UNIVERSITÀ DEGLI STUDI DI NAPOLI "FEDERICO II"

FACOLTÀ DI BIOLOGIA

Dottorato di Ricerca in Biologia
XXXIII Ciclo

"POLIFENOLI DA SEMI DI UVA: NUOVE MOLECOLE PER L'INDUSTRIA NUTRACEUTICA"

Tutor:
Prof. Salvatore Cozzolino
Dr. Stefania Crispi

Candidato:
Francesco Di Meo

Summary

Introduction

In the recent years there is a growing industrial interest in the recovery and reuse of waste from production processes in the food industry. Industrial and agro-industrial by-products and waste can be recovered and valorised, transforming them into new materials and products with high added value. Industrial and agro-industrial waste are enhanced through their reuse in the preparation of innovative materials and components.

In fact, to date the deficiency of natural resources, climate change, food loss and waste are decreasing the availability of food and raw materials. Decline of natural resources worldwide, degradation in land, water and biodiversity, poses questions about the ability to meet food and material demands for the next future. Consequently, finding the balance between the increasing demand for resources and sustainable supply is the challenge of the present and the future.

Food supply chains start with agricultural phase, continue with manufacturing, retail and end with consumption. Throughout this cycle, food is lost or wasted because of a variety of reasons, i.e., technical, economic or social problems.

In the twentieth century, the prevalent policy was to increase food production but not to improve the efficiency of the food production system. Food loss or waste were not considered a problem. Only in the 21th century, the increased demand for food, the depletion of natural resources, the restrict energy demands and the minimize of economic costs have led to this problem.

Food processing industries generate an impressive amount of waste, consisting especially of the organic residues of processed raw materials. Most of these materials, defined waste, could represent an added value. The same term "waste" indicates a wide variety of products coming out of the production system. In fact, lately, the new term "waste by-products", indicating products that can be reused, is gaining popularity. The possibility to recover by-products from production processes is very wide and varied and depends on the type of process under consideration. For example, vegetable by-products and plant residues, due to their content of polyphenols, glucosinolates, proteins, pectin, phytochemicals and plant enzymes, may be used to generate innovative products such as dietary fibres, food flavours or food supplements.

Thus, in recent years, the concept of circular economy has become more and more established. Circular economy is a term that defines an economic system designed to be able to regenerate itself, therefore guaranteeing its eco-sustainability. The principal aim is the waste reduction and the reuse of resources.

Wineries constitute one of the most important agro-industrial sectors world-wide. Grapes are one of the most cultivated fruits worldwide, with the production of more than 60 million metric tons per year. *Vitis vinifera* is the most commonly cultivated species for wine production, and according to the FAO, around 80% of its production represents 78 million tons that has been used in the wine sector. Winemaking process generates an ample variety of solid and liquid by-products, including vine shoots, grape marc or grape pomace, wine lees, spent filter cakes, vinasses, and winery wastewater that

must be treated, disposed of or reused properly, in order to avoid negative environmental impact. Large quantities of scraps are composed of rejected or inedible plant tissues with a high amount of water content and organic matter. Therefore, special attention has been given to more advantageous and sustainable options by both the scientific community and the producers, aiming at maximum utilization of all raw materials and by-products derived from the wine industry, ultimately reducing to a minimum the disposal of waste streams.

Since ancient times and particularly in Mediterranean countries, wine has been closely associated with the diet, and its regular consumption has been associated with health benefits. Epidemiological and clinical studies around the world, over the past twenty years, have pointed out that the moderate intake of alcoholic beverages produces positive effects on antioxidant capacity, lipid profile and the coagulation system. Several studies have focused their attention on the components of red wine (mainly polyphenols and especially resveratrol) since the so-called "French paradox" was first described. Although the chemical constituents of grapes and wine may vary, similar beneficial effects have been observed in different varieties of red wine related to their higher polyphenolic content. Among food industry wastes, grape wastes are partially used in cosmeceutical and nutraceutical fields. However, little is known about the potential use of grape seeds. In fact, if the use of pulp and skin of grapes is already widely spread, the properties of grape seeds are not well characterized yet. Grapevine (*Vitis vinifera* L.) is a plant rich of bioactive compounds that are principally distributed in berry skin, stem, leaves and seeds, rather than in the pulp of the berry. Among the

substances accumulated in grapes, the most important and biologically interesting ones are surely polyphenols. About 70% of the polyphenols contained in grapes are present in the grape seed.

Polyphenols are the most abundant secondary metabolites present in the plant kingdom. They represent a large and diverse group of molecules including two main families, the flavonoids and the non-flavonoids. Plant polyphenols belong to different groups: phenolic acids, phenolic diterpenes, volatile oils, flavonoids and stilbenes. All of them are characterized by multiples of phenol units.

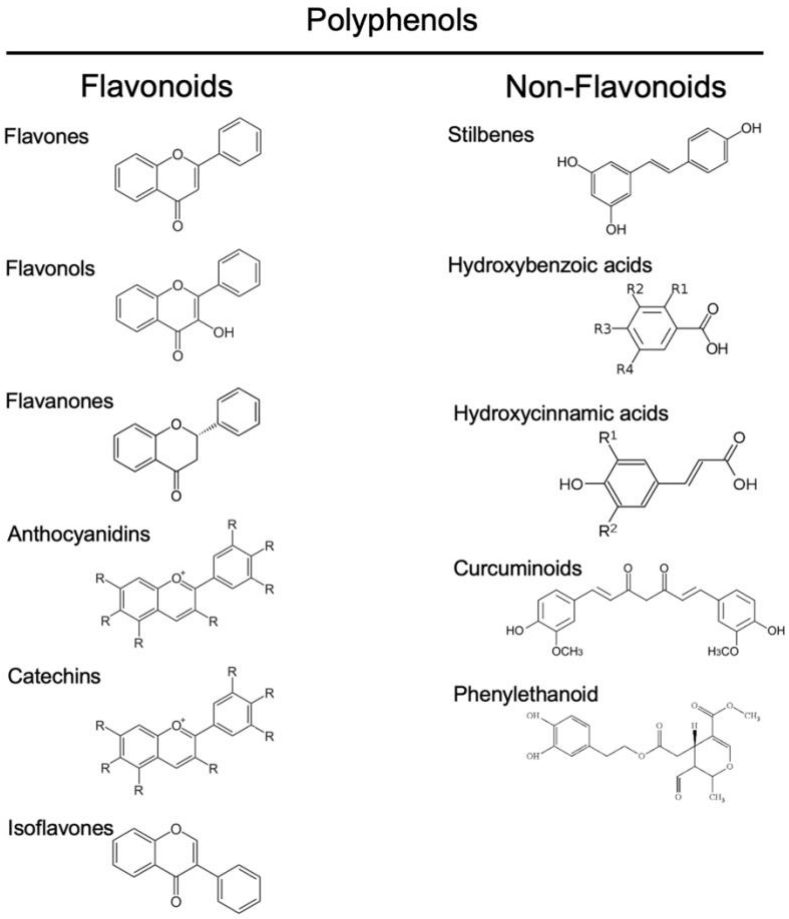

Figure 1.1 *Structures of polyphenols. Polyphenols are classified by the number of phenol rings in flavonoids and non-flavonoids. The figure shows basic skeleton structure of polyphenols. Image from: [1]*

Polyphenols are known for their anti-inflammatory, antimicrobial and antioxidant activities and are of interest in nutraceutical and cosmetic fields [1-3]. Moreover, some of them can also have cytotoxic effects and therefore they can be studied to develop new molecules with pharmacological activity. Polyphenols present in higher quantities in grapes are mainly flavonoids (anthocyanins, flavanols and flavonols), stilbenes and phenolic acids [4]. Anthocyanins and flavonols are mainly found in the skin [5] whereas flavanols and hydroxycinnamic derivatives (among the most common ones we find coumaric acid, caffeic acid and ferulic acid) have been found in higher quantities in grape seeds [6].

Anthocyanins are the main polyphenols in red grapes, whereas flavan-3-ols are more abundant in the white variety [7].

Anthocyanins belong to the flavonoid family. These molecules consist of a benzene molecule fused with a pyran molecule, linked with a phenyl group that can be in turn linked to several substituents.

Anthocyanins are polyhydroxylated polyaromatic compounds capable of reacting with oxidants such as molecular oxygen and free radicals thus reducing the damage that these molecules can cause to cells and tissues [8].

Flavan-3-ols are derivatives of flavans that possess a 2-phenyl-3,4-dihydro-2H-chromen-3-ol skeleton. These compounds include catechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, and proanthocyanidins [9].

However, the effects of polyphenols are often limited by their poor bioavailability [10]. Moreover, *in vivo* studies the dosage of single molecules of polyphenols did not have the expected effect. This can be due to the limited absorption but also to the lack of a synergistic action among the various molecules composing an extract [11].

For this reason, bioactive phytochemicals taken through diet represent a promising opportunity for the development of more effective strategies for the prevention or treatment of cancers that can be utilized as complementary to traditional medicine.

For thousands of years, different cultures have used phytochemicals for treatment of various diseases, nevertheless their active ingredients or mechanisms of action often are not well characterized. Grape seed extract are hopeful bioactive molecules that have demonstrated anticarcinogenic effects *in vitro* and *in vivo* analysis and exhibit no toxicity *in vitro* [12,13].

Phytochemicals can act also as protective agent against oxidative stress. Flavonoids are in particular described as the molecules with a strong bioactivity in brain functions with positive effects on synaptic plasticity and neuronal activity. Among the bioactive phytochemicals, *Ginkgo biloba* is one of the most used worldwide [14]. *Ginkgo biloba* fallen leaves are treated as waste to be incinerated, polluting the air, soil and rivers. In addition, the leaves have become a problem for local businesses and governments in the plantation area to dispose of [15]. Therefore, its recovery to produce nutraceutical extracts may be a solution to this problem. *Ginkgo biloba* has been widely used in the treatment of cardiovascular and cerebrovascular diseases, liver cirrhosis and acute and chronic renal disease. More recently, a

standardized extract of *Ginkgo biloba*, EGb 761, has been found to have neuroprotective effects in several central nervous system and neurodegenerative diseases [14]. EGb 761 (EGb) is a standardised extract of *Ginkgo biloba* leaves that contains a well-defined concentration of flavone glycosides and terpene lactones (24% and 6%, respectively). In fact, EGb contains 6% terpenoids (in which 3.1% are ginkgolides A, B, C, and J and 2.9% is bilobalide), 24% flavonoid glycosides, and 5% to 10% organic acids [16]. The flavonoids act as free radical scavenging, whereas terpenes lactones protect mitochondrial membranes from free radical damage [17]. EGb has been described to have antioxidant properties playing an important role as a free radical scavenger [18]. It has been demonstrated that the antioxidant activity, as a "radical scavenger", is due to its superoxide dismutase-like activity that enables it to scavenge hydroxyl radicals [19]. *Ginkgo biloba* also has the capacity to regulate the oxidative stress. The levels of glutathione, malondialdehyde, superoxide dismutase and nitric oxide, increased after a treatment with EGb [20]. These properties determine beneficial effects in neurodegenerative diseases as Alzheimer [21,22] or Parkinson [23]. However, the mechanism of the action of EGb protection against oxidative stress-induced apoptosis remains to be fully elucidated.

In fact, oxidative stress is one of the main causes of neural damage. EGb 761 exerts neuroprotective action mainly acting as free radical–scavenger. In fact, EGb 761 is able to reduce the endogenous and the induced levels of ROS [24]. This activity is linked to the chemical structure of the flavonoids that allow to not only to react and directly scavenge, the hydroxyl radicals but also to inhibit the formation of new hydroxyl radicals [25].

It is well known that oxidative stress determines the activation of the apoptotic processes, thus playing a crucial role in most of neurological diseases.

The evidence mentioned above prompted us to explore the protective effect of EGb 761 against oxidative stress-induced apoptosis in neuroblastoma cells.

Aim

The main aim of this study was to analyse the anti-cancer potential of grape seeds global semipolar extracts of two Italian grape varieties Aglianico (red) and Falanghina (white) in cancer cell lines. Many studies have shown that extracts from different cultivar can have different efficacy. It is believed that this difference is due to a different chemical composition.

The use of natural extracts represents a new resource for the treatment of cancer, which can be used alongside the already established chemotherapy, radiotherapy and surgery.

One of the major advantages of natural products is represented by the low intensity of side effects, which are usually milder than those associated to the use of chemotherapeutics.

Initially, we tested the efficacy of the two grape seed extracts on mesothelioma cell lines. Malignant mesothelioma is a rather rare and aggressive cancer of the mesothelium, the epithelium lining vital organs such as the lungs and heart and body cavities such as the peritoneal cavity. The worldwide incidence is 2.2 cases per million population [26].

We used three mesothelioma cell lines, MSTO-221H and NCI-H2452, sensitive to standard chemotherapeutic treatment, and IST-Mes2, insensitive to chemotherapeutic treatment.

Usually, phytochemicals anticancer activities are exerted by inducing programmed cell death (apoptosis). Apoptosis can be executed through the intrinsic or the extrinsic pathway. The extrinsic pathway is activated by the bond of death receptors and their ligand, as the receptor FAS and his ligand

FASL. The intrinsic pathway is regulated by B-cell lymphoma-2 (BCL-2) family proteins. BAX (Bcl-2- associated X protein) impairs mitochondrial membranes determining cytochrome *c* release, and this activity is inhibited by binding of BCL-2 to BAX. Unbalance between BCL-2 and BAX favours BAX activity, determining the onset of apoptosis. Therefore, we decided to analyse gene and protein expression of regulatory genes of these pathways.

Once the most promising and effective extract was chosen, we analysed the transcriptional pathways modulated following treatment.

Afterward, we wanted to understand if the anticancer effects were restricted to mesothelioma only or also to other tumours.

Therefore, the effect of grape seed extract was tested in breast cancer. Breast cancer is the most common tumour in women, and one of the three most common cancers worldwide [27] and its morbidity shows an increasing trend year by year [28].

We used a cell line of breast cancer, MDA-MB-231, frequently used as late-stage breast cancer model. This cell line is ER, PR, and E-cadherin negative and expresses mutated p53 [29].

Subsequently, we tested the biological effects of grape seed extract on medulloblastoma cells. Medulloblastoma is the most common paediatric brain tumour and is currently treated by surgery, radiotherapy, and chemotherapy [30]. Although this multi-modal treatment is the most effective, the aggressive nature of this therapy results in a number of serious neurocognitive side effects. We used two different cell lines, DAOY HTB-186 and D283 Med HTB-185, which have a higher degree of stemness than the former.

The industrial characterization of the PhD pushed us to try to understand how to industrialize the process of production of the extract, to understand the common points with the industrial processes already widely developed for the production of other derivatives from grape seeds, and the economic feasibility of the project. Following this route, we have analysed the biological effects of grape seed oil, a commercial product derived from grape seeds.

In the second chapter of this thesis, I have analysed the antioxidant potential of flavonoids present in a standardized *Ginkgo biloba* extract. In fact, oxidative stress occurs in the cell when the antioxidant defence in unable to balance the rate of the reactive oxygen species generated [31].

When present in excess, oxidants elevate the intracellular levels of reactive oxygen species (ROS) and damage cell membrane, proteins and DNA.

We chose to analyze *Ginkgo biloba* protective effects in neurons that are particularly prone to production of ROS and highly susceptible to redox stress, because of their high lipid and metal ion content combined with their high metabolic rate and relatively low concentrations of antioxidants [32]. Neuronal cell death induced by oxidative stress is especially dangerous because adult neurons are post-mitotic cells with limited capacity to proliferate or be replaced. Therefore, in neuronal cells, reduction of oxidative stress could inhibit apoptosis potentially preventing neurodegeneration.

I have analyzed the protective effect of EGb 761 on oxidative stress-induced apoptosis in SK-N-BE cells with the aim to unravel the molecular pathway in which EGb 761 acts as antioxidant. SK-N-BE is a human neuroblastoma

cell line, commonly used as cellular model for research studies aimed to analyze the role of oxidative stress in neurodegeneration [33-35].

Chapter 1

Polyphenols from grape seed extract

Materials and Methods

2.1. Cell culture

Human malignant mesothelioma cell lines MSTO-221H (MSTO) and NCI-H2452 (NCI) were grown in RPMI supplemented with 10% FBS, glutamine (2 mM), sodium pyruvate and antibiotics (0.02 IU/mL-1 penicillin and 0.02 mg/mL-1 streptomycin). Human mesothelioma cells Ist-Mes2 (Mes2) were cultured in DMEM, supplemented with 10% FBS, glutamine (2mM), 1% nonessential amino acids, and antibiotics (0.02 IU/mL-1 penicillin and 0.02 mg/mL-1 streptomycin).

Human mesothelium cell lines, Met5-A, were grown in RPMI supplemented with 10% FBS, glutamine (2 mM), sodium pyruvate and antibiotics (0.02 IU/mL-1 penicillin and 0.02 mg/mL-1 streptomycin).

Human breast adenocarcinoma cell line MDA-MB-231(MDA) were maintained in RPMI 1640 medium supplemented with 10% FBS.

The human mammary epithelial cell line, MCF10A cells, were cultured in DMEM/Ham's F-12 supplemented with 100 ng/ml cholera toxin, 20 ng/ml epidermal growth factor, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, and 10% FBS.

Human medulloblastoma cell lines, DAOY HTB-186 (DAOY) and D283 Med HTB-185 (DAOY) were routinely maintained in complete growth medium Eagle’s Minimum Essential Medium supplemented with 10% FBS with 2 mM glutamine and 100 U penicillin/0.1 mg/mL streptomycin.

2.2. Plant material

Two *Vitis vinifera* cultivars were used in this study: Aglianico del Taburno, , and Falanghina del Beneventano. Berries were collected from a nine-year-old vineyard (145 m a.s.l., Castelvenere, Benevento Province, Southern Italy) during the 2015 growing season. The whole berries were frozen immediately in liquid nitrogen. Three independent pools (biological replicates) of about 100 berries were used for the analyses.

2.3. Extract preparation

To obtain peel and seed semipolar extracts from Aglianico and Falanghina, each tissue was first isolated, minced with liquid nitrogen and then lyophilized. The powder was homogenized with methanol/chloroform (1:4) using TissueLyser (Qiagen). Aqueous phases was then recovered after a 5′ centrifugation (12,000 rpm) and lyophilized again. The powder was then dissolved in DMSO (Sigma) at a concentration of 500 mg/ml and stored at −20° C. The extracts were designed as follows: Aglianico grape peel, AGP; Aglianico grape seed, AGS; Falanghina grape peel, FGP; Falanghina grape seed, FGS.

2.4. Cell proliferation assay

To evaluate the effects of peel and seed semipolar extracts from Aglianico and Falanghina on cell proliferation, approximately 1×10^4 cells were plated in 48-well plates and treated with different concentrations of either peel (up to 1000 μg/ml) or seed (up to 500 μg/ml) extracts for 24, 48 or 24+24h (24+24). In the case of the 24+24 treatment, cells were treated twice: after

overnight incubation and again after 24 h. Then, cells were fixed with 3.7% formaldehyde for 10min, washed with PBS, and stained with 0.5% crystal violet for 10 min. A microplate reader (Cytation3, ASHI) was then used to measure the absorbance of each well at 595 nm. All the experiments were performed in triplicate. Through these experiments, we selected the optimal concentration (350 µg/ml) and extract source (seed) to be used in subsequent experiments.

2.5. Colony forming assay

For colony assay, cells were seeded in six-well plates at a density of 500 cells/well and incubated at 37°C in a 5% CO_2 humidified incubator. The culture medium was replaced every three days and cells were grown for 14days. Then, cells were treated with 350µg/ml of Falanghina (FGS) or Aglianico (AGS) seed extracts for 24 h and grown for additional 14 days using media without seed extracts. Colonies were finally stained with crystal violet and counted. Representative plates were photographed using phase contrast microscope (Leica, Milan, Italy). All the experiments were performed in triplicate.

2.6. Wound healing assays

Approximately 1.5×10^5 cells were plated in six-well plates. After overnight incubation, cells were treated with 350 µg/ml of FGS or AGS extracts for 24, 48 and 24 + 24 h. Wounds were created in confluent cells using a 200 µL pipette tip. To analyse cell migration, at least 10 representative images for each scratch were taken in different scratched area of cells at different time

points of incubation. Images were focused on the centre of the wound field, photographed and measured using ImageJ software. The influence of FGS and AGS on wound closure was compared to scratched cells at time zero hours. All the experiments were performed in triplicate.

2.7. Apoptosis detection by flow cytometry analysis

Approximately 7.5×10^5 cells were plated in 100 mm plates. After overnight incubation, cells were treated with 350 μg/ml of FGS or AGS extracts for either 24, 48 or 24 + 24 h and stained with propidium iodide and annexin V (Annexin V FITC assay, BD Biosciences, Franklin Lakes, NJ), according to the manufacturer's guidelines. Flow cytometry was performed using a FACS-canTM flow cytometry system (Becton Dickinson, San Jose, CA). All the experiments were performed in triplicate.

2.8. RNA extraction and expression analysis of apoptotic pathway genes in MM cells

RNA was extracted using Trizol (Life Technologies) following manufacturer's instructions. For RT-q-PCR assays, 200 ng of total RNA from each sample were retro-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). RT-q-PCR reactions were performed by means of a 7900 HT Real Time PCR (Applied Biosystem). Gene specific primers for the selected genes: BAX: (Forward 5′-TTTGC TTCAGGGTTTCATCCA-3′, Reverse 5′- CTCCATGTTACTGTCCAGTT CGT-3′); (BCL-2: Forward 5′-GTTCCCTTTCCTTCCATCC-3′, Reverse 5′-TAGCCAGTCCAGAGGTGAG-3′); FAS: (Forward 5′-CCCTCCTACCT-

CTGGTTCTTACG-3′, Reverse 5′TCAGTCACTTGGGCATTAACACTT-T-3′); FASL: (Forward 5′-CCTGAAAAAAAGGAGCTGAGGAA-3′, Reverse 5′-GGCATGGACCTTGAGTTGGA-3′); GAPDH: (Forward 5′-CAAGGCTGTGGGCAAGGT-3′, Reverse 5′-GGAAGGCCATGCCAGT-GA-3′); MDM2 (Forward: 5'-AAGGTGGGAGTGATCAAAAGGA-3'; Reverse: 5'-TAGAAACCAAATGTGAAGATGAAGGT-3'); JUN (Forward: 5'-TTTTGCAAGCCTTTCCTGCG-3', Reverse: 5'-TCTTCTCTGCGTGGCT-CTC-3'); DHCR24 (Forward 5'-GCTGGACT-CTCCCCGTGTT-3'; Reverse: 5'-TGCCTGTGCCCATGATCA-3') DHCR7 (Forward: 5'-CGCAGGACTTTAGCCGGT-3',Rev: 5'-TGGCT-TTGGGAATGTTGGG-T-3'); HMGCR (Forward: 5'-CCTTTCCAGA-GCAAGCACATTA-3', Reverse: 5'- TTTCCCTTACTTCATCCTGT-GAGTT-3'); ACAT2 (Forward: 5'-TGTGGCTCCGGAAG-ATGTG-3, Reverse: 5'-GCCTGCTGCCAAGACATGT-3'); FASN (Forward: 5'-CTGCTGCTGGAAGTCACCTATG-3', Reverse: 5'-CGGAGTGAA-TCTGGGTTGAT-G-3'); FDFT1 (Forward: 5'-GGAAGGTGATGCCCAA-GATG-3', Reverse: 5'-ACTGGTCTGATTGAGATACTTGTAGCA-3'); HMGCS1 (Forward: 5'- TGCTGTCTTCAATG-CTGTTAACTG-3', Reverse: 5'-ACCAGGGC-ATACCGTCCAT-3').

Primers were designed at exon-exon junctions using Primer express 2.0 (Applied Biosystems). Target expression level was performed as previously described [36] using GAPDH as housekeeping gene. All the experiments were performed in triplicate.

2.9. Cytochrome *c* release

To determine the cytochrome *c* release from mitochondria to cytosol, cytosolic fractions were isolated after FGS and AGS extracts treatment. 1 × 10^6 cells were resuspended in 100 µL of ice-cold plasma membrane permeabilization buffer (200 µg/ml digitonin, 80 mM KCl in PBS), and incubate on ice for 5 min. Lysates were centrifuged at 800 X g for 5 min at 4 °C, and the supernatants (cytosolic fraction) were then collected. All the experiments were performed in triplicate.

2.10. Western blot

Protein extracts were prepared as previously described [37]. For each lane, 20 µg of total cell lysates were separated in 4–15% Tris–glycine gels (BioRad) at 100 V. Proteins were then transferred to PVDF membranes, probed with the specific primary antibodies, followed by secondary antibodies conjugated with horseradish peroxidase according manufacturer's indications. Primary antibodies used for Western blot include PARP (Cell Signaling, #9542), BCL-2 (Abcam, ab182858), BAX (Santa Cruz Biotechnology, sc-493) and Cytochrome c (Abcam, ab133504), MDM2 (Santa Cruz Biotechnology, sc-965), Phospho-c-Jun (Ser243) (Cell Signaling, #32994), p53 (Cell Signaling, #2524). β-Actin (Cell Signaling, #3700) was used as loading control. All the antibodies were used at working concentration indicated by manufacturers. Protein bands were detected by Clarity western ECL (BioRad) and quantified with ImageJ software. All the experiments were performed in triplicate.

2.13. Statistical analysis

Analysis to evaluate the difference between control and treatments was performed using Graph Pad Prism 9.0 (GraphPad Software, San Diego, CA, USA). For each experiment, overall significance of the differences among means was evaluated using the One-Way ANOVA, while differences of each treatment with the control was evaluated using the Dunnet's multiple comparison test with Bonferroni post hoc correction.

Statistically significant difference compared to untreated cells are: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

All metabolomics data have been statistically validated using an ANOVA with Tukey's t-test analysis.

2.14. Micro-Array analysis

MSTO-211H ($1,5 \times 10^6$) were treated with 350 ug/ml of AGS for 24+24 hours then RNA was extracted using Trizol (Life Technologies) and following manufacturer's instructions. Total RNA samples were quantified with a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific, Waltham, MA, United States).

Expression profiling was performed using 100 ng RNA and the Affymetrix Human Clariom S Assay.

(Affymetrix/Thermo Fisher Scientific), which interrogates over 20000 well-annotated genes. Total RNA was converted in labelled ssDNA and used to hybridize microarray. All the procedures including wash and staining of array were performed following Affymetrix instructions.

After laser scanning .CEL files from array were used for data analysis. Primary data analysis was performed with the Transcriptome Analysis Console software (Thermofisher version 3.1.0.5) including the Robust Multiarray Average module for normalization.

2.15. Pathway and network analysis by IPA.

The list of differentially expressed genes in the MSTO cell line, containing gene identifiers and corresponding expression values, was uploaded into the IPA software (Qiagen). The "core analysis" function included in the software was used to interpret the differentially expressed data, which included biological processes, canonical pathways, upstream transcriptional regulators, and gene networks.

2.16. Oil preparation

The industrial process for the production of grape seeds oil includes a first phase of mechanical separation of grape seeds from the remaining residues of the wine making process. In 100 kg of grapes, 80% is represented by pulp, 12% skin, 4% seeds and the remaining part by stems, and after wine making process, skin and seeds are combined. After separation the grape seeds are dried, at 1.5 atm, at about 60/70° C for about 2 hours. With this process the percentage of water in grape seeds changes from 50 to 10%. At this point the grape seeds pass into a hopper from which oil and a waste product, called flour, comes out. For 100 grams of grape seeds you get about 17 grams of oil and 83 grams of flour.

The grape seed oil is dissolved in DMSO to a final concentration of 0.9%. Cells are treated with the oil for 48 hours. 0.9% DMSO is added to the control cells.

Results

3.1. Seed rather than peel extracts determine cancer growth inhibition

First of all, we determined that the vehicle with which the extracts, DMSO, were prepared did not affect the viability of the treated cells.

Treatments of MSTO, NCI and Mes2 cells with hydroalcoholic semi-polar peel extract did not affect MM cell viability, also when used at high concentration (1000 µg/ml) (**Fig. 3.1**).

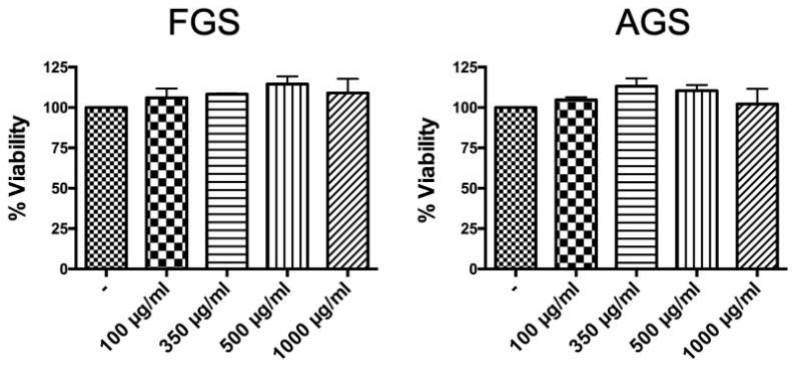

Figure 3.1 *Effect of hydroalcoholic semi-polar peel extract on MSTO cells. The same results were obtained with NCI and Mes2 cells. The bars represent the average ± standard deviation (n=3). Statistically significant difference from the control: **p<0.01, ***p<0.005, ****p<0.001.*

On the contrary, extracts from seeds determined cell growth inhibition in a concentration and time-dependent manner (**Fig. 3.2 a, b**). In particular, Falanghina grape seed extract (FGS) at 350µg/ml was able to significantly decrease MSTO cell viability up to 30% (ANOVA p-value <0.0001). At the

same concentration, Aglianico grape seed extract (AGS) decreased MSTO cell viability up to 40% (ANOVA p-value < 0.0001).

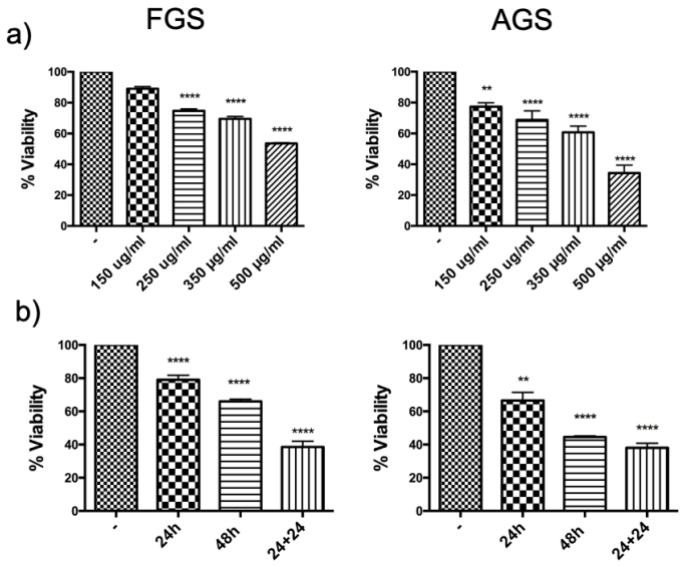

Figure 3.2 *Extracts from seeds determined cell growth inhibition in a concentration and time-dependent manner on MSTO cells); untreated cells:-. The bars represent the average ± standard deviation (n=3). Statistically significant difference from the control: **p<0.01, ***p<0.005, ****p<0.001.*

Since AGS at 350 µg/ml resulted closer to the half minimal (50%) inhibitory concentration (IC), we decided to use this concentration for subsequent experiments. Following 48 h of treatment, MSTO cell death was higher than that observed after 24 h. Moreover, a stronger effect was evident when the 48 h treatment was performed by adding the extract twice, every 24 h (24 + 24 h) (ANOVA p-value < 0.0001). (**Fig. 3.2 b**). Similar results were obtained with NCI and Mes2 cell lines (**Fig. 3.3**).

