDR per well. Incubate at room temperature for 6-7 min.

Aspirate GDR and add 1 ml of cold freezing medium (10% DMSO in KSR).

Detach the colonies using a cell lifter and transfer the aggregates in a 15 ml tube containing cold freezing medium. Pipette once to break up the aggregates and transfer 1 ml in labelled cryovials. Place vials in a precooled box and transfer immediately to -80°C freezer. Move and store the frozen vials in the liquid Nitrogen tank.

*iPS cells thawing protocol*

Thaw Matrigel™ overnight on ice at 4°C.

Dilute Matrigel™ in cold DMEM/F12 medium and coat the 12 well plate with 500 µl of diluted Matrigel™ for 1 h at 37°C.

Thaw the cells by shaking the vial in a water bath set at 37°C. Let only a small pellet to stay frozen.

Add 1 ml of medium to the cells drop wise and gently transfer the volume in a 15 ml tube containing 8 ml of medium.

Centrifuge at 230 g for 3min at room temperature.

Aspirate the supernatant and gently re-suspend the cells in 2 ml of medium supplemented with thiazovidin 2 µM taking care to maintain the cells as aggregates.

Aspirate the Matrigel™ from the well and plate the cells at different dilutions. This gives the flexibility when the cells will be ready.

Move the plate back and forth to distribute cell aggregates and place it in the incubator.

Change the medium the next day without rock inhibitor. Hereafter medium can be refreshed every alternate day if using Essential 8 flex medium.

### *3.2.2 Cerebral organoid*

Cerebral organoids have been generated using the protocol established by Lancaster et al. (Lancaster and Knoblich, 2014) with few modifications as follows.

*Embryoid Body formation*

Cells have to be of high quality without sign of differentiation.

Pre-treat iPS cells with10 µM Rock inhibitor (RI; Y-27632) for 1 hour at 37°C.

Remove the medium from the well and wash the cells with 1 ml D-PBS 1X without calcium and magnesium.

Remove D-PBS and add 1 ml of Accutase per well. Incubate for 25 min at 37°C.

Pipette up and down to obtain a single cell suspension and transfer in a 15 ml tube containing 7 ml of hES medium without bFGF and RI.

Centrifuge at 270 g for 5 min. Carefully remove the supernatant. Avoid disturbing the cell pellet.

Resuspend the cells in 3 ml hES medium supplemented with 50 μM RI and 4 ng/ml bFGF.
Mix 20 μl of cell suspension with an equal volume of trypan blue and count the live cells using an automated cell counter.
Use the average of two replicates to calculate the cell density. Prepare the desired cell dilutions in hES medium supplemented with 50 μM RI and 4 ng/ml bFGF and seed 150 μl in each well of an ultra-low attachment U-shaped 96 well plate.
After 24 hours, small embryoid bodies (EBs) are generated in culture.
At day three of differentiation feed the EBs by carefully removing half of the volume. Add 1 volume of hES medium without RI and bFGF.
Culture EBs for 2-3 more days until they reach a size between 500 and 600 μm.

*Neural Induction*

When EBs reach a diameter of 500-600 μm and show bright and smooth edges, typically at day 6, transfer them into neural induction (NI) medium in ultra-low attachment 24 well plates.
Use wide bore 200 μl tips to aspirate the EB. Wait until the EB settle at the bottom of the tip and let the EB fall in the well of a 24 well plate containing 500 μl of NI medium. Avoid transfer old EB medium.
Feed Neuroepithelial Tissues every other day. For media change tilt the plate and let the tissues settle on the bottom. Carefully remove half of the existing medium and add 500μl of fresh NI medium. Culture Neuroepithelial tissues in NI medium for typically 4-5 days. After neural induction a translucent tissue with radially organized cells should appear.
Move to Matrigel embedding step to allow the growth and differentiation of the neuroepithelium into MB.

*Matrigel Embedding of neuroepithelial tissues in hanging drop*

Matrigel™ embedding step was performed in hanging drops on the lid of 10 cm Petri dishes to avoid the risk of contamination that might arise from the use of parafilm dimples.
Approximately 15 to 20 Matrigel™ embedded MB fit on the lid of a Petri dish. Enough Petri dishes have to be used according to the number of neuroepithelial tissues to be transferred.

Procedure:
Thaw Matrigel™ aliquots on ice at 4°C and warm N2B27 medium without vitamin A (VitA) at 37°C.
Use wide bore tips to transfer tissues one by one from the well to the lid of the 24-well plate.
Carefully, remove the excess of medium surrounding the tissue with an uncut 200 µl tip.
Take 15µl of thawed Matrigel™ using the wide bore tip and suck the tissue in the same tip.
Make a droplet on the lid of a Petri dish filled with 15 ml of PBS 1X (this is needed to avoid that MB dry during the incubation). Be careful that the tissue is positioned in the middle of the droplet.
Invert the lid onto the Petri dish and put back in the incubator. Incubate for 30 min at 37°C to let the Matrigel™ solidify.
Spray the forceps with 70% ethanol and let it evaporate for 1-2 min.
Invert the lid and use a cut 1 ml tip to add the warm N2B27–VitA medium to the embedded tissues. Using the warm medium will help to detach the Matrigel™ droplets from the lid.
Transfer the floating tissues in an ultra-low attachment 6-well plate. Up to four tissues can be cultured in the same well. Continue culturing the tissue droplets in a $CO_2$ incubator.
Change the medium after 24 hours.
Tilt the plate and let the tissues settle on the bottom. Use an uncut 1 ml tip to gently remove the medium from the well. Remove as much medium is possible. Gently add 4ml of fresh medium on the wall of the plate without disturbing the tissues.

*Cerebral organoid differentiation*

After 4 days of culture in N2B27–VitA, cerebral organoids were moved to the final differentiation medium N2B27+VitA as follows.
Tilt the plate and let the organoids seat on the bottom of the well. Carefully remove the N2B27–VITA medium and gently replace it with N2B27+VitA.
From now on, culture the organoids on an orbital shaker set at 100 RPM and perform complete medium change every three/four days.
Cerebral organoids have been harvested at different time points and processed for RNA isolation or immunostaining.

### *3.2.3 RNA isolation and qPCR*

*RNA isolation*

RNA isolation has been performed from whole organoids using RNeasy Mini Kit (Cat No./ID: 74106) from QIAGEN.

MBs were harvested at five time points of differentiation (d11; d32; d60; d90; d120) using a wide bore 200 µl tip for not yet embedded MB or a cut 1ml tip for Matrigel™ embedded MB and 1 MB was transferred in a 2 ml centrifuge tube. Excess culture medium was carefully aspirated and MB were washed with 500 µl of D-PBS 1X. This step was performed on ice. RLT buffer was added to MB (respectively 350 µl for not yet embedded MBs and 700 µl for Matrigel™ embedded MB). Samples were stored at -80°C until they have been processed. Contaminating DNA removal was performed trough DNase treatment.

RNA concentrations were measured using Nanodrop 2000 and RNA integrity and RIN were determined on a Bioanalyzer 2100 system (Agilent Technologies Inc.). Low quality samples were excluded from the further analysis.

*cDNA synthesis*

cDNA was synthesized from 1 µg of RNA using Superscript III reverse transcriptase (RT) (Thermo Fisher Scientific) as follows:

1st mix

- RNA 1 µg 12 µl
- Oligo dT 0,4 µg/ul 0,5 µl
- Random decamers 50 µM 0,5 µl
- dNTPs 10 mM 1 µl

Reaction was performed as follows:
Step1. 65°C x 5 min
Step2. 4°C x 2 min

2nd mix

- 1st mix 14 µl
- Buffer 5X 4 µl
- DTT 100 mM 1 µl

- RNase OUT 40 U/µl 0,5 µl
- SuperScript III RT 200 U/µl 0,5 µl

Reaction was performed as follows:

Step1. 25°C x 5 min
Step2. 50°C x 1 hour
Step3. 70°C x 15 min

cDNAs were stored at -20°C.

*Real-time PCR*

qPCR was carried out in triplicate on a QuantStudio real time PCR system (Thermo Fisher Scientific).

Reaction mix:

- cDNA 12 ng
- SYBR Green PCR master mix 2X (Thermo Fisher Scientific) 5 µl
- Primer Pair 10 µM 0,4 µl
- H2O 1,6 µl
- Final Volume 10 µl

The qPCRs were performed with QuantStudio 12K flex instrument from Applied Biosystem using the thermocycler parameters as follows:

| | |
|---|---|
| Hold stage: | Step1. 95°C x 20 sec |
| PCR stage: | Step1. 95°C x |
| | Step2. 62°C x 30 sec |
| Melt Curve stage: | Step1. 95°C x 15 sec |
| | Step2. 60°C x 1 min |
| | Step3. 95°C x 15 sec |

Normalized relative quantities (RQ) were calculated using Thermo Fisher analysis tool on Cloud with TBP, OAZ1 and RPLPO as reference targets (Hellemans et al., 2007).

Primer list:

| Gene | Forward Primer Sequence (5'-3') | Reverse Primer Sequence (3'-5') | Amp Length |
|---|---|---|---|
| *BRACHYURY* | TGCTTCCCTGAGACCCAGTT | GATCACTTCTTTCCTTTGCATCAAG | 121 bp |
| *BRN2* | CGGCGGATCAAACTGGGATTT | TTGCGCTGCGATCTTGTCTAT | 216 bp |
| *CUX2* | CGAGACCTCCACACTTCGTG | TGTTTTTCCGCCTCATTTCTCTG | 173 bp |
| *DLG4* | TCGGTGACGACCCATCCAT | GCACGTCCACTTCATTTACAAAC | 114 bp |
| *GATA4* | TCCAAACCAGAAAACGGAAG | GAAGGCTCTCACTGCCTGAA | 78 bp |
| *HMOX1* | AAGACTGCGTTCCTGCTCAAC | AAAGCCCTACAGCAACTGTCG | 247 bp |
| *MAP2* | CTCAGCACCGCTAACAGAGG | CATTGGCGCTTCGGACAAG | 95 bp |
| *NANOG* | CCCCAGCCTTTACTCTTCCTA | CCAGGTTGAATTGTTCCAGGTC | 97 bp |
| *NCAM1* | TGTCCGATTCATAGTCCTGTCC | CTCACAGCGATAAGTGCCCTC | 82 bp |
| *OAZ1* | AGCAAGGACAGCTTTGCAGTT | ATGAAGACATGGTCGGCTCG | 68 bp |
| *POU5F1* | CTTGAATCCCGAATGGAAAGGG | GTGTATATCCCAGGGTGATCCTC | 164 bp |
| *OTX1* | GCCTCCCCTTCCAGTCTTTC | GGGCAGAAACACGCCAGTTA | 140 bp |
| *PAX6* | GTGTCCAACGGCTGTGTGAG | CTAGCCAGGTTGCGAAGAAC | 254 bp |
| *RPLPO* | CCTCATATCCGGGGGAATGTG | GCAGCAGCTGGCCACCTTATTG | 95 bp |
| *SYN1* | TGCTCAGCAGTACAACGTACC | GACACTTGCGATGTCCTGGAA | 110 bp |
| *SNCA* | AAGAGGGTGTTCTCTATGTAGGC | GCTCCTCCAACATTTGTCACTT | 106 bp |
| *SOD1* | GGTGGGCCAAAGGATGAAGAG | CCACAAGCCAAACGACTTCC | 227 bp |
| *TBP* | TTCGGAGAGTTCTGGGATTGC | CACGAAGTGCAATGGTCTTTAG | 95 bp |
| *TRDX1* | ATGGGCAATTTATTGGTCCTCAC | CCCAAGTAACGTGGTCTTTCAC | 115 bp |

### *3.2.4 Histology and immunostaining*

MBs were harvested for immunostaining at day 45 and 75 of differentiation. Sample were prepared following the Lancaster protocol with few modifications (Lancaster and Knoblich, 2014) as follows.

*Fixation and sucrose/gelatin embedding*

Use a cut 1 ml tip to transfer MB to a standard 24-well plate. Aspirate the medium and wash once with 1ml D-PBS1X.
Fix the tissue in 4% PFA for 45 to 60 min at room temperature.
Remove the fixation solution and wash three times with 1 ml D-PBS 1X for 10 min at room temperature.
Replace D-PBS with 1 ml 30% sucrose solution in D-PBS and incubate overnight at 4°C.
The day after, thaw gelatin-sucrose solution and warm it at 37°C.
In the meantime, make the molds using aluminum-foil.
Equilibrate the tissue at 37°C for 20 min, then replace the sucrose solution with warm sucrose-gelatin solution. Keep the sucrose-gelatin solution warm to avoid the polymerization.
Put a small amount of warm sucrose-gelatin solution into the mold just to make a layer on the bottom. Then, use a cut 1 ml tip to transfer MB from the well and position in the center of the mold.
Put immediately in dry ice to freeze the tissue and store at -80°C.

*Cryosectioning*

MB were cut in 30 μm sections using the Leica Cryosat. All the sections have been collected on Super-frost Plus glasses and stored at -80°C.

*Protocol for immunostaining on frozen sections on glasses*

Thaw glasses for 15 min at room temperature.
Rehydrate the tissue and remove the sucrose/gelatin solution by rinsing glasses with PBT 0.2%.
Wipe the excess of liquid and draw hydrophobic circles around the slices with the Pap-pen. Let the liquid dry for 2 min.
Perform permeabilization and blocking in 1% Triton-X, 10% FBS, 1% BSA, 0.02% NaN3 in PBS 1X for 1 hour at room temperature.

Dilute primary antibody in 0.2% Tween-20, 1% BSA, 0.02% NaN3 in PBS 1X and incubate overnight at 4°C.
Wash three times in PBT 0.2% for 15 min at room temperature
Dilute Alexa Fluor™-conjugated secondary antibody (Invitrogen) in 0.2% Tween-20, 1% BSA, 0.02% NaN3 in PBS 1X and incubate for1 hour at room temperature (protect from light).
Wash three times in PBT 0.2% for 15 min at room temperature
Dilute stock solution of Hoechst 33342 (10 mg/ml) 1:10000 in PBT 0.2% and incubate 4 min at room temperature (protect from light).
Rinse with PBT (protect from light).
Mount with Antifade medium. Store the glasses at 4°C protected from light.
Images were acquired using either a Leica TCS SP8 or a Zeiss Axiovert microscope and processed with the respective softwares.

List of Antibodies

| **Antigen** | **Host species** | **Cat. n** | **Company** | **Dilution** |
|---|---|---|---|---|
| *TUJ-1* | rabbit | T200 | Sigma | 1:1000 |
| *MAP-2* | mouse | MAB3418 | Millipore | 1:250 |
| *PAX-6* | rabbit | 901301 | Biolegend | 1:300 |
| *NESTIN* | mouse | MAB5326 | Millipore | 1:1000 |
| *CTIP2* | rat | AB18465 | Abcam | 1:300 |
| *BRN2* | rabbit | 12137 | Cell signaling | 1:1000 |

## 3.3 RESULTS

### *3.3.1 Minibrain generation using different seeding cell density conditions*

I used the protocol described by Lancaster and collaborators (2014) to generate Minibrains (MB) from the WT NAS6 iPS cell line.

Since each cell line has a unique behavior in term of proliferation rate and differentiation commitment, I tested three different seeding cell density conditions (S1: 9000 cell/well; S2: 10000 cell/well; S3: 12000 cell/well) in order to find the one that allows the more robust generation of MB.

I followed the differentiation via brightfield images to monitor if MBs reach the proper size and shape at several steps of the differentiation protocol, according to Lancaster et al. (2014).

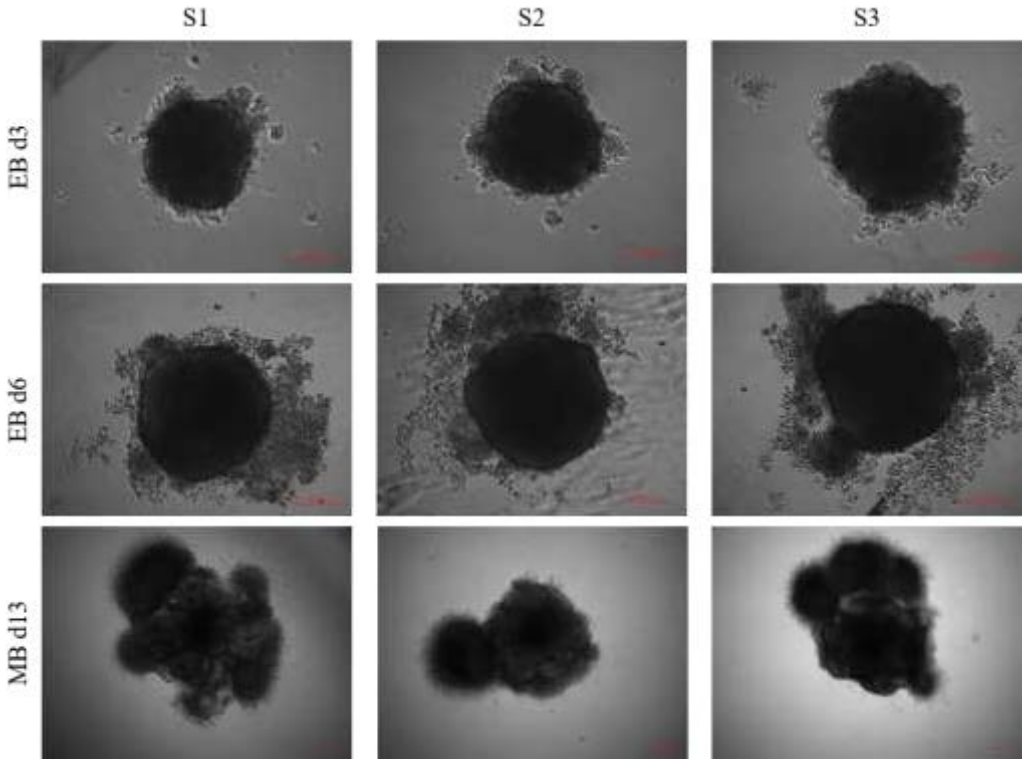


Fig. 3.1 **Seeding cell density conditions for generating WT NAS6 MB.** Cells seeded as single-cell suspension aggregate to form embryoid body (EB) structures. At day 3 of differentiation EB reach a size around 300µm. At day 6 of differentiation EBs reach a size around 600µm. At d13 of differentiation (2 days after Matrigel™ embedding) MB initiate differentiation and start to extrude neurites out of the neuroepithelial tissue. MBs from the three conditions do not show relevant morphological differences at none of the stage of differentiation.

Next, I harvested from 2 to 5 MBs at four time points during differentiation (d11, d32, d60 and d90 of differentiation) from three independent experiments and analyzed the expression of neural specific differentiation markers via qPCR. In particular, I selected pluripotency markers (NANOG, POU5F1), neural precursor markers (PAX6, OTX1) and mature neuron markers, including pan neuronal markers (MAP2, NCAM1, SYN1) and cortical markers (BRN2, CUX2, DLG4) to have an overall comprehension of MB differentiation.

The first data that emerged from the qPCR analysis is the high inter- and intra-experiment gene expression variability in MBs generated from all the three cell density conditions.

The second observation that can be drawn from this analysis is concerning the trend of differentiation. Gene expression for pluripotency markers was almost undetectable already at d11 of differentiation in MBs from all the three cell density conditions.

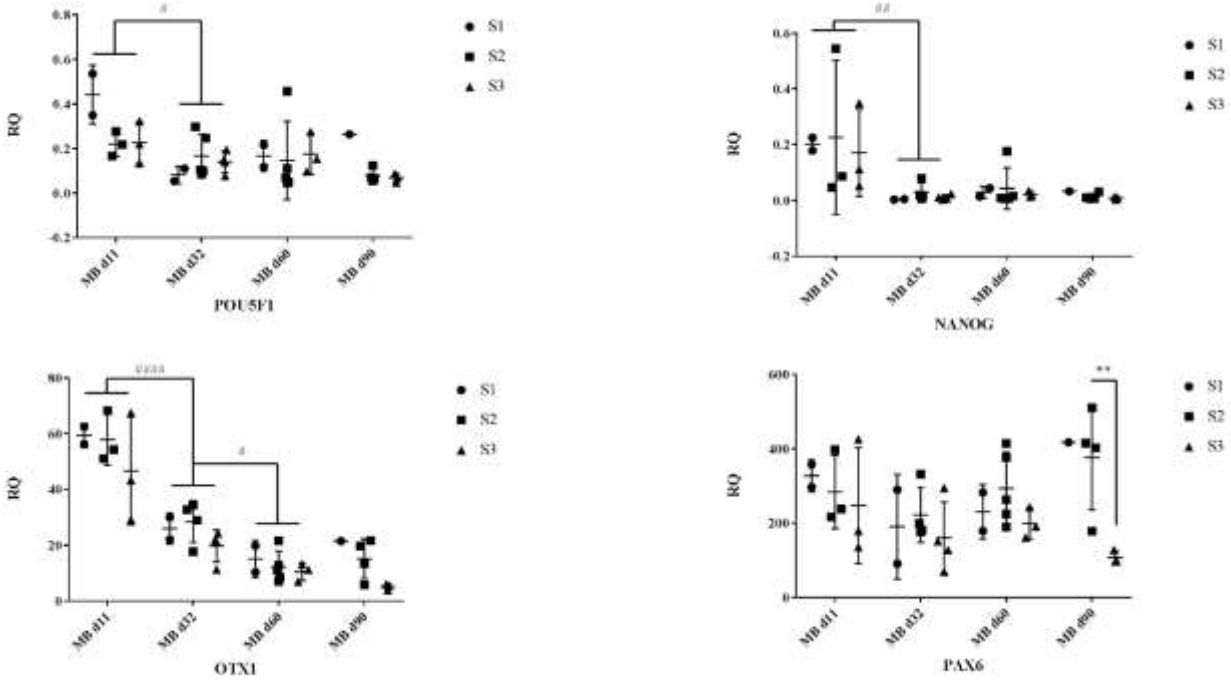


Fig. 3.2 **qPCR analysis for pluripotency markers and neural precursor markers in WT NAS6 MB from seeding density conditions.** Two-way ANOVA followed by Turkey´s multiple comparisons test. # for $p < 0.05$; #### for $p < 0.0001$; ** for $p < 0.005$

On the other hand, neural precursor genes expression was high in MB at d11 of differentiation. Albeit, while OTX1 expression decreased after d11 of differentiation, PAX6 expression stayed high at all the subsequent time points analyzed (d32, d60, d90; Fig. 3.2). Mature neuron specific gene expression increased starting from d32 of differentiation and increased considerably and consistently over the time. In particular, MBs expressed high level of mature

neuron markers (MAP2; NCAM1; SYN1; CUX2; BRN2; DLG4) at d60 of differentiation (Fig. 3.3).

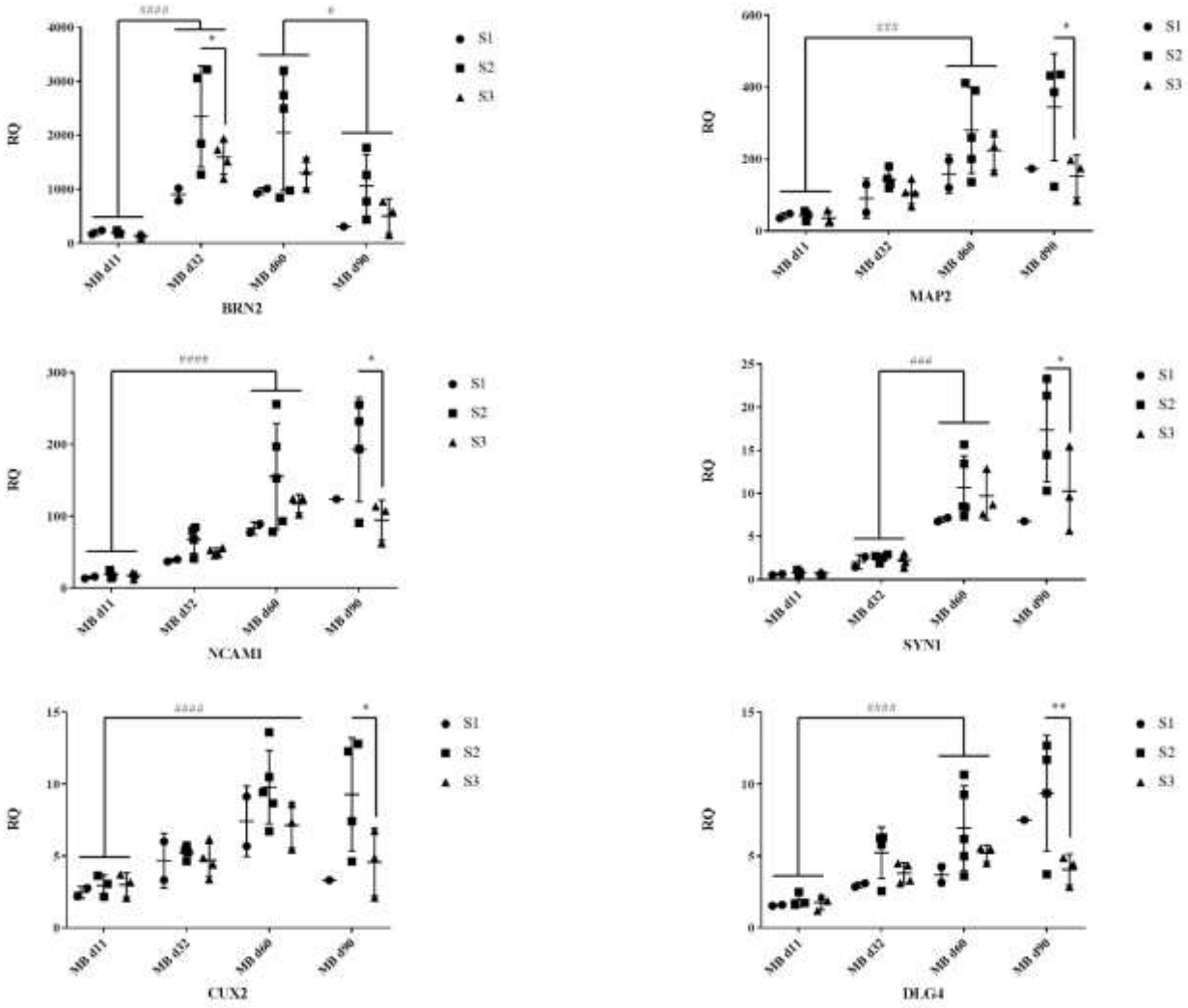


Fig. 3.3 **qPCR analysis for mature neuron markers in WT NAS6 MB from seeding density conditions.** Two-way ANOVA followed by Turkey´s multiple comparisons test. # for $p < 0.05$; #### for $p < 0.0001$; * for $p < 0.05$; ** for $p < 0.005$

The experiments were carried out to find the best seeding cell density conditions. Therefore, I performed a two-way ANOVA statistical test on gene expression data sets. The analysis indicated that the differences among the three cell density conditions were slightly significant only at d90 time point and suggested that the best seeding condition for WT NAS6 iPS cell line is S2 (10000 cell/well).

Experiments for total RNA-seq analysis of MB at different stage of differentiation are currently ongoing. These experiments will allow to analyze the complete expression pattern of MB during differentiation and maturation.

### *3.3.2 MBs contain human brain-like structures*

To understand the internal structural organization of MB, I performed immunostaining on frozen sections for both neural precursor and mature neuron markers.

Staining of MBs at day 75 of differentiation showed that MBs contain structures that resemble the subventricular zone of the human brain (red arrow; Fig. 3.4). Moreover, these structures retained the overall organization in which neural precursor cells ($Nestin^+$) localized in the most inner layers, while mature neurons resided in the outer part of these structures.