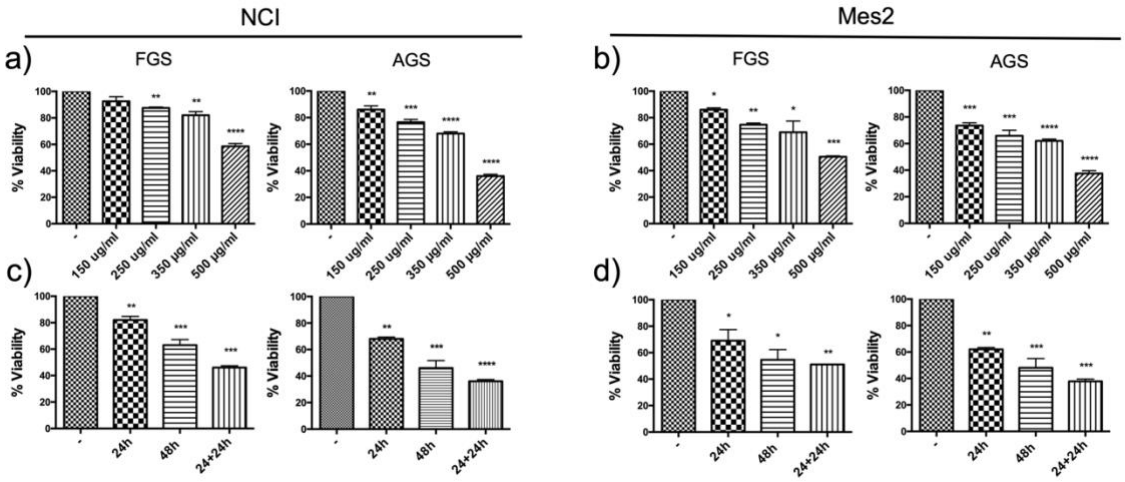

Figure 3.3 *NCI and Mes2 cell viability: a, b) treatments with FGS and AGS extracts; c, d) time course treatment with FGS and AGS extracts (350 µg/ml); untreated cells:-.The bars represent the average ± standard deviation (n=3). Statistically significant difference from the control: **p<0.01, ***p<0.005, ****p<0.001.*

These experiments indicated that seed extracts were more effective than peel extracts. For this reason, subsequent assays were carried out only with seed extracts.

3.2. FGS and AGS impair mesothelioma tumorigenic properties affecting cell proliferation and migration

To evaluate the anticancer potential of FGS and AGS, cell migration and invasive ability were analysed on MSTO, NCI and Mes2 treated independently with the seed extracts of Falanghina and Aglianico. The colony formation assay indicates the cellular self-renewal ability and their long-term proliferative potential. Our results showed that this ability was strongly impaired by seed extracts of both varieties in all the cell lines

analysed. In fact, after colonies growth, a 48h treatment with 350 µg/ml of FGS or AGS almost completely inhibited colony formation in MSTO (67% and 90%, respectively; ANOVA p- value < 0.0004) as well as in NCI (51% and 84%, respectively; ANOVA p-value < 0.0001 and in Mes2 cells (48% and 88%, respectively; ANOVA p-value < 0.0003) (**Fig. 3.4**).

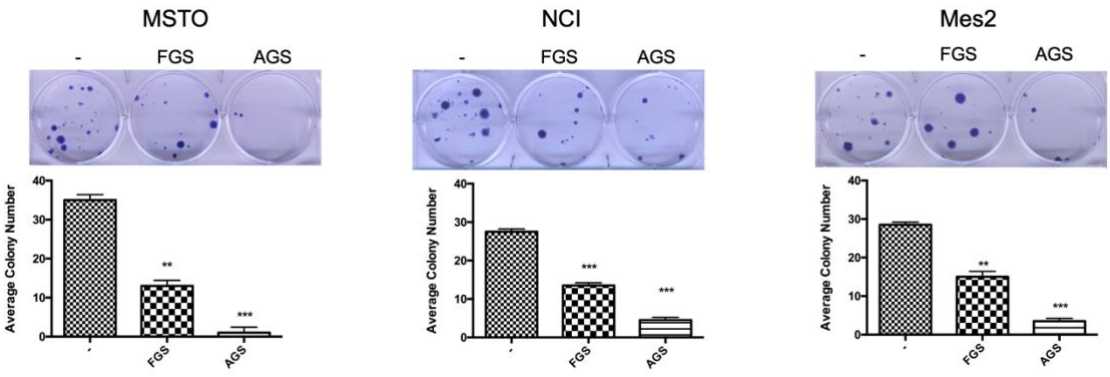

Figure 3.4 *Colony formation assay after FGS and AGS treatment (350 µg/ml); untreated cells :-. Representative plate images after crystal violet staining are shown. Histograms report average of colony number. The bars represent the average ± standard deviation (n=3). Statistically significant difference from the control: **p<0.01, ***p<0.005.*

To measure the cell migration capability in vitro, we also performed a wound healing assay treating scratched cells with FGS or AGS extracts. MSTO cell migration was significantly inhibited by both extracts in a time-dependent manner reaching a gap of about 120% (FGS; ANOVA p-value < 0.0001) or 170% (AGS; ANOVA p-value < 0.0001) compared to the wound gap in the untreated cells. In particular, the wound gap was maximum after 24 + 24 h treatment with 350 µg/ml FGS or AGS extracts (ANOVA p-value < 0.0001)

(**Fig. 3.5**). On the contrary, in the untreated cells the wound gap was closed at the end of the treatment.

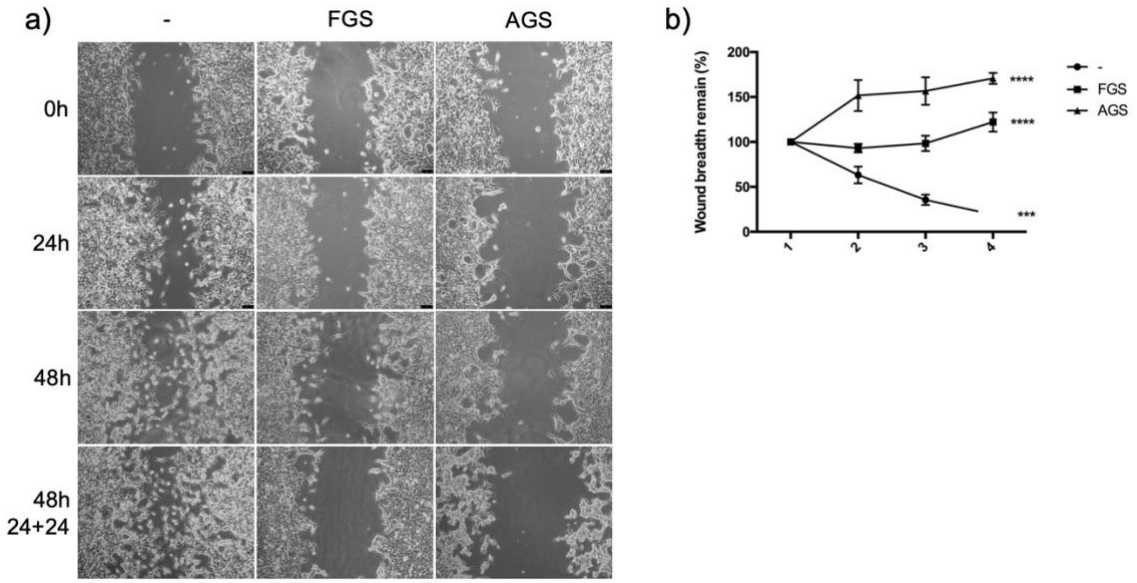

Figure 3.5 *Wound healing assay. The wound closure rate was measured by detecting the closure distance at three different time intervals on MSTO cells untreated (-) or treated with 350 µg/ml of seed extracts. Representative micrographs at phase contrast microscope are shown. (h) Quantification of open wound area.*
*The indicators represent the average ± standard deviation (n=3). Statistically significant difference from the control: **p<0.01, ***p<0.005, ****p<0.001.*

Similar results were obtained with NCI and Mes2 cells (**Fig. 3.6**).

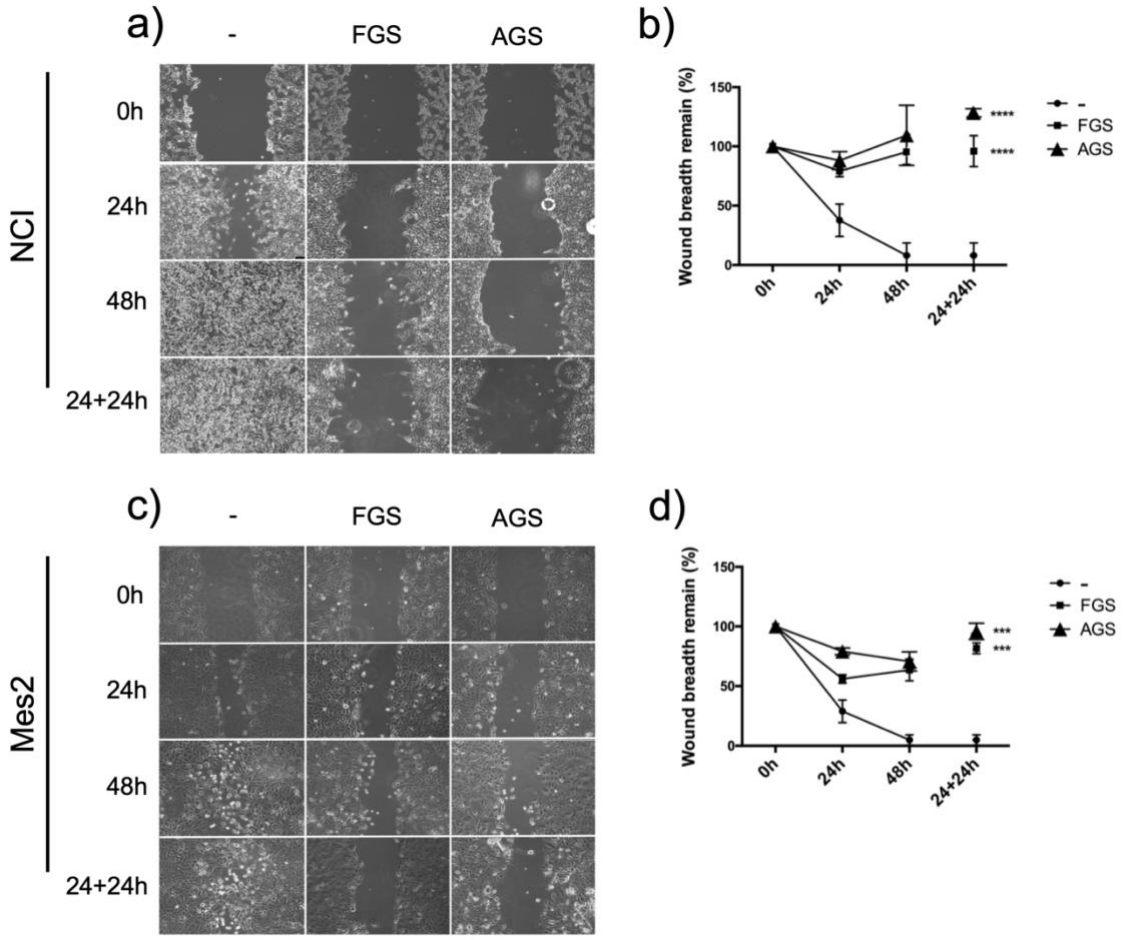

Figure 3.6 *Wound healing assay on NCI (a) and Mes2 (c). The wound closure rate was measured by detecting the closure distance at three different time intervals on untreated cells (-) or cells treated with 350 µg/ml of extracts; representative micrographs of the cell migration from three independent experiments are shown. b) and d) Quantification of open wound area. The indicators represent the average ± standard deviation (n=3). Statistically significant difference from the control: ***p<0.005, ****p<0.001.*

Taken together, these results revealed that grape seed extracts from both varieties determined a comparable inhibition in cell growth and migration.

3.3. FGS and AGS induce apoptosis in mesothelioma cell lines

To confirm whether the loss of viability was due to apoptosis, we analysed cell cycle perturbation and the downstream signalling triggered by grape seed extracts. MSTO treatment with FGS determined a growing apoptotic induction during time. It reached about 20% at 24 + 24 h treatment (**Fig. 3.7 a, b**). AGS treatments on MSTO cell lines resulted in apoptotic increase of about 35% after 24 + 24 h treatment (**Fig. 3.7 c, d**) (ANOVA p-value < 0.0051).

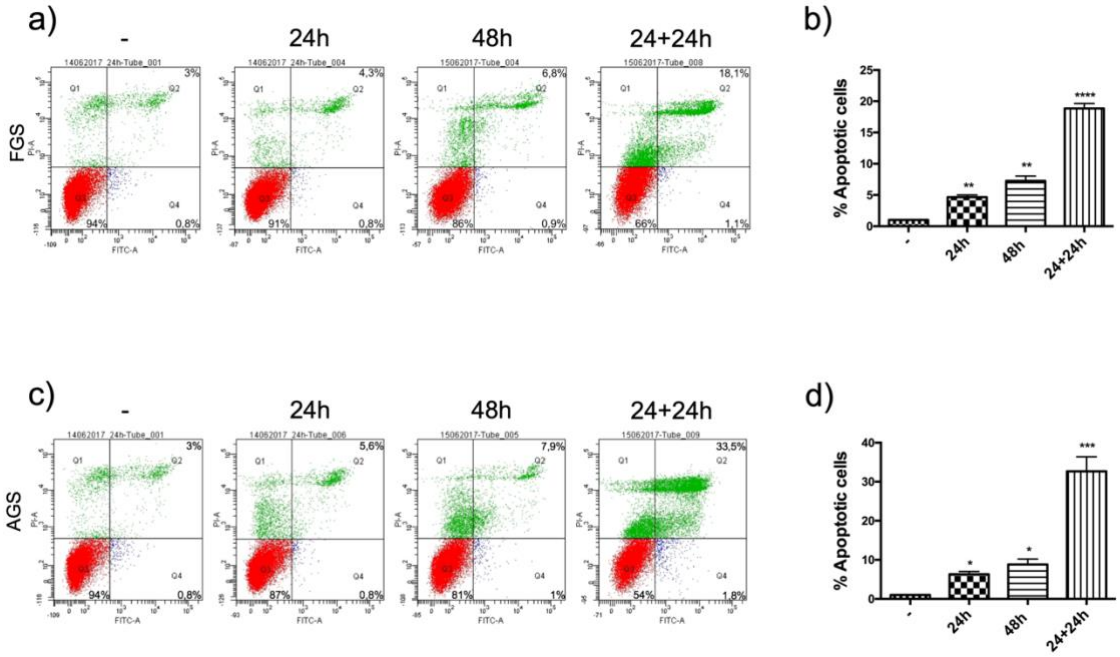

Fig 3.7 *MSTO apoptosis analysis by Annexin V-FITC/PI after treatments with 350 µg/ml of FGS (a) and AGS (c) extracts at three different time intervals; untreated cells :- .Q2 and Q4: early and late apoptotic cells; Q1: necrotic fraction; Q3: live cells. Histograms reporting a data summary of the apoptotic index after FGS (b) or AGS (d) treatment represented in (a) and (c). Bars represent the average ± standard deviation (n=3). Statistically significant difference from the control: *p<0.05, **p<0.01,*

****p<0.005, ****p<0.001.*

The, NCI and Mes2 cell lines showed only slight modifications in apoptosis compared to MSTO. Interestingly, Mes2 cells that did not undergo apoptosis after combined drug treatment with piroxicam and cisplatin [37], resulted more sensitive to both extracts (**Fig. 3.8**), suggesting that grape seed extracts act as broad regulators of apoptotic induction.

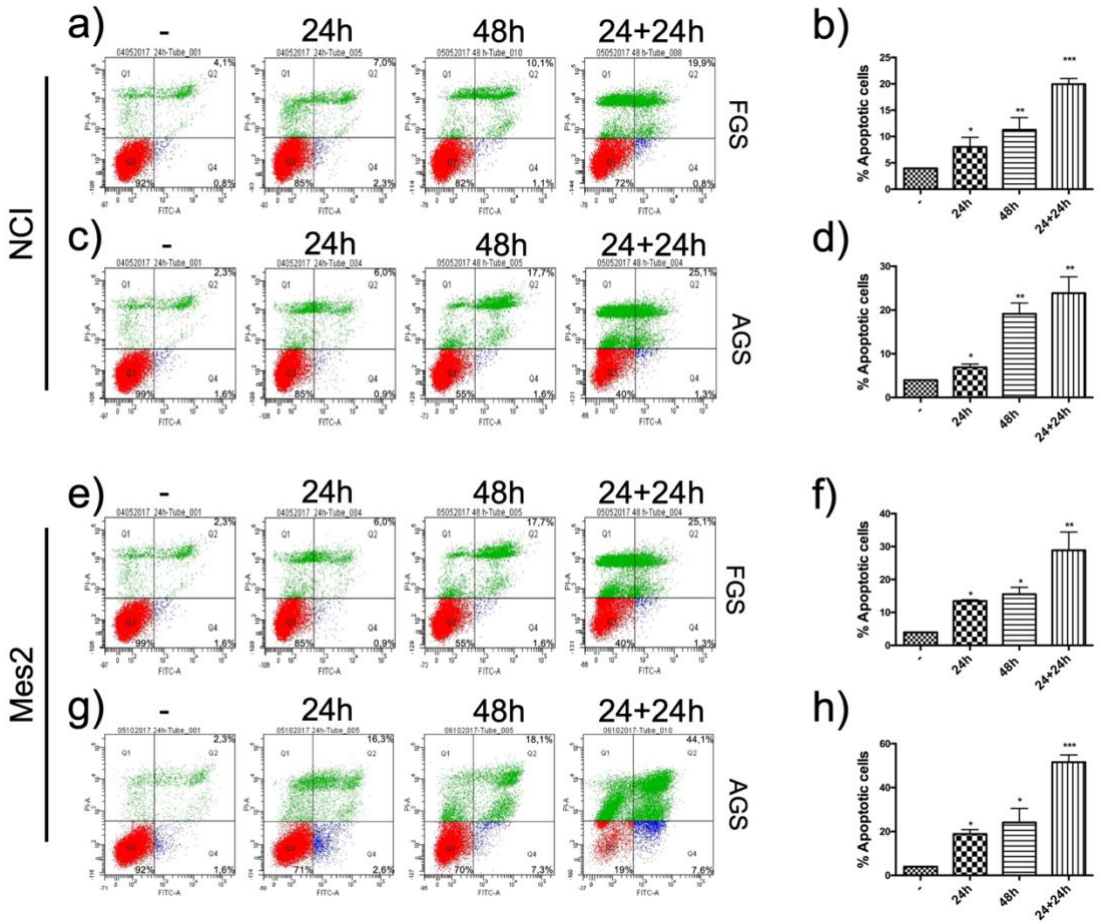

Figure 3.8 *NCI (a, c) and Mes2 (e, g) cells were exposed to 350 mg/ml of FGS or AGS for the indicated time and analyzed by flow cytometry analysis (Annexin V-FITC/PI). Histograms reporting a data summary of the apoptotic index for NCI (b, d) or Mes2 (f,*

*h). The bars represent the average ± standard deviation (n=3). Statistically significant difference from the control: *p<0.05,**p<0.01, ***p<0.005, ****p<0.001.*

3.4. FGS and AGS activate specific pathways involved in apoptosis

To better investigate the molecular mechanism involved in the apoptotic cell death following FGS and AGS treatments, we focused our attention on the apoptotic pathway involved. We first analysed the expression levels of BCL-2 and BAX after FGS and AGS treatments at 24, 48 and 24 + 24 h by q-PCR. As reported in **Fig. 3.9**, the BAX/BCL-2 ratio suggested a time-dependent apoptotic induction in all MM cell lines analysed. The highest BAX/BCL-2 ratio was observed at 24 + 24 h treatment.

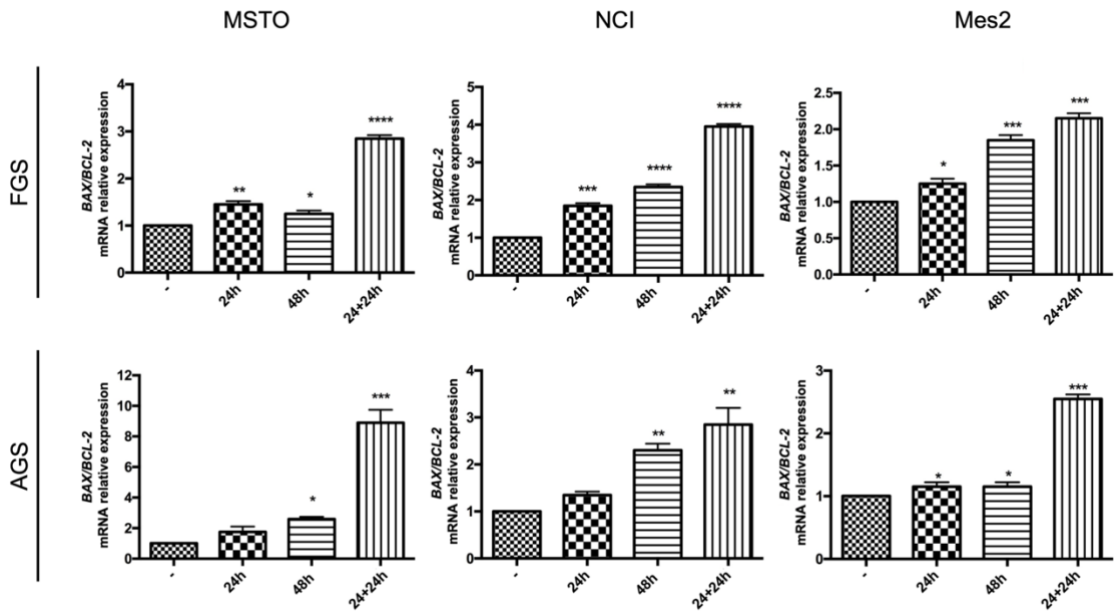

Figure 3.9 *Quantitative analysis of mRNA expression levels of Bax and Bcl-2 in MM cell lines. The bars represent the average ± standard deviation (n=3). Statistically significant difference from the control: *p<0.05, **p<0.01, ***p<0.005, ****p<0.001.*

Then, we investigated if grape seed extracts could activate the extrinsic pathway by measuring expression levels of Fas and FasL, two markers of extrinsic apoptosis induction. Our results provided evidence that Fas was not modulated by FGS and AGS in the cell lines analysed. Furthermore, we were not able to detect any expression of FasL (data not shown). These findings indicated that intrinsic but not extrinsic apoptotic pathway is triggered by FGS and AGS treatments.

3.5. FGS and AGS extracts induce protein expression of cleaved PARP and BAX and cytochrome c release

To gain more insight into the molecular mechanism involved in the apoptotic cell death, we also investigated the effect of FGS and AGS extracts on PARP cleavage as well as on BAX and BCL-2 protein expression. PARP protein is very important for cellular function and survival, and it is a well-known caspase enzyme substrate [38]. Immunoblots clearly showed that, in MSTO cell line, both seed extracts induced PARP cleavage and increased ratio of BAX/ BCL-2 (**Fig. 3.10**), confirming that both extracts are able to induce apoptosis.

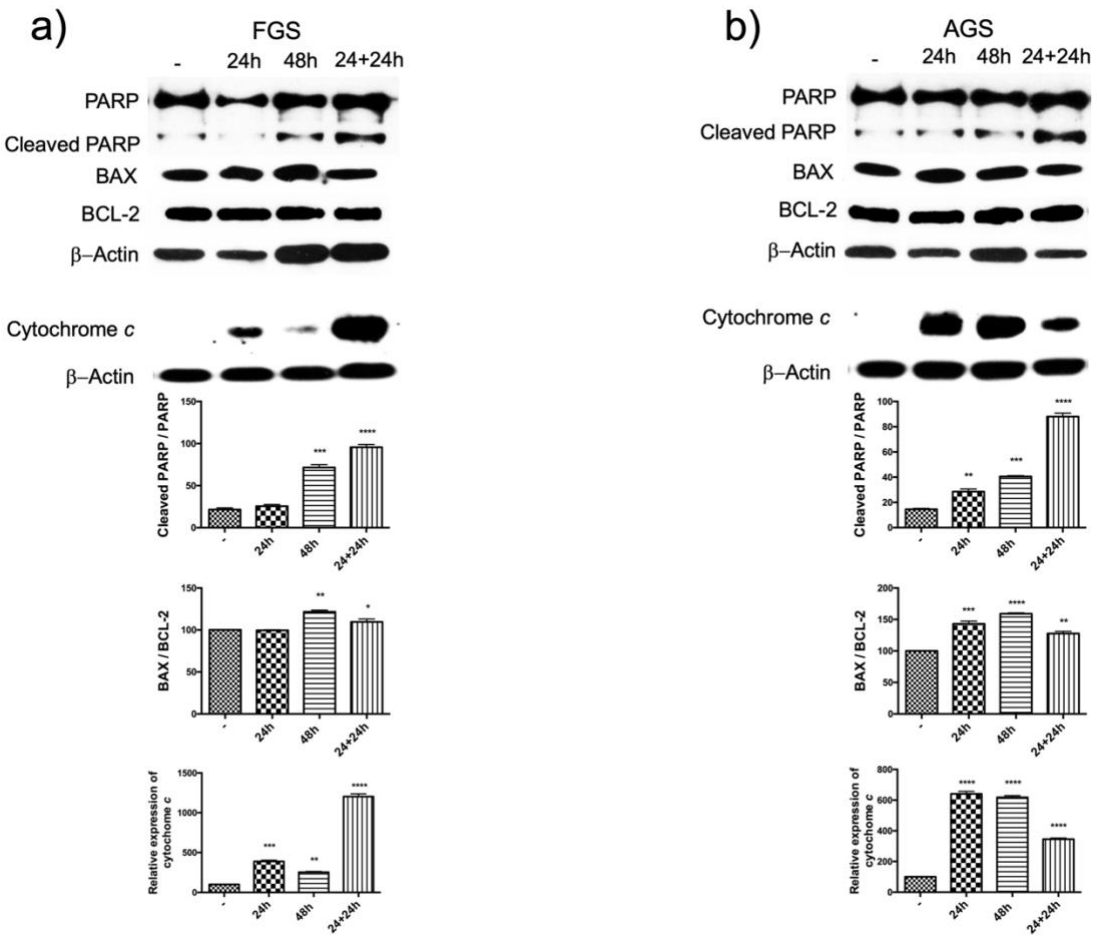

Figure 3.10 *Analysis of cleaved PARP, BAX, BCL-2 and of cytochrome c (cytosolic fraction) on MSTO cells treated with FGS (a) or AGS (b) (350 µg/ml); untreated cells:-. β-Actin protein expression was used as loading control. Histograms report the relative expression level. Statistically significant difference from the control: *p<0.05, **p<0.01, ***p<0.005, ****p<0.001.*

Similar results were obtained with NCI and Mes2 cells (**Fig. 3.11**).

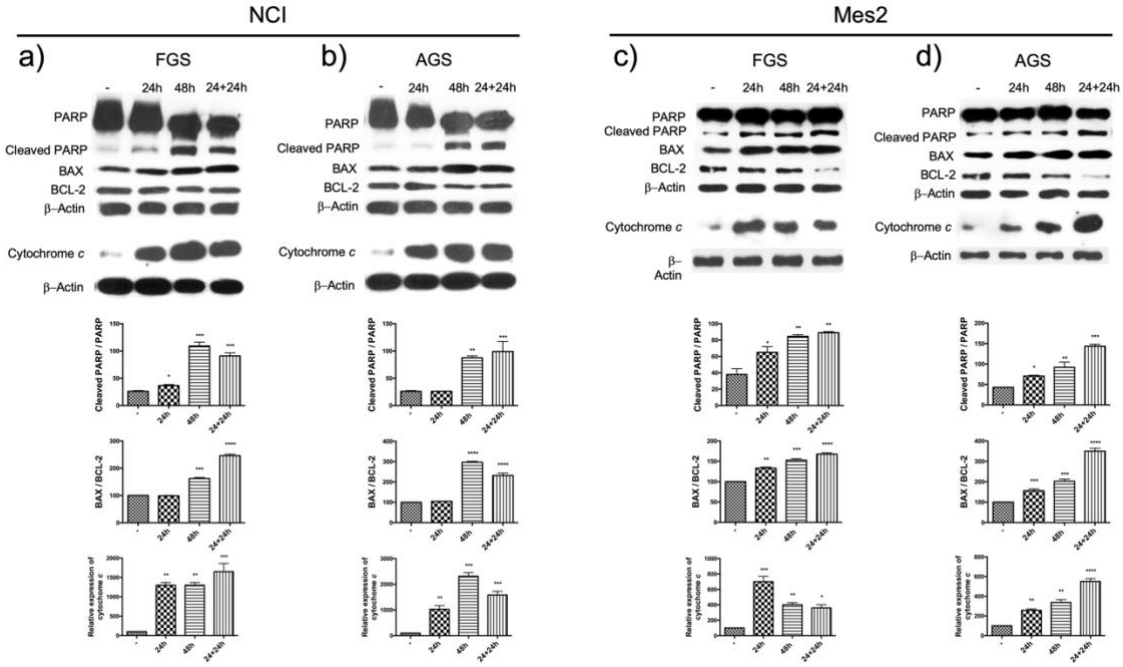

Figure 3.11 *Analysis of cleaved PARP, BAX, BCL-2 and of cytochrome c (cytosolic fraction) on NCI (a, b) and Mes2 (c, d) cells untreated (-) or treated (350 µg/ml) with FGS or AGS at three different time intervals. β-Actin protein expression was used as loading control. Histograms report the relative expression level. Statistically significant difference from the control: *p<0.05, **p<0.01, ***p<0.005, ****p<0.001.*

To further investigate the mechanism of grape extracts-mediated apoptosis, we examined the cytochrome *c* release from mitochondria. Indeed, the apoptotic cell death through the intrinsic mitochondrial pathway is known to induce mitochondrial membrane permeabilization and the subsequent cytochrome c release. As shown in **Fig 3.10**, treatment with grape seed extracts induced cytochrome *c* release in MSTO cell lines. Similar results were also obtained in NCI and Mes2 cells (**Fig. 3.11**). All these findings, together with earlier observations, suggest that FGS and AGS may directly

induce mitochondrial membrane damage and, as consequence, cellular apoptosis.

3.6. Metabolomics and transcriptomic analysis of grape peel and seed samples

Cell treatments with seed extracts were very effective, whereas those with peel extracts did not affect cell viability.

To better investigate on the metabolite content of Aglianico and Falanghina seeds a metabolomic analysis and a transcriptomic analysis was performed.

Analysis of metabolite content [39] identified a total list of 100 negatively and 241 positively charged ions specifically present or over-/down-represented in seed over peel metabolomes of Aglianico, Falanghina, or in both varieties. The number of compounds that were consistently over- and down-accumulated in seed vs peel tissues was 108 and 154 in Aglianico and 76 and 130 in Falanghina are reported in **Table 1**.

Table 1. *Number of statistically significant (p-value ≤ 0.05) over- and down-accumulated metabolites in Aglianico and Falanghina peel and seed extracts.*

	AGS/AGP	FGS/FGP	AGS/FGS	AGP/FGP
Over-accumulated	108	76	69	39
Down-accumulated	154	130	123	198

Subsequent analysis indicated that Aglianico and Falanghina seed metabolomes resulted highly enriched in several secondary compounds,

particularly phenylpropanoid precursors and proanthocyanidin. The results can be visualized in the heatmap (**Fig. 3.12**) [39].

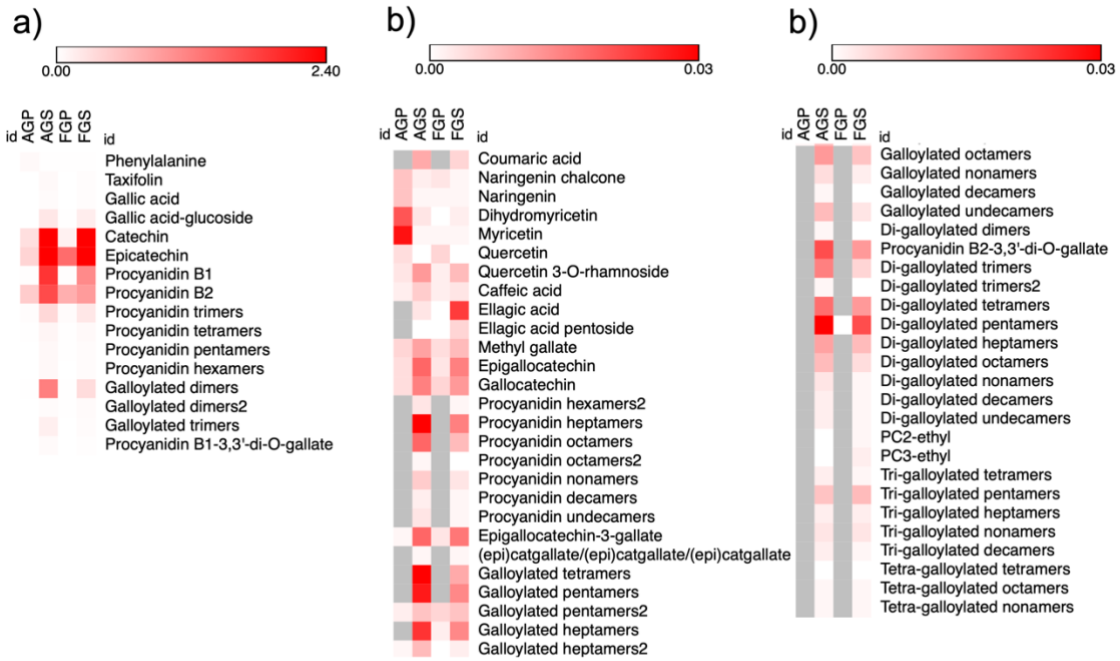

Figure 3.12 *Metabolites have been classified in higher- (a) and lower-represented (b), according to the detected values. Red squares of different shades represent the relative values for each metabolite, expressed as fold on the level of the internal standard. Data are present as average ± standard deviation. Gray squares indicate no detectable expression of the corresponding metabolite.*

According to metabolomic analysis gene expression analysis [39] confirmed an increased expression in seeds of genes involved in the phenylpropanoids and flavonoids biosynthesis.

3.7. Microarray analysis

Microarray results allowed to identify the major molecular pathways involved in the response to AGS treatment. Surprisingly, AGS treatment deregulates the cholesterol biosynthesis pathway, and instead, as we expected, it deregulates a pathway involved in apoptosis, which includes the MDM2, JUN, and p53 genes.

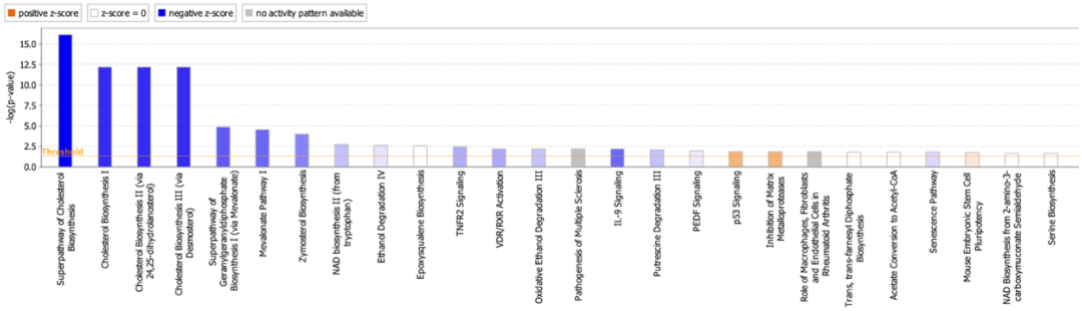

Figure 3.13 *Top Canonical Pathway results from IPA*

Enriched canonical pathways detected using IPA. A total of 67 enriched canonical pathways were identified by applying the -log (P-value) are shown in **Fig. 3.15**. The 'superpathway of cholesterol biosynthesis' was the highest ranking signalling pathway with a -log (P-value) of 2.5. Taking Z-score >2 as the threshold of significant activation.

In addition, the IPA analysis showed that genes deregulated within the class "Diseases and Disorders" are categorized as belonging to cancer, cell death, proliferation and cell viability.

A deeper bioinformatic analysis of cholesterol biosynthesis genes and of those involved in apoptosis, allowed to build a functional network including

a subset of genes in common or having a connection between the two pathways.

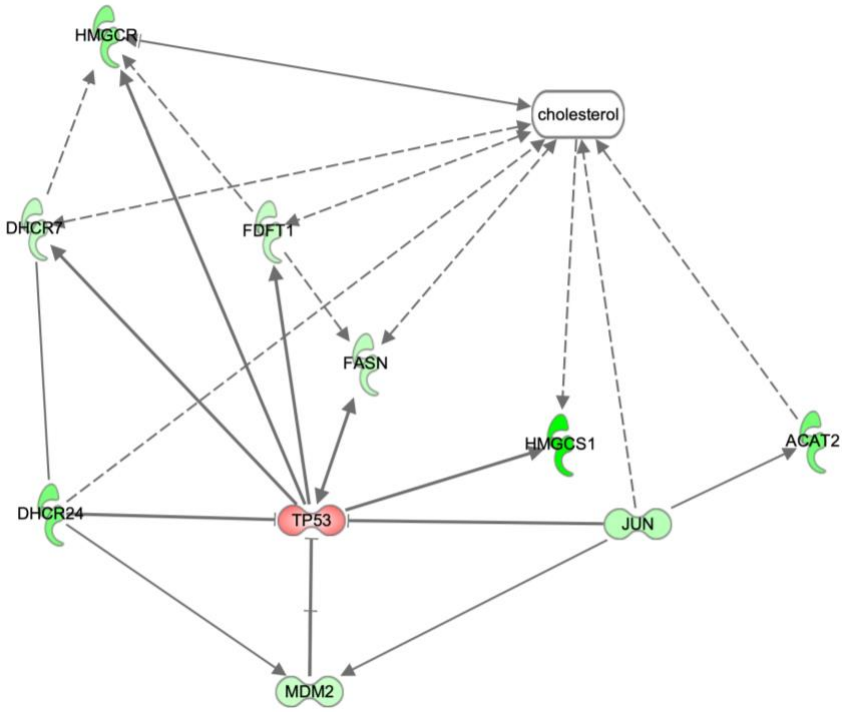

Figure 3.14 *Integrated Network. Biosynthesis and apoptosis pathway genes united in a single network linking them together.*

Specifically, the cholesterol biosynthesis genes chosen were: DHCR24, DHCR7, HMGCR1, ACAT2, FDFT1, HMGCS1, and FASN. All these genes encode proteins involved in the long pathway leading to cholesterol or fatty acid biosynthesis.

DHCR24 and DHCR7, encode for enzymes involved in the last stages of cholesterol formation. DHCR24 is also capable to reduce the activity of p53, directly by reducing its activation, but also indirectly, by increasing the interaction between MDM2 and p53, thus leading to the ubiquitination of the latter.

The expression of these genes connecting the two signalling pathways was biologically validated in all three mesothelioma cell lines by q-PCR. Gene expression analysis confirmed that is downregulated after the treatment with grape seed extract (**Fig. 3.15**)

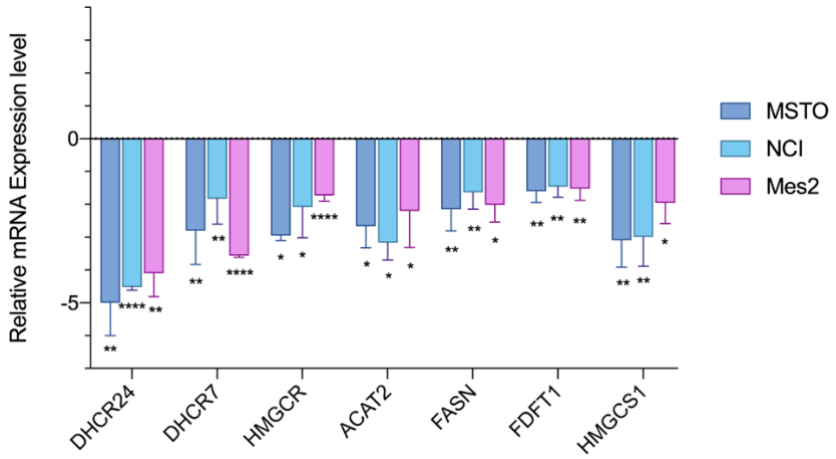

Figure 3.15 *Quantitative analysis of mRNA expression levels of genes of cholesterol synthesis in MM cell lines. The bars represent the average ± standard deviation (n = 3). Statistically significant difference from the control: *p < 0.05, **p < 0.01, ****p < 0.001.*

Among the genes responsible for apoptosis activation, we selected MDM2, JUN and p53. Validation by q-PCR confirmed the results of transcriptomic analysis. In fact, MDM2 and JUN are downregulated, while p53 is upregulated (**Fig. 3.16**)

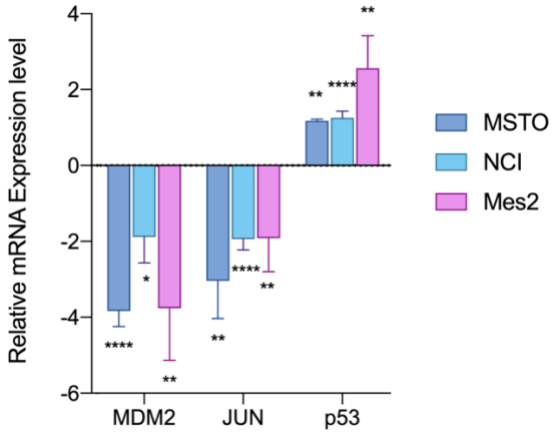

Figure 3.16 *Quantitative analysis of mRNA expression levels of genes of apoptotic pathway in MM cell lines. The bars represent the average ± standard deviation (n = 3). Statistically significant difference from the control: *p < 0.05, **p < 0.01, ****p < 0.001.*

The modulation of gene expression found in the MSTO cell line was also confirmed in the other two mesothelioma lines tested, NCI and Mes2.

3.8. AGS and grape seed oil determine growth inhibition in mesothelioma and breast cancer cells.

During the stage carried out in the company, I have studied the grape seed oil production process in order to compare this process with the extraction protocol set up in our laboratory and analysed the resulting product by comparing the biological activity of the oil with the extract.

For this purpose, MSTO cells were treated with 0.9% grape seed oil in DMSO for 48 hours. The results obtained have shown that grape seed oil is capable of reducing the viability of mesothelioma cells, but less than Aglianico extract (**Fig. 3.17**).

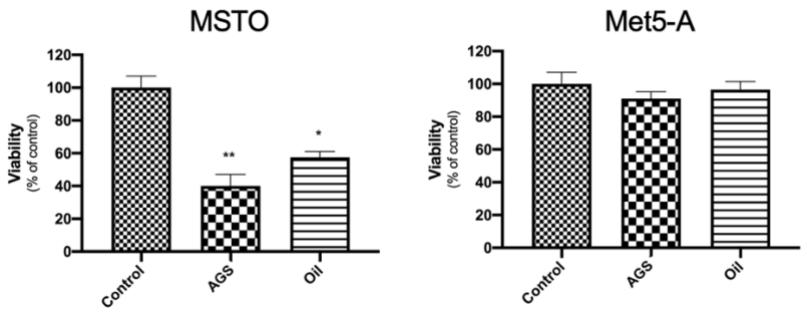

Figure 3.17 *Bioactivity of grape seed extracts and grape seed oil on human mesothelioma cells, MSTO, and human mesothelium cells, Met5-A**.*** *The bars represent the average ± standard deviation (n = 3). Statistically significant difference from the control: *p < 0.05, **p < 0.01.*

At this point we tried to understand if the biological activity of Aglianico semipolar extracts and grape seed oil was due to the activation of particular pathways specifically activated in mesothelioma cells or if they could be activated in other tumours as well. Both treatments were therefore initially tested using a breast cancer cell line (MDA-MB-23). In addition, it was verified that the extract had no effect on non-tumour cell counterparts (MCF-10A).

As showed in **Fig. 3.18** both AGS and grape seed oil, are able to reduce cell viability, also in different tumour cell line. However, the treatment with grape seed oil is less effective than AGS treatment in reducing viability.

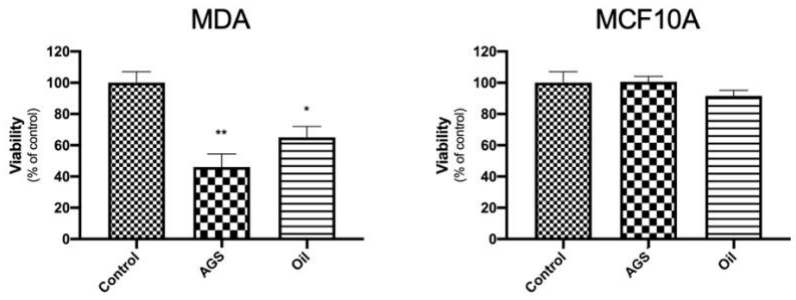

Figure 3.18 *Bioactivity of grape seed extracts and grape seed oil on human breast cells, MDA, and mammary epithelial cells, MCF10A. The bars represent the average ± standard deviation (n = 3). Statistically significant difference from the control: *p < 0.05, **p < 0.01.*

3.9. AGS and grape seed oil bioactivity on mesothelioma and breast cancer cells.

To determine if the reduction in viability was due to apoptosis, we analysed cell cycle perturbation and the downstream signalling triggered by grape seed extracts and grape seed oil. As shown in the **Figure 3.19** MSTO treatment with oil does not determined apoptotic induction, as detected for AGS.

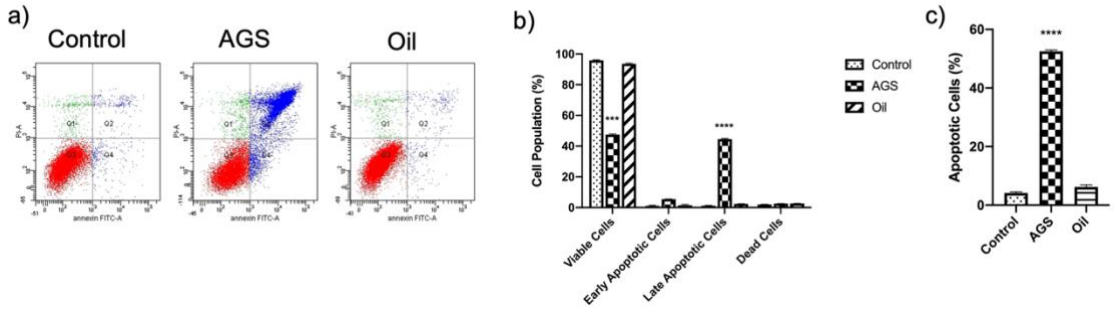

Figure 3.19 *MSTO apoptosis analysis by Annexin V-FITC/PI after treatments with AGS or grape seed oil; untreated cells:-.Q2 and Q4: early and late apoptotic cells; Q1:*

*necrotic fraction; Q3: live cells. Histograms reporting a data summary of the cell population (b) and apoptotic index after treatment represented in (c). Bars represent the average ± standard deviation (n = 3). Statistically significant difference from the control: **p < 0.01, ***p < 0.005, ****p < 0.001.*

Also, in MDA cells treated with AGS the apoptotic process is activated, meanwhile the treatment with the oil does not determine apoptotic induction (**Fig. 3.20**).

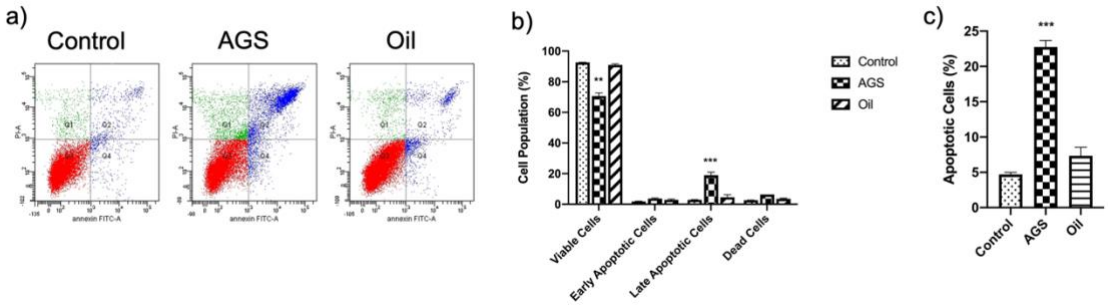

Figure 3.20 *MDA apoptosis analysis by Annexin V-FITC/PI after treatments with AGS or grape seed oil; untreated cells:-. Q2 and Q4: early and late apoptotic cells; Q1: necrotic fraction; Q3: live cells. Histograms reporting a data summary of the cell population (b) and apoptotic index after treatment represented in (c). Bars represent the average ± standard deviation (n = 3). Statistically significant difference from the control: ***p < 0.005, ****p < 0.001.*

Therefore, we also compared gene expression of genes after oil treatment and after AGS treatment in MSTO cells. As shown in **Fig. 3.21**, oil treatment modulates gene expression in the same direction as AGS treatment but with lower efficacy.

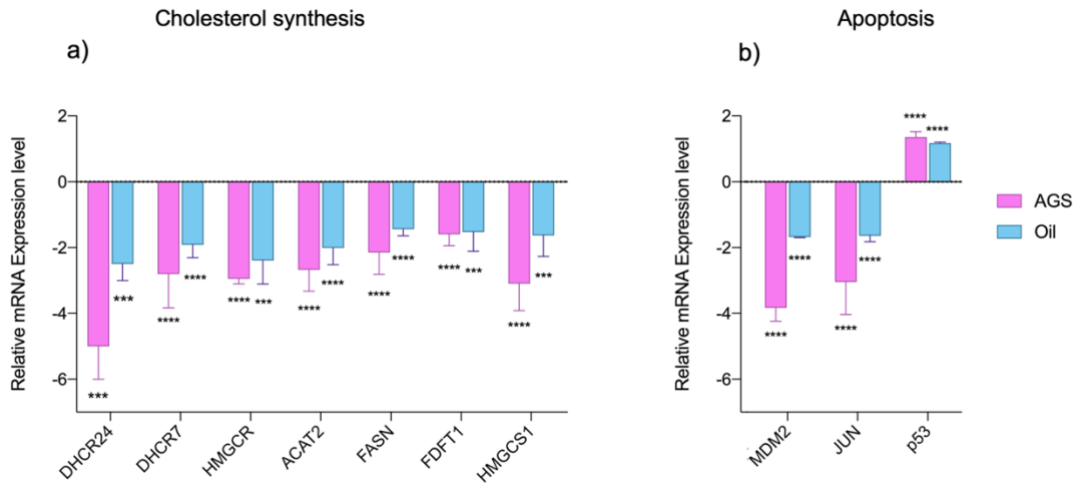

Figure 3.21 *Quantitative analysis of mRNA expression levels of genes of cholesterol synthesis and apoptotic pathway in MSTO cell lines. The bars represent the average ± standard deviation (n = 3). Statistically significant difference from the control: ***p < 0.005, ****p < 0.001.*

At this point we analysed the expression of genes present in the integrated network in MDA cells after treatment with AGS or Oil. While the modulation of genes responsible for the apoptotic pathway is the same that we found in mesothelioma cells, in breast cancer cells the cholesterol biosynthesis pathway did not result modulated.

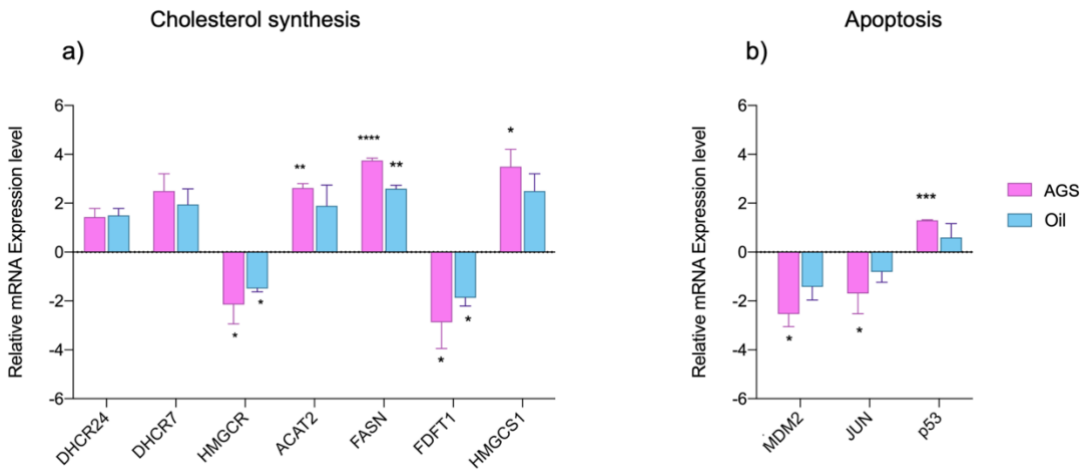

Figure 3.22 *Quantitative analysis of mRNA expression levels of genes of cholesterol synthesis and apoptotic pathway in MSTO cell lines. The bars represent the average ± standard deviation (n = 3). Statistically significant difference from the control: ***p < 0.005, ****p < 0.001.*

3.10. AGS bioactivity on medulloblastoma cells

To find out if the extract could be also active in other cancers, we have tested the effect of the extract on two medulloblastoma cell lines. The analysis of two cell lines, DAOY and D283, has shown that, also in this case, AGS is able to reduce viability and to induce apoptosis.

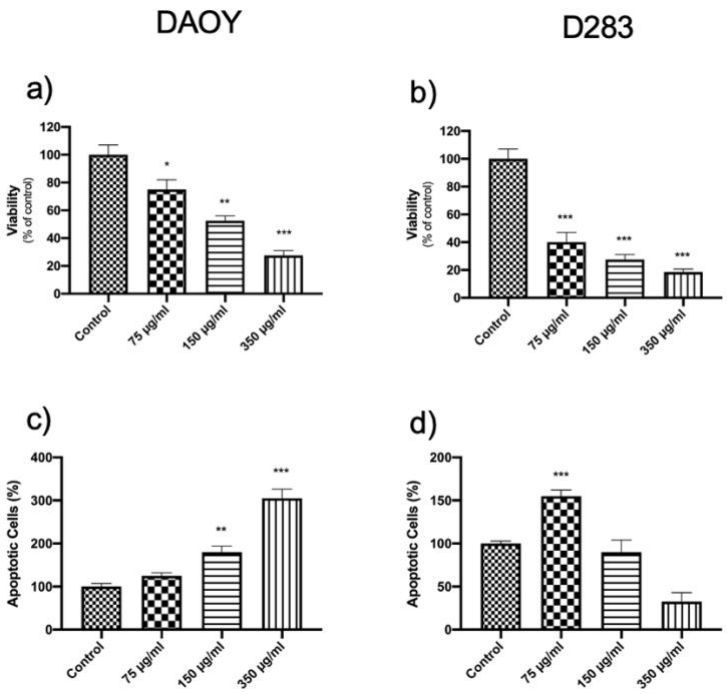

Figure 3.23 *Bioactivity of grape seed extracts and grape seed oil on human medulloblastoma cells, DAOY (a) and D283 (b). Apoptotic cells after AGS treatment in DAOY (c) and D283 (d). The bars represent the average ± standard deviation (n = 3). Statistically significant difference from the control:*p<0.05, **p<0,01 ***p < 0.005, ****p < 0.001.*

Subsequently, we analysed also in these cells the expression of genes present in the functional network previously identified. The analysis by q-PCR showed that in all cell lines, the genes that regulate apoptosis have the same modulation found in mesothelioma cells. Cholesterol biosynthesis, however, does not seem to be modulated in the same way, in fact, while it is downregulated in DAOY cells, as well as in mesothelioma cells, it does not occur in breast and D283 cancer cells. This allows us to hypothesize that the

inhibition of cholesterol biosynthesis is a pathway specifically activated only in some tumours.

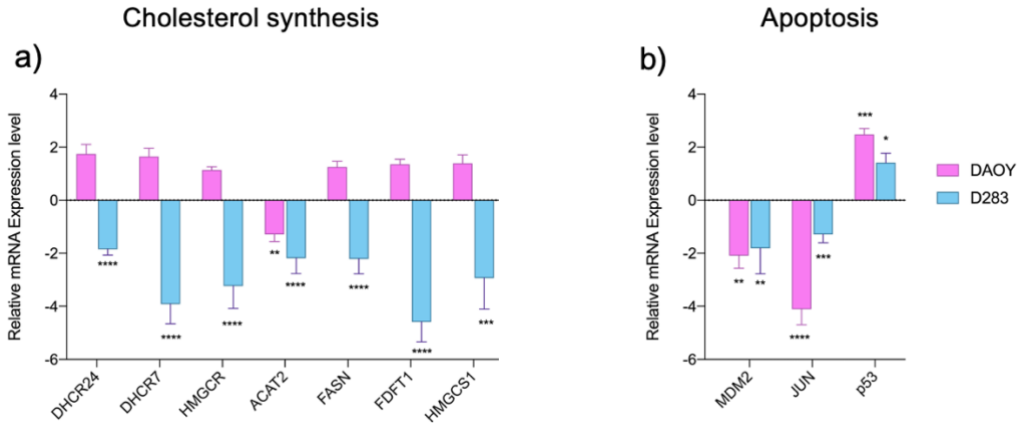

Figure 3.24 *Quantitative analysis of mRNA expression levels of genes of cholesterol synthesis and apoptotic pathway in medulloblastoma cell lines. The bars represent the average ± standard deviation (n = 3). Statistically significant difference from the control:*p<0.05, **p<0,01 ***p < 0.005, ****p < 0.001.*

3.11. Protein expression analysis

Modulation of apoptosis by genes present in the pathway has been confirmed also by protein expression analysis by means of western blot. As shown in **Fig. 3.25** MDM2 protein expression is decreased, as is the expression of phosphorylated c-JUN protein, the active form of c-JUN protein. Furthermore, p53 protein expression was found to be increased in all tumor lines tested.

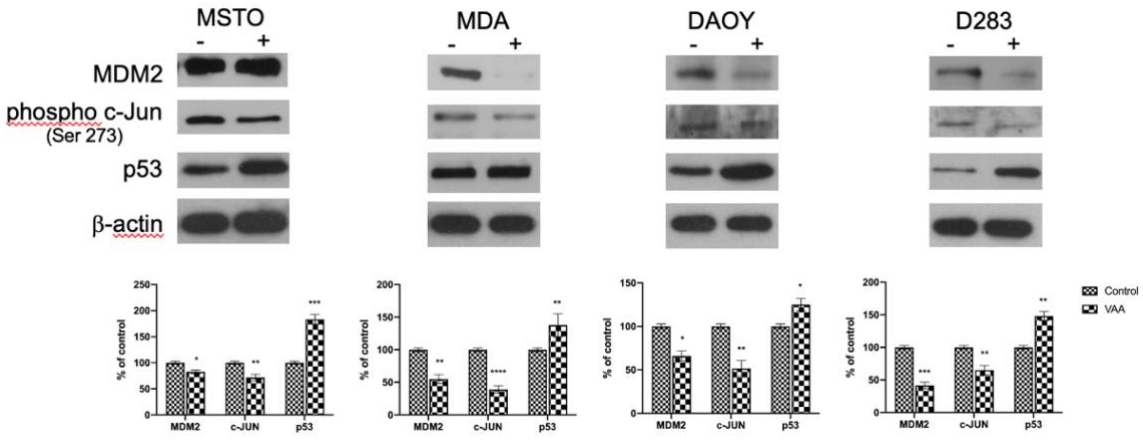

Figure 3.25 *Protein expression levels of MDM2, phosho c-JUN and p53 after AGS treatment analysed by Western blot analysis. Histograms report the expression of MDM2, phosho c-JUN and p53 normalized expression. β-Actin was used as loading control. The bars represent the average ± standard deviation (n = 3). Statistically significant difference from the control:*p<0.05, **p<0,01 ***p < 0.005, ****p < 0.001.*

Finally, the molecular apoptotic pathway activated after AGS treatment in the other tumoral lines was investigated by q-PCR. **Fig. 3.26** indicates that in MDA cells, there is modulation of BAX and BCL2 genes similarly to that found mesothelioma cell lines. Surprisingly, in medulloblastoma cells, BAX and BCL2 genes are not modulated in the same direction, but the expression of death receptor gene FAS, is modulated.

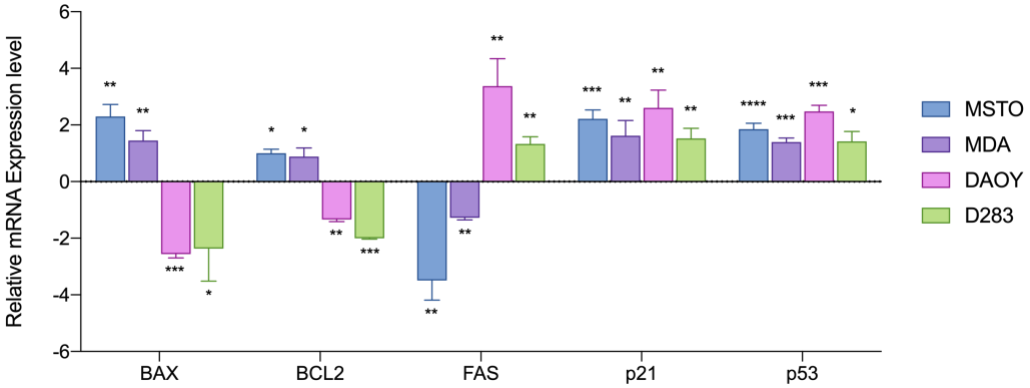

Figure 3.26 *Quantitative analysis of mRNA expression levels of Bax, Bcl-2, Fas, p21 and p53 after AGS treatment. The bars represent ± the average ± SD of independent experiments (n = 3). Statistically significant difference compared to untreated cells:*p<0.05, **p<0,01 ***p < 0.005, ****p < 0.001.*

Discussion

The development of alternative and more effective therapies in malignant mesothelioma is deemed urgent since to date the standard therapeutic modalities, including chemotherapy, surgery and radiotherapy have yielded un- satisfactory outcomes. The main objective of our research was to evaluate the anticancer efficacy of semi-polar extracts from peel and seed tissues of two Italian grape varieties, Aglianico and Falanghina, and to determine the mechanisms of action using different human mesothelioma cell lines. Our results suggest that Aglianico and Falanghina peel extracts do not exert proapoptotic effects on MM cells. This agrees with previous studies reporting only chemopreventive or adjuvant effect of peel extracts, mainly for the presence of resveratrol [40]. Additional studies are needed to validate if our peel extracts could act as adjuvant on MM cells when combined to chemotherapeutics. Our data also point out seeds as potential source of phytochemicals with anticancer activities in MM. Indeed, they were able to induce apoptosis in different MM cell lines, including those insensitive to standard chemotherapeutic treatments [37]. Semi-polar seed extracts from both Aglianico (AGS) and Falanghina (FGS) affected tumorigenic properties of human mesothelioma cells in a time- and dose-dependent manner, with Aglianico showing the strongest effect. Colony formation assay showed that FGS and AGS reduced the capacity of mesothelioma cells to form colonies, indicating that these extracts impair cellular self-renewal ability and proliferative cell rate. In addition, as assessed by a wound-healing assay, FGS

and AGS displayed the capacity to significantly inhibit cell migration capability.

We provided evidence that cell viability reduction after FGS and AGS treatments was due to apoptosis. Indeed, we found that FGS and AGS trigger a time–dependent increase of the BAX/BCL-2 ratio and of cytochrome c release in all mesothelioma cell lines analysed, with the highest threshold reached after the 24 + 24 h treatment. BAX and BCL-2 are two members of the BCL family with an opposite role in regulating mitochondrial function and cytochrome c release. Alterations of mitochondria homeostasis represent one of the main pathways involved in apoptosis. In non-apoptotic cells, BAX is mainly located in the cytosol, while BCL-2 is found in the mitochondrial membranes. Upon apoptosis induction, BAX translocates into mitochondria triggering permeabilization of mitochondrial outer membrane with cytochrome *c* release. The apoptotic factors are then released into the cytoplasm and transferred to the nucleus, where they induce apoptosis. The pro-survival BCL-2 acts as antiapoptotic protein by preventing mitochondrial outer membrane permeabilization and inhibiting the transfer of apoptosis-inducing factors to the nucleus. BAX/BCL-2 ratio regulates the mitochondrial intrinsic apoptosis pathway with subsequent cytochrome *c* release in the cytoplasm.

Several studies described the anticancer activity of grape seed standardized extracts against different human cancer cell lines such as breast, colon, lung and skin [41] due to the presence of specific metabolites residues (gallate esters, pyrogallol) [42-44]. Our *in vitro* study showed, for the first time, the effectiveness of complex semi-polar extracts from Aglianico and Falanghina

seeds, suggesting that a specific combination of active compounds characterizes a grape variety and influences its biological activity. Other studies using semipolar fractions extracted from mature red grape seeds showed that they affected cell viability and enhanced Flouracil toxicity of 5-FU in Caco-2 cells [44]. Therefore, to gain a first clue about the source of this bioactivity, we exploited high-resolution metabolomics to investigate the composition of the compounds accumulated in the peel and seed extracts of the two grape varieties. Overall, resulted highly enriched in proanthocyanidin molecules, which were present at very low levels or totally undetected in the peel samples of both varieties. Interestingly, Aglianico seeds displayed higher total proanthocyanidins. This finding agrees with the stronger inhibitory effect of Aglianico compared to Falanghina seeds on MM cell lines. Transcriptional data confirmed these results indicating that proanthocyanidins represent the main class of compounds accumulated in seed extracts.