PAX6 is a neural precursor marker that *in vivo* is expressed in both outer Radial Glia (oRG) in the outer subventricular zone and in intermediate progenitors. The oRG is the major source of cortical neurons *in vivo*. Similarly, $PAX6^+$ cells in MB localized in subventricular-like structures as well as they were spread through the organoid. Cortical neurons, instead, were located outside of these subventricular-like structures in MBs ($CTIP2^+$ and $BRN2^+$ cells (arrowhead); Fig. 3.4). These results indicated a parallel between the organization of MBs and the *in vivo* brain also suggesting the concomitant differentiation and migration of neurons in the subventricular-like zone in an inside-out fashion. Differentiating cortical neurons, moreover, organized to form layers typical of the human brain cortex in which it was possible to identify early cortical neurons in the deeper layer ($CTIP2^+$ cells; Fig. 3.4) and late cortical neurons in the upper layers ($BRN2^+$ cells; Fig. 3.4) although cortical layering in MB was incomplete.

I identified $BRN2^+$ cells also in the subventricular-like structure (arrow; Fig. 3.4) and this might be because BRN2 has been shown to be expressed also in neural progenitor cells (Dominguez et al., 2013). All together, these data indicated that MBs contain areas that resemble the human brain structures, such as the subventricular-like zones, and the neural populations originated in the MBs have characteristic localizations similar to those observed *in vivo*.

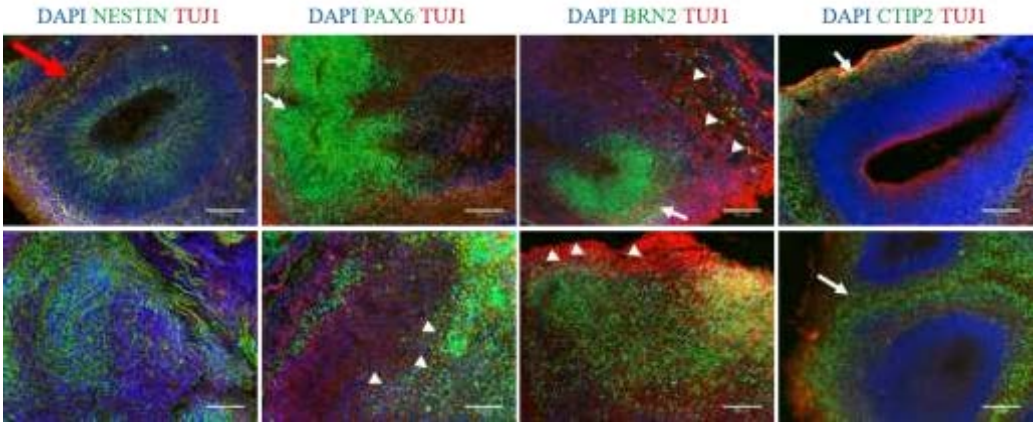


Fig. 3.4 **Immunostaining analysis on WT NAS6 MBs at day 75 of differentiation.** Representative images of neural sub-population localization. The red arrow indicates the subventricular-like structure that is possible to identify in the MBs. Scale bars: 50 μm

### *3.3.3 Protocol adaptation with SMAD inhibitors*

The protocol for generation of cerebral organoid was characterized by a high batch to batch variability that was reflected also by the high gene expression variability that I observed (described above).

Beside the high variability that characterizes MB generation, the presence of cell types derived from other embryonic origins in 3 and 6 months old MBs has been reported (Quadrato et al., 2017). This recent study reported that, among the neural subpopulations, MBs contained mesodermal cells indicating that the neural commitment was not exclusive. This may be due by the absence of any growth factor to stimulate neural differentiation. On the other hand, it is also known that ectoderm specification is the result of the default differentiation pathway. Mesoderm and endoderm are generated during embryonic gastrulation upon accomplishment of epithelial to mesenchymal transition (EMT). During gastrulation, epiblast cells undergo EMT through conversion of cell-adhesion proteins (E-cadherins). This process is known to be mediated by the activation of the Wnt/β-catenin and the TGF-beta/BMP-induced SMAD signaling pathways. The efficacy of SMAD inhibitors has already been reported in 2D hiPS neural differentiation protocols (Chambers et al., 2009).

Thus, I thought that the use of SMAD inhibitors, hereafter referred to as small molecules, could have been useful to inhibit differentiation of meso-endodermal

cells and push differentiation towards neural fate, ultimately resulting in a more reproducible MB generation.

Therefore, I tested the efficacy of SMAD inhibition during early stages of 3D MB differentiation.

I selected the first 6 days of differentiation to test the effect of small molecules treatment. During this time frame, cells are fed with basal hES medium and it is possible that some cells escape the default neural commitment and are committed for meso-endodermal specification.

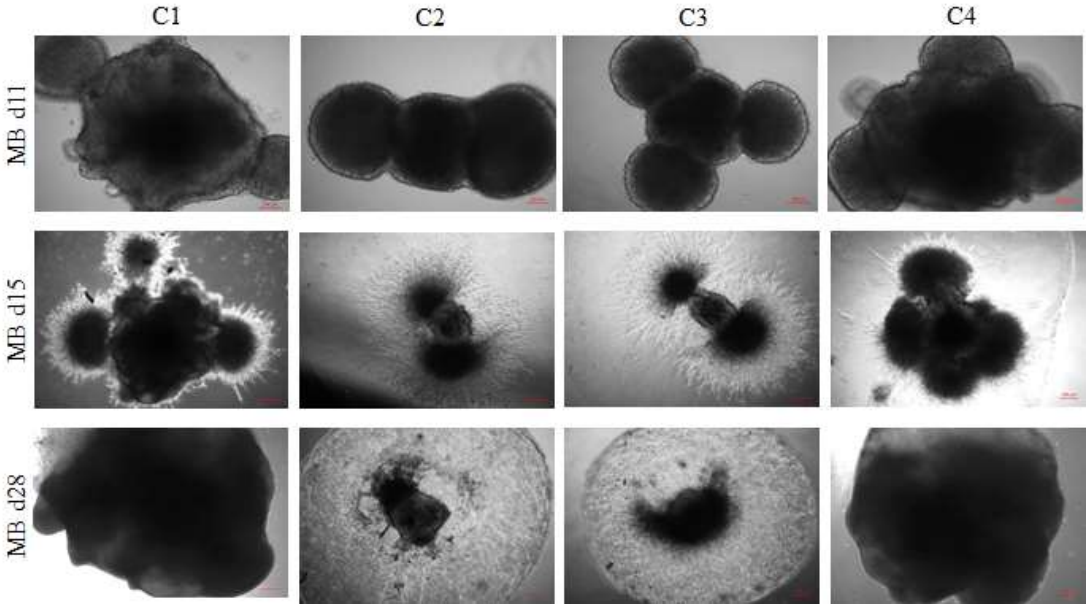


Fig. 3.5 **SMAD inhibitors treatment during early stages of MB differentiation.** Upper panels: Brightfield images at day 11 of differentiation before Matrigel™ embedding. Small molecules treatment from day 0 (C2 and C3) generated neural tissues with a smoother shape compared to untreated (C1) and day 3 (C4) treatment conditions. Middle panels: At day 15 of differentiation, C2 and C3 neural tissues started to show sign of a hard pushing to neural fate. Lower panels: At day 28 of differentiation, C2 and C3 neural tissues were fully differentiated in the Matrigel™ droplets and was not possible to identify the organoid structure, while C1 and C4 neural tissue did.

I tested four different treatment conditions (C1: untreated condition; C2: treatment from day 0 to day 3 with 10 μM SB431542 and 1 μM Dorsomorphin; C3: treatment from day 0 to day 6 with 10 μM SB431542 and 1 μM Dorsomorphin; C4: treatment from day 3 to day 6 with 10 μM SB431542 and 1 μM Dorsomorphin).

Brightfield images at day 28 of differentiation showed that C2 and C3 treatment conditions pushed the cells to a neural fate in such a way that it did not allow the formation of structures that resemble cerebral organoids. Instead, they formed 3D neuron cultures with visible signs of degradation. In contrast, C4 treatment condition allowed the formation of MB (Fig. 3.5).

Therefore, I excluded C2 and C3 conditions and proceeded with the C1 and C4 treatment conditions for the following analysis.

I tested the expression of early mesodermal (Brachyury) and endodermal (GATA4) markers in 5 MBs from two independent experiments harvested at three different time points (d11, d32 and d60) during differentiation.

The two-way ANOVA analysis indicated that the difference in Brachyury and GATA4 genes expression between C1 and C4 harvested MBs was significant only at d11 of differentiation (Fig. 3.6). This is possibly due by the fact that the chosen genes are specific for early meso-endodermal differentiation. Therefore, I will perform gene expression analysis for mature meso-endodermal markers in order to confirm the efficacy of small molecules treatment also at later time points of MB differentiation.

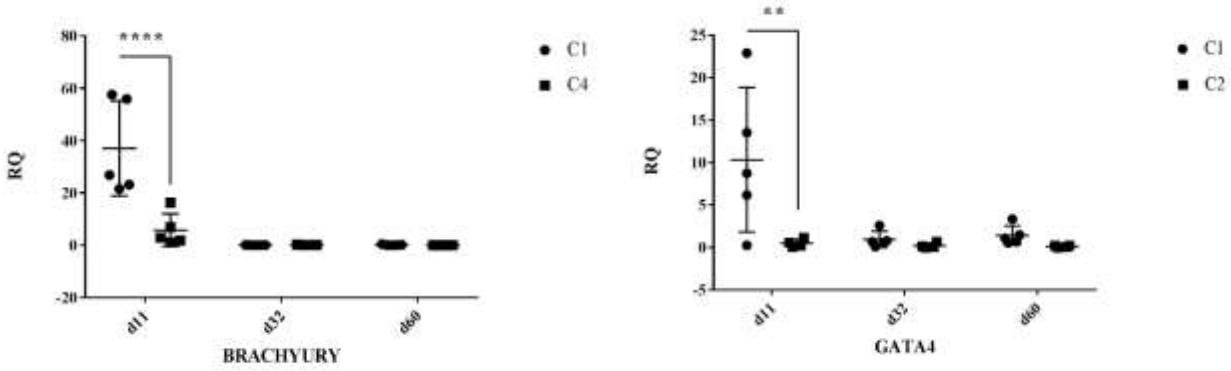


Fig. 3.6 **qPCR analysis for early mesodermal and endodermal markers.** Two-way ANOVA followed by Sidak´s multiple comparisons test. ** for $p < 0.005$; **** for $p < 0.0001$.

Nevertheless, the reduction of early meso-endodermal markers at d11 of differentiation was promising for the efficacy of the small molecules treatment over the possibility to standardize MB differentiation (Fig. 3.6).

I continued to analyze the expression of neural differentiation markers in MB at five different time points (d11, d32, d60, d90 and d120) of differentiation.

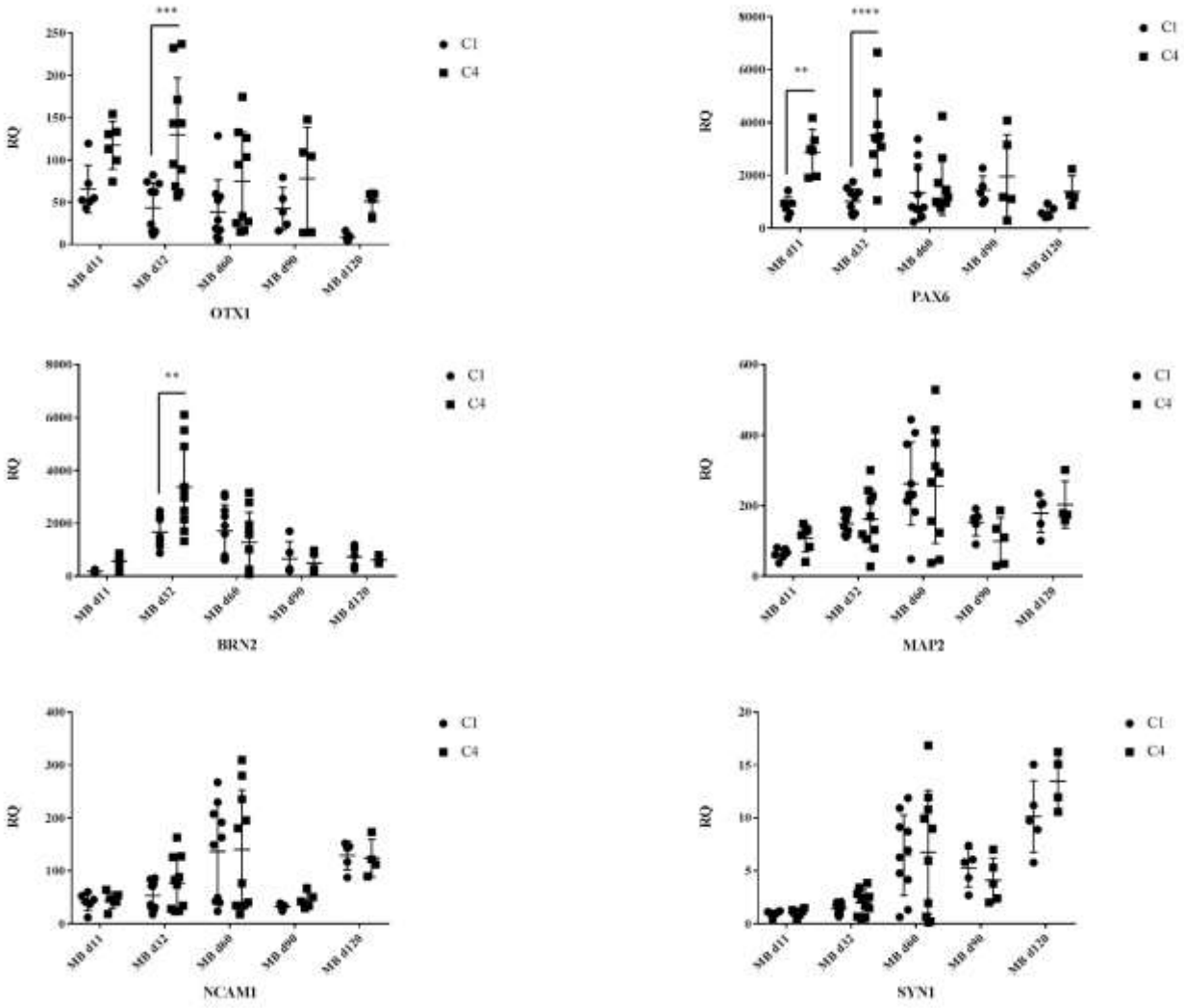


Fig. 3.7 **qPCR analysis for neural differentiation markers.** Two-way ANOVA followed by Sidak´s multiple comparisons test. ** for $p < 0.005$; *** for $p < 0.0005$; **** for $p < 0.0001$

I performed qPCR analysis for neural precursor markers (OTX1, PAX6 and BRN2) and mature neuron markers (MAP2, NCAM1 and SYN1) in 5 to 10 MBs from two independent experiments. The results showed that small molecules treatment induces an increase in neural precursor genes expression (OTX1, PAX6, BRN2; Fig. 3.7). The two-way ANOVA test confirmed the significance of the increased gene expression in C4-MBs especially at d11 and d32 of differentiation. On the other hand, small molecules treatment did not prompt any significant increase in mature neuron genes expression (MAP2, NCAM1, SYN1;

Fig. 3.7). Moreover, small molecule treatment did not improve the high variability in gene expression observed in MB generation.

### *3.3.4 Minibrain as model system for Parkinson´s diseases*

One of the key pathological characteristics of most neurodegenerative diseases is the formation of insoluble protein aggregates that can spread throughout the brain. Protein aggregation and spreading is challenging to replicate *in vitro*, but it has been possible in several transgenic mouse models. However, the use of mouse models to find modifiers of the aggregation is very time consuming and expensive. 3D MBs might be used to assess the aggregation and spreading of insoluble aggregates in lines carrying mutations in genes implicated in neurodegenerative disease.

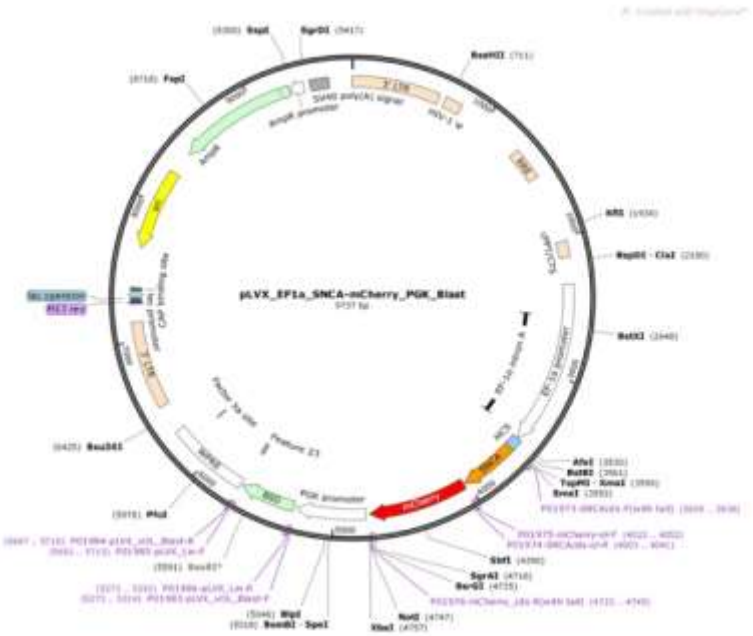


Fig. 3.8 **SNCA-RFP lentiviral vector map.** mCherry-tagged SNCA gene is cloned under EF1 constitutive promoter to lead a stable expression of SNCA protein. mCherry tag allows SNCA visualization via microscopy imaging and cell sorting via Fluorescence activated cell sorting (FACS).

SNCA has been recognized as genetic risk factor for both Mendelian and sporadic forms of PD. α-syn protein is the main component of Lewy bodies

aggregates and plays a central role in disease process. Nevertheless, the mechanisms that lead to α-syn protein aggregation and toxicity are still unknown.

In order to assess these mechanisms, I generated MB from a SNCA-overexpressing iPS cell line. Brightfield images of SNCA overexpressing MB at various stages of differentiation showed that this mutant iPS cell line is able to generate MBs in culture (Fig. 3.9). Further, I analyzed the expression of neural differentiation markers to understand whether I could find differences between WT NAS6 and SNCA overexpressing MBs. I analyzed via qPCR the expression of neural precursor markers (OTX1, PAX6, BRN2) and mature neuron markers (MAP2, NCAM1) in 2 SNCA overexpressing MBs harvested from two independent experiments at three time points (d11, d32, d60) of differentiation and compared to 7 WT NAS6 MBs from three independent experiments.

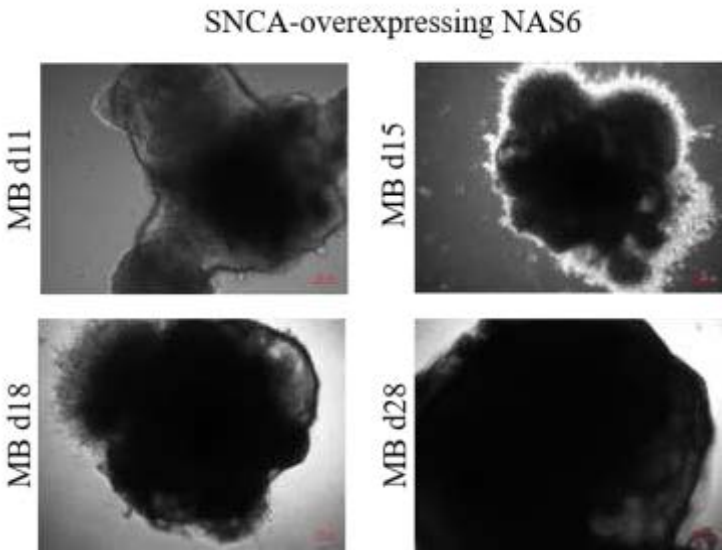


Fig. 3.9 **SNCA overexpressing NAS6 iPS-derived MB.** Brightfield images of SNCA overexpressing MBs at various time points during differentiation.

The analysis showed the same trend of gene expression in both WT NAS6 and SNCA overexpressing MB during the differentiation. Moreover, high variability in gene expression was observed in SNCA overexpressing MBs as well (Fig. 3.10). Interestingly, qPCR analysis showed reduced level of the neural precursor/cortical marker BRN2 at d32 and d60 of differentiation in SNCA overexpressing MBs compared to WT NAS6 MBs (Fig. 3.10). Moreover, a reduction in mature neuron genes (MAP2, NCAM1) expression has been

observed in SNCA overexpressing MB at d60 of differentiation compared to aged-matched WT NAS6 MBs (Fig. 3.10).

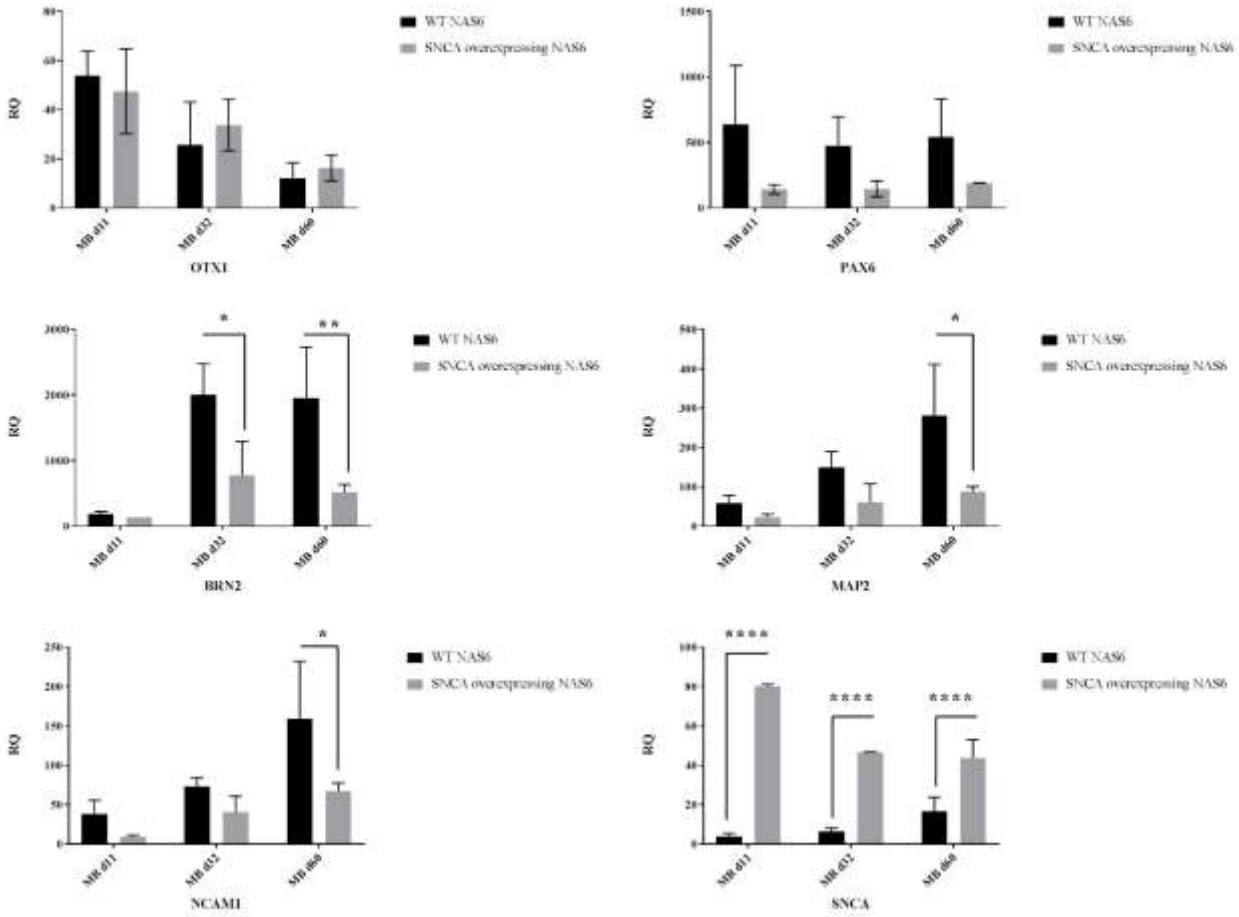


Fig. 3.10 **qPCR analysis for neural differentiation markers in WT NAS6 and SNCA overexpressing NAS6 MBs.** Two-way ANOVA followed by Sidak´s multiple comparisons test. * for $p < 0.05$; ** for $p = 0.005$; **** for $p < 0.0001$

Despite the small number of MBs analyzed, the differences in neural markers gene expression between WT NAS6 and SNCA overexpressing MB are significant (BRN2, MAP2, NCAM1; Fig. 3.10). Further analysis will be performed with a larger number of MBs in order to confirm the observed results. On the other hand, immunostaining analysis for neural markers did not show clear structural differences between WT NAS6 and SNCA overexpressing MB (Fig. 3.11). Indeed, subventricular-like structures were clearly recognizable in SNCA overexpressing MB as well as in WT NAS6 MBs. Nevertheless, despite

that a quantitative estimation of neural populations has not been performed, $PAX6^+$ and $CTIP2^+$ cells were present in lower amount in SNCA overexpressing MB compared to WT NAS6 MB (Fig. 3.11) even though, BRN2 staining did not show big alteration neither in amount or localization of $BRN2^+$ cells compared to WT NAS6 MB.

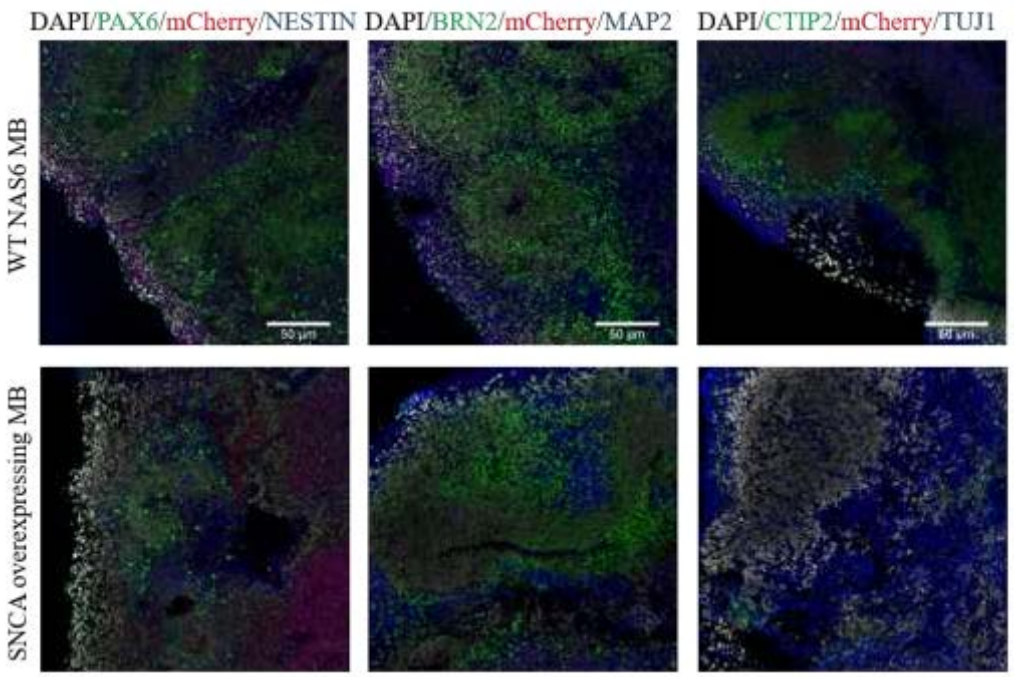


Fig. 3.11 **Immunostaining analysis WT NAS6 and SNCA overexpressing MBs at day 45 of differentiation.** SNCA overexpressing MB (lower panels) contained less $PAX6^+$ and $CTIP2^+$ cells compared to WT NAS6 MBs (upper panels). No differences were observed in the amounts of $BRN2^+$ cells between WT NAS6 and SNCA overexpressing MBs. mCherry fluorescence was poorly detected in SNCA overexpressing MBs (lower panels). Scale bars: 50 μm.