With respect to the detected bioactivity of AGS and FGS extracts, metabolomics data suggest that phenylpropanoids, particularly proanthocyanidins, are responsible or at least greatly contribute to the anticancer activity; in this context, we cannot exclude that additional compounds may take part in this process, and future fractionation studies will be carried out to unravel the molecules whose accumulation is mostly associated to the bioactivity.

This is the first study indicating that grape seed semi-polar extracts are effective in MM cells. Moreover, studies on the molecular mechanisms identified that apoptotic intrinsic signalling pathways is involved. Our

findings will provide the reference for future analyses to identify the metabolic fraction/s mainly responsible/s for this bioactivity, thus to produce more effective extracts; in addition, this study will allow planning more detailed in vivo experiments relevant to the treatment of MM and possibly for clinical applications.

Analysis of transcriptomic pathways showed that the main pathways involved by AGS treatment were those of cholesterol biosynthesis. This result was extremely surprising, while we expected a major involvement of pathways related to the cell death of cancer cells. Cholesterol plays a fundamental role in the cellular structure, in fact it is an indispensable component of the cellular membrane, so its deficiency can cause serious dysfunction in the cells. p53 protein plays a key role in controlling the initiation and development of cancer by regulating cell cycle arrest, apoptosis, senescence, and DNA repair. MDM2 protein is a major negative regulator of p53 that inhibits the activity of p53 and decreases its protein stability [45]. The dysregulation of the p53-MDM2 pathway, involving p53 mutations or deletions and/or MDM2 amplification and overexpression, is the most commonly observed molecular modification in various human cancers [46-48]. c-JUN, the protein encoded by the JUN gene, blocks p53 activity, its over expression represses p53 expression and accelerates cell proliferation [49]. The connection between the two pathways is represented by the DHCR24 gene. This gene encodes for an enzyme involved in the long chain of cholesterol synthesis. This enzyme, 24-Dehydrocholesterol reductase catalyses the reduction of the delta-24 double bond of sterol intermediates during the final steps of cholesterol biosynthesis. Therefore, a

reduced expression of DHCR24 induces a consequent reduction of cholesterol synthesis [50]. On the other hand, the same DHCR24 protein blocks the activity of p53. DHCR24 acts in two different ways, in fact it is able to directly reduce the activation of p53 [51], preventing the acetylation of this protein, that in turn stabilizes and increases the activity of p53.

At the same time DHCR24 is also capable of preventing the binding between p53 and MDM2. This binding promotes the elimination of p53 by ubiquitination. It is reported that overexpression of DHCR24 reduces p53 levels [52]. Thus, in our case, a reduction in DHCR24, MDM2, and JUN leads to an over expression of the onco-suppressor p53, a very common mechanism of action observed in treatment with natural products against cancer [53]. What is very interesting is that the effect of grape seed treatment is the same even in Mes2 mesothelioma cells that are resistant to standard chemotherapy treatment due to the lack of p21.

The extraction protocol from grape seeds developed in the laboratory allowed us to obtain a very small amount of extract. During the period in the company, I was able to study the process of industrial production of another product obtained from grape seeds, grape seed oil, an oil marketed and used in cooking. This allowed me to see what the commonalities were between the laboratory extraction protocol and the industrial process, and how the latter could be adapted to obtain a product as similar as possible to the laboratory extract but on an industrial scale.

Having studied the production process, we investigated the biological effects of grape seed oil on cells and compared with effect of AGS. We have seen that oil is capable of reducing viability in both mesothelioma and breast

cancer cells, but that unlike AGS this reduction in viability is not due to induction of the apoptotic process.

Modulation of gene expression in the integrated network is also less effective than treatment with AGS. These results could be due to the fact that the oil we used is a commercial product produced from grape seeds of different cultivars, as we have previously seen different cultivars such as Aglianico and Falanghina have different biological effects. Therefore, it would be appropriate for future research to use an oil produced only from Aglianico seeds.

Results obtained with medulloblastoma cells have confirmed that treatment with AGS reduces viability and induces apoptosis also in other tumour types, leading us to suppose that the mechanism of action is also valid for other tumour types and not linked to some typical feature of a single tumour type. This assumption has been validated by modulation of gene expression of the apoptosis pathway in medulloblastoma cells. In fact, while the cholesterol synthesis pathway is only modulated in certain tumour types, the pathway leading to p53 activation is conserved in all cell lines tested. Further confirmation is the modulation of protein expression level in all the lines analysed, where there is a reduction in p53 inhibitors, MDM2 and JUN, and an increase in p53 itself.

The expression of BAX, BCL2 and FAS genes in the different cell lines highlights even more the central role of p53. In fact, if the apoptotic process is triggered by different pathways in different cells, all of them converge in the activation of p53.

Chapter 2

Polyphenols from Ginkgo biloba extract

Materials and methods

4.1. Cell Culture and Chemicals

Human neuroblastoma cell line, SK-N-BE(2) (CRL-2271, ATCC®, LGC Standards S.r.l., Milan Italy) were cultured at 37 °C in a 5% CO_2 humidified incubator in either RPMI-1640 medium (Euroclone spa, 20016 Pero, MI) supplemented with 10% fetal bovine serum (FBS), glutamine (2 mM), sodium pyruvate and antibiotics (0.02 mg/mL streptomycin and 0.02 IU/mL penicillin).

Ginkgo biloba L. extract EGb 761 (EGb) was a gift from Schwabe (Schwabe Pharma Italia Srl, Egna, Italy). EGb stock solution contained 250 mg/mL of extract was dissolved in dimethyl sulfoxide (DMSO). Hydrogen peroxide (H_2O_2) (Sigma-Aldrich, St. Louis, MI, USA) was used as oxygen stress inducer.

4.2. Cell Proliferation Assay

For each experiment, approximately 1.5×10^5 cells/well in 6-well plates were plated and treated as described and untreated cells were used as control. To identify the H_2O_2 concentration able to determine about 50% of viability decrease, SK-N-BE cells were treated with 25, 50, 75, and 100 mM of H_2O_2 for 24 h. When specified cells were treated with 25 µg/mL EGb for 24 h, the medium was replaced before H_2O_2 treatment.

To evaluate the effect of EGb on cell viability, cells were treated with 10, 25, and 50 µg/mL for 24 h. To estimate the protective effect of EGb cells were treated for 24 h with 25 µg/mL of EGb, then insulted with 75 µM of H_2O_2 for

additional 24 h. EGb was dissolved in DMSO. Untreated samples were exposed to 0.1% DMSO and were used as control.

For each experiment after treatment cells were collected and counted with Trypan Blue solution. (T6146, Sigma-Aldrich, St. Louis, MI, USA).

All the experiments were performed in triplicate. Data are expressed as the mean ± SD.

4.3. Propidium Iodide and DAPI Staining Assay

In 6-well plates approximately 1.5×10^5 cells/well were plated and treated with EGb and H_2O_2 as previously described. After treatment cells were stained with 10 mg/mL of Propidium Iodide (PI) (Bioshop, Burlington, ON L7L 6A4, Canada) and DAPI (4′,6-diamidine-2′-phenyl indole dihydro chloride, Roche, Mannheim, Germany). Representative images were taken using fluorescent microscope (DMI8, Leica, Instruments, Germany) and florescence was quantified using Leica Application Suite X software (Leica, Milan, Italy). All the experiments were performed in triplicate. Data are expressed as the mean ± SD.

4.4. Mitochondria Membrane Potential Measurement

Mitochondria membrane potentials (MMP) were measured by JC-10 (Sigma-Aldrich, St. Louis, MI, USA) following the manufacturer's instructions. Loss of MMP was indicated by a progressive JC-10 dislocation from mitochondria to the cytosol. Cells were photographed using fluorescent microscope (DMI8, Leica, Instruments, Germany). Red (540/570 nm) and green (485/534 nm) florescence was quantified by Leica Application Suite X (LAS

X) (Leica, Milan, Italy). All the experiments were performed in triplicate. Data are expressed as the mean ± SD.

4.5. RNA Extraction and q-PCR

RNA extraction and q-PCR were essentially performed as previously described. Brefly, Total RNA was isolated from each sample with Trizol (Thermo Fisher Scientific, Waltham, MA USA), as indicated by manufacturer. For each sample to analyse, cDNA was than obtained starting from 200 ng of total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Thermo Fisher Scientific, Waltham, MA USA). The described selected genes using gene specific primers BAX: Forward 5′-TTTGCTTCAGGGTTTCATCCA-3′: Reverse 5′- CTCCATGTT-ACTGTCCAGTTCGT-3′; BCL- 2: Forward 5′-GTTCCCTTTC-CTTCCATCC-3′; Reverse 5′-TAGCCAGTCCAGAGGTGAG-3′; p53: Forward 5'-TCTGTCCCTTCCCAGAAAACC-3'; Reverse 5'-CAAGAA-GCCCAGACGGAAAC-3′; GAPDH: Forward 5′-CAAGGCT-GTGGGC-AAGGT-3′; Reverse 5′-GGAA GGCCATGCCAGTGA-3'.

All primers were selected using a specific software (Primer express 2.0, Applied Biosystems, Foster city, CA, USA) and all of them specifically covered the exon-exon junctions. The analysis of gene expression was done as described in [37] and GAPDH gene was used as internal control. q-PCRs were done using the 7900 HT Real Time PCR (Applied Biosystem) and for each experimental condition a triplicate was performed. Data obtained are expressed as the mean ± SD.

4.6. Western Blot

For each experimental condition and from each sample total, protein extracts were obtained, as described in [54]. For the analysis, 20 μg of each sample were loaded on Tris–glycine gradient gels (4% to 15% gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and separated at 100 V. To probe proteins with specific primary antibodies antibodies, they were transferred to PVDF membranes (Biorad Laboratories, Inc., Hercules, CA, USA). All the secondary antibodies used were horseradish peroxidase conjugated. All the antibodies were used as indicated by manufacturer. The following primary antibodies were used for Western blot: PARP (Cell Signaling, #9542), BCL-2 (Abcam, ab182858), BAX (Santa Cruz Biotechnology, sc-493), Acetyl-p53 Lys382 (Cell Signaling, #9542). As the internal control we used β-Actin (Cell Signaling, #3700), which was used as the loading control. To detect protein levels, Clarity western ECL (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used. The quantization was then obtained using ImageJ software vJ1, an open source tool. For each experimental condition a triplicate was performed, and results are expressed as the mean ± SD.

4.7. Statistical Analysis

To perform calculations on sample size the on line available software GPower was used. Sample size was determined using as parameters: $1 - \beta = 0.80$, $\alpha = 5\%$. For each experiment, statistical analysis was done using Graph Pad Prism 9.0 (GraphPad Software, San Diego, CA, USA) to analyse the significance of the differences between control and treatments. We evaluated the differences among means applying the one-way ANOVA. Bonferroni's

multiple comparison test with Bonferroni post hoc correction was used to analyse the differences of each treatment respect to the control.

Statistically significant difference compared to DMSO treated cells are: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Results

5.1. EGb Protects SK-N-BE Cells Against Oxidative Stress Induced Apoptotic Cell Death

We first determined, in a dose-response curve at 24 h, the amount of H_2O_2 that had lethal effect on SK-N-BE human neuroblastoma cells. Oxidative stress induced cell death was around 50% when cells were treated with 75 µM H_2O_2 (**Fig 5.1**).

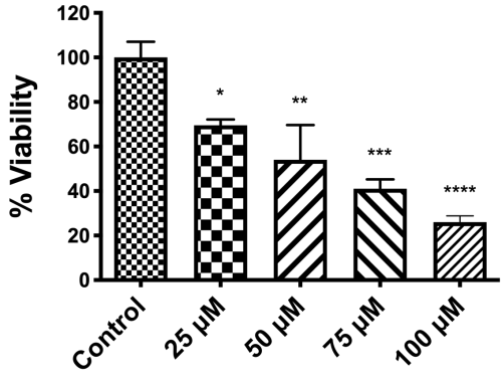

Figure 5.1 *H_2O_2 affects SK-N-BE cell viability. Cell viability decrease after treatments with different concentration of H_2O_2. The bars represent ± the average ± SD of independent experiments (n = 3). Statistically significant difference compared to control cells: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.*

To verify if 75 µM H_2O_2 was able to induce apoptosis on SK-N-BE cells, untreated and H_2O_2 treated cells, without fixation and permeabilization, were stained with Annexin V - FITC and PI. The analysis by fluorescence microscopy confirmed that H_2O_2, at this concentration, induced apoptosis

(**Fig 5.2 a**). Indeed, a strong increased number of stained Annexin V - FITC and PI cells were present when cells were treated with H_2O_2 (**Fig. 5.2 b**).

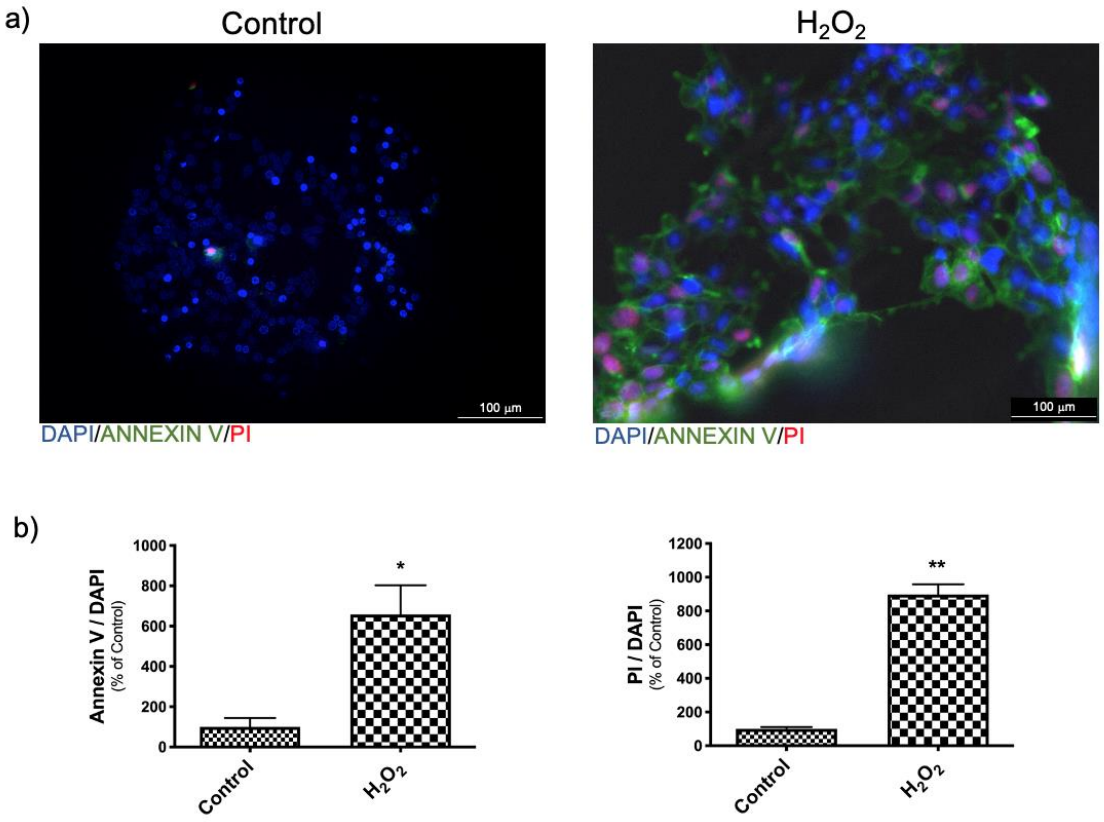

Figure 5.2 *Representative images of DAPI, Annexin V-FITC and PI triple fluorescence staining showing cellular apoptosis after H_2O_2 treatment. DAPI: blue; Annexin V: green; PI: red. Histograms reports quantification of fluorescence of DAPI, Annexin V, and PI. The bars represent ± the average ± SD of independent experiments (n = 3). Statistically significant difference compared to control cells: * $p \leq 0.05$, ** $p \leq 0.01$.*

To ascertain that EGb did not induce cell death, SK-N-BE cells were treated with various concentrations of EGb for 24 h. Results showed that EGb at all used concentrations did not reduce cell viability (**Fig 5.3**).

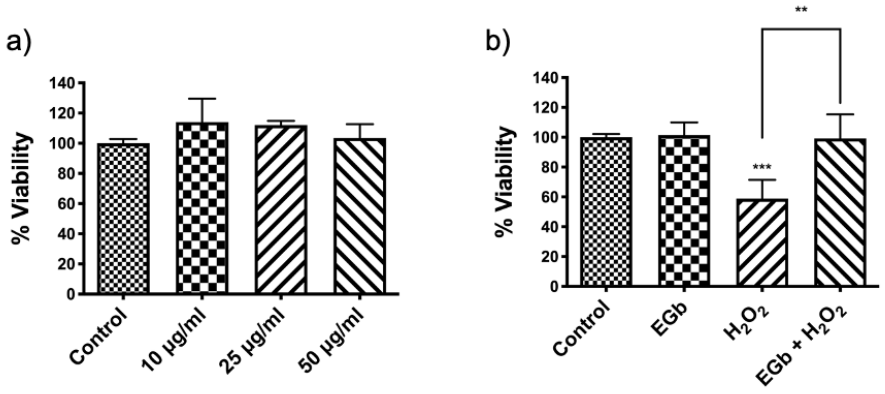

Figure 5.3 *Effects of EGb on cell viability. Cell viability after treatments with different concentration of EGb. The bars represent ± the average ± SD of independent experiments (n = 3). Statistically significant difference compared to control cells: ** $p \leq 0.01$, *** $p \leq 0.005$.*

To determine whether EGb played a role in protecting SK-N-BE from H_2O_2-induced cell death, cells were pre-treated for 24 h with EGb (25 µg/mL) and then challenged with H_2O_2 (75 µM) for the following 24 h. Analysis of cell vitality revealed that the oxidant sensitivity of SK-N-BE cells was completely reverted by pre-treatment with EGb (**Fig 5.4**).

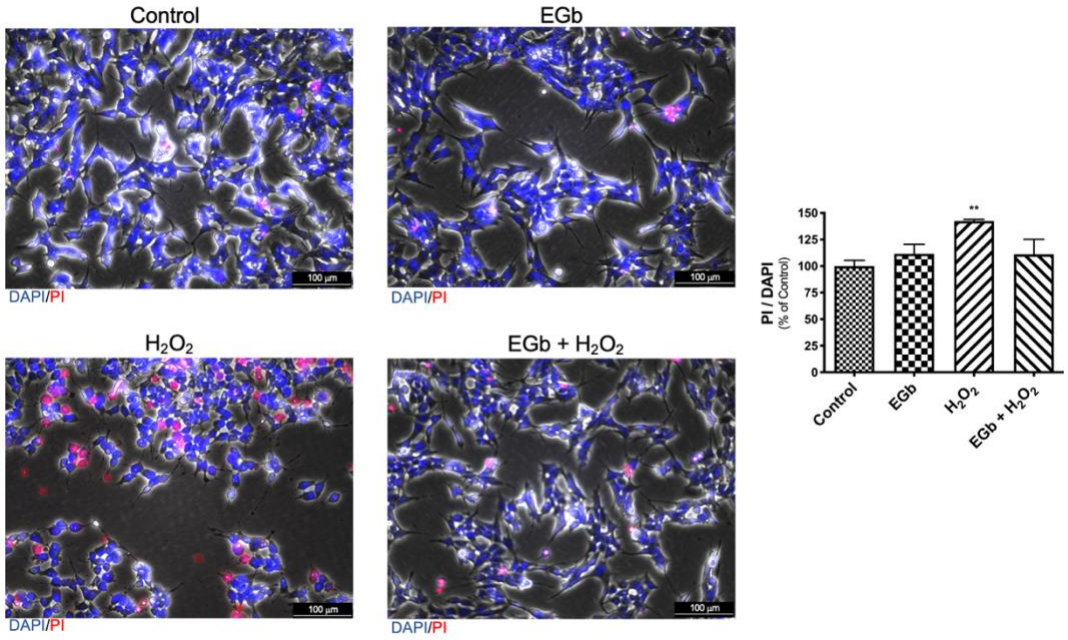

Figure 5.4 *Cell viability after treatments with 25 mg/mL of EGb, 75 mM of H_2O_2 or a combination of them. Cells treated with DMSO were used as control. Fluorescent microscopic image of DAPI/PI stained cells; DAPI: blue; PI: red. Histogram reports quantification of fluorescence of DAPI and PI. The bars represent ± the average ± SD of independent experiments (n = 3). Statistically significant difference compared to control cells: ** $p \leq 0.01$*

Concomitant addition of EGb and H_2O_2 or addition of EGb 24 h later H_2O_2 treatment did not result in a reversion of lethality. Results were confirmed by analysis with PI and DAPI staining, as shown by fluorescence microscopy analysis. Indeed, a comparable number of the PI positive cells were present in untreated, EGb treated and EGb- H_2O_2 treated cells, where a higher number were present in presence of H_2O_2 alone.

5.2. EGb Protects SK-N-BE Cells Against Oxidative Stress Induced Apoptosis

To confirm that EGb could protect cells against apoptotic cell death induced by oxidative stress, we first analysed the presence of poly(ADP-ribose) polymerase (PARP) cleavage, an hallmark of apoptosis. As expected, PARP cleavage increased after H_2O_2 treatment, although the cells were completely protected from oxidative stress-induced apoptosis in presence of EGb (**Fig 5.5**). Then, to study the protective mechanism of EGb against oxidative stress-induced apoptosis, we investigated the molecular signalling pathway involved in the apoptotic cell death analysing p53 expression.

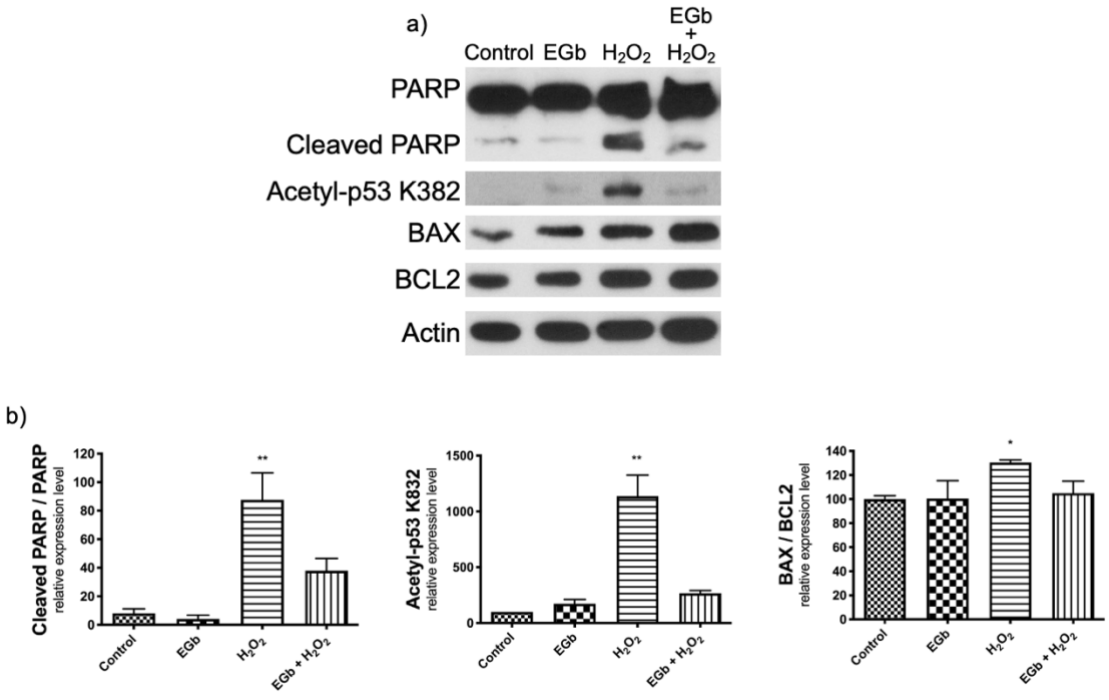

Figure 5.5 *EGb protects SK-N-BE from apoptosis. Western blot analysis of protein expression of cleaved PARP, Acetylated-p53/K382, BAX and Bcl2 in SK-N-BE cells after treatments (a). expression of Acetylated-p53/K382, cleaved PARP and BAX/Bcl2*

*ratio normalized expression is reported in the histograms (b). β-Actin was used as loading control. The bars represent ± the average ± SD of independent experiments (n = 3). Statistically significant difference compared to control cells: * p ≤ 0.05, ** p ≤ 0.01. Cells treated with DMSO were used as control.*

The tumour suppressor protein p53, modulating cell homeostasis, has a determinant role in cell fate.

Oxidative stress, leading to post-translational modifications of p53, allows it to regulate genes that can activate cell survival or cell death processes [25].

Gene expression analysis, by q-PCR, revealed that p53 was not modulated by oxidative stress as well as by EGb (**Fig 5.6**).

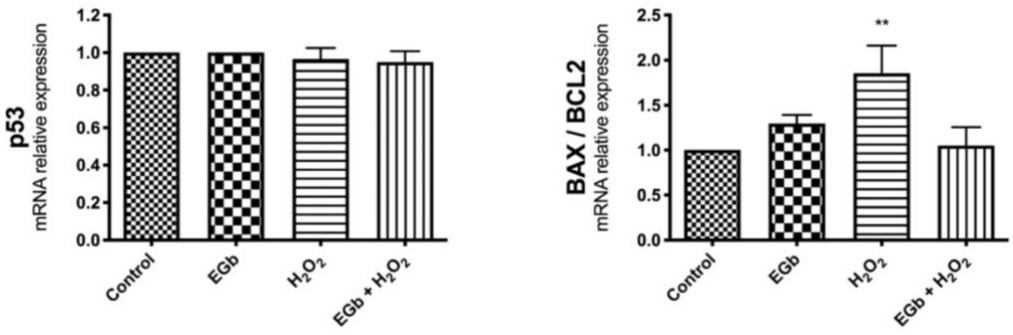

Figure 5.6 *Quantitative analysis of mRNA expression levels of p53, BAX and Bcl-2 in SK-N-BE cells after treatments. Histograms report the expression of p53 and BAX/Bcl2 ratio. The bars represent ± the average ± SD of independent experiments (n = 3). Statistically significant difference compared to control cells: **p ≤ 0.01. Cells treated with DMSO were used as control.*

It is known that increased p53-acetylation at lysine 382 (K382) promotes p53-dependent pro-apoptotic activity in cancer cells [55]. Thus, we analysed by western blot analysis whether these post-translational modifications of

p53 could account for the apoptotic reduction observed in presence of EGb. Results clearly demonstrate that K382 acetyl-p53 was strongly increased following H_2O_2 insult, however p53-acetylation was inhibited in presence of EGb (**Fig 5.5**).

Oxidative stress activates the mitochondrial intrinsic pathway of apoptosis [56]. p53, interacting with members of the Bcl-2 family, directly participating to the activation of the intrinsic apoptosis pathway [56]. We focused our attention on the ratio between two members of the Bcl-2 family, BAX and BCL-2, which are markers of cell susceptibility to intrinsic apoptosis. Protein expression analysis evidenced an increased BAX/Bcl-2 ratio in H_2O_2 treated SK-N-BE while pre-treatment with EGb restored a normal ratio (**Fig 5.5**). These results were also confirmed by gene expression analysis, by q-PCR of the corresponding genes (**Fig. 5.6**).

5.3. EGb mitigated the H_2O_2 induced decrease in mitochondrial membrane potential

Increased BAX/Bcl-2 ratio suggested that mitochondria are involved in apoptosis. Indeed, it is well known that, during intrinsic apoptosis, the mitochondrial membrane potential (MMP) collapses, triggering other downstream events in the apoptotic cascade. Thus, we investigated by JC-10 assay the change of MMP following H_2O_2 or EGb treatment of SK-N-BE cells. Results showed that untreated cells displayed intact, well-polarized mitochondria marked by a red punctate fluorescence. On contrary, H_2O_2 treated cells showed a reduction of the red fluorescence and an increase of the green one, indicating loss of MPM because of the progressive JC-10

dislocation from mitochondria to the cytosol. On the contrary, EGb treatment restored the fluorescence to the values of untreated cells. (**Fig 5.7**).

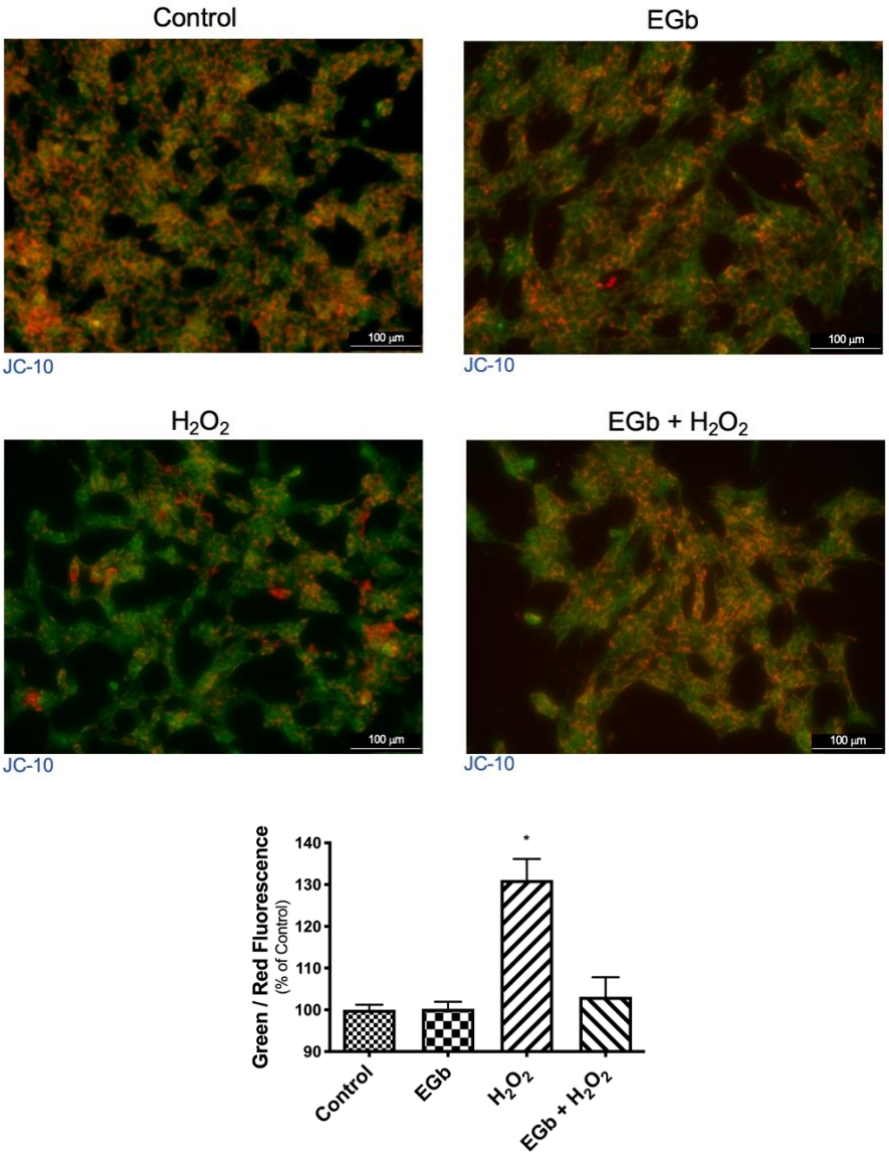

Figure 5.7 *EGb reduces the decrease of mitochondrial membrane potential. Fluorescence analysis of mitochondria in control or EGb treated cells with or without H_2O_2. Histogram reports quantification of fluorescence of Red (540/570 nm) and green (485/534 nm). The bars represent ± the average ± SD of independent experiments (n =*

*3). Statistically significant difference compared to control cells: * $p \leq 0.05$). Cells treated with DMSO were used as control.*

5.4. EGb exhibits intracellular anti-apoptotic effect

To verify whether EGb acts as antioxidant into cells, or if it was able to directly scavenge H_2O_2 in the culture medium, SK-N-BE cells were treated for 24 h with EGb, then the culture medium was replaced and cells were challenged with H_2O_2. Results showed that that EGb determined antioxidant protection on cell viability independently by its presence in the culture medium. In fact, pre-treatment with EGb was per se sufficient to attenuate the H_2O_2-induced cell death in SK-N-BE cells (**Fig 5.8**).