Moreover, the presence of mCherry-tag would have allowed to detect SNCA overexpressing cells in SNCA overexpressing MBs. mCherry fluorescence was not detectable in WT NAS6 MB and very few $mCherry^+$ cells have been found in SNCA overexpressing MB (Fig. 3.11). qPCR analysis for SNCA expression in WT NAS6 MBs showed that SNCA started to be detectable at d32 of differentiation and increased at d60 of differentiation. On the other hand, as expected, SNCA expression in SNCA overexpressing MBs was greater than the age-matched WT counterpart at all the time points analyzed, although it decreased over the time (Fig. 3.10).

A possible explanation about these observations on SNCA in SNCA overexpressing MBs might arise by the fact that the starting SNCA overexpressing iPS cell line was a mixed population of cells containing different numbers of random integrations. During MB differentiation, processes such as proliferation, differentiation and apoptosis take place and it might be that some cells are more prone than others to die. In particular, the cells with the highest number of integrations could have died during differentiation, thus leaving only the cells carrying few integrations contributing to MB generation.

mCherry-tag might be used to fluorescence activated cell (FAC)-sort SNCA overexpressing iPS cells according to the number of virus integrations thus generating at least three sub-populations (low, medium and high) of SNCA-expressing cells. This might be a possible strategy to further characterize SNCA overexpressing MB and in particular to better understand the effect of SNCA overexpression in MB generation.

### *3.3.5 Oxidative stress status in WT NAS6 and SNCA overexpressing Minibrain*

Oxidative stress is a well-known issue in neurodegenerative disease, including PD, in which it is thought to play an important role.

Moreover, during last decade a possible interplay between SNCA expression and Nrf2/ARE antioxidant pathway has been proposed. Indeed, it has been shown that Nrf2 deletion in SNCA overexpressing mouse models for PD aggravates neurodegeneration (Lastres-Becker et al., 2012). Furthermore, the authors showed the activation of the Nrf2/ARE pathway in PD postmortem brain tissues. In particular, they observed an increase in astrocytic and microglia expression of HMOX1 suggesting a possible role for Nrf2 in counteracting α-syn toxicity (Lastres-Becker et al., 2012).

Nrf2/ARE pathway activation in astrocytes seems to be sufficient to protect against neurodegeneration in MPTP treated PD mouse models (Chen et al., 2009). On the other hand, it has also been shown that MPTP treatment induced HMOX1 expression in Substantia nigra and ventral midbrain, suggesting that also neural Nrf2 activation might play an important role in α-syn -mediated oxidative stress (Innamorato et al., 2010).

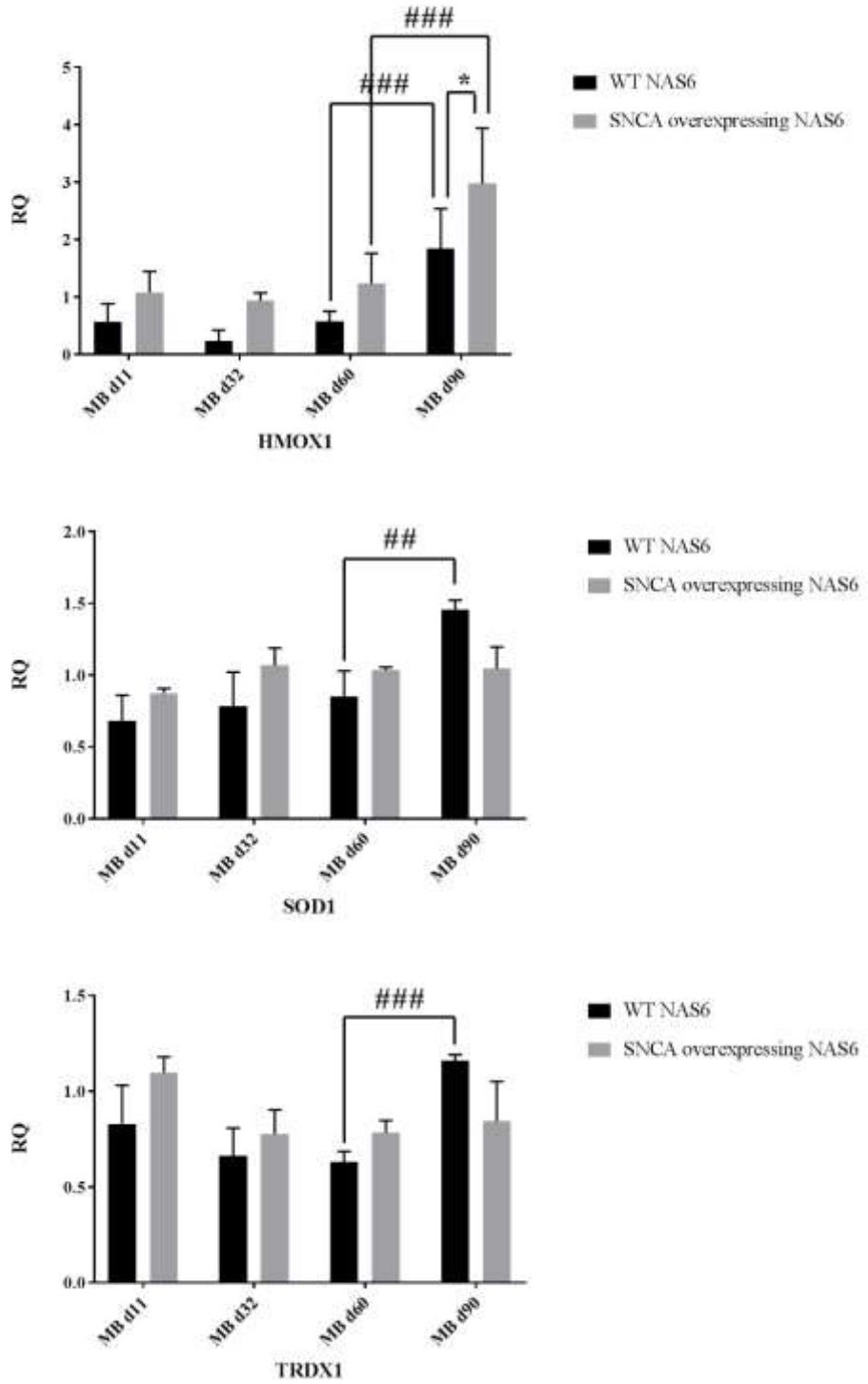


Fig 3.12 **qPCR analysis for oxidative stress markers on WT NAS6 and SNCA overexpressing NAS6 MBs.** Two-way ANOVA followed by Sidak´s multiple comparisons test.

* for $p < 0.002$; Two-way ANOVA followed by Turkey´s test. ## for $p < 0.005$; ### for $p < 0.001$.

Indeed, HMOX1 has been shown to accumulate in postmortem PD brain (Schipper et al., 1998) especially in dopaminergic neurons of Substantia nigra *pars compacta* where it colocalized in Lewy bodies.

The expression of HMOX1 and other Nrf2 regulated antioxidant genes, such as Glutathione synthetase (GSS) and Thioredoxin reductase 1 (TRDX1) was induced in mouse dopaminergic neurons upon stimulation with $H_2O_2$ (Yoo et al., 2003).

Likewise, studies performed in mouse models of PD showed the positive effect of Nrf2/ARE signaling pathway activation over the pathology progression (Lastres-Becker et al., 2016).

Therefore, I analyzed the expression of three out of the hundreds Nrf2/ARE-regulated antioxidant genes: HMOX1, TRDX1, SOD1 (also shown to colocalize in Lewy bodies (Nishiyama et al., 1995) and to physically interact with α-syn protein both *in vitro* and *in vivo* (Helferich et al., 2015)). In particular, I measured their expression in SNCA overexpressing MBs from two independent experiments and compared this to WT NAS6 MBs gene expression at four time points (d11, d32, d60, d90) of differentiation.

qPCR analysis showed a significant increase in HMOX1 gene expression at d90 of differentiation in both WT NAS6 and SNCA overexpressing MBs. Moreover, HMOX1 expression in d90 SNCA overexpressing MBs was increased compared to aged-matched WT NAS6 MBs (Fig 3.12). On the other hand, no significant differences were observed in the expression of SOD1 and TRDX1 between WT NAS6 and SNCA overexpressing MBs at any of the time points analyzed. Nevertheless, SOD1 expression increased in d90 WT NAS6 MBs (Fig 3.12).

## 3.4 CONCLUSIONS

3D cerebral organoids are emerging as a very promising tool to model and study human brain development and diseases. Nevertheless, this new technology still needs to be improved in terms of stability and reproducibility of the generated 3D cultures.

I generated MBs from a WT hiPS cell line in order to characterize their differentiation and growth. Seeding density condition is considered a crucial factor that may influence neural differentiation in 2D cultures and this may affect 3D culture as well.

Therefore, I tested three seeding conditions and found the best among those for the WT NAS6 iPS cell line and performed the characterization at gene expression level, using a set of markers to assess neural differentiation. From the gene expression analysis via qPCR I showed the general process of differentiation. MBs up to one month of differentiation were still immature as they expressed high levels of neural precursor markers (Fig 3.2). After two months in culture they started to be more differentiated as shown by the expression of mature neuron markers (Fig 3.3). Moreover, at d60 of differentiation MBs started to exhibit signs of neural maturation. For example, SYN1 gene expression is normally associated with the presence of mature neurons characterized by firing potential. SYN1 expression started to be significantly detectable at d60 of MB differentiation and increased at the later time point (d90) analyzed (Fig 3.3).

In parallel to the characterization at the gene expression level, I performed immunostaining analysis. This analysis allowed visualizing the internal organization of MB and showed that MBs contain structures that resemble those of the human brain, such as the subventricular zone. Inside these subventricular-like structures cell populations organized in layers suggestive of the concomitant differentiation and migration of neural cells through the layers (Fig 3.4) in a fashion similar to that observed *in vivo*.

One of the main limitations of this system is the high variability that characterizes MBs generation. This is possibly due to the minimal induction to neural fate that relies on the assumption that stem cell differentiation in absence of external stimuli follows the default neural pathway. The presence of mesodermal cells, however, indicated that not all the cells follow the neural differentiation pathway (Quadrato et al., 2017). On the contrary, some of them seem to escape the default neural differentiation and get specified for meso-endodermal lineages. Treatment from day 3 of differentiation with SMAD inhibitors revealed to be useful to reduce the amount of meso-endodermal population in MBs as shown by the expression analysis of early mesodermal and endodermal markers that were strongly reduced at d11 time point of differentiation (Fig. 3.5). However, the treatment did not result in a robust

improvement of the differentiation protocol. MBs derived using small molecules treatment condition still exhibited high gene expression variability, as emerged by the qPCR analysis for neural differentiation markers at various time points during differentiation. Therefore, other strategies need to be taken into account in order to make MB generation more stable and reproducible.

A possible route might be to synchronize the iPS cell cycle before seeding. Cells in culture may reside in different stages of cell cycle progression. Therefore, some cells might be more prone than others to move toward an asymmetric division instead of the symmetric one that characterizes non-differentiating/proliferating stem cells. Synchronized cells, instead, are all in the same stage of cell cycle progression and should all react the same way when induced to differentiate.

Synchronized iPS cells can be obtained via treatment with a microtubule destabilizer agent, such as Nocodazole (Yang et al., 2016). This agent interferes with microtubule polymerization and cause arrest in the G2/M phase of the cell cycle. Thus, cell-cycle synchronization might reduce the propensity of the cells toward a cell division rather than another and maybe contribute to reduce MBs variability.

I generated MBs from an iPS cell line that constitutively overexpresses SNCA gene thus mimicking the effect of the SNCA gene triplication that can cause a familiar form of PD. The SNCA overexpressing iPS cell line was able to generate MBs in culture even though qPCR analysis showed reduced levels of mature neuron markers at d32 and d60 of differentiation compared to WT NAS6 MBs (Fig 3.10). However, no significant differences were observed in neural precursor markers expression (Fig 3.10). Alterations in SNCA overexpressing MB differentiation were also observed through immunostaining analysis, despite no structural differences have been seen in SNCA overexpressing MB compared to WT NAS6 MB. These results suggest a possible impairment of the differentiation of SNCA overexpressing MBs that might involve the later stages of differentiation rather than the early one.

The analysis of oxidative stress markers showed an increase in HMOX1 expression over the time of differentiation. Moreover, SNCA overexpressing MBs exhibited higher level of HMOX1 mRNA at d90 of differentiation compared to WT NAS6 MBs (Fig 3.12). On the other hand, TRDX1 and SOD1 expression did not show any significant difference between WT NAS6 and SNCA overexpressing NAS6 MBs. However, this analysis has been performed

in not stress-induced conditions where MBs were cultured in antioxidant containing medium that might hide possible differences in oxidative state. Therefore, it might be important to perform experiments in which oxidative stress is actively induced either via antioxidant depletion or via treatment with oxidant agents.

In line with previous studies (Lastres-Becker et al., 2012), these results on HMOX1 expression suggest an interplay between α-syn and Nrf2/ARE pathway. Several evidences indicated that mutations in SNCA gene trigger oxidative stress and mitochondrial damage (Junn and Mouradian, 2002; Martin et al., 2006; Shavali et al., 2008). Mitochondria are often impaired in neurodegenerative diseases (Gan et al., 2018) and the activation of Nrf2 pathway has been shown to rescue the α-syn induced oxidative stress-mediated mitochondrial energy metabolisms impairment (Fu et al., 2018). Several evidences support the role of Nrf2 in maintenance of mitochondrial homeostasis and structure. Impairment in mitochondrial complex 1 activity has been observed in Nrf2 Knock-out (Nfr2-KO; Kovac et al., 2015) and the mitochondrial protein DJ1, known to maintain the normal activity of mitochondrial complex 1 and known genetic risk factor for PD (Bonifati et al., 2003; Símon-Sáncez et al., 2009), has been shown to stabilize Nrf2 (Clements et al., 2006) although the molecular mechanisms are still poorly understood (Dinkova-Kostova et al., 2015)

Therefore, mitochondria might be the missing link between SNCA overexpression and Nrf2 pathway activation. It might be interesting to measure the ROS production and ATP synthesis rate to evaluate the mitochondrial activity in SNCA overexpressing MBs. Furthermore, lentiviral transfection of SNCA overexpressing MBs with Nrf2 short hairpin RNA (shRNA), in order to mediate the pathway inactivation, might be helpful to understand the role of Nrf2/ARE pathway on mitochondrial homeostasis in SNCA overexpressing MBs.

Thus, even though an effort still needs to be done in order to improve the stability and robustness of differentiation protocol, all together these results encourage the use of 3D MB culture to study human brain development and disease. Moreover, despite the limitations of this model system, it has emerged and reinforced the great potential of MBs also to model some aspects of neurodegenerative disease, such as PD.

## Acknowledgements

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The experiments have been performed under the supervision of Ashutosh Dhingra.

# Chapter 4

# DISCUSSION

Neurodegenerative diseases such as PD, AD, HD and FTD are usually late-onset and share many common altered mechanisms such as protein aggregation, synaptic dysfunction, decreased autophagy/lysosomal proteolysis, mitochondrial dysfunction and oxidative stress (Gan et al., 2018). These alterations are often interconnected. For example, decreased autophagic activity may lead to increased ROS production via mitochondrial turnover impairment. The link between autophagy and oxidative stress has been suggested in the pathophysiology of PD (Nakanishi et al., 2001; Giordano et al., 2014) and the activation of autophagy has been hypothesized to be beneficial for the reduction of oxidative stress propagation and to delay neurodegeneration (Giordano et al., 2014). There are numerous evidences that highlight the causative role of oxidative stress in neurodegenerative diseases (Okun et al., 2007; Sanders et al., 2013; Kouti et al., 2013; Popa-Wagner et al., 2013) and antioxidant-based drugs have been considered for therapeutic applications in the treatment of neurodegenerative diseases (Liu et al., 2017). However, there are still open questions, about the main oxidant molecules involved and how these oxidants affect the molecular mechanisms altered in neurodegenerative diseases, that need to be answered in order to identify potential antioxidant agents for the treatment of these pathologies (Patel, 2016). Oxidative stress has also been observed in developmental and childhood-onset brain diseases (Hayashi et al., 2012; Dasuri et al., 2013; Gan et al., 2018) such as in RTT (Filosa et al., 2015). A whole exome sequencing study revealed that mutations in MeCP2 protein gene correlate with variants in genes related to oxidative stress (Grillo et al., 2013). Moreover, MeCP2 regulates the expression of oxidative stress related genes, including those associated to ATP synthesis and mitochondrial functioning (Pecorelli et al., 2013), suggesting a possible role of mitochondria in redox impairment observed in RTT patients. Since mitochondria are the major sources of ROS, an impairment in their normal functioning can result in oxidative burden. Of note, treatments directed to rescue systemic oxidative stress, such as ω-3

polyunsaturated fatty acids (ω-3 PUFAs), Trolox and Curcumin, showed a general amelioration of neural symptoms (De Felice, et al., 2012; Filosa et al., 2015). To date, many mouse models have been generated in order to better understand the molecular mechanisms altered in Rett syndrome and to find the correlation between oxidative stress and MeCP2 depletion (Filosa et al., 2015). These models allowed to reproduce many of the alterations observed in RTT patients, including the systemic oxidative stress. They also provided the opportunity to hypothesize that redox imbalance and oxidative damage might precede the onset of symptoms. Moreover, MeCP2 reactivation in mice rescues the redox imbalance, thus giving strength to the possible role of MeCP2 protein in cellular response regulation against oxidative stress (De Felice et al., 2014).

However, animal models are far to faithfully model the human pathologies and the current human diseases research is facing the limitations that the existing model systems present. Animal models, especially rodents, offer great possibilities in term of genetic engineer potentials and they allow studying biological processes in fully developed organs *in vivo*. However, the differences between rodents and human species might be relevant when studying aspects of human development and diseases. Moreover, their use in research is challenging due to cost expensiveness and ethical issues.

The introduction of stem cells represent a breakthrough in human diseases research. ES cells have been isolated for the first time from mouse embryos in 1980s (Evans et al., 1981; Martin, 1981) and from donated human embryos in 1998 (Thomson et al., 1998). Since then, ES cells have constituted an important tool for genetic engineering and a valuable model system for basic research on pluripotency and developmental biology as well as for biomedical research. The availability of a wide range of differentiation protocols allows to study the molecular mechanisms that drive cell differentiation and take place during development. Moreover, ES-derivatives have been used in preclinical studies as potential therapeutic strategies for spinal cord injury (Hans S. Keirstead et al., 2005), eye-diseases (Lu et al., 2009), infarct-injured hearts (Shiba et al., 2012), diabetes (Pagliuca et al., 2014), PD (Ambasudhan et al., 2014), cancers and immunological diseases (Chivu-Economescu and Rubach, 2017). The introduction of human patient derived-iPS cells (Takahashi et al, 2007) opened the way for new opportunities in regenerative medicine. Patient derived-iPS cells, indeed, maintain the genetic background of the donor patient and are more suitable than human ES cells for transplantation because they avoid immune

rejection issues (Soldner and Jaenisch, 2018). Upon gene correction, iPS cells can be differentiated into, ideally, all the cell types of the body making it feasible to reintroduce the specific cell types that have been lost during the progression of a specific diseases, such as the dopaminergic neurons in PD patients (Trounson and DeWitt, 2016). Beside that, stem cells represent a powerful model system to study human diseases. Indeed, by taking advantage from mutant ES cell lines or patient-derived iPS cells, it is possible to investigate on those mechanisms that have been altered in specific cell types during pathologies (Soldner and Jaenisch, 2018). Furthermore, one of the major advantages of stem cell technology is the possibility to perform high throughput screenings in order to test the effectiveness of drugs and compounds on a disease-associated phenotype for therapeutical applications (Soldner and Jaenisch, 2018).

More recently, the introduction of 3D cultures offered a straightforward opportunity to recreate and study a plethora of phenomena in complex *in vitro* systems. Stem cell-derived 3D cultures resemble better than 2D systems the microenvironment needed for cell differentiation, thus offering major reliability than 2D cultures for human developmental studies. Given their complexity, in which several human brain-like structures grow and connect among each other, 3D Minibrains represent an unprecedented tool to study *in vitro* some mechanisms of neurological diseases progression that were not possible to investigate in 2D systems and would have required the use of animal models. Moreover, since they are *in vitro* systems, 3D cultures retain the scalability potential that would apply to high-throughput strategies for drug screening. Even though an effort still needs to be done in order to make Minibrain generation more complete and reproducible, 3D Minibrains offer the opportunity to fill the gap existing between *in vivo* animal models and *in vitro* human 2D systems.

The use of ES and iPS cells and the possibility to differentiate them into complex 3D organoids is promising to move forward in the comprehension of human brain diseases and, hopefully, shorten the way for therapeutic strategies that might at least improve the lifespan and life quality of Rett disease patients, as well as for neurodegenerative diseases.

# Chapter 5

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## APPENDIX: Publications

1) Maalej, A., M. Forte, Z. Bouallagui, S. Donato, L. Mita, D. G. Mita, S. Filosa, S. Crispi and S. Sayadi (2017). "Olive compounds attenuate oxidative damage induced in HEK-293 cells via MAPK signaling pathway." J Funct Foods 39(Pt A): 18-27.
2) Di Meo, F., S. Donato, A. Di Pardo, V. Maglione, S. Filosa and S. Crispi (2018). "New Therapeutic Drugs from Bioactive Natural Molecules: The Role of Gut Microbiota Metabolism in Neurodegenerative Diseases." Curr Drug Metab 19(6): 478-489.
3) Stellavato, A., A. V. A. Pirozzi, S. Donato, I. Scognamiglio, S. Reale, A. Di Pardo, S. Filosa, V. Vassallo, G. Bellia, M. De Rosa and C. Schiraldi (2018). "Positive Effects against UV-A Induced Damage and Oxidative Stress on an In Vitro Cell Model Using a Hyaluronic Acid Based Formulation Containing Amino Acids, Vitamins, and Minerals." Biomed Res Int 2018: 8481243.

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# Olive compounds attenuate oxidative damage induced in HEK-293 cells via MAPK signaling pathway

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ABSTRACT

The protective effects of ethanolic olive fruit extract (OFE) and purified oleuropein (Ole) have been evaluated against $H_2O_2$-induced oxidative damage and apoptosis in human embryonic kidney (HEK-293) cells. Olive compounds demonstrated a protective effect against $H_2O_2$-induced cell death resulting in total rescue of cell viability. This effect was accompanied by a marked decrease in intracellular reactive oxygen species (ROS) and lipid peroxidation as evidenced by malondialdehyde (MDA) production. Consequently, olive compounds reduced the apoptotic cell death. This was confirmed by flow cytometry analysis for multicaspases and by western blot and real-time PCR analyses for the expression of key effectors involved in the apoptotic process. Our data suggest that the anti-apoptotic effect could be due to the activation of the Erk/p38 signaling pathway and to the modulation of Bcl-2/Bax ratio. These findings provide new insight into the mechanisms underlying the protective effects of OFE and Ole against oxidative damage-induced apoptosis.



## 1. Introduction

Reactive oxygen species (ROS) resulting from oxidative stress are unstable and can damage vital components of the cell leading to alterations in cell membrane fluidity, permeability as well as DNA breakdown (Fernández-Blanco, Font, & Ruiz, 2015). It is well known that hydrogen peroxide ($H_2O_2$) is one of the strongest oxidants triggering, both *in vivo* and *in vitro*, ROS accumulation (Frei & Higdon, 2003; Liu et al., 2014). Oxidative stress is responsible for renal diseases, ranging from acute (Baliga, Ueda, Walker, & Shah, 1999) to chronic renal failure with associated inflammation (Handelman et al., 2001). However, the protective effect of antioxidants against such diseases has been poorly analyzed (Rodrigo & Bosco, 2006). In addition, contrast media induced nephropathy, are known to be a serious complication of radio diagnostic procedures. In fact, contrast media exposure significantly increases the oxidative stress in kidney, resulting in an increase in TBARS and decrease in GSH. Many attempts have been made to select antioxidant molecules able to protect the kidney from this damage (Sterling, Tehrani, & Rudnick, 2008).

It has been reported that some phenolic compounds contribute to modulating the systemic antioxidant defense through enhancing the plasma antioxidant capacity in humans (Carrizzo et al., 2013; Duthie et al., 1998).

Mitochondria being one of the major sources of ROS are associated with an important signaling pathway in apoptosis (Crispo et al., 2010). The main events involved in this pathway are the release of cytochrome *c* and the decrease of mitochondrial membrane potential. Thereby, the activation of caspases and Bcl-2 takes place (Tang & Tai, 2007). It has been reported that oxidants can trigger the activation of numerous signaling pathways including MAPK (mitogen-activated protein kinases) (Martindale & Holbrook, 2002). These proteins are made of three major subfamilies that have structural differences: Erk (the extracellular signal-regulated kinase), p38 kinase and JNK (the c-Jun N-terminal kinase). Such kinases are activated by phosphorylation of both threonine and tyrosine residues. Activated MAPK phosphorylate their specific substrates on threonine and/or serine residues leading to the activation of several transcription factors (Park, Yoo, Kim, Kwon, & Kim, 2005). Furthermore, the p38 pathway has been

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associated with cell growth and differentiation, cytokines production, inflammation, and cell death (Rodríguez-González, Wilkins-Rodríguez, Argueta-Donohué, Aguirre-García, & Gutiérrez-Kobeh, 2016). On this line, experimental studies on animals or cultured human cell lines showed that olive compounds protect against the development of chronic diseases such as cancers, diabetes and cardiovascular diseases through different pathways. Recent study demonstrated the anticancer potentiel of an olive fruit extract against hepatic HepG2 and colon Caco-2 cancer cells. In fact, this polyphenols-rich extract decreased cell viability, inhibited cell proliferation and induced cell apoptosis through the Akt and MAPKs/Erk pathways (Maalej, Bouallagui, Hadrich, Isoda, & Sayadi, 2017). Similar effects were observed in human breast cancer MCF-7 cells treated with oleuropein or hydroxytyrosol (Han, Talorete, Yamada, & Isoda, 2009). On the other hand, some investigators reported that phenolic compounds with high reducing ability can be not only antioxidants but also pro-oxidants, thus generating reactive oxygen species (ROS) (Lopaczynski & Zeisel, 2001). This could be the possible mechanism of action of antioxidants for tumor suppressive effect (Han et al., 2009). ROS may even activate apoptotic death pathways. In addition, polyphenols can prevent tumor invasion via inhibition of the matrix metalloproteinases (Maeda-Yamamoto et al., 1999). Hadrich, Mahmoudi, et al. (2016) reported the anti-obesity effects of oleuropein, *in vivo,* by increasing adiponectin levels in serum, modulating lipids metabolism and activating AMPK in white adipose tissue in high cholesterol diet-induced obese rat. Hadrich, Garcia, et al. (2016) also suggested that oleuropein plays a beneficial role in glucose metabolism of differentiated C2C12 skeletal muscle cells supporting the therapeutic potential of oleuropein to the treatment of diabetes. More interestingly, antioxidant, antiapoptotic and anti-inflammatory effects were also observed with olive fruit extract and oleuropein administered orally to deltamethrin-induced liver and kidney toxicity rats model (Maalej, Mahmoudi, et al., 2017). However, the effect of olive compounds on $H_2O_2$-induced oxidative injury on kidney cells is still poorly studied. The aim of the present study was to evaluate the protective effect of a Tunisian olive fruit extract (Jerboui fruit extract (OFE)), and purified oleuropein (Ole) against $H_2O_2$-induced cell damage in human embryonic kidney cells HEK-293. Even though this investigation did not show effects on metabolic parameters in renal cells, more attention was paid to study the mechanism underlying the potential protective effect of olive compounds through monitoring their effect on the oxidative status, as well as apoptotic proteins and genes expression.

## 2. Material and methods

### 2.1. Preparation of olive fruit extract and purified oleuropein

Olive fruit from Jerboui cultivar grown in Béja prefecture (Tunisia) was used to prepare extract. Fresh fruits (200 g) with a maturation index of 2 were collected, manually milled, and extracted twice with 350 mL of ethanol (70%) under agitation (200 rpm in an orbital shaker) for overnight at room temperature. The extract was concentrated and washed with hexane to remove the lipid fraction. The resulting extract (OFE) was freeze-dried and stored for further analyses. Oleuropein purification was performed as previously reported by our group (Hadrich, Mahmoudi, et al., 2016). Briefly, 100 g of dried olive leaves were soaked in 500 mL distilled water, stirred at room temperature overnight and filtered. The resulting aqueous phase was then extracted thrice with equal volume of ethyl acetate. The organic phase was dried under vacuum and the residue was chromatographed on silica gel at low pressure using a mixture of methylene chloride and methanol. Finally, the purified fraction was freeze-dried and stored until use.

### 2.2. Phenolic characterization of olive compounds

A high-performance liquid chromatography analysis was developed to identify and quantify the major phenolic compounds of the prepared OFE. The phenolic profile of OFE was obtained using an Agilent series 1260 HPLC-DAD instrument (Agilent Technologies, Waldbronn, Germany) following the method previously reported by Souilem et al. (2014). The separation was carried out on a Zorbax Eclipse XDB-C18 column (4.6 mm I.D. × 250 mm × 3.5 μm particle size). The mobile phase was 0.1% acetic acid in water (A) versus 100% acetonitrile (B) for a total running time of 50 min. The elution conditions applied for phenolic compounds were: 0–22 min, 10–50% B; 22–32 min, 50–100% B; 32–40 min, 100% B; 40–44 min, 100–10% B. The column was subjected to washing and reconditioning step after each analysis using 100% acetonitrile. The column temperature was maintained at 40 °C and the flow rate of the mobile phase was 0.5 mL/min. The injection volume of the OFE sample was 10 μL. Detection was performed using a diode array detector (DAD), and the chromatograms were recorded at λ = 254 nm for oleuropein and at 330 nm for flavonoids (Luteolin glucoside, apigenin glucoside and verbascoside). Quantification was performed using external standards (oleuropein, luteolin glucoside, apigenin glucoside, verbascoside, gallic acid, hydroxytyrosol, tyrosol, caffeic acid, rutin, *p*-Coumaric, ferulic acid, and quercetin) from Sigma-Aldrich.

### 2.3. Cell culture

Human embryonic kidney cells (HEK-293) (CRL-1573, American Type Culture Collection, Manassan, VA) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% dextran-coated charcoal fetal bovine serum (FBS) and 1% penicillin/streptomycin solution and maintained at 37 °C under 5% $CO_2$ and 95% air. When confluent, cells were detached enzymatically with trypsin-ethylenediamine tetra-acetic acid (Trypsin-EDTA) and sub-cultured into a new cell culture flask. The medium was replaced every 2 days.

### 2.4. Cell viability analysis

Cell viability was analyzed by measuring the conversion of MTT into the purple formazan, a property distinctive of cells with active metabolism. Cells were seeded in 96-well plates at a density of $3 \times 10^4$ cells/well and incubated overnight. The plates were treated with olive compounds OFE (50–200 μg/mL) and Ole (10–50 μg/mL) for 18 h. Then, cells were exposed to 400 μM $H_2O_2$ for 6 h as reported in the appropriate sections. Next, the medium was removed, 100 μL new medium and 10 μL MTT solution (5 mg/mL) were added and incubated at 37 °C for 4 h. Finally, 100 μL SDS (10%) was used in order to dissolve the formazan crystals. The optical density (OD) was measured at 570 nm using a microplate reader (Thermo Scientific Varioskan Flash).

### 2.5. Clonogenic assay

Clonogenic assay (colony formation assay) is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony (at least 50 cells) (Rafehi et al., 2011).

HEK-293 cells ($1 \times 10^5$ cells/well) were plated in 10-cm diameter Petri dishes and incubated for 24 h at 37 °C to attach before treatment with OFE or Ole for 18 h. After further incubation for 6 h in the presence of $H_2O_2$ (400 μM), the cell suspension was diluted 1:1000 and seeded into 10-cm diameter Petri dishes for clonogenic assay according to the method previously described by Franken, Rodermond, Stap, Haveman, and Van Bree (2006). Petri dishes were placed in a 5% $CO_2$ incubator until control cells have

formed large clones (one week). Colonies were then fixed with glutaraldehyde (6.0%, v/v) and stained with crystal violet (0.5%, w/v).

### 2.6. TBARS assay

Thiobarbituric acid-reactive substances (TBARS) are markers of lipid peroxidation. We measured their concentrations using the method described by Fki, Sahnoun, and Sayadi (2007) with slight modifications. Briefly, HEK-293 cells were seeded in a 24-well plate at a density of $1.0 \times 10^5$ cells/well and cultivated at 37 °C in 5% $CO_2$ for 24 h. Plates were pre-treated with various concentrations of studied samples for 18 h before exposure to 400 μM of $H_2O_2$ for additional 6 h. Cells were lysed using RIPA buffer (BioBasicInc, Canada) and centrifuged at 3000 rpm for 10 min. The pellet was then resuspended in 500 μL of deionized water and 1 mL of TBA solution (15% trichloroacetic acid, 0.8% thiobarbituric acid, 0.25 N HCl) was added. The mixture was incubated at 95 °C for 15 min. After cooling to room temperature, optical density was measured at 532 nm. Values were reported to a calibration curve prepared with 1,1,3,3-tetraethoxypropane (TEP).

### 2.7. Measurement of intracellular ROS

Intracellular reactive oxygen species (ROS) production was measured using DCFH-DA as previously mentioned by Lou, Jing, Ren, Wei, and Zhang (2012) with slight modifications. In a 24-well plate, $10^5$ cells/well were exposed to OFE or Ole pretreatment for 18 h and then incubated with 400 μM of $H_2O_2$ for 6 h. Cells were then incubated with 20 μM of DCFH-DA for 30 min. Then, cells were rinsed three times with PBS, trypsinized and collected by centrifugation (1000 rpm for 5 min). 200 μL of PBS solution containing 30.000 cells were transferred to a 96-well microplate. The fluorescence intensity was detected using the excitation/emission couple 488/525 nm.

### 2.8. Flow cytometry analysis of apoptotic cells

In order to evaluate the apoptotic rate of HEK-293 cells, multicaspases assay was performed. Cells were seeded in Petri dishes and treated with different concentrations of OFE (100 and 200 μg/mL) or Ole (25 and 50 μg/mL). Cells were then collected, washed twice with PBS, and stained with SR-VAD-FMK (sulforhodamine-valyl-alanyl-aspartyl-fluoromethylketone) and 7-amino-actinomycin D (7-AAD) following the manufacturer instructions (Cat. No. 4500–0530, EMD Millipore Corporation, Hayward, CA 94545). Apoptotic cell populations were quantified using Guava PCA system (Guava Technologies).

### 2.9. Western blot analysis

Protein extraction was performed after cell treatment by means of ice-cold RIPA buffer (Bio Basic Inc., Canada), supplemented with protease inhibitor cocktail (Roche Life science, Germany) for 5 min followed by centrifugation (12.000g) for 10 min at 4 °C. Total protein concentration was determined using the Bradford reagent. The expression of target proteins; Bax, Bcl-2, p21, p38, P-Erk, Erk, P-Akt, Akt, Cox-2 and β-actin, was determined by western blot analysis using monoclonal antibodies (Cell Signaling Technology, Danvers, MA USA). Briefly, for each sample 30 μg proteins were separated on SDS–polyacrylamide gel and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat dry milk in TBS (Tris-buffered saline) with 0.1% Tween 20 (TBS-T) for 30 min at room temperature and incubated overnight at 4 °C with primary antibodies. Antibodies were detected using the HRP-conjugated secondary antibody for 1 h at room temperature. β-actin was used to normalize the amount of loaded proteins. Immunoreactive bands were visualized using enhanced chemiluminescence reagents (ECL) and densitometric analysis was performed using "Image J" image analysis software. The experiments were performed in triplicates.

### 2.10. RNA extraction and qPCR analysis

Total RNA from HEK-293 cells treated with OFE or Ole were extracted using Trizol (Life Technologies). DNA residues were removed using DNAseI (Life Technologies) and RNA was quantified using Experion system (BioRad). For qPCR assays, 200 ng of RNA from each sample were retro-transcribed using the High Capacity c-DNA Reverse Transcription Kit (Applied Biosystems) following manufacturer instructions. Q-PCR was performed using a 7900 HT Real Time PCR (Life Technologies) and SYBR® Select Master Mix 2X assay (Applied Biosystems, Foster City, CA, USA). All primers were designed using the Primer Express 3.0 software (Applied Biosystems) (Table 1). The amount of c-DNA target was calculated using the average value of the ΔCT between the target and the control (GADPH) gene, and was expressed as $2^{-\Delta\Delta CT}$ (Livak & Schmittgen, 2001), where $\Delta\Delta CT = (CT_{Target} - CT_{GADPH})_{Treatment} - (CT_{Target} - CT_{GADPH})_{Control}$. Three separate experiments (n = 3) were performed for qPCR and each sample was tested in triplicate in an individual run.

### 2.11. Statistical analysis

All experiments were conducted with three independent experiments, and values were expressed as means ± standard deviation (SD). The data were statistically analyzed by one-way ANOVA, using Tukey's multiple comparisons test and applying a significance level of $p \leq 0.05$.

## 3. Results

### 3.1. Compositional characterization of OFE and Ole

Using HPLC analysis, it was possible to reveal the presence of several phenolic compounds the chromatograms were recorded at λ = 254 nm for oleuropein, and at 330 nm for flavonoids (verbascoside, luteolin glucoside and apigenin glucoside). HPLC chromatograms of the OFE (Olive fruit extract) and Ole (purified oleuropein) are depicted in Fig. 1. Peaks were identified by comparing of the retention time and UV spectra of olive compounds with those of standards in the same conditions. Oleuropein (18.93 min) was the major phenolic compounds identified in OFE (Fig. 1A) while verbascoside (15.29 min), luteolin glucoside (15.76 min), and apigenin glucoside (17.46 min), were the three main flavonoids (Fig. 1B). The chromatogram of Fig. 1C depicts a sharp monomodal peak of oleuropein at a retention time of 18.93 min. Oleuropein purity, determined by LC-MS-MS, was 98%.

**Table 1**
Primer sequences for qPCR amplification.

| Gene | | Primer sequence |
|---|---|---|
| *Sirt 1* | Forward | TGTACGACGAAGACGACGAC |
| | Reverse | TTCATCACCGAACAGAAGGTT |
| *Ask 1* | Forward | CCAGGGCCTCTTCCTGCTA |
| | Reverse | TCTGCTCCTCCCAGAGGATTT |
| *GAPDH* | Forward | CAAGGCTGTGGGCAAGGT |
| | Reverse | GGAAGGCCATGCCAGTGA |

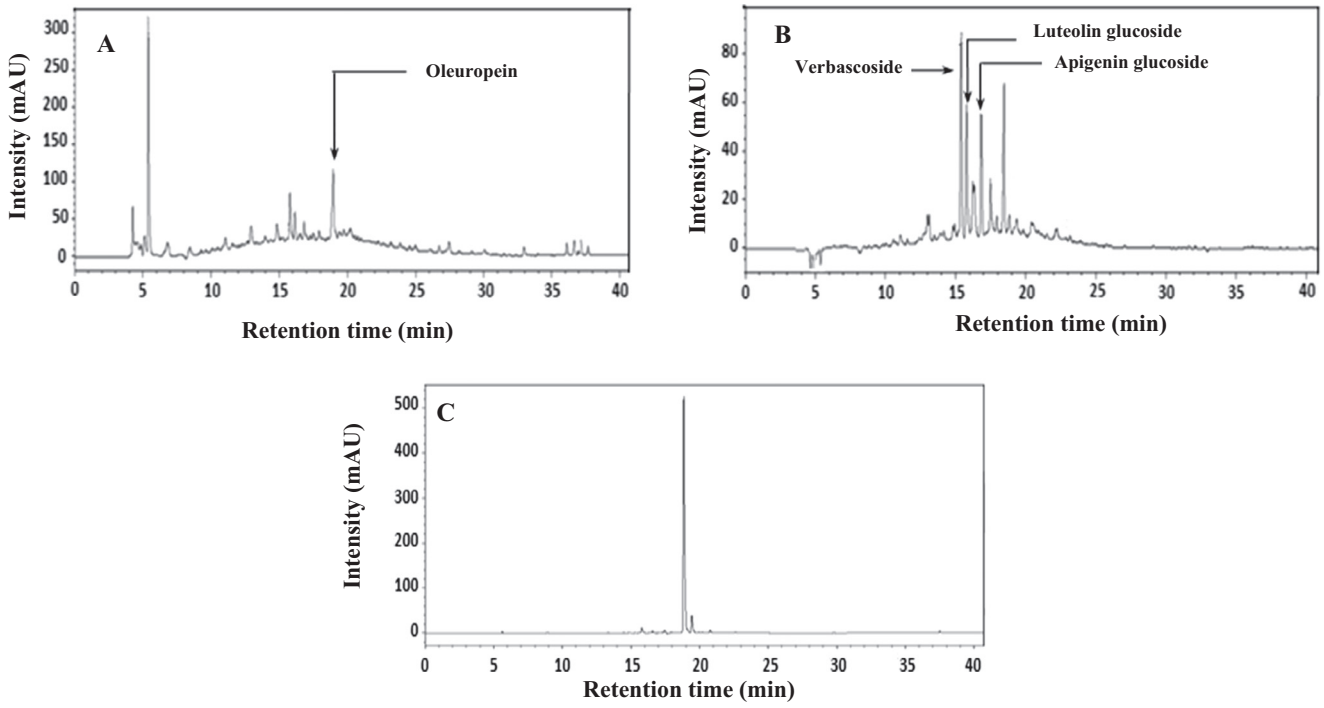


**Fig. 1.** HPLC chromatograms of olive fruit extract (A: λ = 254 nm, B: λ = 330 nm) and purified oleuropein (C: λ = 254 nm). The chromatograms were recorded at λ = 254 nm for oleuropein (18.93 min) and at 330 nm for flavonoids: verbascoside (15.29 min), Luteolin glucoside (15.76 min) and apigenin glucoside (17.46 min).

### *3.2. OFE and Ole prevent the $H_2O_2$-induced decrease in cell viability and proliferation of HEK-293 cells*

To determine the adequate concentrations of OFE and Ole to be used in the experiments, HEK-293 cells were incubated with different concentrations of OFE and Ole for 24 h. The results indicated that low concentrations of both OFE (e.g., 50, 100, 200 μg/mL) and Ole (e.g., 10, 25, 50 μg/mL) did not show any toxic effect (data not shown).

Treatment with $H_2O_2$ at the concentration of 400 μM was shown to have a significant toxic effect (45.35% ± 2.08) after 6 h of exposure.

For combined treatments, cells were pre-incubated with OFE (50, 100 and 200 μg/mL) or Ole (10, 25 and 50 μg/mL) for 18 h then treated with 400 μM of $H_2O_2$ for additional 6 h. As shown in Fig. 2A, OFE at doses 100 and 200 μg/mL strongly prevented $H_2O_2$-induced toxicity while there was a moderate effect at 50 μg/mL. Similarly, a protective effect was also exhibited by Ole treatment at concentrations of 10, 25 and 50 μg/mL.

Cells proliferation was further evaluated by clonogenic assay (Fig. 2B). The colony-forming efficiency was significantly restored in cells pretreated with OFE or Ole than in $H_2O_2$-treated cells. Therefore, the two highest protective concentrations of OFE (100 and 200 μg/mL) and Ole (25 and 50 μg/mL) were selected for use in further experiments.

### *3.3. OFE and Ole protect cells from ROS generation and lipid peroxidation*

ROS and TBARS assays were carried out for monitoring reactive oxygen species and lipid peroxidation generation as the major index of oxidative stress. As shown in Fig. 3A and B, exposure of HEK-293 cells to $H_2O_2$ led to an increase in both ROS and TBARS levels in comparison to the control cells.

Interestingly, both OFE (100 and 200 μg/mL) and Ole (25 and 50 μg/mL) significantly reduced $H_2O_2$-induced ROS and TBARS formation in a dose-dependent manner (Fig. 3A and B).

### *3.4. OFE and Ole reduce $H_2O_2$-induced apoptosis*

To elucidate whether the observed reduction in cell viability induced by $H_2O_2$ treatment was associated with apoptosis, we examined the apoptotic cell populations using multicaspases assay by means of flow cytometry. As expected, results showed an increase of apoptotic cells after $H_2O_2$ exposure (46.16% (± 2.97); $P < .01$). On the contrary, OFE (200 μg/mL) and oleuropein (50 μg/mL) pretreatments strongly decreased the number of apoptotic cells up to 6.18% (± 1.03) and 6.66% (± 2.06) ($P < .01$), respectively (Fig. 4). Interestingly, no significant difference was detected between cells treated with 200 μg/mL of OFE and those treated with 50 μg/mL of Ole.

### *3.5. Reduction of $H_2O_2$-induced apoptosis through the intrinsic pathway*

As described above, OFE at 200 μg/mL and Ole at 50 μg/mL exerted the most significant protective effects on $H_2O_2$-induced oxidative damage. Hence, these concentrations were used for further experiments.

To examine the effectiveness of OFE and Ole in attenuating $H_2O_2$-induced apoptosis, we analyzed Bax and Bcl-2 proteins expression by western blotting analysis, since the ratio Bax/Bcl-2 is known to increase in apoptosis. HEK-293 cells were exposed to $H_2O_2$ alone (400 μM for 6 h) or to $H_2O_2$/OFE and to $H_2O_2$/Ole. As shown in Fig. 5 (A, C), Bax/Bcl-2 levels increased in cells exposed to $H_2O_2$ compared to untreated cells. On the contrary, treatment for 18 h with OFE (200 μg/mL) or Ole (50 μg/mL) markedly reduced the Bax/Bcl-2 ratio.

Parallely, western blotting with a monoclonal antibody against p21 protein level was performed and indicated a significant ($P < .01$) increase in the protein expression in response to $H_2O_2$ treatment (400 μM, 30 min) (Fig. 5B, D). This increase was strongly inhibited after OFE (200 μg/mL, 24 h) or Ole (50 μg/mL, 24 h) pretreatment.

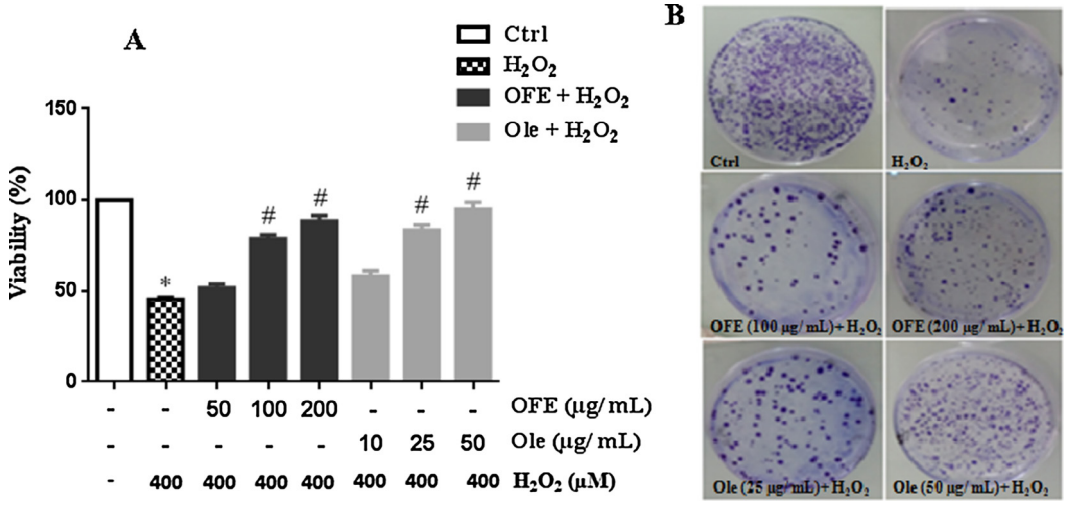


**Fig. 2.** Effect of OFE and Ole on $H_2O_2$-induced decrease in HEK-293 cell viability. Cells were pretreated with OFE (50–200 μg/mL) or Ole (10–50 μg/mL) for 18 h and then exposed to $H_2O_2$ (400 μM) for 6 h. After the treatment, cell viability was determined using MTT assay (A) and cell proliferation was analyzed by clonogenic assay (B). Ctrl: untreated cells or control. Data are expressed by means ± SD (n = 3). * Results were compared to control cells ($p < .001$). # Results were compared to $H_2O_2$-treated cells with alone ($p < .001$).

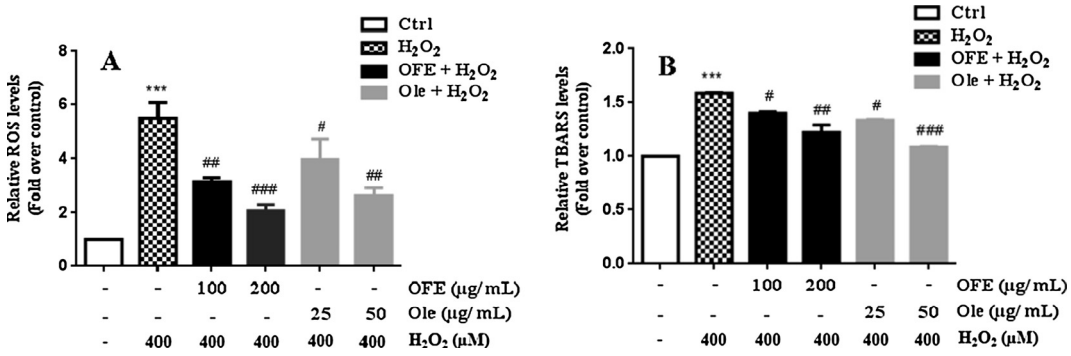


**Fig. 3.** Protective effect of OFE and Ole on intracellular ROS (A) and lipid peroxidation (B) in HEK-293 cells. Cells were incubated with OFE (100 and 200 μg/mL) or Ole (25 and 50 μg/mL) for 18 h and then exposed to 400 μM of $H_2O_2$ for 6 h. Ctrl: untreated cells. Values are presented by means ± SD (n = 3). *** $p < .001$ compared to control cells and ### $p < .001$; ## $p < .01$; # $p < .01$ compared to $H_2O_2$-treated cells.

Since p21 up-regulates Bax and antagonizes the death-protective function of Bcl-2, this result indicates that p21 could promote $H_2O_2$-induced apoptosis modulating the molecular ratio of Bcl-2/Bax in HEK-293 cells.

### 3.6. OFE and Ole attenuate P-p38 and P-Erk MAPK proteins levels in response to $H_2O_2$ treatment

The MAPK subfamilies are activated in response to oxidant injury, thus contributing to apoptosis onset following oxidative insults. In order to evaluate the potential implication of MAPK signaling pathway in the protective effect of OFE or Ole against $H_2O_2$-induced apoptosis in HEK-293 cells, we assessed the expression of Erk 1/2 and p38, two subfamilies of MAPK. Fig. 6A reveals that $H_2O_2$ treatment (400 μM for 30 min) increased the ratio phospho/total compared to untreated cells. In addition, the phosphorylated p38 was markedly increased in $H_2O_2$-treated cells above the basal level. Interestingly, the pretreatment with OFE or Ole (200 and 50 μg/mL for 24 h, respectively) induced a significant decrease in p38 and Erk 1/2 phosphorylation (Fig. 6A) compared to $H_2O_2$-treated cells.

### 3.7. Involvement of Akt signaling in hydrogen peroxide-induced damage

Akt, one of the major signaling enzymes involved in cell survival against oxidative stress, is able to suppress apoptosis and promote cell survival. The influence of OFE and its phenolic component Ole on $H_2O_2$-induced Akt dephosphorylation was determined using an anti-phospho-Akt monoclonal antibody which detects phosphorylated Akt at Ser473. Hydrogen peroxide treatment (400 μM, 30 min) resulted in a marked decrease in Akt phosphorylation with respect to untreated cells (Fig. 6A). However, pre-treatment with OFE (200 μg/mL, 18 h) prior to hydrogen peroxide treatment prevents the inhibition of Akt phosphorylation. Similar results were observed with 50 μg/mL of Ole pre-treatment.

### 3.8. OFE and Ole regulated the mRNA expression levels of apoptosis-related factor

To confirm the action of OFE and Ole on the stress response pathway, we analyzed the expression level of other members known to be involved in apoptosis mediated by $H_2O_2$. In particular, we analyzed the expression of Sirt 1 and Ask 1 by quantitative PCR analysis.

Sirt 1, a member of the Sirtuin family, is known to control Bax-mediated apoptosis through Ku70 (Cohen et al., 2004; Yeung et al., 2004). Whereas, Ask 1 (apoptosis signal-regulating kinase 1) encodes for a serine/threonine protein kinase ubiquitously expressed, which activates MKK3/6-p38 signaling pathways. This gene has been reported to be activated in response to $H_2O_2$ treatment.

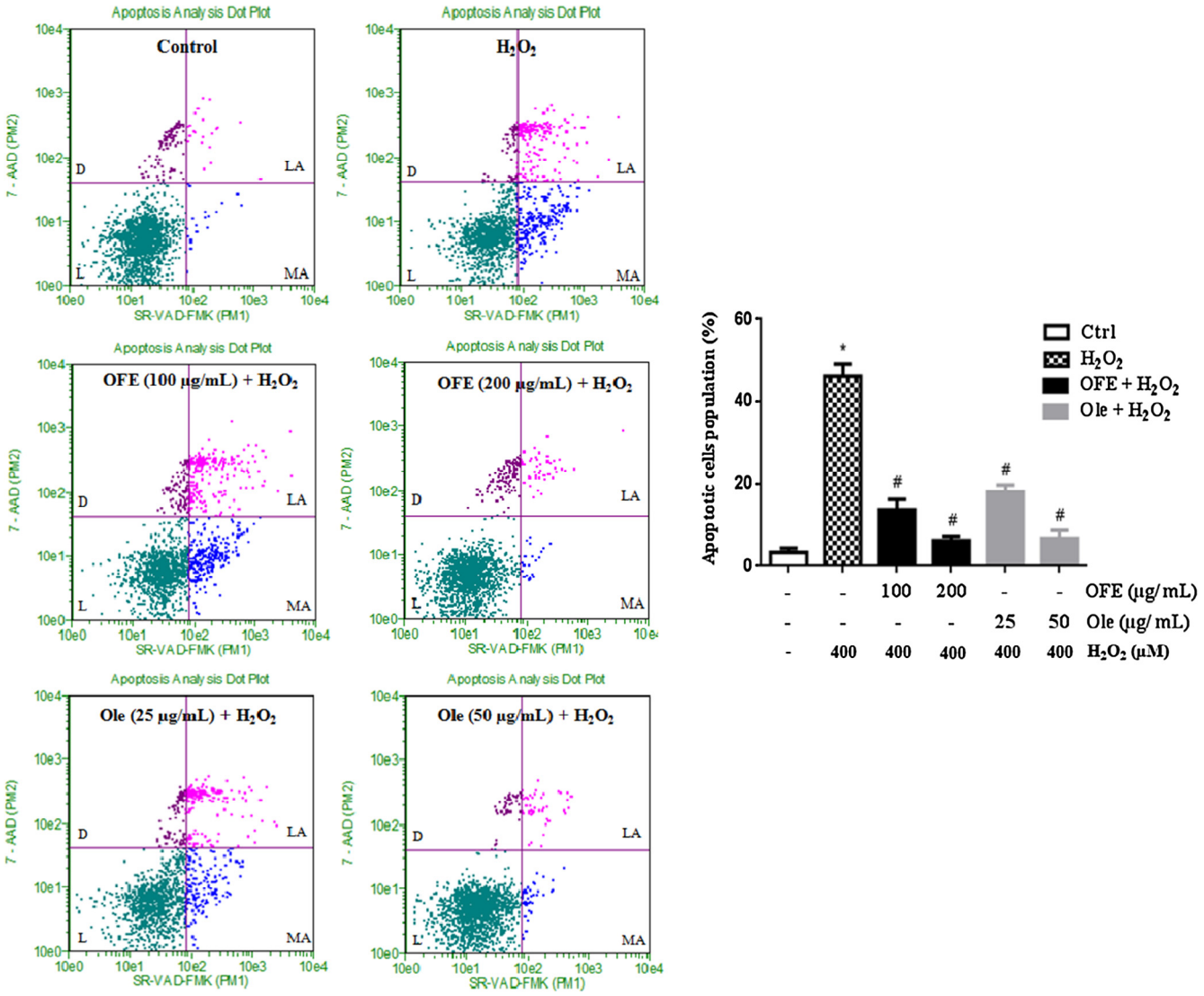


**Fig. 4.** Flow cytometry analysis of apoptotic cells. Live cells (quadrant L), Mid and late apoptotic cells (quadrants MA and LA) and dead cells (quadrant D) detected by flow cytometry after 6 h of exposure to $H_2O_2$ alone or cells pretreated for 18 h with OFE (100 and 200 µg/mL) or Ole (25 and 50 µg/mL) were analyzed. Ctrl: untreated cells. Results were compared to control cells: $^{*}$ $p < .01$; $^{**}$ $p < .001$ and to cells treated with $H_2O_2$ alone: $^{\#}$ $p < .01$; $^{\#\#}$ $p < .001$.