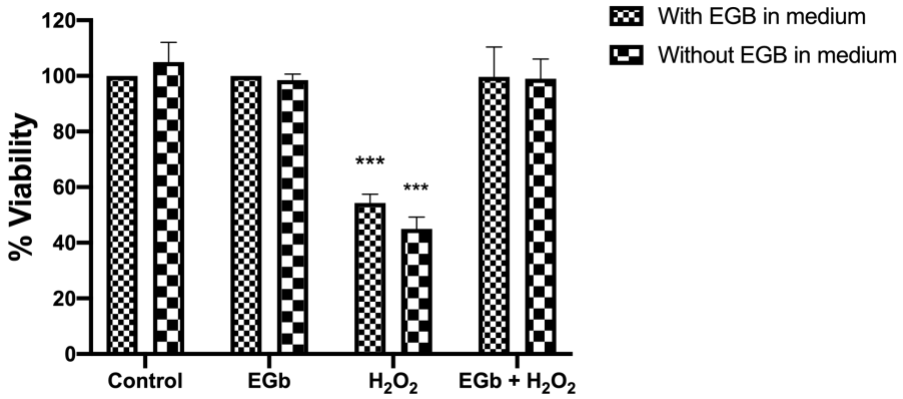

Figure 5.8 *Intracellular effect of EGb. Cell viability analysis of SK-N-BE with or without EGb in the medium after H_2O_2 oxidative insult. The bars represent ± the average ± SD of independent experiments (n = 3). Statistically significant difference compared to control cells: *** $p \leq 0.005$). Cells treated with DMSO were used as control.*

Moreover, in these conditions we observed a reduced cleavage of PARP protein, a reduced amount of K382 acetyl-p53 and a reduced BAX/Bcl-2 ratio

(**Fig 5.9**), confirming that EGb was able to protect SNKBE cells by apoptotic cell death exerting an intracellular antioxidant action.

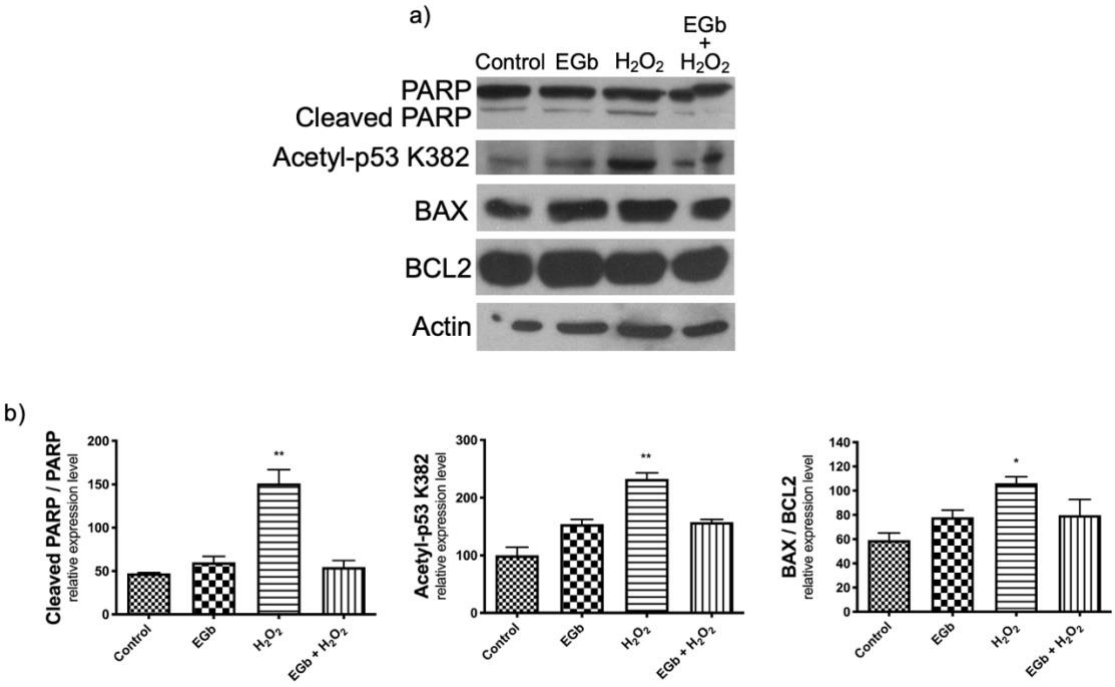

Figure 5.9 *Protein expression analysis of cleaved PARP, Acetylated-p53/K382, BAX and Bcl2 in SK-N-BE cells after treatments by Western blot analysis (a). Histograms report the expression of Acetylated-p53/K382, cleaved PARP and BAX/Bcl2 ratio normalized expression (b). β-Actin was used as loading control. The bars represent ± the average ± SD of independent experiments (n = 3). Statistically significant difference compared to control cells: * $p \leq 0.05$, ** $p \leq 0.01$). Cells treated with DMSO were used as control.*

Discussion

The incidence of neurological disorders—the most dreaded maladies of older people—are expected to increase over the next few decades due to prolonged life expectancy [57]. To date, more than 1 in 10 individuals over 65 years are affected by neurodegenerative diseases and the numbers will continue to increase with age. Until now, no effective treatments have been described to cure these diseases and the costs for their management represent one of the leading medical and societal challenges faced by society [58]. For these reasons recent investigations have been focused to understanding their pathogenesis and to the development of novel therapeutics. *Ginkgo biloba*, a plant that has been used for thousands of years, is considered one of the more promising natural drugs. The extracts, obtained from *Ginkgo biloba* leaves, have been recently used also in clinical studies.

Most of the studies on the EGb concern its neuroprotective effects, against ageing. Standardized *Ginkgo biloba* extracts (EGb) have been used for treatment and prevention of different neurological disorders as Alzheimer's disease [21,22], Parkinson's disease [23,59], cerebral vascular deficit, and dementia [24,60].

Indeed, the brain is especially sensitive to the effects of ageing. This tissue being primarily composed of postmitotic cells—neurons and oligodendrocytes—is more vulnerable than proliferating cells to macromolecular damages, especially to DNA [61]. DNA damages in neurons accumulate from development throughout life. To escape this process, postmitotic neurons adopt selective mechanisms aimed to specifically repair genes actively transcribed [62].

Oxidative stress is one of the main causes of neural damage. EGb exerts neuroprotective action mainly acting as free radical–scavenger In fact, EGb is able to reduce the endogenous and the induced levels of ROS [63]. Moreover, EGb can directly upregulate antioxidant enzymes such as superoxide dismutase and catalase [64]. This activity is linked to the chemical structure of the flavonoids that allow to not only react and directly scavenge the hydroxyl radicals, but also to inhibit the formation of new hydroxyl radicals [65]. It is well known that oxidative stress determines the activation of the apoptotic processes, thus playing a pivotal role in most of neurological diseases. EGb can act on multiple cellular pathways with the final goal to balance the existing apoptotic machinery. In fact, EGb prevents mitochondrial membrane damage reducing the release of cytochrome c from the mitochondria, upregulates the antiapoptotic protein Bcl-2-and inhibit PARP cleavage [66].

The neuroprotective effect of EGb 761 has been reported in different neuronal cell lines in which it acts by inhibiting oxidative stress induced apoptosis [67-69] or the activation of mitochondrial intrinsic apoptosis [67,68] . A recent *in vivo* study reported that EGb 761 protected from brain injury by suppressing neuronal apoptosis [14]. Moreover, some studies reported the protective effect of *Ginkgo biloba* extract in people affected by neurodegenerative diseases [70,71].

In this study we analysed the protective effect of EGb on oxidative stress-induced apoptosis in SK-N-BE cells with the aim to unravel the molecular pathway in which EGb acts as antioxidant. Human neuroblastoma cell line N-type have neuronal morphology [72] and have been commonly used as

model for research in neuroscience and in particular in studies related to oxidative stress and neurodegenerative diseases [33-35,73].

Our results demonstrated that the standardized extract EGb 761 significantly protected neuroblastoma cells from oxidative stress blocking apoptosis in a p53-dependent pathway. Interestingly, according to previous studies we found that EGB was able to inhibit p53 acetylation at lysine 382. It is known that p53 activity depends on the acetylation of specific lysine [74]. In addition, the acetylation of the C-terminal K382 lysine is crucial for p53 activation [75] since it results in the activation of PUMA promoter—a member of Bcl-2 family [76]. PUMA, promoting BAX multimerization and mitochondrial translocation, induces apoptosis [77]. Accordingly, our results show that EGb protects against mitochondrial membranes depolarization with a consequent reduction of BAX/Bcl-2 ratio. These results were supported by reduction of PARP cleavage with increased viability.

Previous studies reported that *Gingko biloba* extracts in cancer cells is able to induce apoptosis in a p53-dependent pathway by increasing the levels of p53 acetylation that, in turn, determines cell cycle arrest and apoptosis. On the contrary, our results demonstrated that the standardized extract EGb 761 significantly protected neuroblastoma cells from oxidative stress blocking apoptosis in a p53-dependent pathway. These results claim the different activity of EGb when used as neuroprotective or as anticancer drug [78].

Conclusion

During this PhD period, my studies have demonstrated that hydro-alcoholic extracts of Aglianico and Falanghina grape seeds induce reduction of key tumorigenic characteristics of mesothelioma cells, such as viability, proliferation, and migration capacity. All the analysis performed determined that treatment with both extracts induces apoptosis in different mesothelioma cell lines via the intrinsic apoptosis pathway, by modulating proteins that regulate mitochondrial membrane permeability and by promoting cytochrome *c* leakage into the cytoplasm.

More interestingly, transcriptome analysis identified that the cholesterol biosynthesis pathway is the pathway most affected by Aglianico extract treatment and that it is linked to the p53-mediated apoptosis induction pathway.

AGS treatment also reduces cell viability and induces apoptosis in different tumour cell lines such as breast cancer and medulloblastoma cell lines. Also, in these cells a key role is represented by activation of the onco-suppressor p53 and by downregulation of its inhibitors, MDM2 and JUN.

As a continuation of the work, it would be important to test the efficacy of Aglianico extract also *in vivo* using a mouse tumour model. If the efficacy will be proved that it would be appropriate to fractionate the extract to identify the more active fraction responsible of the biological activity.

Neurodegenerative disorders include a range of conditions that share common degenerative pathways, although they manifest clinical differences. Increased oxidative stress has been described in almost all neurodegenerative disorders. In neurons, imbalance between the accumulation of free radicals and antioxidant defences seems to be the link between cell death and progression of neurodegenerative diseases. Oxidative stress can trigger apoptosis in neuronal cells and excessive death of one or more populations of neurons, resulting in a neurodegenerative disease [79].

Our data suggest that EGb 761 blocking the onset of p53-dependent apoptotic pathway induced by oxidative stress, could be considered as antioxidant nutraceutical to be potentially used for the prevention and treatment of neurodegenerative diseases. This hypothesis could be strengthened with a larger number of randomized clinical trials.

These studies unravel two possible uses of polyphenols. In fact, if grape seed extracts are able to induce apoptosis in cancer cells, on the other hand polyphenols of different origin, as those extracted from *Ginkgo biloba* leaves, are able to reduce the effects of oxidative stress and could prevent neurodegenerative diseases.

Polyphenols of natural origin are many and can have very different actions. This is the reason why they represent a good future perspective for the treatment of many diseases and disorders. Moreover, they are safe and have a very low toxicity, so, it is easy to test their efficacy in pre-clinical and clinical studies.

References

1. Di Meo, F.; Donato, S.; Di Pardo, A.; Maglione, V.; Filosa, S.; Crispi, S. New Therapeutic Drugs from Bioactive Natural Molecules: The Role of Gut Microbiota Metabolism in Neurodegenerative Diseases. *Curr Drug Metab* **2018**, *19*, 478-489, doi:10.2174/1389200219666180404094147.

2. Di Meo, F.; Margarucci, S.; Galderisi, U.; Crispi, S.; Peluso, G. Curcumin, Gut Microbiota, and Neuroprotection. *Nutrients* **2019**, *11*, doi:10.3390/nu11102426.

3. Di Meo, F.; Cuciniello, R.; Margarucci, S.; Bergamo, P.; Petillo, O.; Peluso, G.; Filosa, S.; Crispi, S. Prevents Oxidative Stress-Induced Apoptosis Blocking p53 Activation in Neuroblastoma Cells. *Antioxidants (Basel)* **2020**, *9*, doi:10.3390/antiox9040279.

4. Hosu, A.; Cristea, V.M.; Cimpoiu, C. Analysis of total phenolic, flavonoids, anthocyanins and tannins content in Romanian red wines: prediction of antioxidant activities and classification of wines using artificial neural networks. *Food Chem* **2014**, *150*, 113-118, doi:10.1016/j.foodchem.2013.10.153.

5. Khoo, H.E.; Azlan, A.; Tang, S.T.; Lim, S.M. Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits. *Food Nutr Res* **2017**, *61*, 1361779, doi:10.1080/16546628.2017.1361779.

6. Weidner, S.; Rybarczyk, A.; Karamać, M.; Król, A.; Mostek, A.; Gręboszz, J.; Amarowicz, R. Differences in the phenolic composition and antioxidant properties between Vitis coignetiae and Vitis vinifera seeds extracts. *Molecules* **2013**, *18*, 3410-3426, doi:10.3390/molecules18033410.

7. Xia, E.Q.; Deng, G.F.; Guo, Y.J.; Li, H.B. Biological activities of polyphenols from grapes. *Int J Mol Sci* **2010**, *11*, 622-646, doi:10.3390/ijms11020622.

8. Higgins, J.A.; Zainol, M.; Brown, K.; Jones, G.D. Anthocyans as tertiary chemopreventive agents in bladder cancer: anti-oxidant mechanisms and interaction with mitomycin C. *Mutagenesis* **2014**, *29*, 227-235, doi:10.1093/mutage/geu009.

9. Márquez Campos, E.; Stehle, P.; Simon, M.C. Microbial Metabolites of Flavan-3-Ols and Their Biological Activity. *Nutrients* **2019**, *11*, doi:10.3390/nu11102260.

10. Teng, H.; Chen, L. Polyphenols and bioavailability: an update. *Crit Rev Food Sci Nutr* **2019**, *59*, 2040-2051, doi:10.1080/10408398.2018.1437023.

11. Aslam, S.; Jahan, N.; Rahman, K.; Zafar, F.; Ashraf, M.Y. Synergistic interactions of polyphenols and their effect on antiradical potential. *Pak J Pharm Sci* **2017**, *30*, 1297-1304.

12. Nandakumar, V.; Singh, T.; Katiyar, S.K. Multi-targeted prevention and therapy of cancer by proanthocyanidins. *Cancer Lett* **2008**, *269*, 378-387, doi:10.1016/j.canlet.2008.03.049.

13. Mittal, A.; Elmets, C.A.; Katiyar, S.K. Dietary feeding of proanthocyanidins from grape seeds prevents photocarcinogenesis in SKH-1 hairless mice: relationship to decreased fat and lipid peroxidation. *Carcinogenesis* **2003**, *24*, 1379-1388, doi:10.1093/carcin/bgg095.

14. Yu, T.; Fan, Y.; Xu, Y.; Xu, L.; Xu, G.; Cao, F.; Jiang, H. Standardized Ginkgo biloba extract EGb 761(R) attenuates early brain injury following subarachnoid hemorrhage via suppressing neuronal apoptosis through the activation of Akt signaling. *Biomed Pharmacother* **2018**, *107*, 329-337, doi:10.1016/j.biopha.2018.08.012.

15. Zhou, G.; Ma, J.; Tang, Y.; Wang, X.; Zhang, J.; Duan, J.A. Multi-Response Optimization of Ultrasonic Assisted Enzymatic Extraction Followed by Macroporous Resin Purification for Maximal Recovery of Flavonoids and Ginkgolides from Waste. *Molecules* **2018**, *23*, doi:10.3390/molecules23051029.

16. Chávez-Morales, R.M.; Jaramillo-Juárez, F.; Rodríguez-Vázquez, M.L.; Martínez-Saldaña, M.C.; Del Río, F.A.P.; Garfias-López, J.A. The Ginkgo biloba extract (GbE) protects the kidney from damage produced by a single and low dose of carbon tetrachloride in adult male rats. *Exp Toxicol Pathol* **2017**, *69*, 430-434, doi:10.1016/j.etp.2017.04.003.

17. Eckert, A.; Keil, U.; Scherping, I.; Hauptmann, S.; Muller, W.E. Stabilization of mitochondrial membrane potential and improvement of neuronal energy metabolism by Ginkgo biloba extract EGb 761. *Ann N Y Acad Sci* **2005**, *1056*, 474-485, doi:10.1196/annals.1352.023.

18. Chen, C.; Wei, T.; Gao, Z.; Zhao, B.; Hou, J.; Xu, H.; Xin, W.; Packer, L. Different effects of the constituents of EGb761 on apoptosis in rat cerebellar granule cells induced by hydroxyl radicals. *Biochem Mol Biol Int* **1999**, *47*, 397-405, doi:10.1080/15216549900201423.

19. Gohil, K.; Packer, L. Bioflavonoid-rich botanical extracts show antioxidant and gene regulatory activity. *Ann N Y Acad Sci* **2002**, *957*, 70-77, doi:10.1111/j.1749-6632.2002.tb02906.x.

20. Zhou, H.C.; Tam, N.F.Y.; Lin, Y.M.; Ding, Z.H.; Chai, W.M.; Wei, S.D. Relationships between Degree of Polymerization and Antioxidant Activities: A Study on Proanthocyanidins from the Leaves of a Medicinal Mangrove Plant Ceriops tagal. *Plos One* **2014**, *9*, doi:ARTN e107606
10.1371/journal.pone.0107606.

21. Rapp, M.; Burkart, M.; Kohlmann, T.; Bohlken, J. Similar treatment outcomes with Ginkgo biloba extract EGb 761 and donepezil in Alzheimer's dementia in very old age: A retrospective observational study. *Int J Clin Pharmacol Ther* **2018**, *56*, 130-133, doi:10.5414/CP203103.

22. Kaur, N.; Dhiman, M.; Perez-Polo, J.R.; Mantha, A.K. Ginkgolide B revamps neuroprotective role of apurinic/apyrimidinic endonuclease 1 and mitochondrial oxidative phosphorylation against Abeta25-35 - induced neurotoxicity in human neuroblastoma cells. *J Neurosci Res* **2015**, *93*, 938-947, doi:10.1002/jnr.23565.

23. Vijayakumaran, S.; Nakamura, Y.; Henley, J.M.; Pountney, D.L. Ginkgolic acid promotes autophagy-dependent clearance of intracellular alpha-synuclein aggregates. *Mol Cell Neurosci* **2019**, *101*, 103416, doi:10.1016/j.mcn.2019.103416.

24. Kandiah, N.; Ong, P.A.; Yuda, T.; Ng, L.L.; Mamun, K.; Merchant, R.A.; Chen, C.; Dominguez, J.; Marasigan, S.; Ampil, E.; et al. Treatment of dementia and mild cognitive impairment with or without cerebrovascular disease: Expert consensus on the use of Ginkgo biloba extract, EGb 761((R)). *CNS Neurosci Ther* **2019**, *25*, 288-298, doi:10.1111/cns.13095.

25. Beyfuss, K.; Hood, D.A. A systematic review of p53 regulation of oxidative stress in skeletal muscle. *Redox Rep* **2018**, *23*, 100-117, doi:10.1080/13510002.2017.1416773.

26. Carbone, M.; Adusumilli, P.S.; Alexander, H.R.; Baas, P.; Bardelli, F.; Bononi, A.; Bueno, R.; Felley-Bosco, E.; Galateau-Salle, F.; Jablons, D.; et al. Mesothelioma: Scientific clues for prevention, diagnosis, and therapy. *CA Cancer J Clin* **2019**, *69*, 402-429, doi:10.3322/caac.21572.

27. Harbeck, N.; Gnant, M. Breast cancer. *Lancet* **2017**, *389*, 1134-1150, doi:10.1016/S0140-6736(16)31891-8.

28. Anastasiadi, Z.; Lianos, G.D.; Ignatiadou, E.; Harissis, H.V.; Mitsis, M. Breast cancer in young women: an overview. *Updates Surg* **2017**, *69*, 313-317, doi:10.1007/s13304-017-0424-1.

29. Huang, Z.; Yu, P.; Tang, J. Characterization of Triple-Negative Breast Cancer MDA-MB-231 Cell Spheroid Model. *Onco Targets Ther* **2020**, *13*, 5395-5405, doi:10.2147/OTT.S249756.

30. Ostrom, Q.T.; Patil, N.; Cioffi, G.; Waite, K.; Kruchko, C.; Barnholtz-Sloan, J.S. Corrigendum to: CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2013-2017. *Neuro Oncol* **2020**, doi:10.1093/neuonc/noaa269.

31. Bergamini, C.M.; Gambetti, S.; Dondi, A.; Cervellati, C. Oxygen, reactive oxygen species and tissue damage. *Curr Pharm Des* **2004**, *10*, 1611-1626, doi:10.2174/1381612043384664.

32. Filosa, S.; Pecorelli, A.; D'Esposito, M.; Valacchi, G.; Hajek, J. Exploring the possible link between MeCP2 and oxidative stress in Rett syndrome. *Free Radic Biol Med* **2015**, *88*, 81-90, doi:10.1016/j.freeradbiomed.2015.04.019.

33. Krishna, A.; Biryukov, M.; Trefois, C.; Antony, P.M.; Hussong, R.; Lin, J.; Heinaniemi, M.; Glusman, G.; Koglsberger, S.; Boyd, O.; et al. Systems genomics evaluation of the SH-SY5Y neuroblastoma cell line as a model for Parkinson's disease. *BMC Genomics* **2014**, *15*, 1154, doi:10.1186/1471-2164-15-1154.

34. Mata, I.F.; Jang, Y.; Kim, C.H.; Hanna, D.S.; Dorschner, M.O.; Samii, A.; Agarwal, P.; Roberts, J.W.; Klepitskaya, O.; Shprecher, D.R.; et al. The RAB39B p.G192R mutation causes X-linked dominant Parkinson's disease. *Mol Neurodegener* **2015**, *10*, 50, doi:10.1186/s13024-015-0045-4.

35. Maraldi, T.; Riccio, M.; Zambonin, L.; Vinceti, M.; De Pol, A.; Hakim, G. Low levels of selenium compounds are selectively toxic for a human neuron cell line through ROS/RNS increase and apoptotic process activation. *Neurotoxicology* **2011**, *32*, 180-187, doi:10.1016/j.neuro.2010.10.008.

36. Crispi, S.; Calogero, R.A.; Santini, M.; Mellone, P.; Vincenzi, B.; Citro, G.; Vicidomini, G.; Fasano, S.; Meccariello, R.; Cobellis, G.; et al. Global Gene Expression Profiling Of Human Pleural Mesotheliomas: Identification of Matrix Metalloproteinase 14 (MMP-14) as Potential Tumour Target. *Plos One* **2009**, *4*, doi:ARTN e7016 10.1371/journal.pone.0007016.

37. Baldi, A.; Piccolo, M.T.; Boccellino, M.R.; Donizetti, A.; Cardillo, I.; La Porta, R.; Quagliuolo, L.; Spugnini, E.P.; Cordero, F.; Citro, G.; et al. Apoptosis Induced by Piroxicam plus Cisplatin Combined Treatment Is Triggered by p21 in Mesothelioma. *Plos One* **2011**, *6*, doi:ARTN e23569

10.1371/journal.pone.0023569.

38. Fischer, U.; Janicke, R.U.; Schulze-Osthoff, K. Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ* **2003**, *10*, 76-100, doi:10.1038/sj.cdd.4401160.

39. Di Meo, F.; Aversano, R.; Diretto, G.; Demurtas, O.; Villano, C.; Cozzolino, S.; Filosa, S.; Carputo, D.; Crisp, S. Anti-cancer activity of grape seed semi-polar extracts in human mesothelioma cell lines. *Journal of Functional Foods* **2019**, *61*.

40. Wang, Y.; Lee, K.W.; Chan, F.L.; Chen, S.A.; Leung, L.K. The red wine polyphenol resveratrol displays bilevel inhibition on aromatase in breast cancer cells. *Toxicological Sciences* **2006**, *92*, 71-77, doi:10.1093/toxsci/kfj190.

41. Mantena, S.K.; Baliga, M.S.; Katiyar, S.K. Grape seed proanthocyanidins induce apoptosis and inhibit metastasis of highly metastatic breast carcinoma cells. *Carcinogenesis* **2006**, *27*, 1682-1691, doi:10.1093/carcin/bgl030.

42. Chou, S.C.; Kaur, M.; Thompson, J.A.; Agarwal, R.; Agarwal, C. Influence of Gallate Esterification on the Activity of Procyanidin B2 in Androgen-Dependent Human Prostate Carcinoma LNCaP Cells. *Pharmaceutical Research* **2010**, *27*, 619-627, doi:10.1007/s11095-009-0037-6.

43. Mitsuhashi, S.; Saito, A.; Nakajima, N.; Shima, H.; Ubukata, M. Pyrogallol structure in polyphenols is involved in apoptosis-induction on HEK293T and K562 cells. *Molecules* **2008**, *13*, 2998-3006, doi:10.3390/molecules13122998.

44. Cheah, K.Y.; Howarth, G.S.; Bindon, K.A.; Kennedy, J.A.; Bastian, S.E. Low molecular weight procyanidins from grape seeds enhance the impact of 5-Fluorouracil chemotherapy on Caco-2 human colon cancer cells. *PLoS One* **2014**, *9*, e98921, doi:10.1371/journal.pone.0098921.

45. Moll, U.M.; Petrenko, O. The MDM2-p53 interaction. *Mol Cancer Res* **2003**, *1*, 1001-1008.

46. Momand, J.; Jung, D.; Wilczynski, S.; Niland, J. The MDM2 gene amplification database. *Nucleic Acids Res* **1998**, *26*, 3453-3459, doi:10.1093/nar/26.15.3453.

47. Onel, K.; Cordon-Cardo, C. MDM2 and prognosis. *Mol Cancer Res* **2004**, *2*, 1-8.

48. Ware, P.L.; Snow, A.N.; Gvalani, M.; Pettenati, M.J.; Qasem, S.A. MDM2 copy numbers in well-differentiated and dedifferentiated liposarcoma: characterizing progression to high-grade tumors. *Am J Clin Pathol* **2014**, *141*, 334-341, doi:10.1309/AJCPLYU89XHSNHQO.

49. Schreiber, M.; Kolbus, A.; Piu, F.; Szabowski, A.; Möhle-Steinlein, U.; Tian, J.; Karin, M.; Angel, P.; Wagner, E.F. Control of cell cycle progression by c-Jun is p53 dependent. *Genes Dev* **1999**, *13*, 607-619, doi:10.1101/gad.13.5.607.

50. Waterham, H.R.; Koster, J.; Romeijn, G.J.; Hennekam, R.C.; Vreken, P.; Andersson, H.C.; FitzPatrick, D.R.; Kelley, R.I.; Wanders, R.J. Mutations in the 3beta-hydroxysterol Delta24-reductase gene cause desmosterolosis, an autosomal recessive disorder of cholesterol biosynthesis. *Am J Hum Genet* **2001**, *69*, 685-694, doi:10.1086/323473.

51. Tsukiyama-Kohara, K. Role of oxidative stress in hepatocarcinogenesis induced by hepatitis C virus. *Int J Mol Sci* **2012**, *13*, 15271-15278, doi:10.3390/ijms131115271.

52. Nishimura, T.; Kohara, M.; Izumi, K.; Kasama, Y.; Hirata, Y.; Huang, Y.; Shuda, M.; Mukaidani, C.; Takano, T.; Tokunaga, Y.; et al. Hepatitis C virus impairs p53 via persistent overexpression of 3beta-hydroxysterol Delta24-reductase. *J Biol Chem* **2009**, *284*, 36442-36452, doi:10.1074/jbc.M109.043232.

53. Qin, J.J.; Li, X.; Hunt, C.; Wang, W.; Wang, H.; Zhang, R. Natural products targeting the p53-MDM2 pathway and mutant p53: Recent advances and implications in cancer medicine. *Genes Dis* **2018**, *5*, 204-219, doi:10.1016/j.gendis.2018.07.002.

54. Crispi, S.; Cardillo, I.; Spugnini, E.P.; Citro, G.; Menegozzo, S.; Baldi, A. Biological agents involved in malignant mesothelioma: relevance as biomarkers or therapeutic targets. *Curr Cancer Drug Targets* **2010**, *10*, 19-26.

55. Reed, S.M.; Quelle, D.E. p53 Acetylation: Regulation and Consequences. *Cancers (Basel)* **2014**, *7*, 30-69, doi:10.3390/cancers7010030.

56. Redza-Dutordoir, M.; Averill-Bates, D.A. Activation of apoptosis signalling pathways by reactive oxygen species. *Biochim Biophys Acta* **2016**, *1863*, 2977-2992, doi:10.1016/j.bbamcr.2016.09.012.

57. Wyss-Coray, T. Ageing, neurodegeneration and brain rejuvenation. *Nature* **2016**, *539*, 180-186, doi:10.1038/nature20411.

58. Hou, Y.; Dan, X.; Babbar, M.; Wei, Y.; Hasselbalch, S.G.; Croteau, D.L.; Bohr, V.A. Ageing as a risk factor for neurodegenerative disease. *Nat Rev Neurol* **2019**, *15*, 565-581, doi:10.1038/s41582-019-0244-7.

59. Rojas, P.; Montes, P.; Rojas, C.; Serrano-Garcia, N.; Rojas-Castaneda, J.C. Effect of a phytopharmaceutical medicine, Ginko biloba extract 761, in an animal model of Parkinson's disease: therapeutic perspectives. *Nutrition* **2012**, *28*, 1081-1088, doi:10.1016/j.nut.2012.03.007.

60. Spiegel, R.; Kalla, R.; Mantokoudis, G.; Maire, R.; Mueller, H.; Hoerr, R.; Ihl, R. Ginkgo biloba extract EGb 761((R)) alleviates neurosensory symptoms in patients with dementia: a meta-analysis of treatment effects on tinnitus and dizziness in randomized, placebo-controlled trials. *Clin Interv Aging* **2018**, *13*, 1121-1127, doi:10.2147/CIA.S157877.

61. Madabhushi, R.; Pan, L.; Tsai, L.H. DNA damage and its links to neurodegeneration. *Neuron* **2014**, *83*, 266-282, doi:10.1016/j.neuron.2014.06.034.

62. Chow, H.M.; Herrup, K. Genomic integrity and the ageing brain. *Nat Rev Neurosci* **2015**, *16*, 672-684, doi:10.1038/nrn4020.

63. Smith, J.V.; Luo, Y. Studies on molecular mechanisms of Ginkgo biloba extract. *Appl Microbiol Biotechnol* **2004**, *64*, 465-472, doi:10.1007/s00253-003-1527-9.

64. Bridi, R.; Crossetti, F.P.; Steffen, V.M.; Henriques, A.T. The antioxidant activity of standardized extract of Ginkgo biloba (EGb 761) in rats. *Phytother Res* **2001**, *15*, 449-451, doi:10.1002/ptr.814.

65. Zimmermann, M.; Colciaghi, F.; Cattabeni, F.; Di Luca, M. Ginkgo biloba extract: from molecular mechanisms to the treatment of Alzhelmer's disease. *Cell Mol Biol (Noisy-le-grand)* **2002**, *48*, 613-623.

66. Smith, J.V.; Burdick, A.J.; Golik, P.; Khan, I.; Wallace, D.; Luo, Y. Anti-apoptotic properties of Ginkgo biloba extract EGb 761 in differentiated PC12 cells. *Cellular and Molecular Biology* **2002**, *48*, 699-707.

67. Shi, C.; Zhao, L.; Zhu, B.; Li, Q.; Yew, D.T.; Yao, Z.; Xu, J. Dosage effects of EGb761 on hydrogen peroxide-induced cell death in SH-SY5Y cells. *Chem Biol Interact* **2009**, *180*, 389-397, doi:10.1016/j.cbi.2009.04.008.

68. Jiang, X.; Nie, B.; Fu, S.; Hu, J.; Yin, L.; Lin, L.; Wang, X.; Lu, P.; Xu, X.M. EGb761 protects hydrogen peroxide-induced death of spinal cord neurons through inhibition of intracellular ROS production and modulation of apoptotic regulating genes. *J Mol Neurosci* **2009**, *38*, 103-113, doi:10.1007/s12031-008-9140-0.

69. Ni, Y.; Zhao, B.; Hou, J.; Xin, W. Preventive effect of Ginkgo biloba extract on apoptosis in rat cerebellar neuronal cells induced by hydroxyl radicals. *Neurosci Lett* **1996**, *214*, 115-118, doi:10.1016/0304-3940(96)12897-4.