Based on these data, we wonder if an increase in mRNA level of these genes could be found in response to hydrogen peroxide treatment. Our results indicated that OFE and Ole treatments were able to rescue the up-regulation of Ask 1 and Sirt 1 mRNA levels induced by $H_2O_2$ treatment (Fig. 7). Indeed, $H_2O_2$ treatment (400 µM, 30 min) significantly increased Sirt 1 and Ask 1 mRNA of about 2.5 and 2.97-fold, respectively compared to untreated cells. The addition of OFE (200 µg/mL) or Ole (50 µg/mL) for 24 h significantly reduced the mRNA levels of both genes.

### *3.9. Protection against $H_2O_2$-induced inflammation through modulating Cox-2 protein expression*

Finally, we investigated whether the protective effect of OFE and Ole could have potential anti-inflammatory activities by modulating Cyclooxygenase-2 (Cox-2) expression. Cox-2 is an important enzyme that mediates inflammatory processes. The up-regulation of Cox-2 has been associated with inflammatory disorders (Surh et al., 2001).

As shown in Fig. 6B, the expression of Cox-2 protein significantly increased in response to hydrogen peroxide treatment. Again, OFE (200 µg/mL) or Ole (50 µg/mL) pre-treatment for 24 h induced a significant decrease of Cox-2 levels in HEK-293 cells compared to those treated with $H_2O_2$ alone (400 µM, 30 min).

## 4. Discussion

Recently, it has been reported that oxidants and oxidative modifications are among the main factors leading to cell and tissue damage. Indeed, it has been shown that hydrogen peroxide contributes to cellular damage in different cell lines (Choi, Cho, Seo, Lee, & Park, 2016; Ding, Wang, Li, & Ma, 2016), and in particular they cause renal cellular injury leading to nephrotoxicity (Salahudeen, Clark, & Nath, 1991). Moreover, it is well known that in kidney, oxidative stress can be increased by contrast media used for radio diagnostic procedures, determining acute nephropathy. Hence, Ole and OFE were selected for analyzing their abilities to minimise $H_2O_2$-induced oxidative stress in HEK-293 cells.

In this study, we first analyzed the effects of various concentrations of OFE (10, 50, 100, 200, 400 and 600 µg/mL) and Ole (0.5, 5, 25, 50, 100 and 200 µg/mL) on HEK-293 cell line proliferation (data not shown). Interestingly, treatment for 24 h with increasing con-

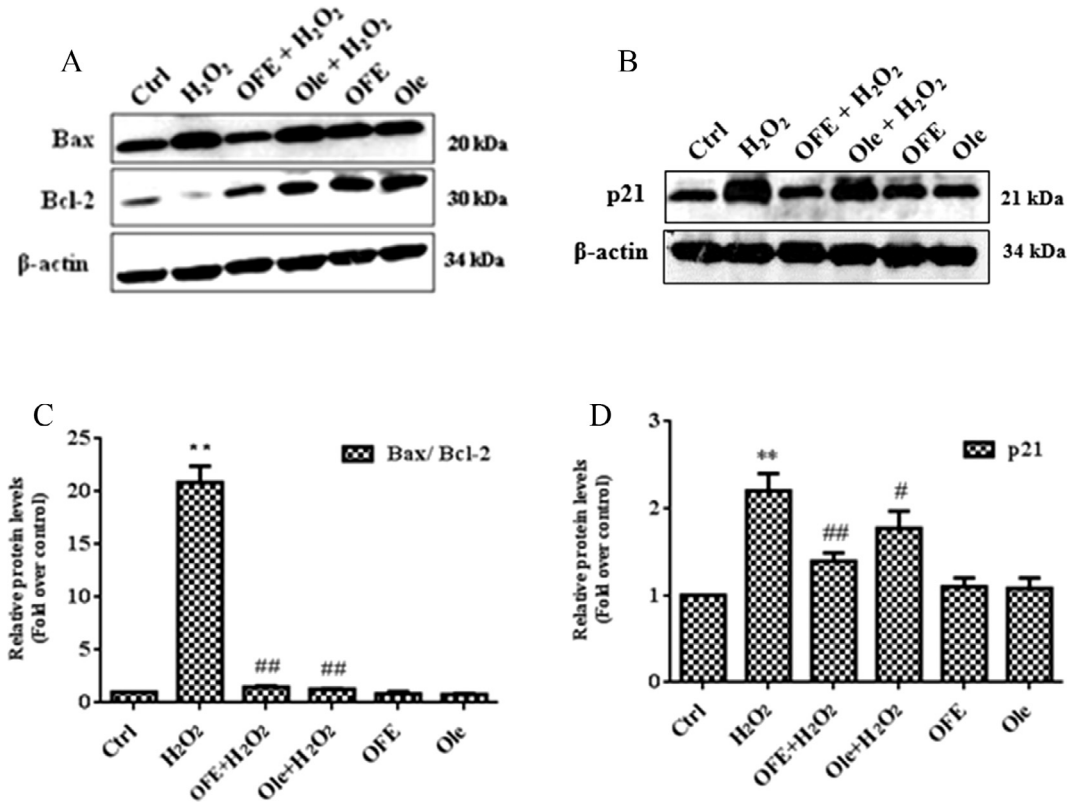


**Fig. 5.** Effect of OFE and Ole on Bax/Bcl-2 ratio and p21 in $H_2O_2$-induced HEK-293 cells. Total protein from HEK-293 cells treated with OFE or Ole were used in western blot analysis to detect the expression of Bax and Bcl-2. A: cells pretreated with OFE (200 μg/mL) or Ole (50 μg/mL) for 18 h prior to $H_2O_2$ treatment (6 h). B: cells pretreated with OFE (200 μg/mL) or Ole (50 μg/mL) for 24 h prior to $H_2O_2$ treatment (30 min) to analyze the expression of p21. In all the experiments, β-actin was used as loading control. C and D: Histograms of relative expression level refer to proteins normalized expression and derived by the analysis of three independent experiments. The values are expressed as the mean ± SD. * indicates a significant difference compared with control cells ($P < .05$). # indicates a significant difference compared with the $H_2O_2$-treated cells ($P < .05$).

centration caused a dose-dependent inhibition of cell growth. The highest concentrations of OFE and Ole with no cytotoxic effect were chosen to evaluate their ability to protect cells from hydrogen peroxide-induced damage and apoptosis in HEK-293 cells.

Then, after using $H_2O_2$ to induce cell damage, we investigated the protective effects of OFE, a Tunisian olive fruit extract, and its phenolic compound oleuropein. The MTT assay showed that peroxide treatment caused loss of viability in HEK-293 cells compared to untreated cells. These findings are similar to previous studies reporting viability decrease both in human kidney cells (HEK-293) (Liu et al., 2014) and in kidney tubule epithelial cells (NRK-52E) (Wang, Matsushita, Araki, & Takeda, 2002) treated with $H_2O_2$.

It is well known that oxidative stress caused by hydrogen peroxide treatment enhances the oxidation of proteins and lipids and causes DNA damage leading to cell death (Liu et al., 2014). Moreover, Salahudeen et al. (1991) reported that $H_2O_2$ enhances ROS levels through increasing intracellular calcium and that lipid alterations contribute to $H_2O_2$-mediated renal cells injury.

In this study, we observed that HEK-293 cells exposed to $H_2O_2$ showed an increase of TBARS and ROS levels as well as the level of apoptosis as evidenced by the increase of mid and late apoptotic cell populations. On the contrary, the hydrogen peroxide effect was strongly reverted in presence of OFE or Ole in a dose-dependent manner.

In the same context, it has been reported that mitochondrial dysfunction appears to initiate ROS production and lysosomal membrane permeabilization, thus causing cell damage (Guicciardi, Leist, & Gores, 2004). Different studies reported that $H_2O_2$-mediated mitochondrial dysfunctions and caspases activation leads to endothelial cell apoptosis (Cai, 2005; Fen-fang et al., 2015).

In an attempt to understand the molecular mechanism by which $H_2O_2$ induces apoptosis, we examined the involvement of caspases in the apoptotic process. OFE and Ole alleviated the apoptosis triggered by $H_2O_2$ indicating a protective role of both extracts on renal cell lines. Bax protein is a member of the Bcl-2 family that promotes apoptosis. Recently, it has been reported that Bax can initiate apoptosis through its own activation. Indeed, Bax translocates to the external mitochondrial membrane, rendering it permeable by oligomerizing and releasing some death-promoting factors (Cohen et al., 2004).

Bcl-2 family members have been described also as key regulators of mitochondrial permeability (Cory & Adams, 2002), and it is known that the increase of Bax/Bcl-2 ratio determines the susceptibility of a cell to apoptosis. To understand the potential modulating effect of OFE and Ole on increased Bax/Bcl-2 ratio, we analyzed the expression of both proteins by western blot. The results showed that increased Bax/Bcl-2 ratio, following $H_2O_2$ treatment, was re-established when cells were pretreated by OFE or Ole. In addition, we observed that OFE and Ole treatments modulate the over expression of p21. This protein, a cyclin-dependent kinase inhibitor 1, is known to up-regulate Bax protein expression and to antagonize the death-protective function of Bcl-2 in response to cell damage (Kang, Kim, & Choi, 1999). These results allowed us to suggest that p21 promotes $H_2O_2$-induced apoptosis via enhancing the expression of Bax, thus modulating the molecular ratio of Bcl-2/Bax in HEK-293 cells.

MAPK (Mitogen-Activated Protein Kinases) play a significant role in many biological processes, including cell adhesion, cell differentiation and apoptosis. In particular, they are involved in coordinating the response to ROS formation, thus influencing cell fate.

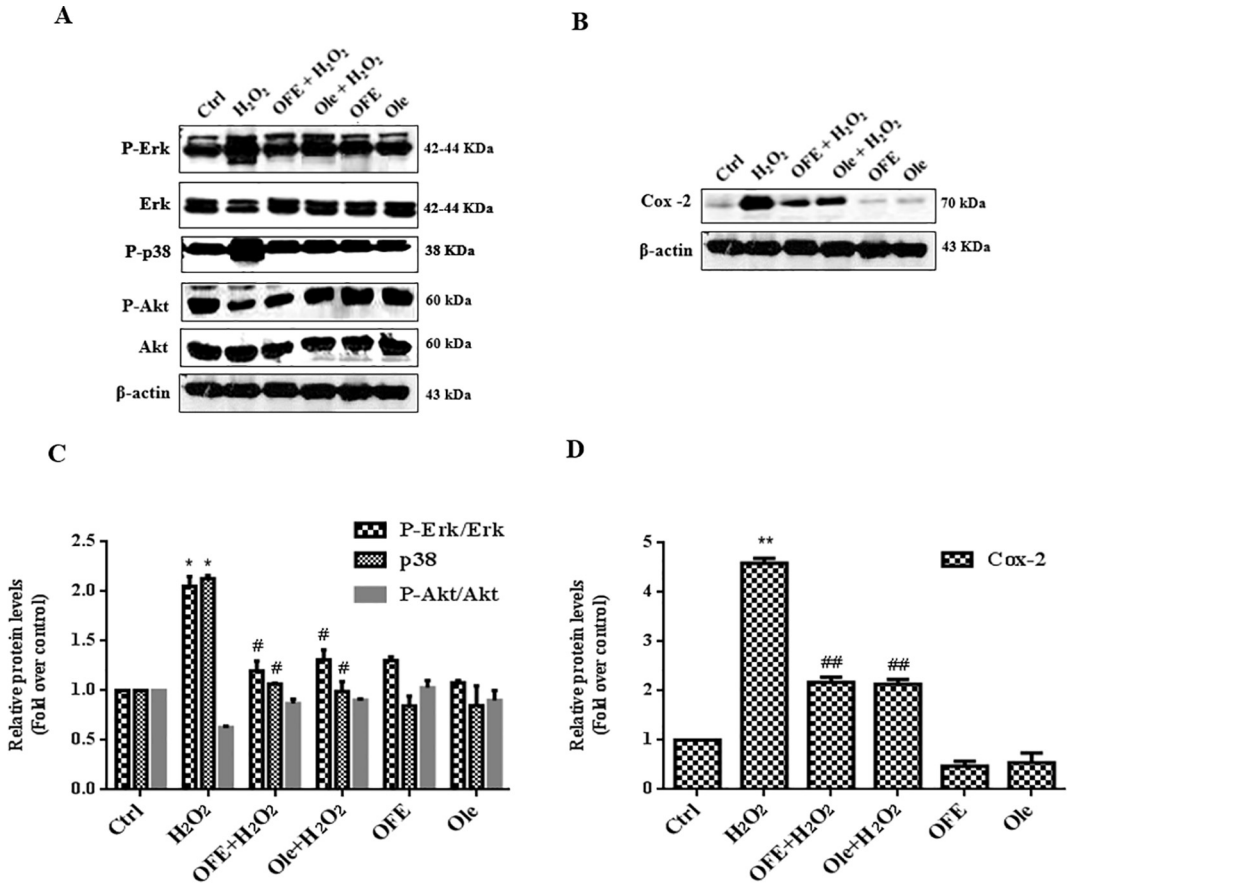


**Fig. 6.** Effect of OFE and Ole on phosphorylated Erk and p38 MAPK and Akt. An equal amount of total protein from untreated cells (Ctrl) or those treated with OFE (200 μg/mL) or Ole (50 μg/mL) for 24 h prior to $H_2O_2$ treatment (30 min) were used in western blot analysis to analyze the expression of P-Erk, Erk, P-p38, P-Akt, Akt (A), and Cox-2 (B). β-actin was used as loading control. (C) and (D) Histograms of relative expression level refer to proteins normalized expression and derived by the analysis of three independent experiments. The values are expressed as the mean ± SD. * indicates a significant difference compared to control cells ($P < .05$). # indicates a significant difference compared to $H_2O_2$-treated cells ($P < .05$).

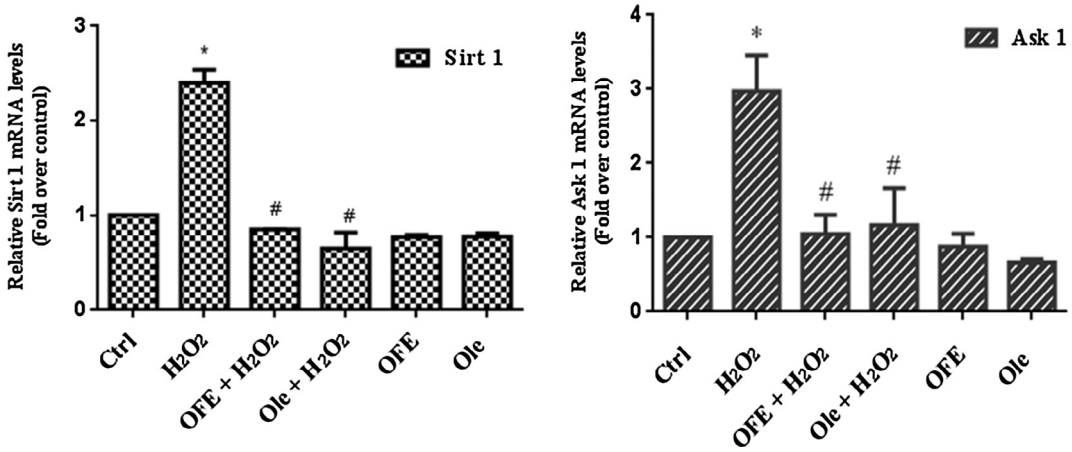


**Fig. 7.** The Gene expression in HEK-293 cells. mRNA was extracted from cells treated with $H_2O_2$ (400 μM, 30 min) alone or following pre-treatment for 24 h with OFE (200 μg/mL) or Ole (50 μg/mL) and analyzed by Real Time PCR. Sirt 1 (A) and Ask 1 (B) relative mRNA levels were normalized using GAPDH as housekeeping gene. Ctrl: control or untreated cells. * $p < .05$; ** $p < .01$ compared to control cells. ### $p < .001$; ## $p < .01$; # $p < .05$ compared to $H_2O_2$-treated cells.

The MAPK subfamilies ERK, JNK and p38 have been shown to be activated in response to oxidant injury contributing to apoptosis onset following oxidative insults (Fico et al., 2004).

In the present work, the expression of both Erk 1/2 and p38 was analyzed. Our results showed that the protection mediated by OFE and Ole could involve the MAPK signaling cascades. In fact, the inhibition of both kinases reduced apoptosis triggered by $H_2O_2$ treatment. These observations are in agreement with previous studies showing that the ability to preserve cells from oxidative damage, exerted by hydroxytyrosol in porcine renal epithelial cell line (Incani et al., 2010), by hesperetin in fibroblasts (Pollard, Whiteman, & Spencer, 2006) or also by quercetin to protect

cardiomyoblasts (Angeloni, Spencer, Leoncini, Biagi, & Hrelia, 2007), is due to the Erk1/2 pathway activation.

Although Erk1/2 phosphorylation has been observed to be unaffected in response to peroxide treatment in cardiomyoblasts (Angeloni et al., 2007), its level was shown to increase in renal cells (Zhuang & Schnellmann, 2006).

Activation of Akt, one of the major signaling enzymes involved in cells survival against oxidative stress, has been shown to suppress apoptosis and promote cell survival (Kang et al., 2010). Additionally, it has been revealed that Akt is involved in cell death regulation (Fresno Vara et al., 2004) through different cell survival mechanisms or apoptosis, as reported in the renal cellular model (Sinha, Bannergee, Schwartz, Lieberthal, & Levine, 2004). To analyze the mechanism of OFE and Ole mediated cell survival, we examined the phosphorylation of Akt. Western blot analysis showed that both OFE and Ole were able to prevent $H_2O_2$-induced inhibition of Akt phosphorylation. These findings are in agreement with studies performed in other cell lines such as porcine renal epithelial cells and cardiomyoblasts where hydroxytyrosol and quercetin, respectively, were able to modulate Akt phosphorylation level (Angeloni et al., 2007; Incani et al., 2010).

The ability of olive compounds, OFE and Ole, the stress response pathway, was further confirmed by the analysis of the expression level of two genes involved in apoptosis mediated by $H_2O_2$. In particular, we analyzed the expression of Sirt 1 and Ask 1 by quantitative PCR analysis. Sirt 1, a member of the Sirtuin family, is a nicotinamide adenosine dinucleotide-dependent histone deacetylase, that was shown to control Bax-mediated apoptosis through Ku70 (Cohen et al., 2004; Yeung et al., 2004). Besides, it has been reported that ROS accumulation in response to $H_2O_2$ treatment resulted in the activation of the apoptosis signal-regulating kinase 1 (Ask 1). This later encodes for a serine/threonine protein kinase ubiquitously expressed, which activates MKK3/6-p38 signaling pathways. Based on this data, the probable increase of Ask 1 mRNA level in response to peroxide treatment to ROS accumulation was checked. We observed that OFE and Ole treatments were able to rescue the up-regulation of Sirt 1 and Ask 1 induced by $H_2O_2$ treatment. This suggests that both OFE and Ole may inhibit Bax expression by decreasing Sirt 1 expression in response to peroxide treatment.

Since inflammation is closely linked to oxidative damage, substances with strong anti-inflammatory activities are anticipated to exert a protective effect on oxidative diseases. Cyclooxygenase-2 (Cox-2) is an important enzyme that mediates inflammatory processes. The up-regulation of Cox-2 has been associated with inflammatory disorders (Surh et al., 2001). In this study, we showed that OFE and Ole exhibit similar significant protection against hydrogen peroxide-induced cellular inflammation through inhibiting Cox-2 expression. The ability of OFE and Ole to modulate MAPK signaling pathway might contribute to the inhibition of inflammation. In fact, it has been reported that Erk 1/2 and p38 MAP Kinases are key elements of the intracellular signaling cascades leading to NF-κB activation, thus modulating Cox-2 expression (Surh et al., 2001). Our findings well agree with previous studies reporting that quercetin down regulated OTA-induced Cox-2 expression in HepG-2 cells (Ramyaa, Krishnaswamy, & Padma, 2014) and that oleuropein protected mice kidneys from oxidative damage and apoptosis by modulating Jnk 1/2, Erk 1/2 and p38 MAPK, and from inflammation by decreasing Cox-2 expression (Potočnjak, Škoda, Pernjak-Pugel, Peršić, & Domitrović, 2016).

The mode of action of OFE and Ole reported in this study is in agreement with previous findings showing that the olive oil polyphenols exhibit chemopreventive effects through p38/CREB signaling pathway (Corona et al., 2007). Our results showed that OFE and Ole have comparable effects.

In conclusion, our results suggest that OFE and Ole exhibit cytoprotective effect in HEK-293 cells against $H_2O_2$-induced oxidative damage and apoptosis. Furthermore, OFE and Ole-induced cell survival correlates with Akt activity, and the protection of kidney cells against $H_2O_2$-induced apoptosis could be stimulated by the activation of p38 and Erk MAPK. The present results suggest that OFE and Ole prevent oxidative stress-induced apoptosis via regulation of PI3K/Akt and MAPK signaling pathways. All these data suggest that OFE and Ole may be used in the treatment of kidney degenerative diseases. However, further studies are needed to explore the details of their protective pathway as well as their bioavailability and metabolism in human kidney.

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## Conflict of interest

No conflicts to disclose.

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**REVIEW ARTICLE**

# New Therapeutic Drugs from Bioactive Natural Molecules: The Role of Gut Microbiota Metabolism in Neurodegenerative Diseases

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**Abstract:** ***Background:*** The gut-brain axis is considered a neuroendocrine system, which connects the brain and gastrointestinal tract and plays an important role in stress response. The homeostasis of gut-brain axis is important for health conditions and its alterations are associated to neurological disorders and neurodegenerative diseases.

***Methods:*** We selected recent papers analysing the association among alterations in the homeostasis of the gut-brain axis and neurological disorders. In addition, we described how bioactive natural molecules - such as polyphenols – by influencing gut microbiota composition may help rescue neural signalling pathways impaired in neurodegenerative diseases.

***Results:*** Recent studies show that gut microbiota is a dynamic ecosystem that can be altered by external factors such as diet composition, antibiotics or xenobiotics. Gut bacterial community plays a key role in maintaining normal brain functions. Metagenomic analyses have elucidated that the relationship between gut and brain, either in normal or in pathological conditions, reflects the existence of a "microbiota-gut-brain" axis. Gut microbiota composition can be influenced by dietary ingestion of probiotics or natural bioactive molecules such as prebiotics and polyphenols. Their derivatives coming from microbiota metabolism can affect both the gut bacterial composition and brain biochemistry.

***Conclusion:*** This review highlights the role of gut microbiota in regulating regulates brain biochemistry and the role of microbiota metabolites on neuropathologies. Dietary ingestion of probiotics, prebiotics and polyphenols affect gut microbiota composition underlining the key role played by specific metabolites not only in the gut microbiota composition but also in the brain health maintenance.

**Keywords:** Gut-brain axis, gut microbiota, metagenomic analysis, prebiotics, probiotics, polyphenols.

## 1. INTRODUCTION

Recent advances in metagenomic sequencing analysis have allowed unravelling the huge number of microbial genes, which could impact on human health. The human gut microbiota is one of the most densely populated microbial ecosystems in nature and its composition is tightly linked to diet. For example, several studies report that changes in the gut microbiota observed in high fat/sugar diet are linked to the onset of increased gut permeability and low-grade inflammation that determine metabolic disorders [1, 2].

The genomes of entire microbial communities and the functional relationship existing between functional components of the microbiome, microbial metabolism and metabolite production are being elucidated using new sequencing technologies [3].

Human microbiome changes over the entire life and the differences in microbial content result in physiological changes of the host. In addition, microbial interactions play an essentially protective role against infections from pathogens determining a coordinate gene expression in response to different host and environmental signals [4].

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It is known that gut microbiota and the host share a reciprocal link since several human diseases are associated to imbalances in the microbiota composition, and that genome of the host may affect the microbiota composition [5].

The data coming from the different large-scale metagenomic projects such as the NIH Human Microbiome Project (hmpdacc.org), the European Metagenomics of the Human Intestinal Tract consortium (metahit.eu), and the International Human Microbiome Consortium (human-microbiome.org) indicate a high inter-individual diversity in microbiota composition and a strong association between their composition and health / disease state [6].

Metagenomic studies have allowed understanding the mechanisms involved in microbial interactions, which play a key role in infections being able to block pathogens activities. In addition, they contribute to healthy state and the onset of several diseases is tightly linked to dysbiosis [7]. Thus, a better understanding of microbial interactions is fundamental to shed light on microbial pathogenesis and it represents a way to develop new therapeutic strategies as well as to identify new natural products which favour the maintenance of specific microbial species [8]. Furthermore, microbiota interactions trigger the synthesis of secondary metabolites that selectively activate silent biosynthetic pathways [9, 10].

The gut-brain axis is widely recognized as the bidirectional neuroendocrine system between the Gastrointestinal tract (GI) and the Central Nervous System (CNS). It plays an important role in the

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stress response. Alterations in the homeostasis of the gut-brain axis are associated not only with gut inflammation and eating disorders but also with neurological disorders [11, 12].

Communication between the gut and the brain involves multiple overlapping pathways: the Enteric Nervous System (ENS), the neuroimmune, and the neuroendocrine systems. These systems are related to each other and to the CNS and gut by afferent and efferent fibres, respectively. Although molecular mechanisms are not well understood, increasing evidence suggests that communication is realized by the afferent neurons of ENS. These fibres transmit all the modifications in the GI tract to the CNS. Cytokines released by neuroendocrine cells act on inflammation and infection. Finally, neuroendocrine hormones change gut permeability [12]. Key signalling events involved in this functional network include the vagus nerve, metabolites and signalling systems of CNS, neurotransmitters and neurotrophins production.

It is worthy to note that enteric neuropathies can also be caused by mutations in genes that are fundamental in CNS development. These genes are expressed both in central and peripheral nervous systems. Their mutations can affect the proper function of specific cells controlling ENS. Mutations in NLG1 (Neuroligin-1) that have been associated to Schizophrenia also determine enteric neuropathy characterized by the absence of enteric ganglia, along variable lengths of distal colon (Hirschsprung disease) [13]. ARX (Aristaless-Related Homeobox gene) mutations cause lissencephaly or epilepsy [14, 15], and are involved in dysgenesis of enteroendocrine cells resulting in congenital intestinal diarrhea [16]. FLNA (Filamin A), a gene involved in allelic diseases such as cortical malformations is crucial for proper enteric neuron development [17].

By acting on the nervous, endocrine and immune system, gut microbiota can influence brain functions [18]. Also, the neural connection between microbiota and brain is carried out through the vagus nerve: microorganisms stimulate afferent neurons of ENS and then the vagal signal from the gut stimulates the anti-inflammatory response [19, 20].

Microbiota metabolites directly influence the brain functions by acting on neuroactive metabolites production [21]. For example, Short-chain Fatty Acids (SCFAs) can stimulate the release of neuropeptides from the gut endocrine cells. In addition, gut microorganisms can produce both neurotransmitters and neuropeptides [22, 23] (Fig. **1**).

Since individual microbiota play a key role in the pathophysiology of the gut-brain axis, it is more appropriate to use the term "microbiota-gut-brain" axis when referring to the relationship between brain and gut either under normal or pathological conditions. This tight association already demonstrated in both preclinical and clinical studies [20, 24, 25], seems to be involved both in brain disorders and in neurodegenerative diseases.

It is emerging that the regulation of microbiota composition can be realized using plant-derived bioactive molecules such as polyphenols. These compounds have antioxidant properties that protect against different chronic diseases and cancer [26]. Interestingly, polyphenols are metabolised by microbiota and their biological properties depend on the bioactive metabolites produced during this process [27].

The enhancement of bioactivity of natural molecules generated by microbiota metabolism could be viewed as a strategy to improve the beneficial properties of these micronutrients, including the antioxidant ones. Since oxidative stress is implicated in aging and in the onset of human age-related degenerative diseases, including neurodegenerative disorders [28], the connection between the effectiveness of natural products and the composition of gut microbiota/metabolites represents a promising field to develop new strategies to counteract neuronal pathologies.

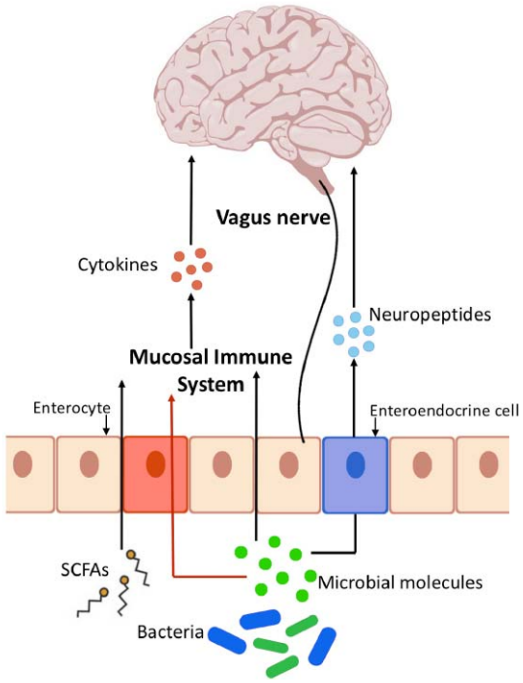


**Fig. (1).** Schematic representation of the microbiota-gut-brain axis. Microbial metabolites can influence brain and host behaviour. The connection between gut microbiota and CNS is mediated by vagus nerve.

We would like to specifically highlight the use of probiotics, prebiotics and polyphenols in the treatment of neurodegenerative diseases and the possible mechanisms of gut microbiota modulation. Understanding how bioactive natural molecules - such as polyphenols - can influence gut microbiota composition may help rescue neural signalling pathways impaired in neurodegenerative diseases.

### 1.1. Impact of Microbiota On Brain Functions

The human gut harbours more than 800 different bacterial species ($10^{14}$ microbial cells) [29] that constitute the microbiota. The metabolic activity of this community is fundamental not only for the digestion of dietary compounds and the supply of nutrients, but also for their transformation into bioactive compounds. The gut microbiota represent a dynamic ecosystem that can be altered by external factors such as diet composition, antibiotics or xenobiotics. Microbiota plays a key role in maintaining physiological homeostasis not only for digestion/host metabolism and prevention of colonization by pathogens, but also for brain functions [30]. Several preclinical and clinical studies have indicated that microbiota can significantly interfere with human cognitive systems [20, 25, 31, 32].

Gut microbiota has a biochemical complexity greater than brain's one. Interestingly, hormones synthesized by the microbiota, act as neurotransmitters within the CNS. This group includes serotonin, melatonin, Gamma-Aminobutyric Acid (GABA) and acetylcholine [33] (Table **1**, Fig. **2**). GABA, the most important inhibitory transmitter in the brain, is produced by *Lactobacillus* and *Bifidobacterium* [22] while noradrenaline, dopamine, and serotonin are mainly produced by *Escherichia* and *Bacillus* [18, 34-36]. *Lactobacilli* have been shown to convert nitrate to Nitric Oxide (NO), which is a signalling molecule widely used in the nervous systems [37].

Gut-microbiota-brain cross-talk can occur not only through neurotransmitters but also using humoral paths [38], as happens for circulating tryptophan.

**Table 1. Examples of neurotrasmitters produced by gut microbiota.**

| Bacterial Strain | Neurotransmitter |
|---|---|
| *E. coli* K-12 | Serotonin |
| *H. alvei* | |
| *K. pneumoniae* | |
| *L. lactis* subsp. *lactis* | |
| *L. lactis* subsp. *cremoris* | |
| *L. plantarum* | |
| *M.morganii* | |
| *S. thermophilus* | |
| *B. mycoides* | Dopamine |
| *B. subtilis* | |
| *B. cereus* | |
| *E. coli* | |
| *E. coli K-12* | |
| *H. alvei* | |
| *K. pneumoniae* | |
| *M. morganii* | |
| *P. vulgaris* | |
| *S. marcescens* | |
| *S. aureus* | |
| *B. mycoides* | Noradrenaline |
| *B. subtilis* | |
| *E. coli K-12* | |
| *P. vulgaris* | |
| *S. marcescens* | |
| *B. adolescentis* | GABA |
| *B. dentium* | |
| *B. dentium* | |
| *B. infantis* | |
| *L. brevis* | |
| *L. rhamnosus* | |
| *L. plantarum* | Acetylcholine |
| *H. alvei* | Histamine |
| *K. pneumoniae* | |
| *L. lactis subsp. cremoris* | |
| *L. lactis subsp. lactis* | |
| *L. plantarum* | |
| *M. morganii* | |
| *S. thermophiles* | |

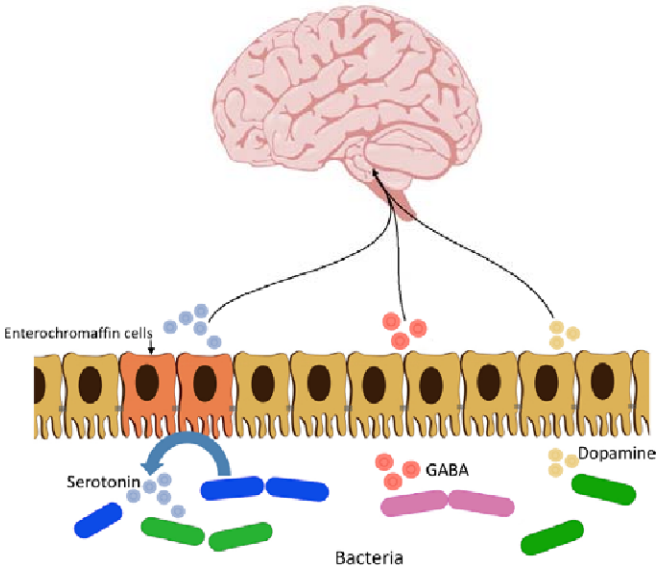


**Fig. (2).** Schematic representation of the neurotransmitters produced by gut microbiota. Microbiota itself can release neurotransmitters that pass the intestinal barrier and reach the brain.

Tryptophan is the precursor of serotonin (5-HT), whose levels are regulated by gut microbiota, and its dysregulation is linked to many disorders both in the brain and in the GI [39].

Serotonin is a biogenic amine that acts as neurotransmitter both in the CNS and in the gut. It plays an important role maintaining mood and cognition [40]. Alterations of serotonin levels can be associated to the onset of gastrointestinal and mood disorders, often with a high co-morbidity. The association between gut microbiota and serotonin has been shown in experiments in which its modulation by using antidepressants, resulted to be effective on irritable bowel syndrome symptoms [41].

The direct regulation of tryptophan on serotonin through microbiota has been demonstrated in germ free animals in which increased levels of circulating tryptophan and decreased levels of serotonin were restored after bacteria colonization of these animals [42].

Bacterial degradation of tryptophan occurs along the kynurenine pathway, the second most prevalent metabolic pathway of tryptophan. Kynurenine is the precursor of kynurenic acid, a neuroactive molecule. Interestingly, a decreased ratio kynurenine/tryptophan is linked to different neurological diseases [39, 43] and genes belonging to kynurenine pathway are regulated in the hippocampus by gut microbiota [44]. Regulation of precursors such as tryptophan and kynurenine strengthen the gut microbiota ability to control CNS and ENS neurotransmission.

Most of the neuroactive molecules are involved in bacteria-bacteria communication. For example, GABA is produced to protect the organism from the acid environment encountered in the stomach [45]. It has been proposed that the function of such small molecules, evolved from bacteria-bacteria communication to bacteria-host communication. In fact, some bacteria species have a receptor-like molecule to take up GABA [46], which along with its receptor is found in host gut epithelia [47]. This suggests that these molecules derived from gut microbiota may actually be involved in "inter-kingdom" signalling processes [48].

Bacteria-bacteria signalling could exert a dual role acting on both gut epithelial cells and ENS in the modulation of the nervous system.

The signature molecules that mediate the microbiota functions are the short-chain fatty acids which represent the most abundant

product of bacterial fermentation [49]. Generally, they act through classical endocrine signalling, since receptors and transporters for SCFAs are expressed in the GI tract [50]. For example, SCFAs modulate the release of serotonin [51] as well as of Peptide YY (PYY), an important neuropeptide acting at multiple levels of the gut-brain axis [52].

In addition, circulating SCFAs, such as butyrate and propionate, can be transferred from the site of production to the CNS by monocarboxylate transporters, which are highly expressed at the Blood-brain Barrier (BBB) where they act as signalling molecules [53].

Systemic administration of SCFA butyrate determines an antidepressant-like behavioural response. Butyrate induces a transient acetylation of histones in frontal cortex and hippocampus, and expression changes of the Brain-derived Neurotrophic Factor (BDNF) [54].

### 1.2. Gut Microbiota in Neurodegeneration

Neurodegenerative diseases are increasing due to the ageing of population. Neurodegeneration is a complex process that can be triggered by the exposure to environmental stressors, such as oxidants, which determine progressive neurons degeneration. This results in an imbalance in the metabolism of gut microbiota that modifies endocrine signalling in the host [55]. Furthermore, it has been suggested that gut dysbiosis may trigger the onset of neurodegenerative diseases including Amyotrophic Lateral Sclerosis (ALS), Alzheimer Disease (AD), Huntington Disease (HD) and Parkinson Disease (PD [56-59]. On the contrary, aged healthy people do not show marked microbiota changes suggesting that a healthy microbiota may be one of the keys of longevity [60, 61].

#### *1.2.1. Amyotrophic Lateral Sclerosis*

ALS is a fatal degenerative neurological condition in which motor neurons are the susceptible neuronal population [56]. The disease is mainly characterized by degeneration of motor neurons in the brain and the spinal cord. ALS develops insidiously with focal weakness but spreads relentlessly to involve most muscles that progressively undergo massive atrophy [62].

ALS has been recognized to involve several non-motor systems. Subclinical involvement of the autonomic system has been also described. Gastrointestinal motor dysfunction can occur in ALS even if patients do not complain of gastrointestinal symptoms [63]. However, still little is known about how this defect may contribute to the pathogenesis of the disease. Studies in animal models further support this evidence and show increased gut permeability associated with a significant reduction in the expression of tight junction proteins and altered microbiota [64, 65]. Interestingly, in ALS animal model the restoration of gastrointestinal microbial homeostasis ameliorated gut integrity and prolonged lifespan.

The link between ALS progression and gut microbiota was demonstrated in a transgenic mouse model [65]. These animals showed increased gut permeability due to defective intestinal tight junction structures. Furthermore, there were a reduced number of epithelial Paneth cells, which acted on the gut microbiota modulating the innate immune response. Dysfunction in the intestinal barrier may promote the passage of toxins into the blood and the innate immune response could be involved in the pathogenesis of ALS [66].

Even if these studies indicate an impairment of microbiota in ALS, to date no human study has investigated whether modifications of the gut microflora populations could be effective in ALS.

#### *1.2.2. Alzheimer Disease*

AD is one of the most common progressive neurodegenerative disorders that constitute the most frequent form of dementia in the elderly population [67]. The disease is pathologically characterized by deposition of Amyloid-$\beta$ (A$\beta$) peptides as A$\beta$ plaques and intracellular neurofibrillary tangles [57, 68] that ultimately lead to a gradual deterioration of brain structure and loss of intellectual function [69].

At first glance AD appears to be confined to the CNS and mainly characterized by impaired cognition, however peripheral defects have been also described [70]. Although several risk factors have been associated with the pathophysiology of AD - aging being the most important one - there is evidence suggesting that compromised GI function and altered gut microbiota may also represent an important determinant [71-74].

Gut microbiota dysbiosis seems to be present also in AD. In fact, different bacterial species are able to produce or aggravate the production of A$\beta$ plaques. AD patients have an increase of Gram-negative bacteria that is coupled with the mucosal disruption triggered in response to this dysbiosis. In AD, a relation has been demonstrated between the onset of pathology and a gut microbiota dysbiosis. The dysbiosis, priming the innate immune system by microbiota, determines a neuroinflammatory response that causes misfolding of neuronal amyloid-$\beta$ and $\alpha$-synuclein [75].

These results allow investigating how probiotic and prebiotic therapies can be used to ameliorate AD symptoms.

#### *1.2.3. Huntington Disease*

HD is the most common dominantly inherited neurodegenerative disorder, mainly characterized by progressive striatal and cortical neurodegeneration and by associated motor, cognitive and behavioural disturbances. The disease-causing mutation is an expansion of a CAG trinucleotide repeat (>36 repeats) encoding a polyglutamine stretch in N-terminal region of Huntingtin (Htt), a ubiquitous protein whose function is still unclear [58]. Although HD is classically described as a central disorder, emerging evidence demonstrates several peripheral dysfunctions associated with the progression of the disease [76]. Expression of Mutated Huntingtin (mHtt) is detected in and out the central nervous system [77] including the enteric neurons [78]. Studies in both animal models and humans reveal gastrointestinal dysfunctions, which may contribute to the worsening of the disease, impacting further the quality of life of patients. Although the underlying molecular mechanism remains unknown, evidence indicates that the consistent loss of body weight - the most important non neurological complication of HD and a direct consequence of GI defects - has been recently associated with an impaired gut mobility and malabsorption [79].

To date, no microbioma analysis has been described in HD patients. Gut microbiota could have a role in HD since presymptomatic HD subjects display clear serum metabolomic shifts that suggest changes in gut microbial-derived metabolites [80].

This study indicates that mHtt affects not only the CNS biochemistry but it has systemic effects, too. In addition, the connections between HD and gut microbiota could be fundamental for HD onset and progression and for the development of novel therapeutic strategies.

#### *1.2.4. Parkinson Disease*

PD is a chronic and long-term degenerative disorder, whose cause is still unknown. A diagnosis of Parkinson's disease is classically established after the manifestation of motor symptoms such as rigidity, bradykinesia, and tremor that are primarily caused by the loss of dopaminergic neurons in the *substantia nigra* of the midbrain [81].

A growing body of evidence supports the hypothesis that Non-motor Symptoms (NMS), especially gastrointestinal dysfunctions, may represent early biomarkers in PD since they are consistently associated with the disease and may precede the classical motor manifestations by decades [82]. In addition to their adverse effects on quality of life, GI dysfunctions make the etiology of PD more complicated. Despite the increased interest in the disease-associated NMS, still little is known about the GI features of PD. It has been

suggested that neurodegeneration, inflammation and intestinal hyperpermeability and microbial dysbiosis could play a key role in the pathophysiology of GI dysfunction in PD [83, 84].

PD patients show gut microbial dysbiosis with a marked impairment of epithelial barrier that has been associated with decreased *Prevotellaceae strains*. These bacteria are the main producers of mucin, a protein that protects the epithelium from pathogens by creating a barrier along the epithelial wall against invading pathogens [85].

In addition, intestinal biopsies of PD patients indicated a reduction of bacteria producing butyrate - an anti-inflammatory molecule (*i.e.*, *Roseburia* and *Faecalibacterium* spp.) - and an increase of proinflammatory bacterial species (*Proteobactera*) [86].

Gut microbiota shifts, in PD, result in a reduction of the gut SCFAs that are implicated in the reduced gastrointestinal motility in affected patients. More interestingly, PD subjects have a low level of butyrate, a Histone Deacetylase (HDAC) inhibitor, which protects dopaminergic neurons from degeneration by upregulating the neurotrophic factors BDNF and the Glial Cell Line-derived Neurotrophic Factor (GDNF) [87].

Novel therapeutic strategies in PD patients, based on gut microbiota modifications, are being successfully tested using fecal transplants from healthy donors [88].

### 1.3. Probiotics and Prebiotics Modulation of Microbiota-Gut-Brain Axis

Recent advances in microbiota analyses indicate that this ecosystem plays a key role in maintaining normal brain functions [89]. The homeostasis of the gut microbiota can be manipulated by changing dietary ingestion of probiotics and prebiotics. These modifications can be viewed as new therapeutic strategies for the treatment of gastrointestinal and CNS-driven disorders.

Probiotics are defined "live organisms which, when administered in adequate amounts, confer a health benefit on the host". Prebiotics are defined as "food ingredients that induce the growth or activity of beneficial microorganisms affecting the composition of the gut microbiota".

*Lactobacillus* and *Bifidobacterium* are the best-recognized microorganisms used as probiotics.

Probiotic health effects are related to the host immunomodulation. For example, *Bifidobacterium infantis* has been shown to normalize the Interleukine (IL) IL-10/IL-12 ratio [90].

Different studies in animal models reported the beneficial effects of prebiotics on the behaviour of the host. For example, depression and anxiety were both reduced in mice after treatment with *Lactobacillus rhamnosus* [20]. Treatment with a specific *Bifidobacterium infantis* strain, rescued the behavioural deficits in the forced swim test in adult rats subjected to the early life stress of maternal separation [32]. This treatment also retrieved basal noradrenaline concentrations in the brainstem [91].

Probiotics can meliorate cognition defects as described in a mouse model of autism [92], in which *Bacteroides fragilis* determined reduction of anxiety, sensorimotor gating, and stereotypical behaviours.

Interestingly, combining probiotic strains results in additive therapeutic efficacy in brain disorders. Combined treatment with *Lactobacillus rhamnosus* and *Lactobacillus helveticus* was shown to rescue memory defects induced by stress in mice [93]. Moreover, healthy human volunteers had beneficial psychological effects when treated with a combination of *Lactobacillus helveticus* and *Bifidobacterium longum* [31].

To date still few studies in humans have given attention to the gut microbial targeting for the treatment of brain diseases; however, the obtained results are really encouraging.

Dietary intake of fermented milk with probiotics was reported to affect the activity of brain regions involved in the central processing control of emotions and sensations [25]. A clinical trial on healthy subjects, showed that treatment with *Lactobacillus helveticus* and *Bifidobacterium* for one month protected from psychological distress [31]. Another study described that ingestion of milk added with probiotics improved mood and cognition in healthy subjects [94].

Dietary prebiotics are non-digestible fibres that selectively stimulate the bacterial growth improving host health [95]. Prebiotics reach the large bowel where they are utilized as substrate by microorganisms to produce energy, metabolites and micronutrients used by the host. In addition, they stimulate the selective growth of certain beneficial species (mainly *Bibifidobacteria* and *Lactobacilli*) or the metabolism of advantageous bacteria that in turn result in specific colonization.

The compounds known as the best prebiotics belong to the Inulin-type Fructans (ITF) Fructo-oligosaccharides (FOS) and to the Galacto-oligosaccharides (GOS). Most of the health benefits associated with the prebiotics were described using prebiotics as food ingredients or supplements. The ability of prebiotics to stimulate the growth of specific bacteria strains can result in significant changes in the gut microbiota composition.

The use of prebiotics represents an additional strategy for modulating the microbiota-gut-brain axis. Few studies have been performed using prebiotics with promising results both in humans and animals [96]. These studies showed an increase of BDNF brain level and of N-methyl-d-aspartate Receptor (NMDAR) signalling [24]. BDNF is a key neurotrophin involved in neuronal growth and survival [97], NMDAR is involved in normal brain function and it is linked to schizophrenia [98]. The results prompt to further investigate the utility of prebiotics in mental health and their potential efficacy for the treatment of psychiatric disorders.

A recent study has described that combined administration of FOS and a modified B immuno-galactooligosaccharides (B-GOS) had neuroendocrine effects.

Administration of B-GOS was shown to exert anti-depressive effects by modulating the Hypothalamic-pituitary-adrenal (HPA) axis, since it reduced cortisol awakening reactivity, usually elevated in individuals at high risk of depression [99].

Several studies in mice and in humans reported that polysaccharides could improve brain functions [100]. The isolichenan, an $\alpha$-glucan from the lichen *Cetrariella islandica*, reverted ethanol-induced memory impairment in mice [101]. The Ambrotose complex, a mixed polysaccharide formulation, significantly improved cognitive function and mood in healthy middle-aged adults [93].

Interestingly, prebiotics not only modulate bacterial growth but they can directly affect signalling molecules in the brain. For example, it has been reported that FOS and GOS increased hippocampal expression of BDNF and NMDAR1 subunit. Furthermore, GOS intake increased hippocampal NR2A subunit, frontal cortex NMDAR1; and plasma PYY [24]. Thus, like probiotics, also prebiotics can modulate brain chemistry by increasing BDNF expression through gut hormones activity. Additional studies to understand whether the brain modulation induced by prebiotics leads to cognitive and mood outcomes are needed, with the aim to consider probiotic-based approaches as novel therapeutic strategies [102].

### 1.4. Polyphenols: Chemistry and Bioavailability

Polyphenols are a large group of natural compounds, produced by plants as secondary metabolites, which defend against exogenous stresses. They protect plants against Reactive Oxygen Species (ROS), Ultraviolet Radiation (UV), pathogens, parasites and plant predators. The difference in their structures reflects their distinct biological functions.

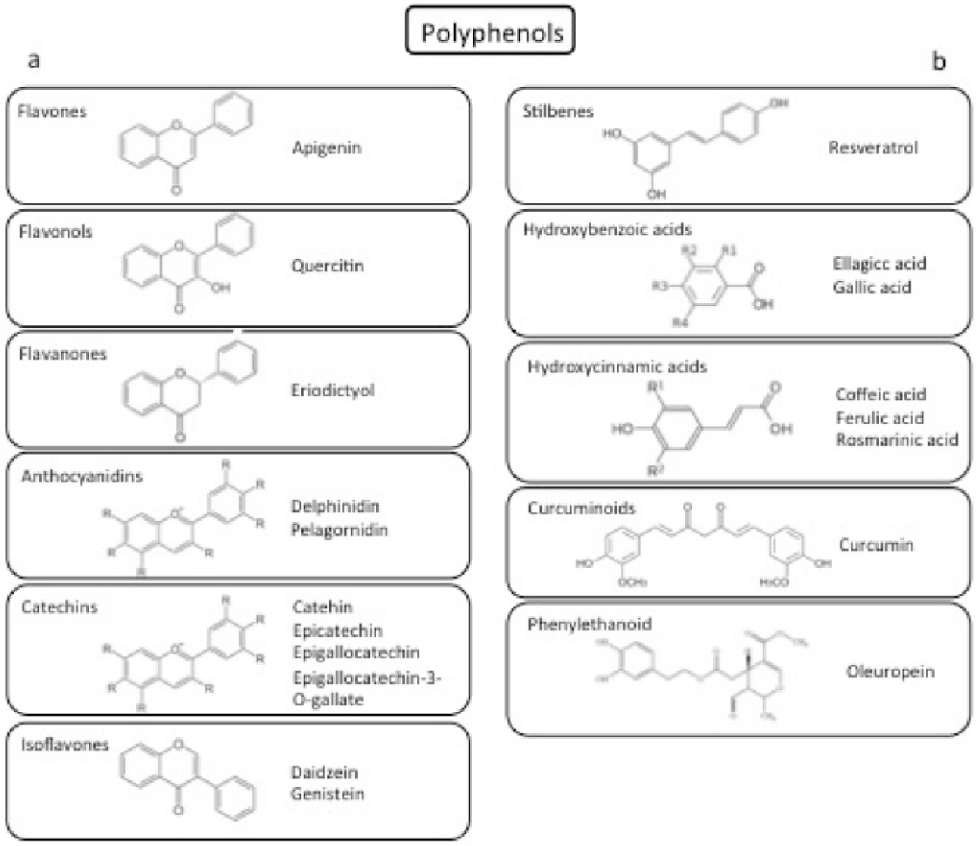


**Fig. (3).** Structures of polyphenols. Polyphenols are classified by the number of phenol rings in flavonoids (**a**) and non-flavonoids (**b**). The figure shows chemical structures and some typical representatives.

Polyphenols are characterized by the presence of at least one phenol ring with the attached hydroxyl group(s). They can be differentiated in flavonoids and non-flavonoids based on the number of aromatic rings and on the number of elements bound to the ring, and include simple molecules such as phenolic acids and complex molecules such as tannins (Fig. **3**). These molecules can be conjugated to organic acids and sugars [103].

Polyphenols act as natural antioxidants thanks to the metal-chelating and free radical scavenger properties. The scavenging activity is linked to their structure. In fact, compounds with similar structures exhibited similar antioxidant activity. The antioxidant activity increase is related to the number of hydroxyl groups directly, and to glycosylation indirectly [104].

Recently, there is a growing interest in the importance of the antioxidant activities of polyphenols and in their possible addition to food. In fact, several epidemiological studies in humans associated diet with chronic diseases, and indicated that prevention could be realized following correct lifestyles [105]. To this respect, the attention to the use of polyphenols as antioxidant has greatly increased.

Polyphenols bioactivity is related to absorption rate, metabolism and bioavailability. These activities depend on the direct interaction with other dietary nutrients, such as proteins, carbohydrates, fat and fibres. In addition, the biological activity of the derivative metabolites may differ from the native compound.

Polyphenols absorption through the gut barrier can be increased after specific conjugation such as methylation, sulfation, and glucuronidation. These processes facilitate biliary and urinary elimination by increasing their hydrophilicity [106]. Polyphenols metabolites found in blood and target organs derive from gut microbiota activity.

Conjugation processes are needed to reduce the potential toxic effects of polyphenols. These reactions are extremely efficient, since aglycones are generally absent in blood, or present in low concentrations, after consumption of nutritional doses. Thus, circulating polyphenols - as conjugated derivatives - can penetrate almost all tissues and exert biological activity.

To entry the brain, polyphenols must cross the gut enterocytes and the BBB. This process depends not only on the polyphenol chemical structure, but also on their interaction with BBB efflux transporter [107]. Interestingly, most plant polyphenols are ligands for brain efflux transporters and penetrate the brain in large amounts [108]. For this reason, a functional BBB might favour polyphenols-mediated neuroprotection.

### 1.5. Polyphenols and Neuroprotection

Neurodegenerative diseases are characterized by neuron loss and neurodegeneration. It is thought that the onset of neurodegeneration starts long before the disease appears, when many neurons have already been destroyed. This can explain why therapeutic approaches have little or no effect.

Neurodegeneration may be induced by neuroinflammation and oxidative stress. Oxidative stress is a cell state in which ROS production overcomes the cellular antioxidant capacity. ROS are generated during cellular metabolism mainly by mitochondria. ROS excess determines severe cellular damage through lipid peroxidation, protein oxidation and plasma membrane damage. ROS activity also acts on both DNA and RNA oxidation [109, 110].

Oxidative stress is thought to contribute to the pathogenesis of a number of human diseases, in particular of neurodegenerative diseases. Nervous tissue is very sensitive to oxidative stress both for the high metabolic activity and oxygen consumption, and for the

low levels of antioxidants enzymes (superoxide dismutase, catalase) and molecules (glutathione) [111, 112].

Oxidative stress triggers the synthesis of superoxide and NO (Nitric Oxide), two neurotransmission regulators. Changes in the production and/or metabolism of these molecules result in pathologic consequences [113, 114].