70. Savaskan, E.; Mueller, H.; Hoerr, R.; von Gunten, A.; Gauthier, S. Treatment effects of Ginkgo biloba extract EGb 761® on the spectrum of behavioral and psychological symptoms of dementia: meta-analysis of randomized controlled trials. *Int Psychogeriatr* **2018**, *30*, 285-293, doi:10.1017/S1041610217001892.

71. Gauthier, S.; Schlaefke, S. Efficacy and tolerability of Ginkgo biloba extract EGb 761® in dementia: a systematic review and meta-analysis of randomized placebo-controlled trials. *Clin Interv Aging* **2014**, *9*, 2065-2077, doi:10.2147/CIA.S72728.

72. Corey, J.M.; Gertz, C.C.; Sutton, T.J.; Chen, Q.; Mycek, K.B.; Wang, B.S.; Martin, A.A.; Johnson, S.L.; Feldman, E.L. Patterning N-type and S-type neuroblastoma cells with Pluronic F108 and ECM proteins. *J Biomed Mater Res A* **2010**, *93*, 673-686, doi:10.1002/jbm.a.32485.

73. Testa, G.; Staurenghi, E.; Giannelli, S.; Gargiulo, S.; Guglielmotto, M.; Tabaton, M.; Tamagno, E.; Gamba, P.; Leonarduzzi, G. A silver lining for 24-hydroxycholesterol in Alzheimer's disease: The involvement of the neuroprotective enzyme sirtuin 1. *Redox Biol* **2018**, *17*, 423-431, doi:10.1016/j.redox.2018.05.009.

74. Knights, C.D.; Catania, J.; Di Giovanni, S.; Muratoglu, S.; Perez, R.; Swartzbeck, A.; Quong, A.A.; Zhang, X.; Beerman, T.; Pestell, R.G.; et al. Distinct p53 acetylation cassettes differentially influence gene-expression patterns and cell fate. *J Cell Biol* **2006**, *173*, 533-544, doi:10.1083/jcb.200512059.

75. Olsson, A.; Manzl, C.; Strasser, A.; Villunger, A. How important are post-translational modifications in p53 for selectivity in target-gene transcription and tumour suppression? *Cell Death Differ* **2007**, *14*, 1561-1575, doi:10.1038/sj.cdd.4402196.

76. Brochier, C.; Dennis, G.; Rivieccio, M.A.; McLaughlin, K.; Coppola, G.; Ratan, R.R.; Langley, B. Specific acetylation of p53 by HDAC inhibition prevents DNA damage-induced apoptosis in neurons. *J Neurosci* **2013**, *33*, 8621-8632, doi:10.1523/JNEUROSCI.5214-12.2013.

77. Ming, L.; Wang, P.; Bank, A.; Yu, J.; Zhang, L. PUMA Dissociates Bax and Bcl-X(L) to induce apoptosis in colon cancer cells. *J Biol Chem* **2006**, *281*, 16034-16042, doi:10.1074/jbc.M513587200.

78. Park, H.J.; Kim, M.M. Flavonoids in Ginkgo biloba fallen leaves induce apoptosis through modulation of p53 activation in melanoma cells. *Oncol Rep* **2015**, *33*, 433-438, doi:10.3892/or.2014.3602.

79. Mattson, M.P. Apoptosis in neurodegenerative disorders. *Nat Rev Mol Cell Biol* **2000**, *1*, 120-129, doi:10.1038/35040009.

Dott. Francesco Di Meo peer reviewed papers published during the PhD period:

1) Di Meo F, Valentino A, Petillo O, Peluso G, Filosa S, Crispi S. Bioactive Polyphenols and Neuromodulation: Molecular Mechanisms in Neurodegeneration. Int J Mol Sci. 2020 Apr 7;21(7):2564. doi: 10.3390/ijms21072564.

2) Di Meo F, Cuciniello R, Margarucci S, Bergamo P, Petillo O, Peluso G, Filosa S, Crispi S. Ginkgo biloba Prevents Oxidative Stress-Induced Apoptosis Blocking p53 Activation in Neuroblastoma Cells. Antioxidants (Basel). 2020 Mar 26;9(4):279. doi: 10.3390/antiox9040279.

3) Di Meo F, Margarucci S, Galderisi U, Crispi S, Peluso G. Curcumin, Gut Microbiota, and Neuroprotection. Nutrients. 2019 Oct 11;11(10):2426. doi: 10.3390/nu11102426.

4) Di Meo F, Aversano R, Diretto G, Demurtas O, Villano C, Cozzolino S, Filosa S, Carputo D, Crispi S. Anti-cancer activity of grape seed semi-polar extracts in human mesothelioma cell lines. Journal of Functional Foods. October 2019, 103515. DOI: 10.1016/j.jff.2019.103515

5) Di Meo F, Filosa S, Madonna M, Giello G, Di Pardo A, Maglione V, Baldi A, Crispi S. Curcumin C3 complex®/Bioperine® has antineoplastic activity in mesothelioma: an in vitro and in vivo analysis. J Exp Clin Cancer Res. 2019 Aug 16;38(1):360. doi: 10.1186/s13046-019-1368-8.

6) Di Meo F, Donato S, Di Pardo A, Maglione V, Filosa S, Crispi S. New Therapeutic Drugs from Bioactive Natural Molecules: The Role of Gut Microbiota Metabolism in Neurodegenerative Diseases. Curr Drug Metab. 2018;19(6):478-489. doi: 10.2174/1389200219666180404094147.

7) Filosa S, Di Meo F, Crispi S. Polyphenols-gut microbiota interplay and brain neuromodulation. Neural Regen Res. 2018 Dec;13(12):2055-2059. doi: 10.4103/1673-5374.241429.

Journal of Functional Foods 61 (2019) 103515

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Anti-cancer activity of grape seed semi-polar extracts in human mesothelioma cell lines

Francesco Di Meo[a,b], Riccardo Aversano[c,*], Gianfranco Diretto[d], Olivia Costantina Demurtas[d], Clizia Villano[c], Salvatore Cozzolino[b], Stefania Filosa[a,e], Domenico Carputo[c], Stefania Crispi[a,*]

[a] Institute of Biosciences and BioResources-UOS Naples CNR, via P. Castellino, 111-80131 Naples, Italy
[b] Department of Biology, University of Naples Federico II, Complesso Universitario Monte Sant'Angelo, via Cinthia, Naples, Italy
[c] Department of Agricultural Sciences, University of Naples Federico II, Via Università 100, Portici, Italy
[d] Department of Sustainability (SSPT), Biotechnology Laboratory, ENEA, Casaccia Research Center, Via Anguillarese 301, Rome, Italy
[e] IRCCS Neuromed, Pozzilli, IS, Italy

ARTICLE INFO

Keywords:
Mesothelioma
Apoptosis
Polyphenols
Grape seed extracts
Anticancer
Secondary metabolites
Phytochemicals

ABSTRACT

Malignant mesothelioma is a tumor that affects pleural surface and has very poor prognosis. The standard therapeutic modalities for this cancer have yielded unsatisfactory outcomes, therefore the development of alternative and effective therapies is currently an urgent requirement.

Grapevine is a plant rich of bioactive compounds, known for its therapeutic effects. Here, we describe the anti-cancer activity of grape seeds semi-polar extracts of two Italian grape varieties (Aglianico and Falanghina) in mesothelioma *in vitro*. Seed extracts from both varieties induced intrinsic apoptosis in a dose and time-dependent manner in three different human mesothelioma cell lines. Global metabolic analysis of this fraction revealed a higher accumulation of phenylpropanoid precursors and proanthocyanidins and expression of genes involved in the aforementioned pathways.

These findings suggest that new phenolic molecules from grape seeds could be viewed as new drugs to be used alone or in combinations with standard chemotherapeutics in mesothelioma treatment.

1. Introduction

Malignant Mesothelioma (MM) is a rare and aggressive form of cancer associated with exposure to asbestos fibers that affects the mesothelium surface of the pleural cavity. The prognosis of MM is very poor due to long latency development (30–40 years), to diagnosis at a very late stage and to its high chemo-resistance (Crispi et al., 2010). Previously, our group has investigated the *in vitro* and *in vivo* efficacy of a piroxicam and cisplatin combined treatment against MM. This treatment determined a marked tumor growth inhibition and an extended survival both in mouse models (Spugnini et al., 2006) and in pets (Spugnini et al., 2008). In addition our group revealed that combined treatment was ineffective in specific mesothelioma cell lines (Baldi et al., 2011).

It should be pointed out that serious limitations in cancer treatments are due to drug toxicity and to development of tumor-resistance. For this reason, research is needed on the identification of novel therapeutic approaches using natural compounds showing cytotoxicity exclusively against cancer cells. In this perspective, phytochemicals represent good candidates to be used alone or associated with standard chemotherapy. In fact, these molecules display an adjuvant effect resulting in tumor growth inhibition and in chemoprotective action towards the healthy cells (Piccolo, Menale, & Crispi, 2015).

A wide range of phytochemicals has been reported to positively affect normal cell growth, proliferation and differentiation. Some bioactive compounds can prevent the occurrence of cancer, reduce cellular oxidative damage and help in the maintenance of intracellular antioxidant defences through their free radical scavenging properties (Vallejo, Salazar, & Grijalva, 2017). Among them, polyphenols, terpenes and alkaloids are the most important molecules able to reduce tumorigenesis and revert cancer related dysfunctions (Seca & Pinto, 2018). Grapevine (*Vitis vinifera* L.) is a plant rich of bioactive compounds already used to develop cosmeceuticals and nutraceuticals (Gollucke, Peres, Odair, & Ribeiro, 2013). They are mainly distributed in berry skin, stem, leaf and seed, rather than in the pulp of the berry. As there is an increasing worldwide interest in the recovery and reuse of residues from agro-industrial processes there is an additional interest in these wine industry by-products. Their transformation makes it possible

* Corresponding authors.
E-mail addresses: raversan@unina.it (R. Aversano), stefania.crispi@ibbr.cnr.it (S. Crispi).

https://doi.org/10.1016/j.jff.2019.103515
Received 18 March 2019; Received in revised form 7 August 2019; Accepted 11 August 2019
1756-4646/ © 2019 Published by Elsevier Ltd.

to obtain raw materials that can be reconverted into a different finished product. In this scenario, the development of new technologies to recover these residues perfectly matches with the growing interest of the cosmeceutical and nutraceutical industry for the production of bioactive compounds.

Mounting evidence indicate that grape-derived phytochemicals have healthy and chemopreventive effects (Katiyar & Athar, 2013). Moreover, some polyphenols exert cytotoxic effects and are of great interest for developing new molecules with pharmacological activity (Ferri et al., 2016). It has been reported that berry peel contains a substantial amount of phenolic compounds, antioxidants and total anthocyanins that can be used in different therapeutic procedures (Ghafoor, Park, & Choi, 2010). However, most of grape polyphenols are present in the seeds, ranging from 60% to 70% of total extractable compounds. In this context, a strong link has been postulated between these bioactivities and a series of non-polar (lipids) and semi-polar (phenolics) molecules highly accumulated in grape seeds (Bazan-Salinas, Matias-Perez, Perez-Campos, Mayoral, & Garcia-Montalvo, 2016). Among advantageous metabolites, a very relevant role has been assigned to proanthocyanidins, oligomeric flavonoids generated downstream of phenolic acids, chalcones, flavanones and flavonols (Gascuel, Diretto, Monforte, Fortes, & Granell, 2017).

Several studies describe the anticancer effects of molecules extracted from grape tissues (Kaur, Agarwal, & Agarwal, 2009). However, none is referred to the effect on MM. Several natural compounds such as epigallocatechin gallate, resveratrol and curcumin displayed adjuvant effect with standard chemotherapy both in MM (Lee et al., 2014; Martinotti, Ranzato, Parodi, Vitale, & Burlando, 2014; Serri et al., 2017) and in other tumours (Chen, Landen, Li, Alvarez, & Tollefsbol, 2013). Recently, it has been reported that Cabernet Sauvignon seed fractions show cytotoxicity on colon cancer cells and can synergize with the chemotherapy drug Fluorouracil by amplifying its efficacy (Cheah, Howarth, Bindon, Kennedy, & Bastian, 2014).

Phytochemicals anticancer activities are exerted by inducing programmed cell death (apoptosis). Dysregulation of apoptosis leads to different human diseases, including cancer. Apoptosis can be executed through the intrinsic or the extrinsic pathway. The intrinsic pathway is regulated by B-cell lymphoma-2 (BCL-2) family proteins. BAX (Bcl-2-associated X protein) impairs mitochondrial membranes determining cytochrome *c* release, and this activity is inhibited by binding of BCL-2 to BAX. Unbalance between BCL-2 and BAX favors BAX activity, determining the onset of apoptosis. Mitochondria play an essential role in this pathway by releasing apoptogenic proteins into the cytoplasm. The extrinsic pathway is activated by extracellular death ligands. Their binding to the cognate death receptors leads to activation of apoptosis (Ashkenazi & Dixit, 1998). Both apoptotic pathways lead to caspases machinery activation determining the cleavage of PARP (poly(ADP-ribose) polymerase) protein that is required for the late apoptotic events (Fischer, Janicke, & Schulze-Osthoff, 2003).

In this study, we describe the anti-cancer potential of grape seeds global semipolar extracts of two Italian grape varieties Aglianico (red) and Falanghina (white) in MM. Grape seed extracts showed anti-cancer activity in three different mesothelioma cell lines MSTO, two lines (MSTO-221H and NCI-H2452) responding to the standard treatment and one line (Ist-Mes2) does not responding to it. Their efficacy was explored analyzing the apoptotic pathways and the reduction of cancer properties. In addition, global metabolic profiling and targeted transcriptional investigations allowed to identify the phytochemical content of our grape extracts and highlighted the significance of the varietal genetic makeup, respectively. Overall, our data demonstrate that our seed extracts induce apoptosis in a dose and time-dependent manner also in MM suggesting that new molecules could be further investigated for novel MM treatment.

2. Materials and methods

2.1. Cell culture

Human mesothelioma cell lines MSTO-221H (MSTO) and NCI-H2452 (NCI) were grown in RPMI supplemented with 10% FBS, glutamine (2 mM), sodium pyruvate and antibiotics (0.02 IU/mL-1 penicillin and 0.02 mg/mL-1 streptomycin). Human mesothelioma cells Ist-Mes2 (Mes2) were cultured in DMEM, supplemented with 10% FBS, glutamine (2 mM), 1% nonessential amino acids, and antibiotics (0.02 IU/mL-1 penicillin and 0.02 mg/mL-1 streptomycin).

2.2. Plant material

Two *V. vinifera* cultivars were used in this study: Aglianico del Taburno, clone Ampelos TEA 22, and Falanghina del Beneventano, clone Ampelos EVA 1, both grafted onto rootstock 1103 Paulsen – *V. berlandieri* × *V. rupestris* – clone ISV 1. Berries were collected from a nine-year-old vineyard (145 m a.s.l., Castelvenere, Benevento Province, Southern Italy) during the 2015 growing season. The technological ripening stage was identified and, for each variety, 30 clusters were harvested from different positions of the vineyard and from random positions on the plant to ensure the representation of the entire vineyard (Rinaldi et al., 2017). Ten berries were randomly selected from different parts of the cluster, avoiding those with visible damages and/or signs of pathogen infection, and pooled with berries from the other plants of the same variety. The whole berries were frozen immediately in liquid nitrogen. Three independent pools (biological replicates) of about 100 berries were used for the analyses.

2.3. Extract preparation

To obtain peel and seed semipolar extracts from Aglianico and Falanghina, each tissue was first isolated, minced with liquid nitrogen and then lyophilized. The powder was homogenized with methanol/cloroform (1:4) using TissueLyser (Qiagen). Aqueous phases was then recovered after a 5′ centrifugation (12,000 rpm) and lyophilized again. The powder was then dissolved in DMSO (Sigma) at a concentration of 500 mg/ml and stored at −20° C. The extracts were designed as follows: Aglianico grape peel, AGP; Aglianico grape seed, AGS; Falanghina grape peel, FGP; Falanghina grape seed, FGS.

2.4. Cell proliferation assay

To evaluate the effects of peel and seed semipolar extracts from Aglianico and Falanghina on cell proliferation, approximately 1×10^4 cells were plated in 48-well plates and treated with different concentrations of either peel (up to 1000 μg/ml) or seed (up to 500 μg/ml) extracts for 24, 48 or 24 + 24 h (24 + 24). In the case of the 24 + 24 treatment, cells were treated twice: after overnight incubation and again after 24 h. Then, cells were fixed with 3.7% formaldehyde for 10 min, washed with PBS, and stained with 0.5% crystal violet for 10 min. A microplate reader (Cytation3, ASHI) was then used to measure the absorbance of each well at 595 nm. All the experiments were performed in triplicate. Through this experiments, we selected the optimal concentration (350 μg/ml) and extract source (seed) to be used in subsequent experiments.

2.5. Colony forming assay

For colony assay, cells were seeded in six-well plates at a density of 500 cells/well and incubated at 37 °C in a 5% CO_2 humidified incubator. The culture medium was replaced every three days and cells were grown for 14 days. Then, cells were treated with 350 μg/ml of Falanghina (FGS) or Aglianico (AGS) seed extracts for 24 h and grown for additional 14 days using media without seed extracts. Colonies were

finally stained with crystal violet and counted. Representative plates were photographed using phase contrast microscope (Leica, Milan, Italy). All the experiments were performed in triplicate.

2.6. Wound healing assays

Approximately 1.5×10^5 cells were plated in six-well plates. After overnight incubation, cells were treated with 350 μg/ml of FGS or AGS extracts for 24, 48 and 24 + 24 h. Wounds were created in confluent cells using a 200 μL pipette tip. To analyse cell migration, at least 10 representative images for each scratch were taken in different scratched area of cells at different time points of incubation. Images were focused on the centre of the wound field, photographed and measured using ImageJ software. The influence of FGS and AGS on wound closure was compared to scratched cells at time zero hours.

All the experiments were performed in triplicate."

2.7. Apoptosis detection by flow cytometry analysis

Approximately 7.5×10^5 cells were plated in 100 mm plates. After overnight incubation, cells were treated with 350 μg/ml of FGS or AGS extracts for either 24 48 or 24 + 24 h and stained with propidium iodide and annexin V (Annexin V FITC assay, BD Biosciences, Franklin Lakes, NJ), according to the manufacturer's guidelines. Flow cytometry was performed using a FACS-can™ flow cytometry system (Becton Dickinson, San Jose, CA). All the experiments were performed in triplicate.

2.8. RNA extraction and expression analysis of apoptotic pathway genes in MM cells

RNA was extracted using Trizol (Life Technologies) following manufacturer's instructions. For RT-qPCR assays, 200 ng of total RNA from each sample were retro-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). RT-qPCR reactions were performed by means of a 7900 HT Real Time PCR (Applied Biosystem). Gene specific primers for the selected genes (*BAX*: Forward 5′-TTTGC TTCAGGGTTTCATCCA-3′, Reverse 5′- CTCCATGTTACTGTCCAGTT CGT-3′; *BCL-2*: Forward 5′-GTTCCCTTTCCTTCCATCC-3′, Reverse 5′-TAGCCAGTCCAGAGGTGAG-3′; *FAS*: Forward 5′-CCCTCCTACCTCT GGTTCTTACG-3′, Reverse 5′TCAGTCACTTGGGCATTAACACTTT-3′; *FASL*: Forward 5′-CCTGAAAAAAAGGAGCTGAGGAA-3′, Reverse 5′-GGCATGGACCTTGAGTTGGA-3′; *GAPDH*: Forward 5′-CAAGGCTGT GGGCAAGGT-3′, Reverse 5′-GGAAGGCCATGCCAGTGA-3′) were designed at exon-exon junctions using Primer express 2.0 (Applied Biosystems). Target expression level was performed as previously described (Crispi et al., 2009) using *GAPDH* as housekeeping gene. All the experiments were performed in triplicate.

2.9. Cytochrome c release

To determine the cytochrome *c* release from mitochondria to cytosol, cytosolic fractions were isolated after FGS and AGS extracts treatment. 1×10^6 cells were resuspended in 100 μL of ice-cold plasma membrane permeabilization buffer (200 μg/ml digitonin, 80 mM KCl in PBS), and incubate on ice for 5 min. Lysates were centrifuged at 800 X g for 5 min at 4 °C, and the supernatants (cytosolic fraction) were then collected. All the experiments were performed in triplicate.

2.10. Western blot

Protein extracts were prepared as previously described (Baldi et al., 2011). For each lane, 20 μg of total cell lysates were separated in 4–15% Tris–glycine gels (BioRad) at 100 V. Proteins were then transferred to PVDF membranes, probed with the specific primary antibodies, followed by secondary antibodies conjugated with horseradish peroxidase according manufacturer's indications. Primary antibodies used for Western blot include PARP (Cell Signaling, #9542), BCL-2 (Abcam, ab182858), BAX (Santa Cruz Biotechnology, sc-493) and Cytochrome *c* (Abcam, ab133504). β-Actin (Cell Signaling, #3700) was used as loading control. All the antibodies were used at working concentration indicated by manufacturers. Protein bands were detected by Clarity western ECL (BioRad) and quantified with ImageJ software. All the experiments were performed in triplicate.

2.11. LC-HRMS of peel and seed semi-polar metabolomes

Global semi-polar extracts of Aglianico and Falanghina berry peels and seeds were produced as previously described and analysed using an Ultimate UHPLC system with a photodiode array detector (Dionex), and a Q-exactive quadrupole Orbitrap mass spectrometry system (ThermoFisher Scientific) (LC-HRMS), as reported before with slight modifications at metabolite ionization level. More in detail, a heated electrospray ionization (HESI) source was used, with nitrogen as sheath and auxiliary gas (40 and 30 units, respectively). The vaporizer and capillary temperatures were set at 280 and 300 °C, respectively. The discharge current was set to 4.5 μA, and S-lens RF level set at 50. The acquisition was carried in the mass range 110–1600 *m/z*, both in positive and in negative ion mode with the following parameters: resolution 70,000, microscan 1, AGC target $1 \times e^6$ and maximum injection time 50. An untargeted metabolomics analysis was performed using the software SIEVE (Thermofisher scientific) to identify, first of all, all the compounds present in the extracts (using the "component extraction" package); subsequently, all the differentially accumulated ions in the designed mass chromatogram comparison (AGP/FGP, AGS/FGS, AGS/AGP, FGS/FGP) have been unravelled using the "differential analysis" package. Differentially accumulated (DA) metabolites were annotated through a metabolomics public database interrogation. All the tentative identities were then validated: (a) using literature sources, by comparing chromatographic and MS properties of each compounds with authentic standards, if available; (b) on the basis of the *m/z* accurate masses, as reported in the Pubchem database for monoisotopic masses, or in the Metabolomics Fiehn Lab Mass Spectrometry Adduct Calculator for adduct ions. Analysis of mass fragmentation was performed as reported in Metlin (http://metlin.scripps.edu/landing_page.php?pgcontent=mainPage), or by the comparison of experimental and theoretical mass fragmentation performed using MassFrontier 7.0 software (Thermofisher scientific). Metabolites in the final targeted list were quantified relative to the formononetin internal standard levels. At least three biological replicates were analyzed. Differentially accumulated compounds were highlighted using a Tukey's *t*-test. Heatmaps of phenylpropanoids were carried out as previously described (Aversano et al., 2017).

2.12. RNA extraction and expression analysis of phenylpropanoid biosynthesis genes in grape cell tissues

Total RNA was isolated from 40 mg of ground material of peel and seed of both varieties. The SpectrumTM Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA) was used following the manufacturer's protocol with some modifications. Quantity and quality of the isolated RNA were measured using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA). For cDNA synthesis, 100 ng of each RNA sample was reverse transcribed using the SuperScript III cDNA Synthesis Kit (Life Technologies) following the manufacturer's protocol. Expression analysis was conducted by RT-qPCR using a SYBR Green method on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). All PCR reactions were performed in 15 μL volume, containing 330 nM of each primer, 2 μL of 5-fold diluted cDNA and 7.5 μL of SYBR Green Mix (Applied Biosystems, Foster City, CA, USA). The SDS 2.3 and RQ Manager 1.2 software (both Applied

Biosystems, Foster City, CA, USA) were used for data elaboration. Genes under investigation were: *VvPAL* (phenylalanine ammonia-lyase), *VvCHS1,2,3* (chalcone synthase), *VvF3′H* (flavonoid-3′-O-hydroxylase), *VvF3′5′H* (flavonoid-3′,5′-hydroxylase), *VvFLS5* (flavonol synthase), Vv*DFR* (dihydroflavonol-4-reductase), *VvLDOX* (leucoanthocyanidin dioxygenase), *VvANR* (anthocyanidin reductase), *VvLAR2* (leucoanthocyanidin reductase) and five transcriptional factors (*MybYBPA1, MybYBPA2, MybYBA1, MybYBC1-L1, MybYBC1-L3*) involved in phenylpropanoid and flavonoid synthesis. Primer pairs used in the quantitative analysis were previously reported (Rinaldi et al., 2017). The expression of each target gene in Aglianico was normalized with the expression level of the housekeeping gene (Actin) against Falanghina samples using the Livak method obtaining the values in $\log_2$(FC). For each sample, three technical and three biological replicates were analysed.

2.13. Statistical analysis

Analysis to evaluate the difference between control and treatments was performed using Graph Pad Prism 6.0 (GraphPad Software, San Diego, CA, USA). For each experiment, overall significance of the differences among means was evaluated using the One-Way ANOVA, while differences of each treatment with the control was evaluated using the Dunnet's multiple comparison test with Bonferroni *post hoc* correction.

Statistically significant difference compared to untreated cells are: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

All metabolomics data have been statistically validated using an ANOVA with Tukey's *t*-test analysis.

3. Results

3.1. Seed rather than peel extracts determine cancer growth inhibition

Treatments of MSTO, NCI and Mes2 cells with hydroalcoholic semipolar peel extract did not affect MM cell viability, also when used at high concentration (1000 μg/ml) (Fig. 1a and data not shown). On the contrary, extracts from seeds determined cell growth inhibition in a concentration- and time-dependent manner (Fig. 1b, c). In particular, Falanghina grape seed extract (FGS) at 350 μg/ml was able to significantly decrease MSTO cell viability up to 30% (ANOVA p-value < 0.0001). At the same concentration, Aglianico grape seed extract (AGS) decreased MSTO cell viability up to 40% (ANOVA p-value < 0.0001). Since AGS at 350 μg/ml resulted closer to the half minimal (50%) inhibitory concentration (IC), we decided to use this concentration for subsequent experiments. Following 48 h of treatment, MSTO cell death was higher than that observed after 24 h. Moreover, a stronger effect was evident when the 48 h treatment was performed by adding the extract twice, every 24 h (24 + 24 h) (ANOVA p-value < 0.0001). (Fig. 1c). Similar results were obtained with NCI and Mes2 cell lines (Fig. S1).

These experiments indicated that seed extracts were more effective than peel extracts. For this reason, subsequent assays were carried out only with seed extracts.

3.2. FGS and AGS impair mesothelioma tumorigenic properties affecting cell proliferation and migration

To evaluate the anticancer potential of FGS and AGS, cell migration and invasive ability were analysed on MSTO, NCI and Mes2 treated independently with the seed extracts of Falanghina and Aglianico (Fig. 1). The colony formation assay indicates the cellular self-renewal ability and their long-term proliferative potential. Our results showed that this ability was strongly impaired by seed extracts of both varieties in all the cell lines analysed. In fact, after colonies growth, a 48 h treatment with 350 μg/ml of FGS or AGS almost completely inhibited colony formation in MSTO (67% and 90%, respectively; ANOVA p-value < 0.0004). (Fig. 1d) as well as in NCI (51% and 84%, respectively; ANOVA p-value < 0.0001 (Fig. 1e) and in Mes2 cells (48% and 88%, respectively; ANOVA p-value < 0.0003) (Fig. 1f).

To measure the cell migration capability *in vitro,* we also performed a wound healing assay treating scratched cells with FGS or AGS extracts. MSTO cell migration was significantly inhibited by both extracts in a time-dependent manner reaching a gap of about 120% (FGS; ANOVA p-value < 0.0001) or 170% (AGS; ANOVA p-value < 0.0001) compared to the wound gap in the untreated cells. In particular, the wound gap was maximum after 24 + 24 h treatment with 350 μg/ml FGS or AGS extracts (ANOVA p-value < 0.0001) (Fig. 1g, h). On the contrary, in the untreated cells the wound gap was closed at the end of the treatment. Similar results were obtained with NCI and Mes2 cells (Fig. S2). Taken together, these results revealed that grape seed extracts from both varieties determined a comparable inhibition in cell growth and migration.

3.3. FGS and AGS induce apoptosis in mesothelioma cell lines

To confirm whether the loss of viability was due to apoptosis, we analysed cell cycle perturbation and the downstream signalling triggered by grape seed extracts. MSTO treatment with FGS determined a growing apoptotic induction during time. It reached about 20% at 24 + 24 h treatment (Fig. 2a, b). AGS treatments on MSTO cell lines resulted in apoptotic increase of about 35% after 24 + 24 h treatment (Fig. 2c, d) (ANOVA p-value < 0.0051). The, NCI and Mes2 cell lines showed only slight modifications in apoptosis compared to MSTO. Interestingly, Mes2 cells that did not undergo apoptosis after combined drug treatment with piroxicam and cisplatin (Baldi et al., 2011), resulted more sensitive to both extracts (Fig. S3), suggesting that grape seed extracts act as broad regulators of apoptotic induction.

3.4. FGS and AGS activate specific pathways involved in apoptosis

To better investigate the molecular mechanism involved in the apoptotic cell death following FGS and AGS treatments, we focused our attention on the apoptotic pathway involved. We first analysed the expression levels of *BCL-2* and *BAX* after FGS and AGS treatments at 24, 48 and 24 + 24 h by qPCR. As reported in Fig. 3, the *BAX/BCL-2* ratio suggested a time-dependent apoptotic induction in all MM cell lines analysed. The highest *BAX/BCL-2* ratio was observed at 24 + 24 h treatment. Then, we investigated if grape seed extracts could activate the extrinsic pathway by measuring expression levels of *Fas* and *FasL,* two markers of extrinsic apoptosis induction. Our results provided evidence that *Fas* was not modulated by FGS and AGS in the cell lines analysed. Furthermore, we were not able to detect any expression of *FasL* (data not shown). These findings indicated that intrinsic but not extrinsic apoptotic pathway is triggered by FGS and AGS treatments.

3.5. FGS and AGS extracts induce protein expression of cleaved PARP and BAX and cytochrome c release

To gain more insight into the molecular mechanism involved in the apoptotic cell death, we also investigated the effect of FGS and AGS extracts on PARP cleavage as well as on BAX and BCL-2 protein expression. PARP protein is very important for cellular function and survival, and it is a well-known caspase enzyme substrate (Fischer et al., 2003). Immunoblots clearly showed that, in all the cell lines analysed, both seed extracts induced PARP cleavage and increased ratio of BAX/BCL-2 (Fig. 4; Fig. S4), confirming that both extracts are able to induce apoptosis.