Protection from oxidative stress in the nervous tissue can be achieved increasing the endogenous defence mechanisms acting on diet changes, and using natural antioxidants to target specific mechanisms of oxidative damage [115].

Novel therapeutic strategies, based on protection by antioxidants or on decreased expression of oxidative stress regulatory genes, seem to be effective in ageing and neurodegeneration prevention. These strategies aim to naturally increase the neuronal response to stress by acting on dietary consumption of foods that contain neuroprotective molecules such as polyphenols.

Several studies indicate that polyphenols are able to modulate cellular functions resulting in active neuroprotection. Examples are resveratrol from grape and wine, curcumin from turmeric, and epigallocatechin from green tea.

Resveratrol has been shown to be protective against neurodegenerative diseases and to decrease the oxidative damage in synaptic membranes [116]. Resveratrol protects neurons against amyloid beta-induced toxicity by inhibiting the formation and extension of amyloid beta fibrils and destabilizing the formed ones [117].

Curcumin is the first food-molecule described to have biological activities [118]. Several *in vitro* and *in vivo* data suggest that curcumin exerts a protective effect against neurodegeneration in cerebral ischemia by protecting BBB integrity [119]. It has also an anti-aggregative effect in PD and in AD. Curcumin acts by binding amyloid fibres. This binding inhibits amyloid beta aggregation preventing fibril and oligomer formation [120]. In PD curcumin has the same effect on alpha synuclein, the protein involved in this disease [121].

The neuroprotective role of Epigallocatechin-3-gallate (EGCG) has been described in different studies. Interestingly, EGCG seems to protect neurons activating stress signalling pathways, survival genes and enzymes needed for processing the amyloid precursor protein [122]. EGCG has been shown to protect from age-related cognitive decline [123]. Studies performed in rodents have shown that EGCG is able to improve spatial cognition and learning ability and to reduce cerebral amyloidosis in Alzheimer [124].

All these studies suggest that beneficial polyphenols effects may be linked to the capacity of reducing oxidative/inflammatory stress signalling, and of increasing protective signalling molecules.

Evidence highlights bioavailability of polyphenols metabolites in the systemic circulation [125] while little is known about how they may cross the BBB and enter into the brain.

Recent studies clearly indicate that polyphenols are able to exert their action through complementary and overlapping mechanisms, and that polyphenols metabolites produced by gut microbiota are the true active molecules.

The composition of the human gut microbiota can be modulated *in vivo* by polyphenols and this relationship can, in turn, be effective in brain neuromodulation.

### 1.5. Polyphenols and Gut Microbiota Composition

Polyphenols are characterized by low bioavailability. They often are ingested in modified forms and are recognized as xenobiotics by the human body. In general, small polyphenols molecules - in form of monomers or dimers - after ingestion can be adsorbed in the small intestine. However, the majority of them have structural complexity and they reach the large intestine without modifications. Complex polyphenols are modified into the gut by the microbial community that converts them into low-molecular-weight metabolites that are readily absorbable [126]. Diet is thought to be the most important factor affecting both microbiota composition and its relationships with the host [127].

Gut microbiota and dietary polyphenols affect each other: microbiota enzymatically transforms polyphenols improving bioavailability and health effects, while polyphenols modulate microbiota community composition avoiding the growth of pathogens.

In fact, bioactive dietary compounds as polyphenols can modify microbiota composition and functions by acting on their growth or metabolism [128]. Several studies indicated that aromatic metabolites deriving from polyphenols could modulate microbiota composition exerting selective probiotic effects [129] (Table **2**).

On the other hand, the metabolizing activity of microbiota on polyphenols influences the host exposure to them and can act on their healthy effect. The importance of microbiota for polyphenol metabolism has been shown several years ago in germ-free or antibiotic-treated animals in which these metabolites were not produced [130]. Recent studies reported that flavonoids could be differently metabolized from different bacterial strains [131-133].

It has been reported that specific polyphenols might modify the gut microbial composition, by inhibiting the growth of specific bacterial species and by increasing that of other species. Polyphenols can change microbiota composition acting on the ratio *Bacteroides*/*Firmicutes*, which constitute the majority of the bacteria genus colonizing the distal gut [134]. The ingestion of different polyphenols might result in different microbiota composition since *Bacteroides* have higher number of glycan-degrading enzymes than *Firmicutes*.

Polyphenols metabolism can be different among individuals since everyone has his/her own microbiota compositions. Thus, similar daily intake of polyphenols can have different effects on health in people with different bacterial content. In fact, the major-

**Table 2. Examples of polyphenols catabolites and their host health effects.**

| Polyphenol | Bacterial Catabolite | Health Effects |
|---|---|---|
| Curcumin | Ferulaldehyde | Reduced inflammatory response from LPS-stimulated blood lymphocytes |
| Epigallocatechin-3-O-gallate | Pyrogallol | Antibacterial activity (especially against Gram-negative enterobacteria), acetylcholinesterase inhibition greater than gallic acid parent; inhibition of Vibrio spp. quorum sensing |
| | 4-hydroxyphenylacetic acid | Antimicrobial/antimycotic activity *in vitro* |
| Daidzein | Equol | Phytoestrogen important for heart and bone health and possible colon cancer protectant |
| Resveratrol | Resveratrol 3-O-beta-D-glucoside | Antioxidation and antiproliferation activities *in vitro* |
| Proanthocyanidins | 3,4-dihydroxyphenylpropionic acid | Reduced inflammatory response from LPS-stimulated blood lymphocytes |

ity of dietary polyphenols are glycosides that are transformed into bioactive aglycones by commensal bacterial glycohydrolases. This modification, changing polyphenols bioavailability, can modulate their activities and functional effects on the mammalian tissues (59).

Polyphenols metabolites produced by gut microbiota are better absorbed in the intestine and remain in the plasma for a longer time.

Modification of gut microbiota composition by polyphenols is due to their "prebiotic-like" effects.

Examples are condensed and hydrolysable tannins present in grape seed, pomegranate, green tea as well as anthocyanins present in blueberry and deriving from microbial polyphenols degradation.

Different studies performed both *in vitro* using batch-culture fermentation or gastrointestinal simulators, and *in vivo* using animal models, showed that different polyphenols modulate specific bacterial strains [135]. These studies indicated that polyphenols reduced the number of potential pathogens, such as *C. perfringens* and *C. histolyticum*, while increased beneficial strains as *Bifidobacteri*a and *Lactobacilli*.

Unabsorbed dietary phytochemicals can affect the microbiota composition. These compounds accumulating in the ileal and colorectal lumen exert antimicrobial activities by inhibiting pathogen growth and stimulating the growth of commensal bacteria, including probiotics [136, 137].

The complex interactions between gut microbiota and the host are due to the different metabolic strategies that bacteria are able to undertake in response to diet. Gut microbiota modulates the host metabolism, regulating the expression of genes involved in the digestion of dietary compounds. They are involved in drug response and provide enzymes needed for molecules degradation [138].

Since microbiota composition is associated to health status, the understanding of these interactions is essential to identify genes and bacteria involved in polyphenol metabolism and conversion. This will lead to understanding strategies needed for delivering health benefits by microbiota modulation.

Therefore, gut microbiota plays a dual role, being both a target for nutritional intervention aimed to improve health and a factor able to modulate the polyphenols biological activity.

## CONCLUSION

Emerging evidence coming from the use of recent high-throughput technologies, allows understanding in depth the mechanisms that links gut microbiota and brain. Novel technologies, such as Functional Magnetic Resonance Imaging (fMRI), have led to decipher the gut-brain signalling in humans. This technology was used to demonstrate in humans that ingestion of SCFAs or fermented milk with probiotic affected the activity of brain regions that control central processing of emotions and sensations [139, 140].

These methods clearly have indicated that gut microbiota modulation can be achieved at transcriptional or translational levels and that changing gene expression and metabolites production may directly affect brain functions.

Gut microbiota composition can be influenced by dietary ingestion of probiotics, prebiotics and polyphenols. These results underline the key role played by specific metabolites not only in the gut microbiota composition but also in the brain health maintenance.

These data strongly support the idea that gut microbiota regulates brain biochemistry and that dysbiosis contributes to the onset of neurodegenerative diseases and to other CNS disorders.

Several studies have demonstrated that abnormalities of brain functions are associated with the altered composition of the gut microbiota, and can be partly or completely reversed by re-establishing the right microbiota composition. Probiotics, prebiotics, and polyphenols also as functional foods are fundamental to this rescue.

Modification of microbiota and its metabolites highlights a new scenario for novel therapeutic intervention in neuropathologies. As a consequence, understanding the early interaction between the intestinal microbiota and the host opens new possibilities for therapeutic interventions, mainly in neurodegenerative diseases. Moreover, deciphering the microbiota dysbiosis in neurodegenerative diseases could be useful to diagnose these pathologies at an early stage.

## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| AD | = | Alzheimer Disease |
| ALS | = | Amyotrophic Lateral Sclerosis |
| Aβ | = | Amyloid-β |
| B-GOS | = | Bimuno-galactooligosaccharides |
| BBB | = | Blood-brain Barrier |
| BDNF | = | brain-derived Neurotrophic Factor |
| CNS | = | Central Nervous System |
| EGCG | = | Epigallocatechin-3-gallate |
| ENS | = | Enteric Nervous System |
| fMRI | = | Functional Magnetic Resonance Imaging |
| FOS | = | Fructo-oligosaccharides |
| GABA | = | Gamma-aminobutyric acid |
| GDNF | = | Glial Cell Line-derived Neurotrophic Factor |
| GI | = | Gastrointestinal Tract |
| GOS | = | Galacto-oligosaccharides |
| HD | = | Huntington Disease |
| HDAC | = | Histone Deacetylase |
| HPA axis | = | Hypothalamic-pituitary-adrenal axis |
| 5-HT | = | Serotonin |
| Htt | = | Huntingtin |
| IL | = | Interleukine |
| ITF | = | inulin-type Fructans |
| NMDAR | = | N-methyl-D-aspartate Receptor |
| NMS | = | Non-motor Symptoms |
| NO | = | Nitric Oxide |
| PD | = | Parkinson Disease |
| PYY | = | Plasma Peptide YY |
| ROS | = | Reactive Oxygen Species |
| SCFAs | = | Short-chain Fatty Acids |
| UV | = | Ultraviolet Radiation |

## CONSENT FOR PUBLICATION

Not applicable.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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*Research Article*

# Positive Effects against UV-A Induced Damage and Oxidative Stress on an *In Vitro* Cell Model Using a Hyaluronic Acid Based Formulation Containing Amino Acids, Vitamins, and Minerals

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Ultraviolet (UV) radiations are responsible for skin photoaging inducing alteration of the molecular and cellular pathways resulting in dryness and reduction of skin elasticity. In this study, we investigated, *in vitro*, the antiaging and antioxidant effects of hyaluronan formulations based hydrogel. Skinkò E, an intradermic formulation composed of hyaluronic acid (HA), minerals, amino acids, and vitamins, was compared with the sole HA of the same size. For this purpose, HaCaT cells were subjected to UV-A radiations and $H_2O_2$ exposure and then treated with growth medium (CTR) combined with M-HA or Skinkò E to evaluate their protective ability against stressful conditions. Cells reparation was evaluated using a scratch *in vitro* model and Time-Lapse Video Microscopy. A significant protective effect for Skinkò E was shown with respect to M-HA. In addition, Skinkò E increased cell reparation. Therefore, NF-kB, SOD-2, and HO-1 were significantly reduced at the transcriptional and protein level. Interestingly, $\gamma$-H2AX and protein damage assay confirmed the protection by hyaluronans tested against oxidative stress. G6pdΔ ES cell line, highly susceptible to oxidative stress, was used as a further cellular model to assess the antioxidant effect of Skinkò E. Western blotting analyses showed that the treatment with this new formulation exerts marked antioxidant action in cells exposed to UV-A and $H_2O_2$. Thus, the protective and reparative properties of Skinkò E make it an interesting tool to treat skin aging.

## 1. Introduction

Dermal exposure to UV is responsible for skin photoaging [1]. Ultraviolet A (UV-A) represents 90% of all UV radiations [2]. However, UV-B and UV-A may challenge epidermal cells (keratinocytes, melanocytes, Langerhans', and Merkel's cells) possibly impairing the metabolism of these cells [3]. Besides, UV-A photons tend to damage the skin even after a short exposure [4], inducing the phosphorylation of JNK through an increase of intracellular ROS in human keratinocytes (HaCaT) [5]. Other manuscripts reported the impact of ROS on keratinocytes, proving also in this cell model the specific external stress, which can generate oxygen species in the cells [6]. Also, extensive UV-A exposure to DNA damage similar to UV-B exposure is reported by Mouret and collaborators [7]. These radiations penetrated into the deep area of the

dermis and destroyed the connective collagen and elastin fibres in the tissue, challenging the cellular homeostasis. As a result, the macromolecular assembly of collagen and elastin in the skin may be impaired or modified, thus causing the skin to become thinner and lose elasticity [8]. The process leads to the catabolism of extracellular matrix components (collagens and proteoglycans) with a loss of matrix [9]. This phenomenon is particularly due to matrix metalloproteinases expression. A strong degradation of type I collagen also occurs in irradiated skin compared with nonirradiated skin [10]. In addition, excessive exposure induces alteration of the biomechanical properties of dermal connective tissue resulting in dryness and reduced skin elasticity [11]. These changes are coupled with DNA damage induced by reactive-oxygen species (ROS) production [12] that not only lead to premature aging of the skin but also increase the risk of contracting skin cancer. Moreover, ROS, inducing apoptosis, reduces the number of skin fibroblasts and decreases their regenerative capacity, leading to increased skin sagging [13]. Furthermore, environmental pollutants may contribute to prompt symptoms of extrinsic skin aging, including coarse wrinkles, irregular pigment spots, and elastosis [14]. Thus, the identification of new compounds, which are effective in protecting skin cells, is an important tool to prevent and/or to contrast the damage induced by ultraviolet radiation. Currently, there is a great tendency to use different skin care product formulations, considering their effect on the reduction of free radicals production generated from ultraviolet radiation. To date, stimulation of skin biorejuvenation is facilitated by minimally invasive intradermal injections of biologically active substances [15, 16] such as hyaluronic acid- (HA-) based gels and dermal fillers [17] alone or in combination with other molecules [18, 19]. Reports show that HA accelerates *in vitro* processes related to wound healing [20] and *in vivo* tissue regeneration, increasing the production of extracellular matrix components [21] that reduce the signs of aging [22]. In particular, HA enhances hydration in the extracellular space due to its ability to attract water molecules [23] and induces optimal physiological conditions in the extracellular matrix for cell proliferation [24] and the organization of fibroblasts. Recently, the protective effect of HA has been proposed as an effective tool against skin damage [25]. An intradermic formulation containing linear HA and antioxidants, which is able to suppress oxidative stress-induced apoptosis in skin fibroblasts, can be a potential treatment for maintaining healthy youthful skin. In the present study, we evaluated the hydrodynamic and biological characterization of a compound containing linear HA plus a mixture of vitamins, antioxidants, and minerals named VISCODERM™ Skinkò E [14]. The aim of this work was to understand the mechanism of the action of medium molecular weight HA (M-HA) and the same HA added with vitamins, antioxidants, and minerals on *in vitro* models of oxidative damage.

## 2. Materials and Methods

*2.1. Materials.* The M-HA was obtained in our laboratory by heterogeneous hydrolysis acid of HA 1200 kDa pharma grade, kindly provided by Altergon s.r.l., following the procedure reported in D'Agostino and collaborators (2017) [25]. In particular, size-exclusion chromatography, equipped with a triple detector, permitted the following characterization: 500±80 kDa, Mw/Mn=1,6. Intrinsic Viscosity=1,1±0,2 m3/kg [25]. The compound was then dissolved in phosphate-buffered saline (PBS) (6,4 mg/ml in PBS) and microfiltered (0,22 $\mu$m) in order to be sterilized. Endotoxin content was lower than 0,05 EU/mg. Skinkò E (6,4 mg/mL) was kindly provided by IBSA Farmaceutici Lodi-Italia. This intradermic formulation was released by dissolving HA raw material with an intrinsic viscosity ranging from 0,90 to 1,34 m3/kg. Composition for 5 mL vial, as reported in the commercial leaflet contains, apart from the linear M-HA, biotechnological origin. Buffered preservation media contain (1) inorganic salts: Ammonium molybdate, ammonium metavanadate, calcium chloride, iron sulfate, potassium chloride, copper sulfate, magnesium chloride, manganese sulfate, sodium acetate, sodium hydrogen carbonate, sodium chloride, sodium hydrogen phosphate, sodium metasilicate, sodium selenite, tin chloride, and zinc sulfate; amino acids: alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, and adenine; vitamins: biotin (vitamin H), calcium pantothenate (vitamin B5), choline chloride, folic acid (vitamin B9), inositol, nicotinamide, pyridoxine (vitamin B6), riboflavin (vitamin B2), thiamine (vitamin B1), cyanocobalamin (vitamin B12); antioxidants: lipoic acid; others: inositol, glucose, putrescine, and sodium pyruvate. MHA and Skinkò E were opportunely diluted 1:1 with complete growth medium, supplemented with fetal bovine serum (FBS) to obtain 1% v/v FBS for the time-lapse experiment. For all experiments, Skinkò E and M-HA solutions were diluted 1:2 from stock solutions of 0,64% w/w in cell medium and then used at a 0,32% v/v final concentration.

*2.2. Cell Culture.* The immortalized human keratinocyte cell line (HaCaT) was obtained from Istituto Zooprofilattico, (Brescia, Italy). Cells were maintained at 37°C in a humidified atmosphere containing $CO_2$ (5% v/v) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 1% penicillin/streptomycin. For the experiments, HaCaT cells were incubated with freshly prepared dilutions of the M-HA 0,32% w/v and Skinkò E 0,32% w/v, and in growth medium, for the specified time and concentration range.

A mouse embryonic stem (ES) cell line, which was engineered through the deletion of the glucose-6-phosphate-dehydrogenase gene (G6pdΔ cells) [26] and the wild type (WT) ES cell, was maintained in an undifferentiated state by culture on a monolayer of mitomycin C inactivated fibroblasts, in the presence of leukemia-inhibiting factor (LIF) [27]. Cells were seeded, at equal density on gelatin-coated plates, 48 hours before treatment. Cells were treated with 100 mU/ml (WT ES) or 10 mU/ml mU (G6pdΔ ES) of glucose oxidase (GOX; Sigma-Aldrich) for 30 minutes and

Table 1: Primer sequences used for the qRT-PCR.

| Gene | Forward Primer | Reverse Primer | AT PCR |
|---|---|---|---|
| HPRT | 5'-TGACCTTGATTTATTTTGCATACC-3' | 5'-CGAGCAAGACGTTCAGTCCT-3' | 55°C |
| SOD-2 | 5'-CTGGACAAACCTCAGCCCTA-3' | 5'-TGATGG CTTCCAGCAACTC-3' | 55°C |
| HO-1 | 5'- GCAGTCAGGCAGAGGGTGATAGAAG-3' | 5'- TGGTCCTTGGTGTCATGGGTCAG -3' | 55°C |

then incubated in the presence of M-HA or Skinkò E and harvested after 8 hours.

*2.3. Sample Preparation.* VISCODERM® Skinkò E formulation was concentrated on a polyethylene sulfate membrane with a nominal molecular weight limit (NMWL) of 3 kDa (Amicon Ultra 0.5 mL Centrifugal filter: Ultracel 3 kDa, Millipore). The concentration was diafiltered using 2 volumes of HPW (High Purified Water). Successively, the ultrafiltration (UF) retentate on the 3 kDa centricon tube was recovered and eventually diluted for the hydrodynamic characterization by SEC-TDA; whereas, the permeate 3 kDa was analyzed by uronic acid assay (E.P. ed 5.0: 2.2.25). This last assay quantified the residual HA in the permeate and diafiltered sample that accounted for about the 25% w/w of the initial HA amount. Therefore, the permeate 3 kDa contained all the Skinkò E components and a residual 1,84 mg/mL of HA. This was diluted 1:2, tested as for Skinkò E, and thus, the final concentration of HA in the cell's plate was 3,2 mg/mL for the complete formulation and only 0,92 mg/mL for the permeation. In this case, the HA effect should be reduced with respect to the other compounds present in the formulation.

*2.4. Hydrodynamic Characterization SEC-TDA*. The chromatographic analyses of ultra-filtered retentate Skinkò E and M-HA using size-exclusion chromatography coupled with triple detection array (SEC-TDA by Viscotek Malvern Instruments, UK) were performed. The details of the system and the analytical conditions have been reported by La Gatta et al. (2010) [28]. The molecular size, molecular weight distribution, polydispersity, and the intrinsic viscosity of the samples have been derived for a complete hydrodynamic characterization.

*2.5. Wound Healing In Vitro Model on HaCaT*. The *in vitro* scratch assay procedure has been fully previously described [18, 20]. In brief, 12 wells (precoated with collagen) were seeded with HaCaT ($1{,}5\times10^3$cells/cm$^2$) and incubated for 48 hours until complete confluence was reached, in order to test the effect of M-HA and Skinkò E (0,32% w/v), respectively, on the rate of wound closure, monitored by Time-Lapse Videomicroscopy (TLVM). Fresh serum-supplemented medium with 1% v/v FBS was used as a control. The images of the scratched monolayers were captured every 60 minutes and analyzed using OKO Vision 4.3 software (Okolab, Italy) [25].

*2.6. Analysis of SOD-2 and HO-1 through qRT-PCR.* To test *in vitro* antioxidant activity, HaCaT cells were treated with M-HA and Skinkò E for 4 hours, with and without preexposure to U-VA radiation ($\lambda_{max}$ 365 nm) for 4 minutes [29]. For the analysis of gene expression, total RNA was extracted, using Trizol, according to the manufacturer's procedures (Invitrogen, Milan, Italy). Retrotranscription was performed using Reverse Transcription System Kit (Promega, Milan, Italy). PCR was then performed using iQ™ SYBR1-Green Supermix (Bio-Rad Laboratories s.r.l., Milan, Italy) to analyze the expression levels of superoxide dismutase-2 (SOD-2), heme oxygenase 1 (HO-1), and hypoxanthine guanine phosphoribosyl transferase (HPRT), using the specific primer pairs designed with Beacon Designer software (Bio-Rad Laboratories s.r.l., Milan, Italy). Primer sequences have been reported in Table 1 and the amplification conditions have been fully described in Stellavato and collaborators (2016) [30]. All PCR reactions were performed in triplicate and the relative expression of specific mRNA with respect to the hypoxanthine guanine phosphoribosyl transferase (HPRT) housekeeping gene. The comparative threshold method $2^{-\Delta\Delta Ct}$ ($\Delta\Delta$Ct = difference of $\Delta$Ct between HA treated cells and control) has been used to calculate the fold-change of genes and the results have been expressed as normalized fold expression, compared with controls using the Bio-Rad iQ™5 software (Bio-Rad Laboratories s.r.l.).

*2.7. Western Blotting for NF-kB and HO-1 as Inflammatory Markers.* For the evaluation of western blotting, HaCaT cells were treated with M-HA and Skinkò E for 24 h with and without preexposure to U-VA radiation ($\lambda_{max}$ 365 nm) for 4 minutes [29]. The proteins were extracted using a RIPA lysis buffer and the concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Milan Italy). Equal amounts of proteins (30 $\mu$g) were loaded on a SDS-PAGE and transferred them to a nitrocellulose membrane [31]. The filters were incubated with antibodies against the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB; rabbit polyclonal IgG C-20; 1:200 v/v ), heme oxygenase 1 (HO-1; goat polyclonal IgG C-18; 1:200 v/v), and actin (actin; goat polyclonal IgG I-19; 1:500) at room temperature for 2 hours. Membranes were washed three times for 10 minutes and incubated with a 1:5000 dilution of horseradish peroxidase-conjugated anti-rabbit antibodies and with a 1:10000 dilution of horseradish peroxidase-conjugated anti-goat antibodies for 1 hour, respectively. All antibodies are purchased from Santa Cruz Biotechnology, CA, USA. Blots were developed using the ECL system according to the manufacturer's protocols (Amersham Biosciences). Extracted proteins from Skinkò E treated WT and G6pd$\Delta$ ES cells [32] and untreated cells were analyzed using PARP antibody (New England Biolabs.). Actin antibody was used as the gel loading control. The same procedure of western blotting was followed to analyze $\gamma$-H2AX (primary antibody

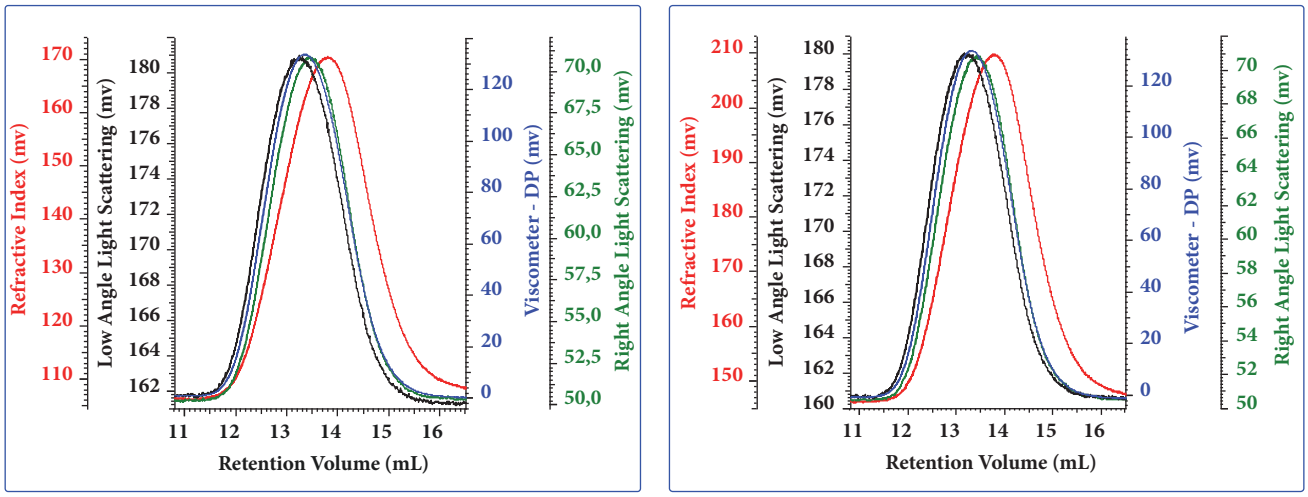


Figure 1: Hydrodynamic characterization by SEC-TDA is reported for (a) Skinkò E UF retentate and (b) M-HA.

anti-$\gamma$H2AX: Cat No. 05-636 from Millipore, MA, USA) as specific biomarker of DNA damage prompted by oxidative stress.

*2.8. Antioxidant In Vitro Activity Using T-Bars Assay on HaCaT.* Reactive aldehydes were assessed by measuring thiobarbituric acid-reactive substances (TBARS), as described previously [32]. The effect of Skinkò E on HaCaT cells (2,0 x$10^5$) was tested in four different experimental setups:

(1) To test the effect on the poststress process, the cells were pretreated for 30 minutes with 50 $\mu$M $H_2O_2$ and then incubated with M-HA and Skinkò E (0,32% v/v) for 24 hours.

(2) To test the antioxidant activity, M-HA and Skinkò E were applied simultaneously with 50 $\mu$M $H_2O_2$ (30 min) or exposed to a U-VA radiation ($\lambda_{max}$ 365 nm) for 4 minutes.

(3) To test the effect of the permeate (3 kDa) Skinkò E formulation, with respect to complete Skinkò E formulation on HaCaT cells, the cells were pretreated with 50 $\mu$M $H_2O_2$ or exposed to a U-VA radiation ($\lambda_{max}$ 365 nm).

The protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Milan Italy). A TBARs assay (thiobarbituric acid-reactive substances) was performed on aliquots of membranes which were extracted (10 $\mu$l) and added to 2 ml of TBA-TCA (TCA 15% w/v, TBA 0,3% w/v in HCl 0,12 N) solution at 100°C for 30 minutes. The chromogen, produced by the stoichiometric reaction of aldehydes (malondialdehyde (MDA)) with thiobarbituric acid (TBA), was quantified by spectrophotometric reading at a wavelength of 532 nm. The amount of TBARs was expressed as a percentage of lipid peroxidation and then normalized with respect to the control.

## 3. Results

*3.1. Molecular Weight, Polydispersity, and Intrinsic Viscosity Determination by SEC-TDA.* The Skinkò E product (Viscoderm Skinkò E VEVU04), analyzed by SEC-TDA, contains HA with an intrinsic viscosity of 0,97±0,2 $m^3$/kg, which is within the expected range (as reported by the producer). A comparative analysis of Skinkò E and M-HA (produced by our laboratory) showed similarities to the chromatographic profile (see Figures 1(a)-1(b)) and derived analytical software parameters. Detailed analyses of molecular distribution highlighted a molecular weight fraction above 700 kDa of 14%, whereas over 40% resulted above 400 kDa for both Skinkò E and M-HA. These results confirmed comparable molecular weight distributions between samples, thus permitting a correct biological/cellular comparison between the treatments.

*3.2. In vitro Scratch Assay Using Time-Lapse Video Microscopy (TVLM).* Wound healing experiments showed that the scratch closure occurred at a faster rate in the presence of Skinkò E and M-HA (the control cells being in normal medium). Between the two M-HA based samples analysed, the Skinkò E acted faster than M-HA. In particular, in wound closure, the sole M-HA achieved 70% of reparation at 14 hours compared with 18 hours for the control. Surprisingly, a higher performance was found for Skinkò E. In fact, samples treated with Skinkò E reached the same repair target (70%) within 9 hours (see Figure 2), corresponding to a 2-fold increase of the reparation rate with respect to the control and also a significant 30% improvement with respect to the sole M-HA.

*3.3. Gene and Protein Expression Analyses.* M-HA and Skinkò E showed the capability of preventing UV-A stress, *in vitro,* in experiments accomplished using a model recently described by Almeida and collaborators (2015) [25]. SOD-2 and HO-1 were markedly reduced at the transcriptional level. In particular, SOD-2 and HO-1 gene expression, as reported in

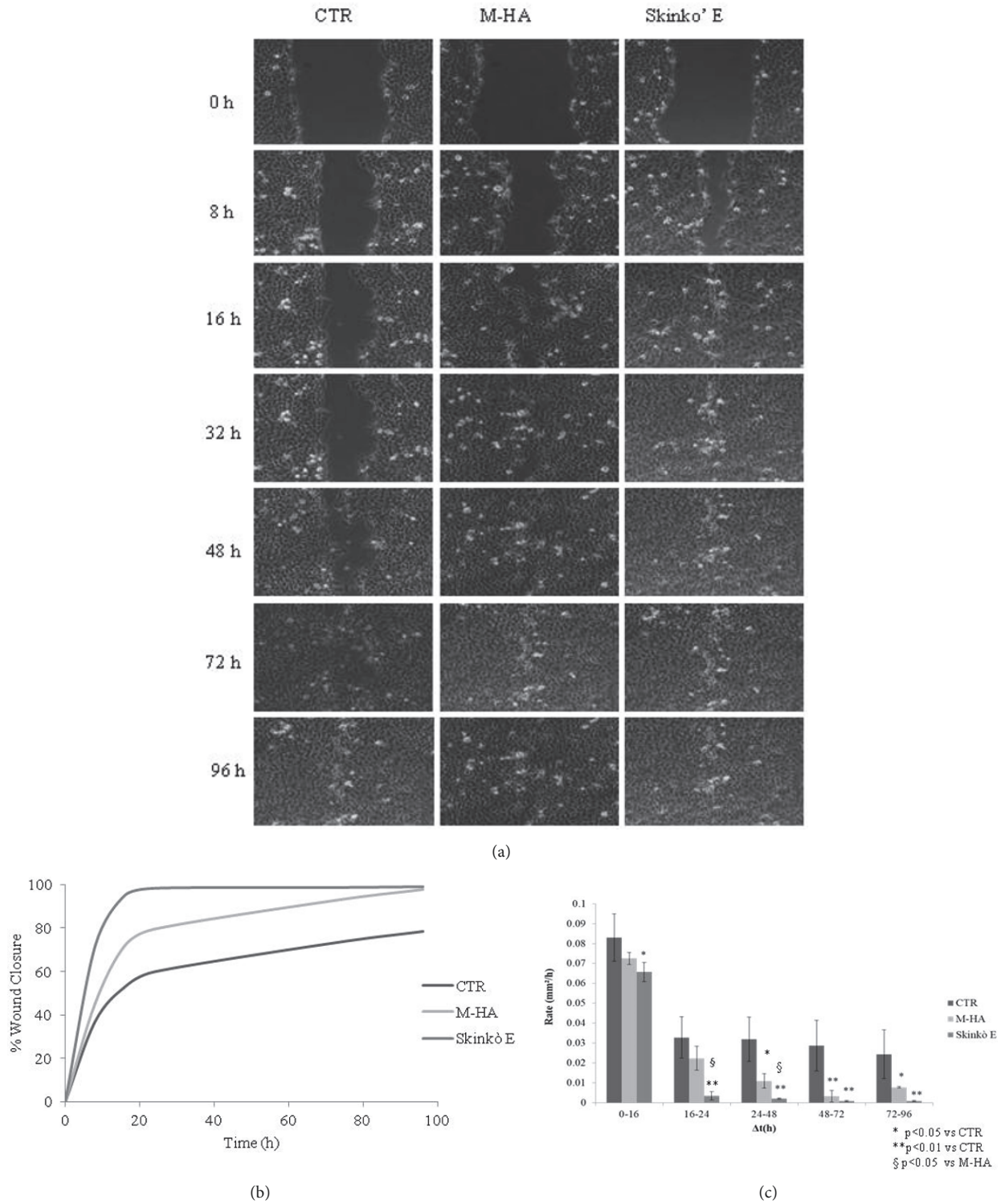


Figure 2: (a) Representative micrograph pictures of HaCaT scratch assays. (b) Repair area percentage for the control and in the presence of treatments: M-HA and Skinkò E; the curves are averages of three different experiments with standard deviation within 5% of the value. (c) Rate of reparation within 96 hours. Skinkò E and M-HA treatments are statistically significant $^{*}(p<0.05)$ with respect to CTR, Skinkò E and M-HA treatments are statistically significant $^{**}(p<0.01)$ with respect to CTR, and Skinkò E is statistically significant $^{\S}(p<0.05)$ with respect to M-HA treatment.

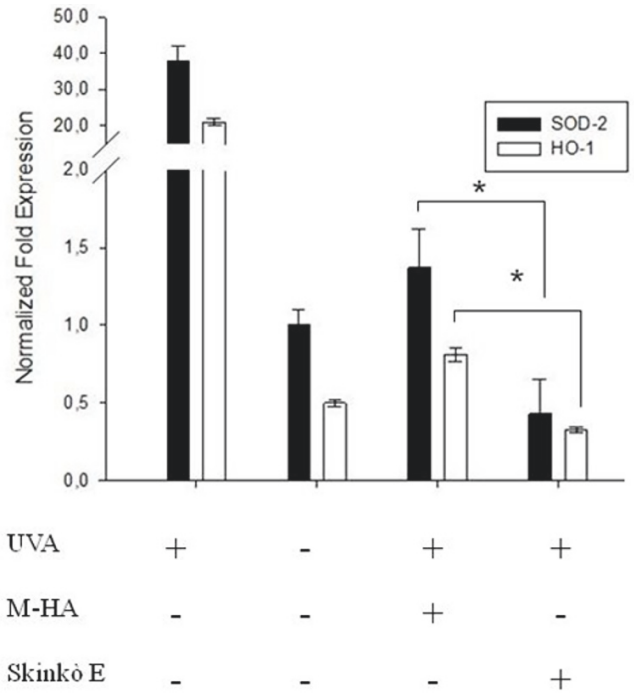


Figure 3: Antioxidant biomarkers (SOD-2 and HO-1) were determined by quantitative real-time PCR on HaCaT UV-A pretreated for 4 minutes and subsequently treated with M-HA 0.32% (w/v) and Skinkò E 0.32% (v/v) for 4 hours. Each column represents the mean and error bars represent the standard deviation (n=3). Test-t was performed to evaluate the significance of M-HA and Skinkò E effects with respect to positive control (UV-A), $^{*}p<0.01$.

Figure 3, were significantly downregulated with respect to UV-A treatment in the presence of Skinkò E. SOD-2 was reduced 2,4-fold, and HO-1 2-fold, with respect to M-HA alone. Similarly, the protein levels of NF-kB and HO-1 (see Figures 4(a)-4(b)) significantly decreased in the presence of M-HA and Skinkò E. Both the treatments were effective but Skinkò E reduced NF-kB and HO-1 expression 3,3-fold and 2,6-fold, respectively, compared with M-HA alone (the data are statistically significant).

*3.4. Oxidative Protein Damage.* As further biomarker of the oxidative stress we evaluated the protein expression of the $\gamma$-H2AX (primary antibody anti-$\gamma$H2AX: Cat No. 05-636 from Millipore, MA, USA), through western blotting analysis following the procedure described in the previous section. The quantification of carbonyl groups introduced into proteins by oxidative reactions was detected by OxyBlot kit (data not shown). Densitometric analysis of $\gamma$-H2AX (Supplementary File (available here)) showed that M-HA and Skinkò E reduced the protein expression with respect to oxidative stress induced. In particular, both the treatments were effective, but Skinkò E reduced $\gamma$-H2AX expression 1,7-fold and 1.3-fold with respect to $H_2O_2$ and UV-A, respectively, compared with M-HA that reduced it 1.5 and 1.1 with respect to both oxidative stresses induced.

*3.5. TBARS Assay.* To assess the antioxidant activity, *in vitro* tests were conducted on M-HA and Skinkò E. Human keratinocytes were treated with oxygen peroxide or exposed to UV-A radiation, and TBARS assay (see Figure 5) showed that the levels of lipid peroxidation dramatically increased in response to both $H_2O_2$ and UV-A exposure, whereas M-HA 0,32% w/v reduced the amount of lipid peroxidation. To better unravel the functionality of the diverse components, Skinkò E was subjected to UF treatment on the 3 kDa membrane and both retentate and permeation were tested separately. Retentate contained over 70% of the total HA present, while permeation clearly contained vitamins and components at low molecular weight and a residual HA that proved randomly able to permeate for about 25-30% of total amount contained in the formulation. This particular behavior is known to occur in linear polyanionic polysaccharides that assume specific enlarged conformations in solution, in order to reduce the repulsion forces in between the dimeric repetitive units. When the cells were exposed, simultaneously, to M-HA, Skinkò E and Skinkò E permeate (3 kDa) in the presence of $H_2O_2$ or U-VA, respectively (see Figures 6(a)-6(b)), the levels of lipid peroxidation were substantially reduced with respect to relative positive control. In particular, Skinkò E and Skinkò E permeation significantly reduced lipid peroxidation 1,3- and 1,5-fold with respect to M-HA alone in HaCaT exposed to UV-A (see Figure 6(b)).

*3.6. Skinkò E Protection against Oxidative Stress-Induced Apoptosis.* To further investigate the protection mechanism of Skinkò E treatments against oxidative stress-induced apoptosis, we used a mouse embryonic stem cell line (ES) [33]. In particular, we used an ES cell line which was extremely sensitive to oxidative stress (G6pdΔ ES cells). G6pdΔ is an engineered ES cell line carrying the deletion of the glucose-6-phosphate-dehydrogenase gene. Indeed, G6PD is essential for the production of high levels of NADPH required for the cell detoxification of reactive-oxygen species [22]. We focused on $H_2O_2$ as the oxidant, WT ES, and G6pdΔ ES cells were treated, respectively, with 100mU/ml or 10mU/ml of glucose oxidase (GOX), an enzyme that produces $H_2O_2$ in the cell culture medium, from glucose and water. As shown in Figure 7, Skinkò E, but not M-HA, reverted the effect of GOX, protecting both WT ES and G6pdΔ ES cells against apoptosis. Since SkinkòE protected both WT and G6PD delta from $H_2O_2$ induced apoptosis, we can speculate that molecules present in the formulation can act as antioxidants, bypassing the function normally performed by NADPH.

*Statistical Analysis.* All the data are represented as the mean ± SD. When there was no variability in the values for a group, a one-sample $t$-test was used to compare the group mean to that value; otherwise a two-sample $t$-test was used to compare group means. $p < 0.05$ was considered significant in each of the comparative analyses.

## 4. Discussion

The present experimental study provided consistent information regarding biochemical changes in cells after *in vitro*

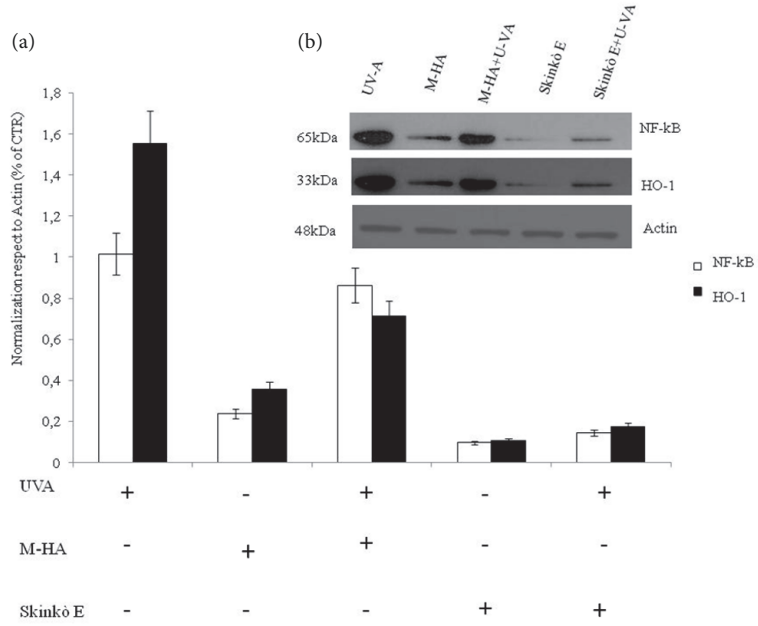


Figure 4: Western blotting analyses showed that M-HA and Skinkò E reduce the inflammatory proteins NF-kB and HO-1. In particular, Skinkò E is more efficient than M-HA on these protein modulations.

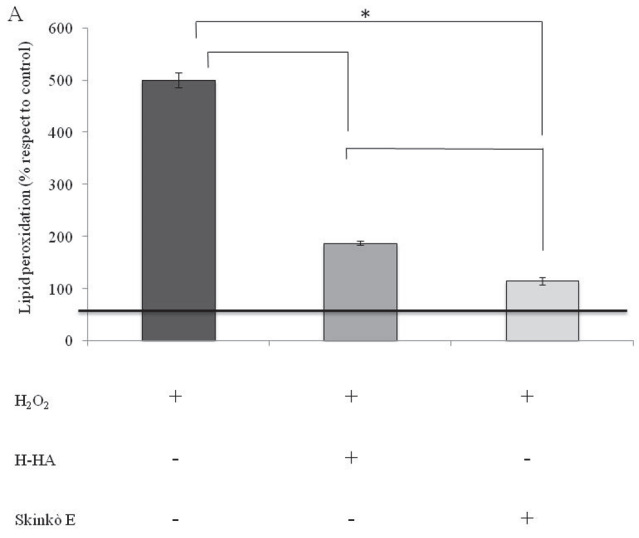


Figure 5: Effect of M-HA 0.32% (w/v) and Skinkò E 0.32% (v/v) on lipid peroxidation after exposure to oxidative stress induced by 50$\mu$M $H_2O_2$ for 30 minutes. M-HA and Skinkò E treatments are statistically significant ($^*p<0.01$) comparing with the positive control ($H_2O_2$). Also, Skinkò E is statistically significant ($^*p<0.01$) with respect to M-HA treatment.

oxidative damage. This included the potential protective effects, such as, antioxidant activity, not only of M-HA, but also of a formulation based on M-HA plus minerals, amino acids, and vitamins. Due to its rich formulation, the injectable solution of hyaluronic acid, complemented with these small molecules (Skinkò E), demonstrated its efficacy against oxidative stress, which is considered the main cause of skin aging or photoaging. To our knowledge, oxidative damage in terms of $H_2O_2$ *in vitro* treatment [32] and UV-A irradiation [33] also contribute to this detrimental condition. To better evaluate the effect of Skinkò E formulation, with respect to the sole M-HA, HaCaT cells were used as the *in vitro* cellular model. Normally, in the skin, oxidative stress is due to reactive-oxygen species (ROS) that can originate both from exogenous factors (i.e., the environment) and from endogenous factors [34, 35]. Specifically *in vitro* tests were carried out to assess the antioxidant activity on M-HA and Skinkò E. Human keratinocytes were treated with oxygen peroxide or exposed to UV-A radiation, showing an increased lipid peroxidation; the latter was significantly reduced by treating the cells with both M-HA and Skinkò E. Interestingly, the beneficial effect was also found when the HaCaT cells were treated with the Skinkò E UF permeation (3 kDa) that consisted of the low molecular weight fraction (vitamins, antioxidants, amino acids, and minerals) [14] and a small amount of residual HA (0,92 mg/ml). In addition, *in vitro* wound healing experiments showed that Skinkò E was superior to the sole HA of the same MW in promoting wound closure, with a relevant and significant improvement that showed in triplicate experiments to accomplish 70% repair in half the time. Beside the UV-A stress *in vitro* model was here tested as discussed by Almeida [29]. Interestingly, this was also prevented by both M-HA and Skinkò E with a marked improvement for the latter that reduced, at transcriptional level, both SOD-2 and HO-1 and protein level NF-kB and HO-1. Since these biomarkers are relevant in assessing the state of the cells, our aim was to highlight the efficacy of the formulations used against the two different oxidative stress insulting conditions tested in this research work. In this respect, our results relative to the decrease of

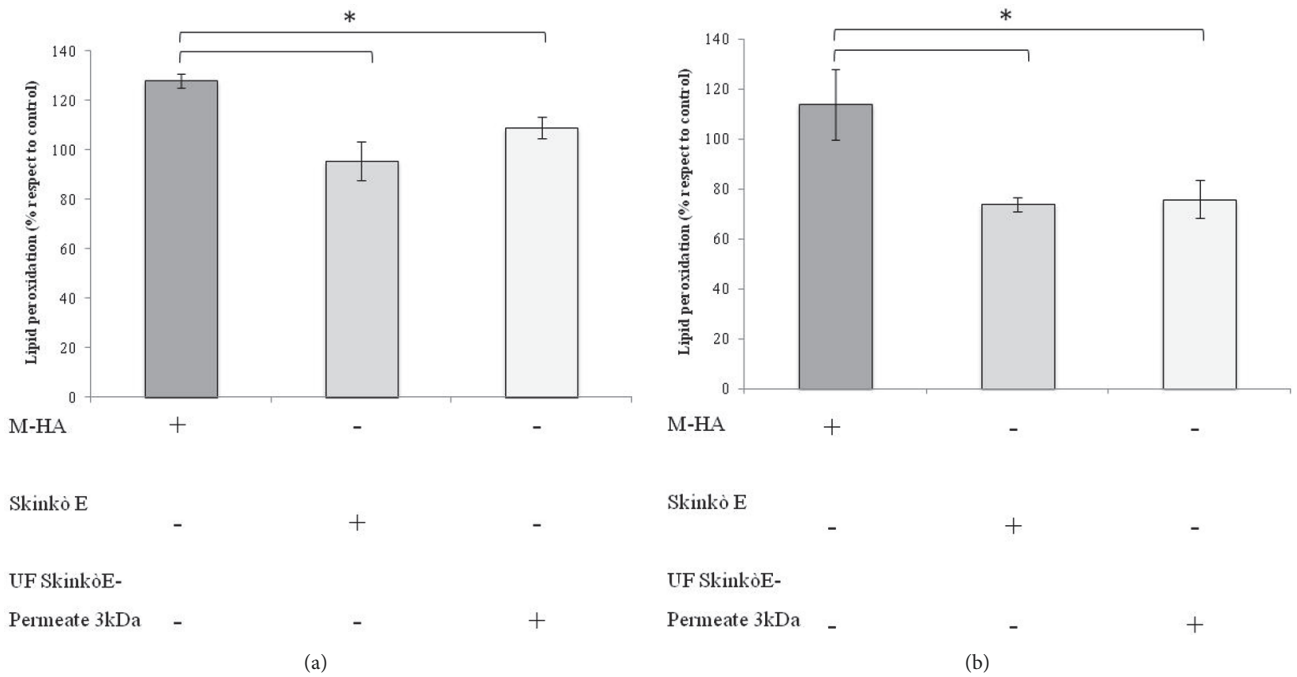


Figure 6: Effect of M-HA 0.32% (w/v), Skinkò E 0.32% (v/v), and permeate (3KDa) Skinkò E on lipid peroxidation after exposure to oxidative stress induced by 50$\mu$M $H_2O_2$ (a) or UV-A irradiation (b) on HaCaT cells. "$*$" in the histogram indicates significant difference at $p<0.01$, comparing with the Skinkò E and UF Skinkò E Permeate 3kDa treatment groups with respect to M-HA. Data of the amount of lipid peroxides (% respect to CTR) are reported as follows: Graph (a): $H_2O_2$=570±14, M-HA=128±7.0, Skinkò E=95.5±7.7, UF Skinkò E Permeate 3kDa=109±4.2. Graph (b): UV-A=480±14.0, M-HA=114±7.0, Skinkò E=74±5.6, UF Skinkò E Permeate 3kDa=76±7.0.

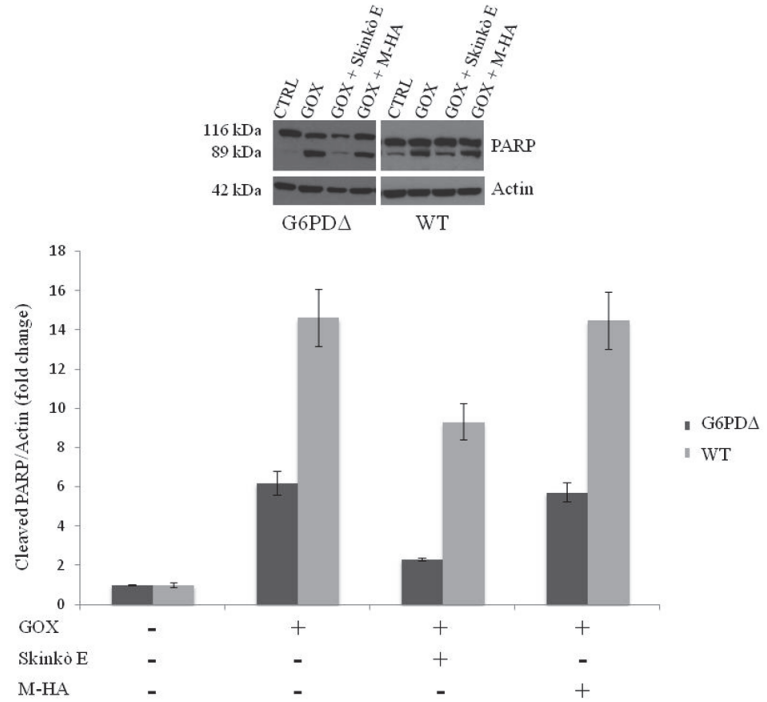


Figure 7: Protective effect of Skinkò E against oxidative stress-induced apoptosis.

protein biomarkers of oxidative stress (e.g., SOD-2) could be explained through the hypothesis that antioxidants enzymes are activated late following the oxidative stress UV-A induced. In our opinion, the cells survived to the treatment with $H_2O_2$ produced SOD-2 as protective enzyme. While in presence of hyaluronans, the cells do not need to activate it, because specifically both M-HA and Skinkò E are able to counteract ROS production, thus reducing the overall stress that the cells experience. Our observations were supported by the work of Leccia and collaborators [36]. Specifically, they reported that antioxidant enzymes are activated relatively late after the initial UV damage and hence could be considered as "late-response" genes and proteins. However, the oxidative protein damage was evaluated supporting a lower detrimental action when cells were exposed to (treated with) M-HA and Skinkò E. In addition, the western blot analyses with $\gamma$-H2AX further support a lower damage to both $H_2O_2$ and UV-A induced stress when the cells received contemporary the treatments. Specifically Skinkò E was permitted to lower damage [37, 38]. The present study confirmed that the use of the intradermal HA solution with other active ingredients may increase keratinocytes function. In particular, amino acids and vitamins present in the Skinkò E formulation are important nutrients to maintain youthful epidermal appearance. For instance, the vitamin B complex is reported to act as free radical scavengers. Also biotin and inositol, important intracellular messengers, play a key role in the synthesis of the matrix proteins forming the cellular scaffolds/ tissue architecture [36]. In addition, antioxidants properties could be also related to the presence of lipoic acid in the formulation tested. The latter, in fact, has been studied for its benefits to skin as potent natural antioxidant [39]. Specifically, lipoic acid improved clinical characteristics related to photoaging of facial skin [40] and also is able to act as protective agent against UV-B induced damage [41]. In *vitro* studies showed its action as ROS scavenger may be accomplished through the direct suppression of the UVR-induced NF-kB activation in human immortalized keratinocytes [42]. In order to test the ability of Skinkò E and M-HA to prevent apoptosis, G6PD delta ES was chosen as cellular model system since they are extremely sensitive to oxidative stress [26]. We have previously demonstrated that it is a reliable model system to analyze apoptosis. Moreover, all the procedures to analyze oxidative stress-induced apoptosis in ES cells have already been set up and described [33]. Using G6pdΔ ES cells, we showed that Skinkò E is also able to protect cells against oxidative induced apoptosis through the production of NADPH, that is, a central enzyme in cell defense against redox insults. Indeed, in order to reduce $H_2O_2$, both essential enzymes, glutathione reductase and catalase, require NADPH; the first enzyme requires NADPH as a cofactor to reduce GSSG in GSH, and the second to form active tetramers out of a single enzymatically inactive subunit. Looking at the Skinkò E formulation, we observed that the three constitutive amino acids of the tripeptide GSH were present in the mix. We can speculate that the presence of these amino acids could induce *de novo* GSH synthesis, supplying cells with new reducing power essential to counteract the oxidative damage. Amino acids and vitamins are also important ingredients for maintaining a youthful appearance. As reported by Tosti [43], the vitamin B complex is essential in different metabolic functions, also acting as free radical scavengers. In addition, the oxidative stress and more generally, the ROS production are among the recognized mechanisms affecting melanocyte disorders which alter skin pigmentation. In particular, Wan and collaborators [44] reported that melanocytes protect skin cells against UV radiation through melanin biosynthesis. Continuous UV radiation exposes melanocytes to oxidative stress and, in this condition, exposed epidermal cells gradually lose this function, resulting in anomalous pigment production [45].

## 5. Conclusions

The present research work suggests a potential role of the Skinkò E formulation on dermal dark spot treatment, in relation to its activity against oxidative stress. Overall the results of this research work confirmed the effectiveness of Skinkò E and of M-HA on cell repair and regeneration through increased cell proliferation and the migration capacity of keratinocytes. It is well known that increased ROS, altering apoptotic pathways, can foster the development of dermatological diseases and aging. Therefore Skinkò E, which protects cells against ROS-induced apoptosis, can play a role in maintaining healthy youthful skin, preventing or delaying skin aging and eventually the onset of skin diseases.

## Conflicts of Interest

Gilberto Bellia is an employee of IBSA Farmaceutici Italia Srl. The other authors declare that they have no conflicts of interest.

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## Supplementary Materials

In order to better analyze oxidative stress, $\gamma$-H2AX (primary antibody anti-$\gamma$H2AX: Cat No. 05-636 from Millipore, MA, USA) was evaluated as specific biomarker of DNA damage prompted by oxidative stress, through western blotting analysis following the experimental procedure reported in Materials and Methods. *(Supplementary Materials)*

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