To further investigate the mechanism of grape extracts-mediated apoptosis, we examined the cytochrome c release from mitochondria. Indeed, the apoptotic cell death through the intrinsic mitochondrial pathway is known to induce mitochondrial membrane permeabilization

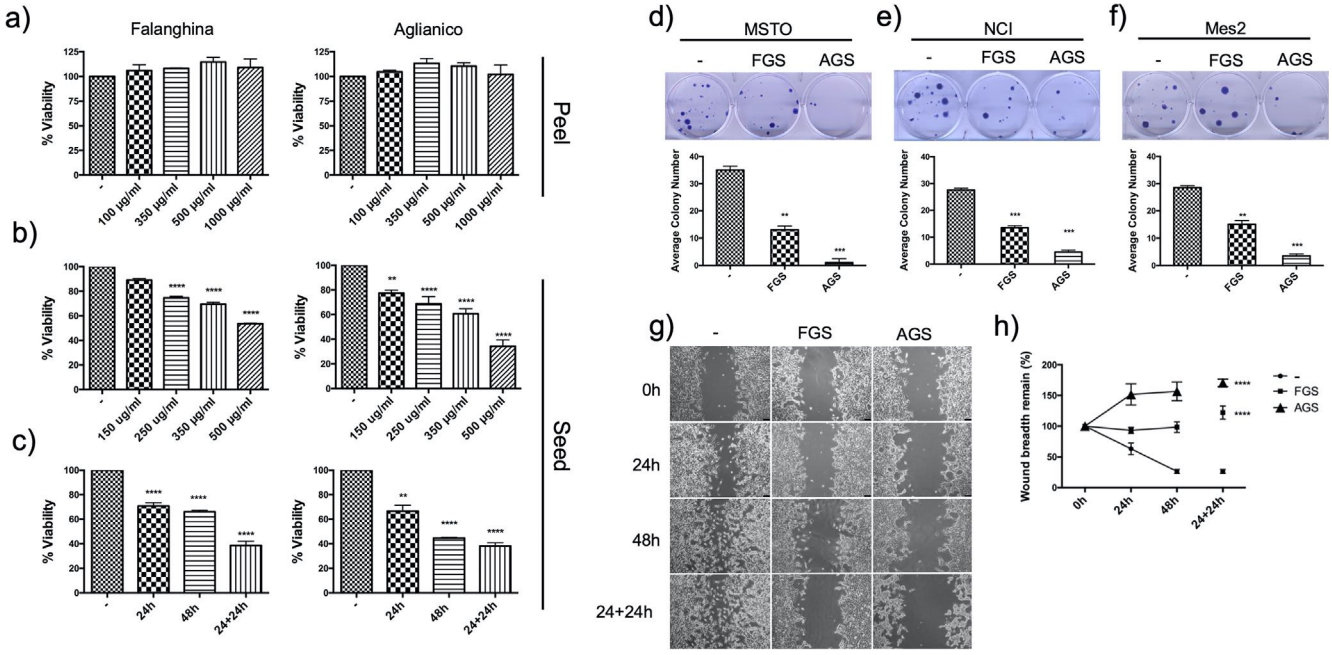

Fig. 1. Bioactivity of grape peel and seed extracts on human mesothelioma cells. MSTO cell viability after treatments with Falanghina and Aglianico extracts: (a) peel extracts; (b) seeds extracts; (c) time course treatment with FGS and AGS extracts (350 µg/ml); untreated cells :-. (d, e, f) Colony formation assay after FGS and AGS treatment (350 µg/ml); untreated cells :-. Representative plate images after crystal violet staining are shown. Histograms report average of colony number. (g) Wound healing assay. The wound closure rate was measured by detecting the closure distance at three different time intervals on MSTO cells untreated (-) or treated with 350 µg/ml of seed extracts. Representative micrographs at phase contrast microscope are shown. (h) Quantification of open wound area. The indicators represent the average ± standard deviation (n = 3). Statistically significant difference from the control: $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and the subsequent cytochrome c release. As shown in Fig. 4, treatment with grape seed extracts induced cytochrome *c* release in MSTO cell lines. Similar results were also obtained in NCI and Mes2 cells (Fig. S4). All these findings, together with earlier observations, suggest that FGS and AGS may directly induce mitochondrial membrane damage and, as consequence, cellular apoptosis.

3.6. Metabolomics analysis of grape peel and seed samples

Cell treatments with seed extracts were very effective, whereas

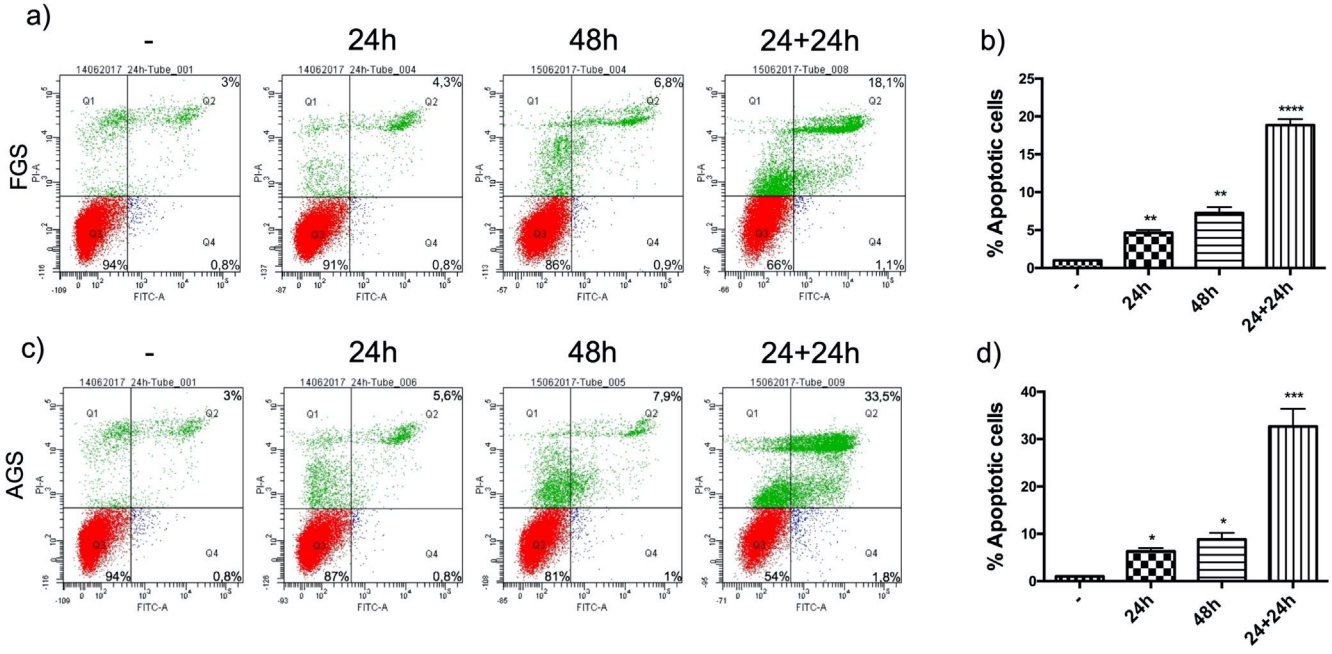

Fig. 2. FGS and AGS treatments induce apoptosis in mesothelioma cells. MSTO apoptosis analysis by Annexin V-FITC/PI after treatments with 350 µg/ml of FGS (a) and AGS (c) extracts at three different time intervals; untreated cells :-. Q2 and Q4: early and late apoptotic cells; Q1: necrotic fraction; Q3: live cells. Histograms reporting a data summary of the apoptotic index after FGS (b) or AGS (d) treatment represented in (a) and (c). Bars represent the average ± standard deviation (n = 3). Statistically significant difference from the control: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$.

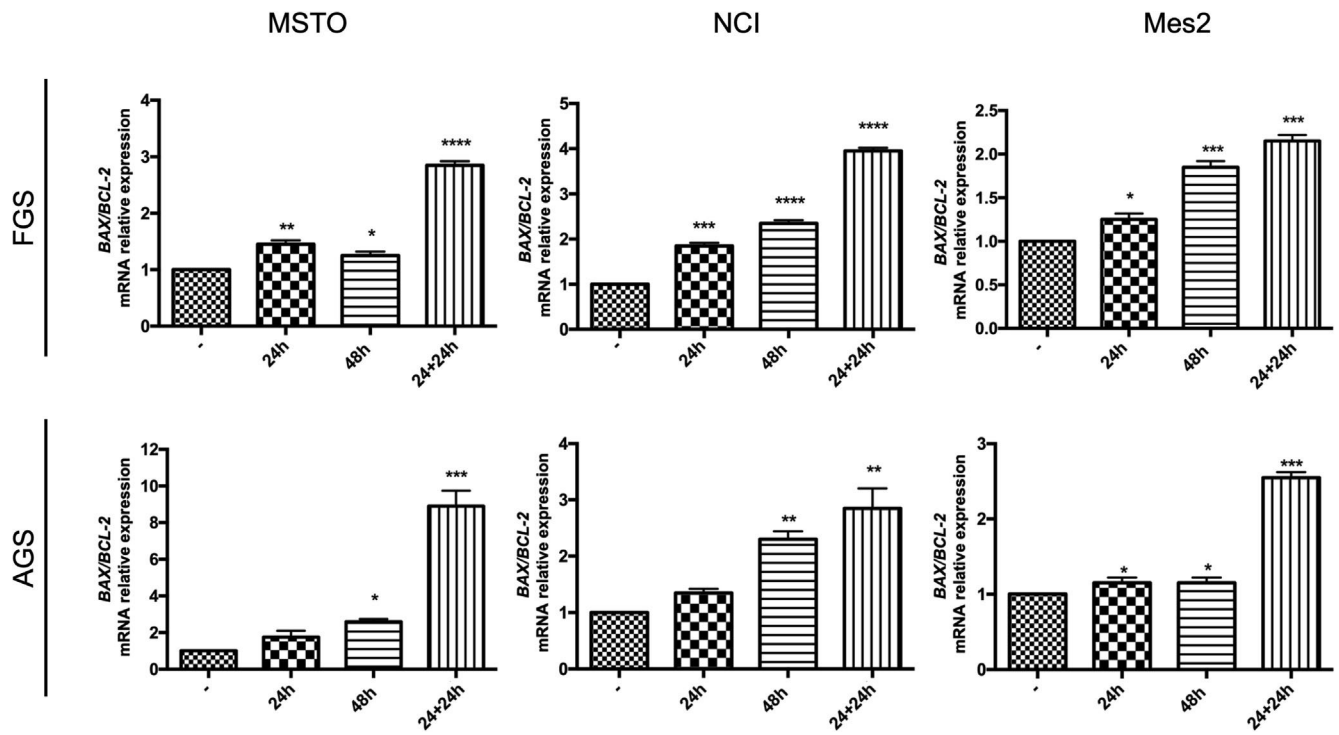

Fig. 3. FGS and AGS treatments trigger apoptosis by activating the intrinsic pathway. Quantitative analysis of mRNA expression levels of *Bax* and *Bcl-2* in MM cell lines. The bars represent the average ± standard deviation (n = 3). Statistically significant difference from the control: *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001.

those with peel extracts did not affect cell viability. To understand the metabolite content differences between seeds and peel, we performed a global metabolomics analysis of the semi-polar fraction of peel and seed extracts of both grape varieties. First, the total ion current (TIC) chromatograms were processed as described in Materials and Methods to retrieve all the compounds detected in the mass chromatogramas ("compound extraction" analysis); subsequently, the differentially accumulated (DA) metabolites in all comparisons (AGS/AGP, FGS/FGP, AGS/FGS, AGP/FGP) were determined and statistically validated using an ANOVA with Tukey's *t*-test. In this way we were able to identify, in an untargeted manner, a total list of 100 negatively and 241 positively charged ions specifically present or over-/down-represented in seed over peel metabolomes of Aglianico, Falanghina, or in both varieties (Table S2). The number of compounds that were consistently over- and down-accumulated in seed vs peel tissues was 108 and 154 in Aglianico and 76 and 130 in Falanghina (Table S1). Overall, a higher number of down-accumulated compounds was observed in all the comparisons under study. In the comparison Aglianico vs Falanghina, the number of compounds that were consistently over- and down-accumulated was, respectively, 69 and 123 in seeds, 39 and 198 in peel (Table S1).

Principal component analysis (PCA) showed a tissue-specific separation, with seed samples from both varieties placing closer in the bottom middle-right side of the plot, although a distinct metabolic attitude was also observed in the peel chromatograms of the red and white varieties (Fig. S5). Subsequently, an *in-batch* interrogation of public and *in house* metabolomics databases was carried out to assign a tentative identification for each metabolite, particularly for the DA ones. Aglianico and Falanghina seed metabolomes resulted highly enriched in several secondary compounds, particularly phenylpropanoid precursors and proanthocyanidin (PA) groups (19 ions): thus, a subset of metabolites was compiled, and a further addition was achieved by including comparison with literature data as well with *in house* databases. The resulting group included 8 PA precursors (as phenylalanine and coumaric acid; chalcones/flavanones like naringenin chalcone, myricetin and quercetin; and caffeic, ellagic and gallic acids) and 53 PAs with different degree of polymerization (from monomers to undecamers), and conjugation with gallic acid (with 1, 2 or 3 units) (Table S3). To better evaluate our findings, a heatmap visualization was used (Fig. 5). In our experimental conditions, within the precursors, gallic acid and its glucoside showed the highest levels in the seeds; in the PA class, catechin, epicatechin, procyanidin B1 and B2, galloylated monomers, dimers and trimers resulted the most abundant compounds (Fig. 5a). Most of proanthocyanidins were only detected in seeds (Fig. 5a and b). To better highlight tissue- and variety-specific metabolite changes (Table S3), for each molecule the ratio between seed vs peel extracts of each variety, as well the ratio between the varieties was calculated. These analyses confirmed the high representation of the whole PA pathway (most of the precursors and, especially, the final products) in seed compared to peel samples. However, while phenylpropanoid precursors were markedly higher in Falanghina seeds vs peels compared to Aglianico (with few exceptions as gallic acid aglycone and glucoside, equal to 32.76 and 76.69 vs 17.26 and 14.70, respectively), a stronger biochemical phenotype was detected in the latter at PA level: for instance, epicatechin (21.43 vs 2.24), procyanidin B1-3,3′-di-O-gallate (839.46 vs 86.65), galloylated trimers (94.38 vs 13.03) and dimers (136.74 vs 26.71). This finding was further evidenced by directly comparing seed extracts from the two varieties (Table S3): most of PA were more abundant in Aglianico with respect to Falanghina. At seed level, the two varieties showed similar trends in the PA precursor pools. Notably, an opposite trend was observed at peel level: with the exceptions of quercetin 3-O-rhamnoside and taxifolin in the precursor, and catechin and galloylated heptamers2 in the PA group, most of the changes were found in Falanghina samples (Table S3).

3.7. Expression of phenylpropanoid biosynthesis genes in Aglianico and Falanghina

The transcript abundance of 16 genes involved in the phenylpropanoids and flavonoids biosynthesis was investigated in peel and seeds of our varieties by RT-qPCR. Fig. 6 reports the log2 expression changes

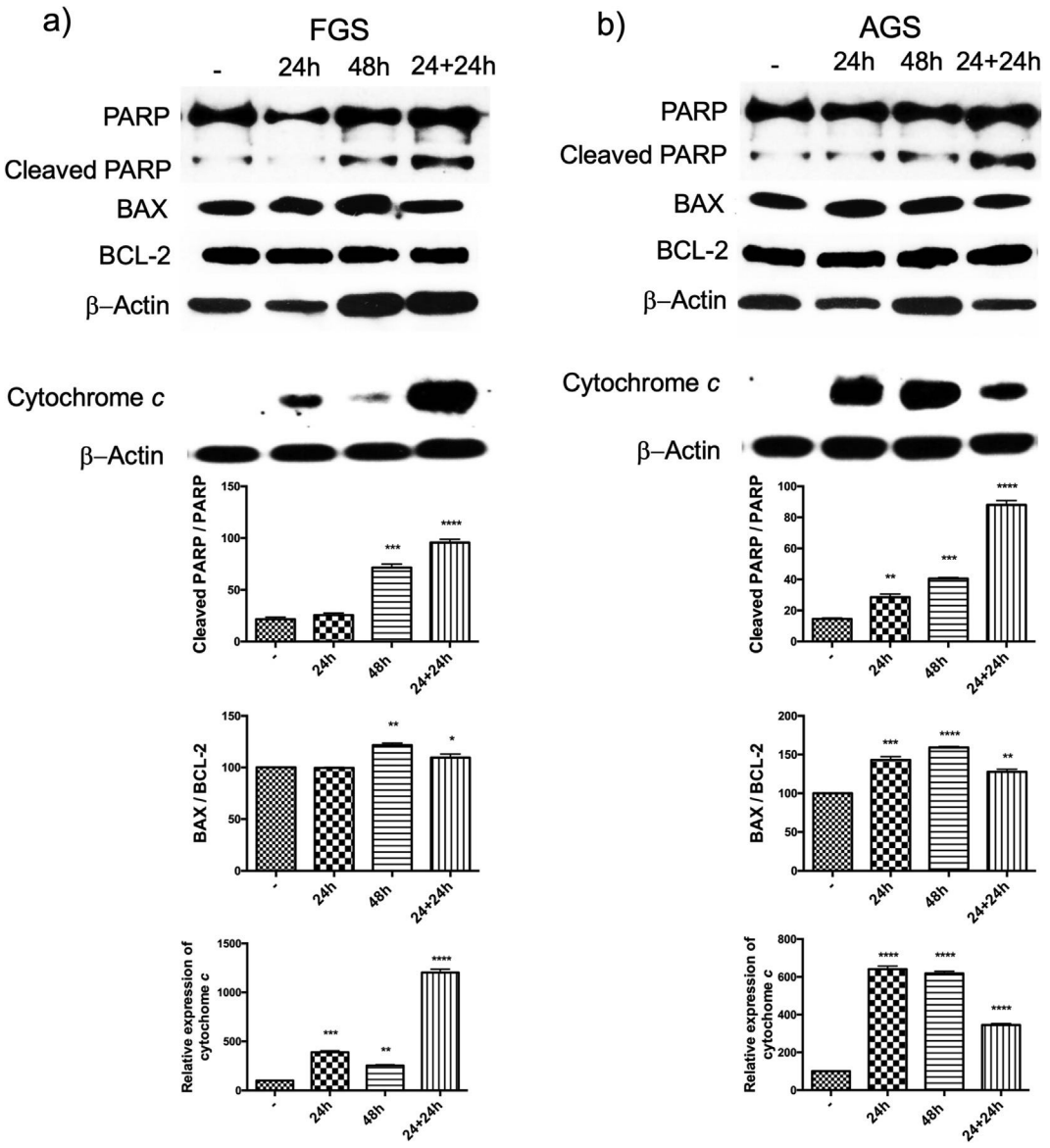

Fig. 4. FGS and AGS increase expression level of apoptotic markers. Analysis of cleaved PARP, BAX, BCL-2 and of cytochrome *c* (cytosolic fraction) on MSTO cells treated with FGS (a) or AGS (b) (350 μg/ml); untreated cells:-. β-Actin protein expression was used as loading control. Histograms report the relative expression level. Statistically significant difference from the control: *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001.

of each target gene in Aglianico compared to Falanghina and, within each variety, in seeds compared to peel. Overall, differences in expression between peel and seeds and between the two varieties were statistically significant for most of genes. In particular, *PAL* was more expressed in Aglianico than in Falanghina, with a greater transcript accumulation in seeds than in peel of both varieties (3.2 FC in Aglianico and 1.6 FC in Falanghina,). Conversely, the three chalcone synthase (*CHS*) genes were generally downregulated in all comparisons, with expression FCs reaching the lowest value (−7.3 FC) in the comparison Aglianico vs Falanghina seeds. After naringenin synthesis, the genes (*F3′H* and *F3′5′H*) participating to the catalysis of the hydroxylated flavanones behaved differently: in Aglianico vs Falanghina comparisons, *F3′H* was downregulated both in peel and in seeds (−2.1 and −1.2 FC, respectively), while *F3′5′H* was upregulated, predominantly in peel, where it reached out a 11.6 FC. In seeds vs peel comparisons, these genes showed an expression greater than 7.0 FC in all samples, with the only exception of *VvF3′5′H* in AGS/AGP (−2.5 FC). The flavonols synthesis gene *FLS5* was overexpressed in all comparisons. Leucoanthocyanidins and anthocyanidins synthesis genes, *DFR* and *LDOX,* performed similarly in both varieties, with a lower expression in the former compared to the latter. On the counterpart, an opposite trend was found between the two genes in seeds vs peel comparisons. Concerning the proanthocyanidins genes, *ANR* and *LAR2* were generally more expressed in Aglianico than in Falanghina, with highest values always detected in seeds. As far as transcription factors are concerned, three of them (*MybA1, MybC1-L1 and MybC1-L3*) showed very similar expression trends in Aglianico (up- in peel and down- in seeds). In this variety, the same genes showed a strong downregulation when comparisons were made between tissues.

4. Discussion

The development of alternative and more effective therapies in MM is deemed urgent since to date the standard therapeutic modalities, including chemotherapy, surgery and radiotherapy have yielded unsatisfactory outcomes. The main objective of our research was to evaluate the anticancer efficacy of semi-polar extracts from peel and seed tissues of two Italian grape varieties, Aglianico and Falanghina,

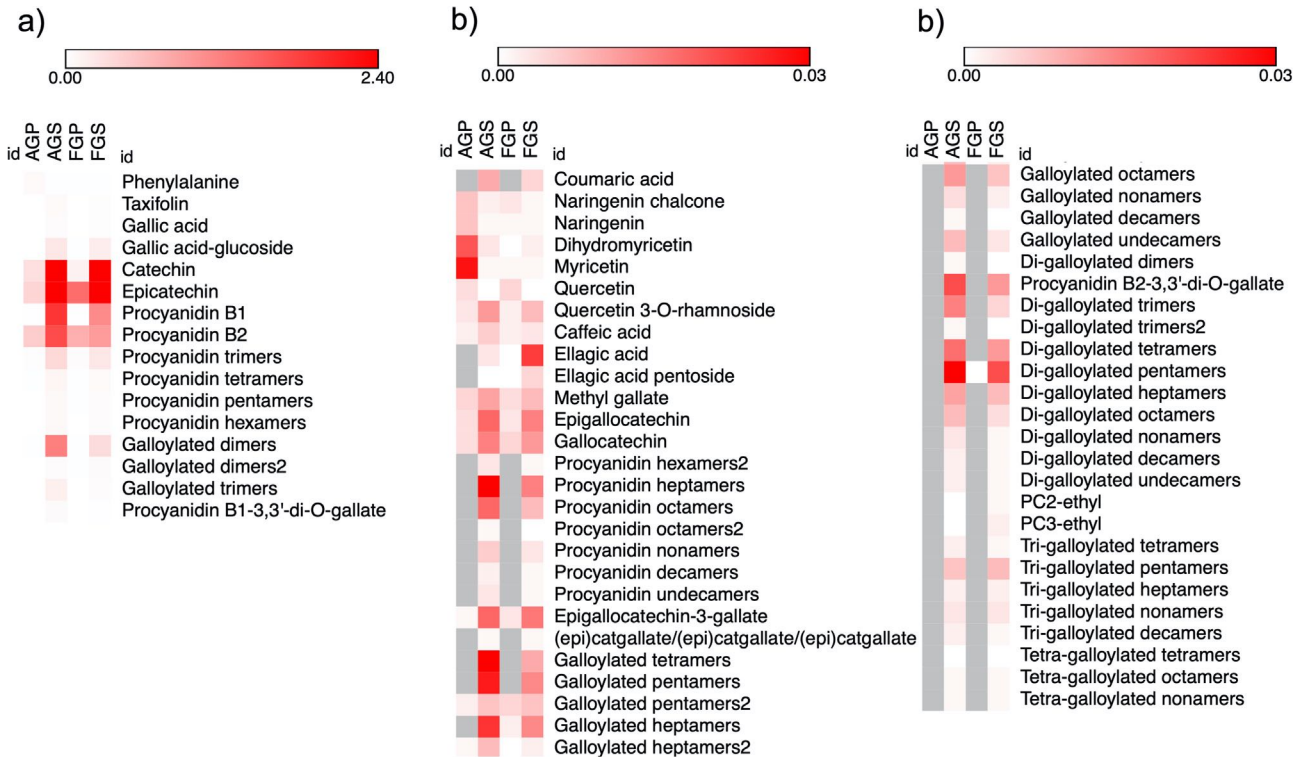

Fig. 5. Heatmap of phenylpropanoid targeted analysis in peels and seeds extracts from Aglianico and Falanghina. Metabolites have been classified in higher- (a) and lower-represented (b), according to the detected values. Red squares of different shades represent the relative values for each metabolite, expressed as fold on the level of the internal standard. Data are present as average ± standard deviation. Gray squares indicate no detectable expression of the corresponding metabolite. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and to determine the mechanisms of action using different human mesothelioma cell lines. Our results suggest that Aglianico and Falanghina peel extracts do not exert proapoptotic effects on MM cells. This agrees with previous studies reporting only chemopreventive or adjuvant effect of peel extracts, mainly for the presence of resveratrol (Wang, Lee, Chan, Chen, & Leung, 2006). Additional studies are needed to validate if our peel extracts could act as adjuvant on MM cells when combined to chemotherapeutics. Our data also point out seeds as potential source of phytochemicals with anticancer activities in MM. Indeed, they were able to induce apoptosis in different MM cell lines, including those insensitive to standard chemoterapeutic treatments (Baldi et al., 2011). Semi-polar seed extracts from both Aglianico (AGS) and Falanghina (FGS) affected tumorigenic properties of human mesothelioma cells in a time- and dose-dependent manner, with Aglianico showing the strongest effect. Colony formation assay showed that FGS and AGS reduced the capacity of mesothelioma cells to form colonies, indicating that these extracts impair cellular self-renewal ability and proliferative cell rate. In addition, as assessed by a wound-healing assay, FGS and AGS displayed the capacity to significantly inhibit cell migration capability.

We provided evidence that cell viability reduction after FGS and AGS treatments was due to apoptosis. Indeed, we found that FGS and AGS trigger a time–dependent increase of the BAX/BCL-2 ratio and of cytochrome *c* release in all mesothelioma cell lines analysed, with the highest threshold reached after the 24 + 24 h treatment. BAX and BCL-2 are two members of the BCL family with an opposite role in regulating mitochondrial function and cytochrome *c* release. Alterations of mitochondria homeostasis represent one of the main pathways involved in apoptosis. In non-apoptotic cells, BAX is mainly located in the cytosol, while BCL-2 is found in the mitochondrial membranes. Upon apoptosis induction, BAX translocates [technical?] into mitochondria triggering permeabilization of mitochondrial outer membrane with cytochrome *c* release. The apoptotic factors are then released into the cytoplasm and transferred to the nucleus, where they induce apoptosis. The pro-survival BCL-2 acts as antiapoptotic protein by preventing mitochondrial outer membrane permeabilization and inhibiting the transfer of apoptosis-inducing factors to the nucleus. BAX/BCL-2 ratio regulates the mitochondrial intrinsic apoptosis pathway with subsequent cytochrome *c* release in the cytoplasm.

Several studies described the anticancer activity of grape seed standardized extracts against different human cancer cell lines such as breast, colon, lung and skin (Mantena, Baliga, & Katiyar, 2006) due to the presence of specific metabolites residues (gallate esters, pyrogallol) (Chou, Kaur, Thompson, Agarwal, & Agarwal, 2010; Mitsuhashi, Saito, Nakajima, Shima, & Ubukata, 2008). (Cheah et al., 2014). Our *in vitro* study showed, for the first time, the effectiveness of complex semi-polar extracts from Aglianico and Falanghina seeds, suggesting that a specific combination of active compounds characterizes a grape variety and influences its biological activity. Other studies using semipolar fractions extracted from mature red grape seeds showed that they affected cell viability and enhanced Flouracil toxicity of 5-FU in Caco-2 cells (Cheah et al., 2014). Therefore, to gain a first clue about the source of this bioactivity, we exploited high-resolution metabolomics to investigate the composition of the compounds accumulated in the peel and seed extracts of the two grape varieties. Overall, resulted highly enriched in proanthocyanidin molecules, which were present at very low levels or totally undetected in the peel samples of both varieties. This is consistent with previous reports (Yang & Chien, 2000). In our study, 53 proanthocyanidins (PA) were detected, with a maximum number of 11 polymerized units. Interestingly, Aglianico seeds displayed higher total PA content and higher polymerized PAs. It has been previously reported that the PA degree of polymerization can affect the biological activity (Zhou et al., 2014). This finding agrees with the stronger inhibitory effect of Aglianico compared to Falanghina seeds on MM cell lines. In addition, a series of PA precursors resulted largely variable within the two tissues and the two varieties: most of them showed higher levels in

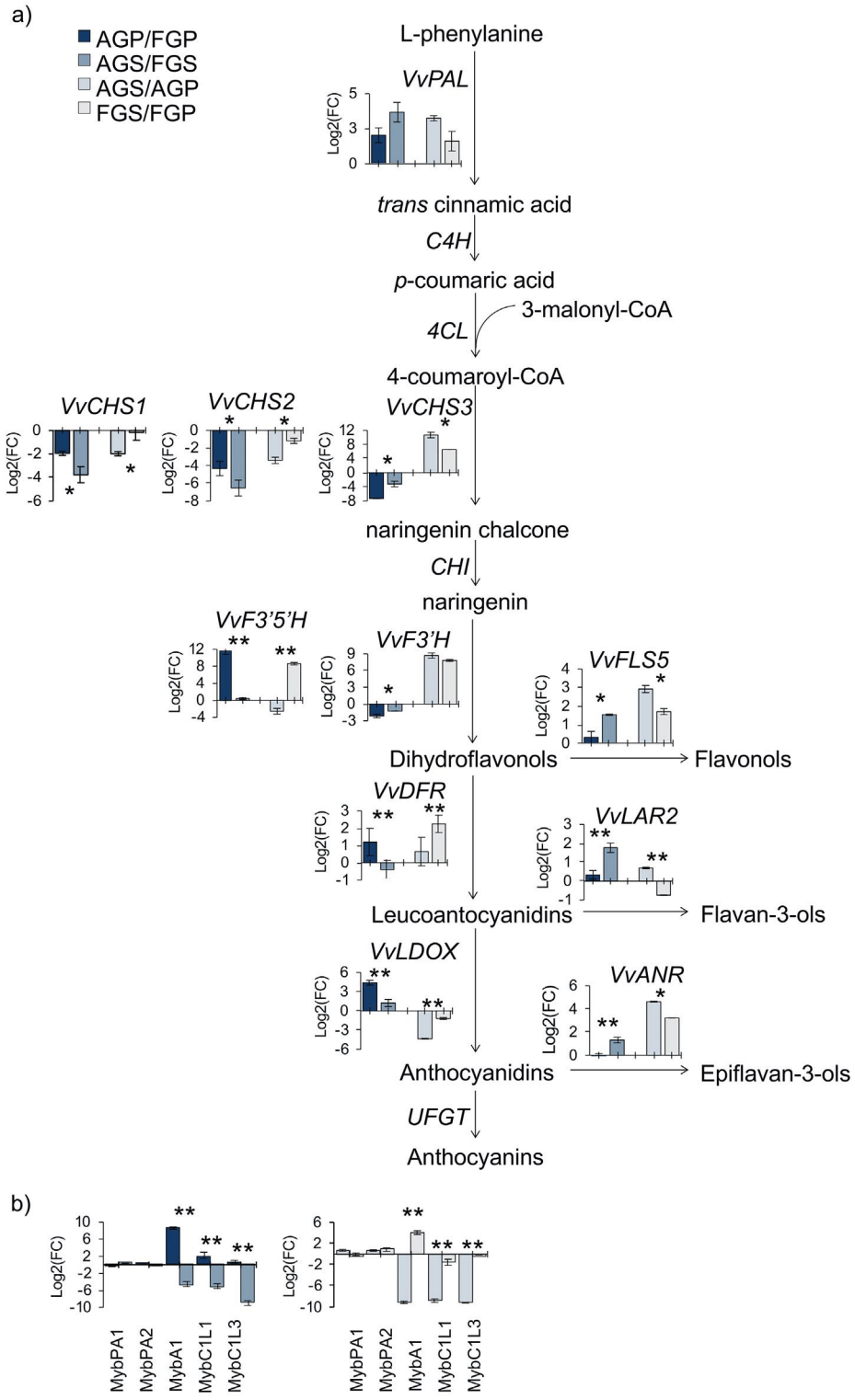

Fig. 6. Expression analysis of proanthocyanidin biosynthesis genes. Representation of the general phenylpropanoid and flavonoid pathways leading to the production of flavonols, proanthocyanidins and anthocyanins. The barplots within the pathway report the expression analysis of (a) 11 structural genes and (b) five transcription factors (*MYBPA1, MYBPA2, MYBA1, MYBC1-L1, MYBC1-L3*) in: the peel of Aglianico compared to that of Falanghina (AGP/FGP); the seeds of Aglianico compared to those of Falanghina (AGS/FGS); the seeds vs peel of Aglianico (AGS/AGP); the seeds vs peel of Falanghina (FGS/FGP). Transcript abundance is represented in log2 Fold Change (FC). Statistically significant variations: *p < 0.05 **p < 0.01.

Aglianico with respect to Falanghina. These data agree with previous studies (Cavallini et al., 2015; Gascuel et al., 2017) highlighting a more active metabolic flux in red compared to white grape varieties, starting from phenylalanine that is the first metabolite in the pathway. With respect to the detected bioactivity of AGS and FGS extracts, our metabolomics data suggest that phenylpropanoids, particularly proanthocyanidins, are responsible or at least greatly contribute to the anti cancer activity; in this context, we cannot exclude that additional compounds may take part in this process, and future fractionation studies will be carried out to unravel the molecules whose accumulation is mostly associated to the bioactivity.

It is known that a dynamic control of the gene expression process represents a major mechanism for metabolite accumulation. For example, the different accumulation in phenylpropanoid end products

such as anthocyanins and PAs, in red vs white grape varieties is dependent on the activity of a series of *Myb* transcription factors, (Bogs, Jaffe, Takos, Walker, & Robinson, 2007; Cavallini et al., 2015; Deluc et al., 2006, 2008; Huang et al., 2014; Kobayashi, Ishimaru, Hiraoka, & Honda, 2002; Terrier et al., 2009; Walker et al., 2007). Similarly, pathway structural genes can also contribute to differential metabolite accumulation. To this aim we analysed in the grape samples the expression of phenylpropanoid structural and regulating genes in order to determine how the transcriptional activities could be associated to the differences in metabolite profiles of Aglianico and Falanghina. Moreover, to quantify the differences in their expressions between seeds and peels, we investigated the expression of five transcription factor paralogs (Azuma, Yakushiji, Koshita, & Kobayashi, 2012; Rinaldi et al., 2017). Overall, the gene expressions were statistically different between tissues (seed vs peel) and varieties (Aglianico vs Falanghina), confirming that their regulation was not only affected by the genetic variety but was also spatially defined (Carrier et al., 2012; Rinaldi et al., 2017; Villano et al., 2017). Out of the 16 genes monitored, only *VvPAL* was expressed similarly in the seed and peel of Aglianico when compared to Falanghina. This is probably due to its role in the phenylpropanoid pathway. Indeed, the phenylalanine ammonia-lyase (*PAL*) catalyzes the conversion of L-phenylalanine into trans-cinnamate, the initial committed step of the multi-branched phenylpropanoid pathway in higher plants. Regarding the late pathway genes, most of them were overexpressed in both tissues, with higher values identified in seeds rather than peels. These data well agree with the metabolic results showing a higher amount of catechin and epicatechin in seed rather then in the peel. Metabolomic and transcriptional data clearly indicated that proanthocyanidins represent the main class of compounds accumulated in seed extracts.

This is the first study indicating that grape seed semi-polar extracts are effective in MM cells. Moreover studies on the molecular mechanisms identified that apoptotic intrinsic signaling pathways is involved. Our findings will provide the reference for future analyses to identify the metabolic fraction/s mainly responsible/s for this bioactivity, thus to produce more effective extracts; in addition, this study will allow planning more detailed *in vivo* experiments relevant to the treatment of MM and possibly for clinical applications.

5. Conclusion

Taken together, our data demonstrated the biological activity of grape seed rather than of peel extracts from Aglianico and Falanghina in different MM cell lines. Our results indicated that seed extracts of both varieties are able to induce apoptosis in a largely dose and time-dependent manner also in cell lines does not responding to standard chemotherapeutics. Global metabolic profiling of peel and seed extracts evidenced that the majority of differentially accumulated compounds belong to the group of the proanthocyanidins, with marked quantitative variations between Falanghina and Aglianico. These results were also confirmed by the expression analysis of genes involved in proanthocyanidin synthesis. These data provide hint about the anti cancer contributing role in the detected bioactivity.

Our multidisciplinary study strengthens the idea that the production of new phenolic molecules from grape seeds can be viewed as a source of new metabolites with biological activity to be used as nutraceuticals or new drugs for MM treatment.

Ethic statement

The authors declare that this research does not include any human subjects and animal experiments,

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

FDM PhD fellowship in Biology is supported by MIUR project PON “Dottorati Innovativi con caratterizzazione industriale” 2017-2018. We thank Dr. Pasquale Barba for his support in FACS analysis. We thank IGB FACS facility and Dr. Maria Rosaria Aletta for bibliographic assistance. We are grateful to Dr Paola Barbarino for proofreading the paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.103515.

References

Ashkenazi, A., & Dixit, V. M. (1998). Death receptors: Signaling and modulation. *Science, 281*, 1305–1308.
Aversano, R., Contaldi, F., Adelfi, M. G., D'Amelia, V., Diretto, G., De Tommasi, N., ... Carputo, D. (2017). Comparative metabolite and genome analysis of tuber-bearing potato species. *Phytochemistry, 137*, 42–51.
Azuma, A., Yakushiji, H., Koshita, Y., & Kobayashi, S. (2012). Flavonoid biosynthesis-related genes in grape skin are differentially regulated by temperature and light conditions. *Planta, 236*, 1067–1080.
Baldi, A., Piccolo, M. T., Boccellino, M. R., Donizetti, A., Cardillo, I., La Porta, R., Quagliuolo, L., Spugnini, E. P., Cordero, F., Citro, G., Menegozzo, M., Calogero, R. A., & Crispi, S. (2011). Apoptosis induced by piroxicam plus cisplatin combined treatment is triggered by p21 in mesothelioma. *PLoS ONE, 6*.
Bazan-Salinas, I. L., Matias-Perez, D., Perez-Campos, E., Mayoral, L. P. C., & Garcia-Montalvo, I. A. (2016). Reduction of platelet aggregation from ingestion of oleic and linoleic acids found in vitis vinifera and arachis hypogaea oils. *American Journal of Therapeutics, 23*, E1315–E1319.
Bogs, J., Jaffe, F. W., Takos, A. M., Walker, A. R., & Robinson, S. P. (2007). The grapevine transcription factor VvMYBPA1 regulates proanthocyanidin synthesis during fruit development. *Plant Physiology, 143*, 1347–1361.
Carrier, G., Le Cunff, L., Dereeper, A., Legrand, D., Sabot, F., Bouchez, O., ... This, P. (2012). Transposable elements are a major cause of somatic polymorphism in vitis vinifera L. *PLoS ONE, 7*.
Cavallini, E., Matus, J. T., Finezzo, L., Zenoni, S., Loyola, R., Guzzo, F., ... Tornielli, G. B. (2015). The phenylpropanoid pathway is controlled at different branches by a set of R2R3-MYB C2 repressors in grapevine. *Plant Physiology, 167*, 1448–U1552.
Cheah, K. Y., Howarth, G. S., Bindon, K. A., Kennedy, J. A., & Bastian, S. E. (2014). Low molecular weight procyanidins from grape seeds enhance the impact of 5-Fluorouracil chemotherapy on Caco-2 human colon cancer cells. *PLoS ONE, 9*, e98921.
Chen, H., Landen, C. N., Li, Y., Alvarez, R. D., & Tollefsbol, T. O. (2013). Enhancement of cisplatin-mediated apoptosis in ovarian cancer cells through potentiating G2/M arrest and p21 upregulation by combinatorial epigallocatechin gallate and sulforaphane. *Journal of Oncology, 2013*, 872957.
Chou, S. C., Kaur, M., Thompson, J. A., Agarwal, R., & Agarwal, C. (2010). Influence of gallate esterification on the activity of procyanidin B2 in androgen-dependent human prostate carcinoma LNCaP cells. *Pharmaceutical Research, 27*, 619–627.
Crispi, S., Calogero, R. A., Santini, M., Mellone, P., Vincenzi, B., Citro, G., ... Menegozzo, M. (2009). Global gene expression profiling of human pleural mesotheliomas: identification of matrix metalloproteinase 14 (MMP-14) as potential tumour target. *PLoS ONE, 4*.
Crispi, S., Cardillo, I., Spugnini, E. P., Citro, G., Menegozzo, S., & Baldi, A. (2010). Biological agents involved in malignant mesothelioma: Relevance as biomarkers or therapeutic targets. *Current Cancer Drug Targets, 10*, 19–26.
Deluc, L., Barrieu, F., Marchive, C., Lauvergeat, V., Decendit, A., Richard, T., ... Hamdi, S. (2006). Characterization of a grapevine R2R3-MYB transcription factor that regulates the phenylpropanoid pathway. *Plant Physiology, 140*, 499–511.
Deluc, L., Bogs, J., Walker, A. R., Ferrier, T., Decendit, A., Merillon, J. M., ... Barrieu, F. (2008). The transcription factor VvMYB5b contributes to the regulation of anthocyanin and proanthocyanidin biosynthesis in developing grape berries. *Plant Physiology, 147*, 2041–2053.
Ferri, M., Bin, S., Vallini, V., Fava, F., Michelini, E., Roda, A., ... Tassoni, A. (2016). Recovery of polyphenols from red grape pomace and assessment of their antioxidant and anti-cholesterol activities. *New Biotechnology, 33*, 338–344.
Fischer, U., Janicke, R. U., & Schulze-Osthoff, K. (2003). Many cuts to ruin: A comprehensive update of caspase substrates. *Cell Death and Differentiation, 10*, 76–100.
Gascuel, Q., Diretto, G., Monforte, A. J., Fortes, A. M., & Granell, A. (2017). Use of natural diversity and biotechnology to increase the quality and nutritional content of tomato and grape. *Frontiers in Plant Science, 8*, 652.
Ghafoor, K., Park, J., & Choi, Y. H. (2010). Optimization of supercritical fluid extraction of bioactive compounds from grape (Vitis labrusca B.) peel by using response surface methodology. *Innovative Food Science & Emerging Technologies, 11*, 485–490.
Gollucke, A. P., Peres, R. C., Odair, A., Jr., & Ribeiro, D. A. (2013). Polyphenols: A

nutraceutical approach against diseases. *Recent Patents on Food Nutrition Agriculture, 5*, 214–219.
Huang, Y. F., Vialet, S., Guiraud, J. L., Torregrosa, L., Bertrand, Y., Cheynier, V., ... Terrier, N. (2014). A negative MYB regulator of proanthocyanidin accumulation, identified through expression quantitative locus mapping in the grape berry. *New Phytologist, 201*, 795–809.
Katiyar, S. K., & Athar, M. (2013). Grape seeds: Ripe for cancer chemoprevention. *Cancer Prevention Research (Phila), 6*, 617–621.
Kaur, M., Agarwal, C., & Agarwal, R. (2009). Anticancer and cancer chemopreventive potential of grape seed extract and other grape-based products. *Journal of Nutrition, 139*, 1806S–1812S.
Kobayashi, S., Ishimaru, M., Hiraoka, K., & Honda, C. (2002). Myb-related genes of the Kyoho grape (Vitis labruscana) regulate anthocyanin biosynthesis. *Planta, 215*, 924–933.
Lee, Y. J., Park, I. S., Lee, Y. J., Shim, J. H., Cho, M. K., Nam, H. S., ... Lee, S. H. (2014). Resveratrol contributes to chemosensitivity of malignant mesothelioma cells with activation of p53. *Food and Chemical Toxicology, 63*, 153–160.
Mantena, S. K., Baliga, M. S., & Katiyar, S. K. (2006). Grape seed proanthocyanidins induce apoptosis and inhibit metastasis of highly metastatic breast carcinoma cells. *Carcinogenesis, 27*, 1682–1691.
Martinotti, S., Ranzato, E., Parodi, M., Vitale, M., & Burlando, B. (2014). Combination of ascorbate/epigallocatechin-3-gallate/gemcitabine synergistically induces cell cycle deregulation and apoptosis in mesothelioma cells. *Toxicology and Applied Pharmacology, 274*, 35–41.
Mitsuhashi, S., Saito, A., Nakajima, N., Shima, H., & Ubukata, M. (2008). Pyrogallol structure in polyphenols is involved in apoptosis-induction on HEK293T and K562 cells. *Molecules, 13*, 2998–3006.
Piccolo, M. T., Menale, C., & Crispi, S. (2015). Combined anticancer therapies: An overview of the latest applications. *Anticancer Agents Medicinal Chemistry, 15*, 408–422.
Rinaldi, A., Villano, C., Lanzillo, C., Tamburrino, A., Jr., Jourdes, M., Teissedre, P. L., ... Aversano, R. (2017). Metabolic and RNA profiling elucidates proanthocyanidins accumulation in Aglianico grape. *Food Chemistry, 233*, 52–59.
Seca, A. M. L., & Pinto, D. C. G. A. (2018). Plant secondary metabolites as anticancer agents: Successes in clinical trials and therapeutic application. *International Journal of Molecular Sciences, 19*.
Serri, C., Argiro, M., Piras, L., Mita, D. G., Saija, A., Mita, L., ... Mayol, L. (2017). Nanoprecipitated curcumin loaded particles: Effect of carrier size and drug complexation with (2-hydroxypropyl)-beta-cyclodextrin on their biological performances. *International Journal of Pharmaceutics, 520*, 21–28.
Spugnini, E. P., Cardillo, I., Verdina, A., Crispi, S., Saviozzi, S., Calogero, R., ... Baldi, A. (2006). Piroxicam and cisplatin in a mouse model of peritoneal mesothelioma. *Clinical Cancer Research, 12*, 6133–6143.
Spugnini, E. P., Crispi, S., Scarabello, A., Caruso, G., Citro, G., & Baldi, A. (2008). Piroxicam and intracavitary platinum-based chemotherapy for the treatment of advanced mesothelioma in pets: Preliminary observations. *Journal of Experimental & Clinical Cancer Research, 27*, 6.
Terrier, N., Torregrosa, L., Ageorges, A., Vialet, S., Verries, C., Cheynier, V., & Romieu, C. (2009). Ectopic expression of VvMybPA2 promotes proanthocyanidin biosynthesis in grapevine and suggests additional targets in the pathway. *Plant Physiology, 149*, 1028–1041.
Vallejo, M. J., Salazar, L., & Grijalva, M. (2017). Oxidative stress modulation and ROS-mediated toxicity in cancer: A review on in vitro models for plant-derived compounds. *Oxidative Medicine and Cellular Longevity, 2017*, 4586068.
Villano, C., Rinaldi, A., Lanzillo, C., Moio, L., Tamburrino, A., Carputo, D., ... Aversano, R. (2017). Polyphenol content and differential expression of flavonoid biosynthetic pathway genes in berries of Aglianico. *Acta Horticulturae, 1188*, 141–148.
Walker, A. R., Lee, E., Bogs, J., McDavid, D. A. J., Thomas, M. R., & Robinson, S. P. (2007). White grapes arose through the mutation of two similar and adjacent regulatory genes. *Plant Journal, 49*, 772–785.
Wang, Y., Lee, K. W., Chan, F. L., Chen, S. A., & Leung, L. K. (2006). The red wine polyphenol resveratrol displays bilevel inhibition on aromatase in breast cancer cells. *Toxicological Sciences, 92*, 71–77.
Yang, Y., & Chien, M. (2000). Characterization of grape procyanidins using high-performance liquid chromatography/mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Journal of Agriculture and Food Chemistry, 48*, 3990–3996.
Zhou, H. C., Tam, N. F. Y., Lin, Y. M., Ding, Z. H., Chai, W. M., & Wei, S. D. (2014). Relationships between degree of polymerization and antioxidant activities: A study on proanthocyanidins from the leaves of a medicinal mangrove plant ceriops tagal. *PLoS ONE, 9*.

antioxidants

MDPI

Article

Ginkgo biloba Prevents Oxidative Stress-Induced Apoptosis Blocking p53 Activation in Neuroblastoma Cells

Francesco Di Meo [1,2,†], Rossana Cuciniello [1,†], Sabrina Margarucci [3], Paolo Bergamo [4], Orsolina Petillo [3], Gianfranco Peluso [3], Stefania Filosa [1,5,*,‡] and Stefania Crispi [1,*,‡]

1 Institute of Biosciences and BioResources-UOS Naples CNR, Via P. Castellino 111, 80131 Naples, Italy; francesco.dimeo.90@gmail.com (F.D.M.); rossanacuciniello@gmail.com (R.C.)
2 Department of Biology, University of Naples Federico II, Complesso Universitario Monte Sant'Angelo Via Cinthia, 80126 Naples, Italy
3 Institute on Terrestrial Ecosystems (IRET) CNR, Via P. Castellino 111, 80131 Naples, Italy; sabrina.margarucci@cnr.it (S.M.); orsolina.petillo@cnr.it (O.P.); gianfranco.peluso@cnr.it (G.P.)
4 Institute of Food Science CNR, Via Roma, 64, 83100 Avellino, Italy; paolo.bergamo@isa.cnr.it
5 IRCCS Neuromed, Localitá Camerelle, 86077 Pozzilli (IS), Italy
* Correspondence: stefania.filosa@ibbr.cnr.it (S.F.); stefania.crispi@ibbr.cnr.it (S.C.)
† Co-first authors.
‡ Co-last authors.

Received: 21 February 2020; Accepted: 25 March 2020; Published: 26 March 2020

Abstract: Oxidative stress has been associated to neuronal cell loss in neurodegenerative diseases. Neurons are post-mitotic cells that are very sensitive to oxidative stress—especially considering their limited capacity to be replaced. Therefore, reduction of oxidative stress, and inhibiting apoptosis, will potentially prevent neurodegeneration. In this study, we investigated the neuroprotective effect of *Ginkgo biloba* extract (EGb 761) against H_2O_2 induced apoptosis in SK-N-BE neuroblastoma cells. We analysed the molecular signalling pathway involved in the apoptotic cell death. H_2O_2 induced an increased acetylation of p53 lysine 382, a reduction in mitochondrial membrane potential, an increased BAX/Bcl-2 ratio and consequently increased Poly (ADP-ribose) polymerase (PARP) cleavage. All these effects were blocked by EGb 761 treatment. Thus, EGb 761, acting as intracellular antioxidant, protects neuroblastoma cells against activation of p53 mediated pathway and intrinsic mitochondrial apoptosis. Our results suggest that EGb 761, protecting against oxidative-stress induced apoptotic cell death, could potentially be used as nutraceutical for the prevention and treatment of neurodegenerative diseases.

Keywords: *ginkgo biloba*; oxidative stress; p53; apoptosis; neuroprotection; neuroblastoma cells

1. Introduction

Oxidative stress occurs in the cell when the antioxidant defence in unable to balance the rate of the reactive oxygen species generated [1]. The cellular response to oxidative stress mainly depends on the injury intensity and duration. Oxidant sources can be external to the cell, such as environmental stress, or they can be generated as a side product of normal aerobic metabolism.

When present in excess, oxidants elevate the intracellular levels of reactive oxygen species (ROS) and damage cell membrane, proteins and DNA. Oxidative stress generates different consequences ranging from stimulation of cell proliferation to cell cycle arrest, stimulation or inhibition of cells migration, and up to cell death by apoptosis or necrosis [2–4].

Among different cell types, neurons are particularly prone to production of ROS and highly susceptible to redox stress, because of their high lipid and metal ion content combined with their high metabolic rate and relatively low concentrations of antioxidants [5]. Oxidative stress is considered a relevant direct or indirect process associated to neuronal cell loss in neurodegenerative diseases such as Parkinson's disease [6] and Alzheimer's disease [7]. Despite the differences in clinical manifestations, neurological disorders show common pathological processes. All of them are characterized by degeneration and progressive loss of distinct neuron subsets in specific areas of the brain. Moreover, they share common neurodegenerative pathways associated with progressive neuronal dysfunction. Most of these pathways seems to be related to oxidative stress, to the imbalance between generation of free radicals and cellular antioxidant defences, and to apoptotic cell death [8]. Neuronal cell death is especially dangerous because adult neurons are post-mitotic cells with limited capacity to proliferate or be replaced. Therefore, in neuronal cells, reduction of oxidative stress could inhibit apoptosis potentially preventing neurodegeneration.

Herbal extracts and phytochemicals can act as protective agent against oxidative stress. Flavonoids are in particular described as the molecules with a strong bioactivity in brain functions with positive effects on synaptic plasticity and neuronal activity. Among the bioactive phytochemicals, *Ginkgo biloba* is one of the most used worldwide [9]. *Ginkgo biloba* has been widely used in the treatment of cardiovascular and cerebrovascular diseases, liver cirrhosis and acute and chronic renal disease. More recently, a standardized extract of *Ginkgo biloba*, EGb 761, has been found to have neuroprotective effects in several central nervous system and neurodegenerative diseases [9].

EGb 761 (EGb) is a standardised extract of *Ginkgo biloba* leaves that contains a well-defined concentration of flavone glycosides and terpene lactones (24% and 6%, respectively). In fact, EGb contains 6% terpenoids (in which 3.1% are ginkgolides A, B, C, and J and 2.9% is bilobalide), 24% flavonoid glycosides, and 5% to 10% organic acids [10]. The flavonoids act as free radical scavenging, whereas terpenes lactones protect mitochondrial membranes from free radical damage [11]. EGb has been described to have antioxidant properties playing an important role as a free radical scavenger [12]. It has demonstrated that the antioxidant activity, as a "radical scavenger", is due to its superoxide dismutase-like activity that enables it to scavenge hydroxyl radicals [13]. Ginkgo biloba also has the capacity to regulate the oxidative stress. The levels of glutathione, malondialdehyde, superoxide dismutase and nitric oxide, increased after a treatment with EGb [14]. These properties determine beneficial effects in neurodegenerative diseases as Alzheimer [15,16] or Parkinson [17,18]. However, the mechanism of the action of EGb protection against oxidative stress-induced apoptosis remains to be fully elucidated.

The evidence mentioned above prompted us to explore the protective effect of EGb against oxidative stress-induced apoptosis in neuroblastoma cells. Our data suggested that this extract could act as cellular scavengers against induced oxidative stress blocking the onset of molecular apoptotic pathway.

2. Materials and Methods

2.1. Cell Culture and Chemicals

Human neuroblastoma cell line, SK-N-BE(2) (CRL-2271, ATCC®, LGC Standards S.r.l., Milan Italy) were cultured at 37 °C in a 5% CO_2 humidified incubator in either RPMI-1640 medium (Euroclone spa, 20016 Pero, MI) supplemented with 10% fetal bovine serum (FBS), glutamine (2 mM), sodium pyruvate and antibiotics (0.02 mg/mL streptomycin and 0.02 IU/mL penicillin).

Ginkgo biloba L. extract EGb 761 (EGb) was a gift from Schwabe (Schwabe Pharma Italia Srl, Egna, Italy). EGb stock solution contained 250 mg/mL of extract was dissolved in dimethyl sulfoxide (DMSO). Hydrogen peroxide (H_2O_2,) (Sigma-Aldrich, St. Louis, MI, USA) was used as oxygen stress inducer.

2.2. Cell Proliferation Assay

For each experiment, approximately 1.5×10^5 cells/well in 6-well plates were plated and treated as described and untreated cells were used as control. To identify the H_2O_2 concentration able to determine about 50% of viability decrease, SK-N-BE cells were treated with 25, 50, 75, and 100 mM of H_2O_2 for 24 h. When specified cells were treated with 25 μg/mL EGb for 24 h, the medium was replaced before H_2O_2 treatment.

To evaluate the effect of EGb on cell viability, cells were treated with 10, 25, and 50 μg/mL for 24 h. To estimate the protective effect of EGb cells were treated for 24 h with 25 μg/mL of EGb, then insulted with 75 μM of H_2O_2 for additional 24 h. EGb was dissolved in DMSO. Untreated samples were exposed to 0.1% DMSO and were used as control.

For each experiment after treatment cells were collected and counted with Trypan Blue solution. (T6146, Sigma-Aldrich, St. Louis, MI, USA).

All the experiments were performed in triplicate. Data are expressed as the mean ± SD.

2.3. Propidium Iodide and DAPI Staining Assay

In 6-well plates approximately 1.5×10^5 cells/well were plated and treated with EGb and H_2O_2 as previously described. After treatment cells were stained with 10 mg/mL of Propidium Iodide (PI) (Bioshop, Burlington, ON L7L 6A4, Canada) and DAPI (4′,6-diamidine-2′-phenyl indole dihydro chloride, Roche, Mannheim, Germany). Representative images were taken using fluorescent microscope (DMI8, Leica, Instruments, Germany) and florescence was quantified using Leica Application Suite X software (Leica, Milan, Italy). All the experiments were performed in triplicate. Data are expressed as the mean ± SD.

2.4. Mitochondria Membrane Potential Measurement

Mitochondria membrane potentials (MMP) were measured by JC-10 (Sigma-Aldrich, St. Louis, MI, USA) following the manufacturer's instructions. Loss of MMP was indicated by a progressive JC-10 dislocation from mitochondria to the cytosol. Cells were photographed using fluorescent microscope (DMI8, Leica, Instruments, Germany). Red (540/570 nm) and green (485/534 nm) florescence was quantified by Leica Application Suite X (LAS X) (Leica, Milan, Italy). All the experiments were performed in triplicate. Data are expressed as the mean ± SD.

2.5. RNA Extraction and q-PCR

RNA extraction and q-PCR were essentially performed as previously described [19]. Brefly, Total RNA was isolated from each sample with Trizol (Thermo Fisher Scientific, Waltham, MA USA), as indicated by manufacturer. For each sample to analyse, cDNA was than obtained starting from 200 ng of total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Thermo Fisher Scientific, Waltham, MA USA). The described selected genes using gene specific primers

BAX: Forward 5′-TTTGCTTCAGGGTTTCATCCA-3′: Reverse 5′- CTCCATGTTACTGTCCA GTTCGT-3′; BCL- 2: Forward 5′-GTTCCCTTTCCTTCCATCC-3′; Reverse 5′-TAGCCA GTCCAGAGG TGAG-3′; p53: Forward 5′-TCTGTCCCTTCCCAGAAAACC-3′; Reverse 5′-CAAGAAGCCCAGAC GGAAAC-3′; GAPDH: Forward 5′-CAAGGCTGTGGGCAAGGT-3′; Reverse 5′-GGAAGGCCA TGCCAGTGA-3′.

All primers were selected using a specific software (Primer express 2.0, Applied Biosystems, Foster city, CA, USA) and all of them specifically covered the exon-exon junctions. The analysis of gene expression was done as described in [20] and GAPDH gene was used as internal control. qPCRs were done using the 7900 HT Real Time PCR (Applied Biosystem) and for each experimental condition a triplicate was performed. Data obtained are expressed as the mean ± SD.

2.6. Western Blot

For each experimental condition and from each sample total, protein extracts were obtained, as described in [19]. For the analysis, 20 µg of each sample were loaded on Tris–glycine gradient gels (4% to 15% gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and separated at 100 V. To probe proteins with specific primary antibodies antibodies, they were transferred to PVDF membranes (Biorad Laboratories, Inc., Hercules, CA, USA). All the secondary antibodies used were horseradish peroxidase conjugated. All the antibodies were used as indicated by manufacturer. The following primary antibodies were used for Western blot: PARP (Cell Signaling, #9542), BCL-2 (Abcam, ab182858), BAX (Santa Cruz Biotechnology, sc-493), Acetyl-p53 Lys382 (Cell Signaling, #9542). As the internal control we used β-Actin (Cell Signaling, #3700), which was used as the loading control. To detect protein levels, Clarity western ECL (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used. The quantization was then obtained using ImageJ software vJ1, an open source tool. For each experimental condition a triplicate was performed, and results are expressed as the mean ± SD.

2.7. Statistical Analysis

To perform calculations on sample size the on line available software GPower was used. Sample size was determined using as parameters: $1 - \beta = 0.80$, $\alpha = 5\%$. For each experiment, statistical analysis was done using Graph Pad Prism 6.0 (GraphPad Software, San Diego, CA, USA) to analyse the significance of the differences between control and treatments. We evaluated the differences among means applying the one-way ANOVA. Bonferroni's multiple comparison test with Bonferroni post hoc correction was used to analyse the differences of each treatment respect to the control.

Statistically significant difference compared to DMSO treated cells are: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

3. Results

3.1. EGb Protects SK-N-BE Cells Against Oxidative Stress Induced Apoptotic Cell Death

We first determined, in a dose-response curve at 24 h, the amount of H_2O_2 that had lethal effect on SK-N-BE human neuroblastoma cells. Oxidative stress induced cell death was around 50% when cells were treated with 75 µM H_2O_2 (Figure 1A). To verify if 75 µM H_2O_2 was able to induce apoptosis on SK-N-BE cells, untreated and H_2O_2 treated cells, without fixation and permeabilization, were stained with AnnexinV-FITC and PI. The analysis by fluorescence microscopy confirmed that H_2O_2, at this concentration, induced apoptosis (Figure 1B). Indeed, a strong increased number of stained AnnexinV-FITC and PI cells were present when cells were treated with H_2O_2 (Figure 1C).

To ascertain that EGb did not induce cell death, SK-N-BE cells were treated with various concentrations of EGb for 24 h. Results showed that EGb at all used concentrations did not reduce cell viability (Figure 2A).

To determine whether EGb played a role in protecting SK-N-BE from H_2O_2 -induced cell death, cells were pre-treated for 24 h with EGb (25 µg/mL) and then challenged with H_2O_2 (75 µM) for the following 24 h. Analysis of cell vitality revealed that the oxidant sensitivity of SK-N-BE cells was completely reverted by pre-treatment with EGb (Figure 2B). Concomitant addition of EGb and H_2O_2 or addition of EGb 24 h later H_2O_2 treatment did not result in a reversion of lethality (Figure S1). Results were confirmed by analysis with PI and DAPI staining, as shown by fluorescence microscopy analysis. Indeed, a comparable number of the PI positive cells were present in untreated, EGb treated and EGb-H_2O_2 treated cells, where a higher number were present in presence of H_2O_2 alone (Figure 2C,D).

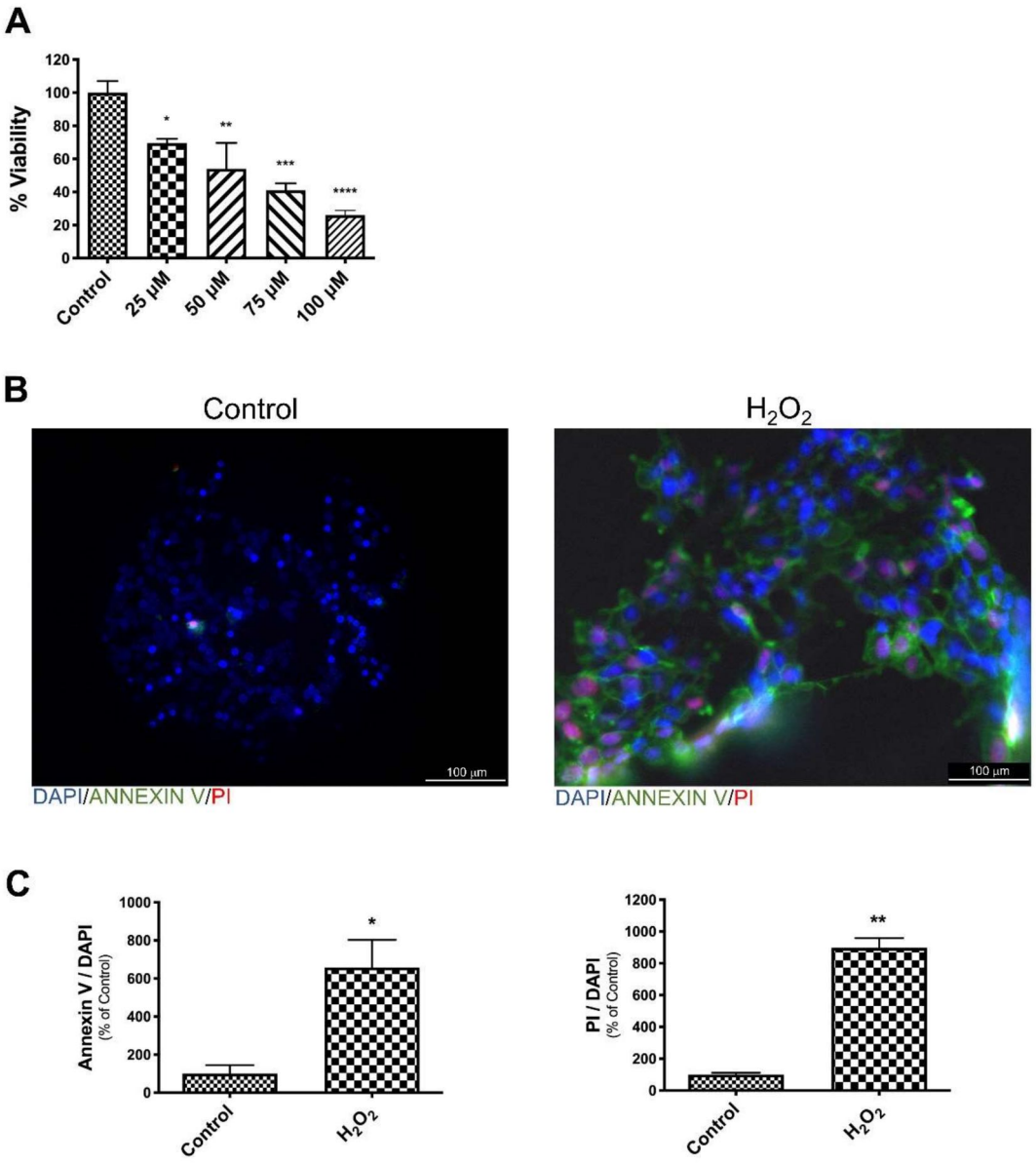

Figure 1. H_2O_2 affects SK-N-BE cell viability. Cell viability decrease after treatments with different concentration of H_2O_2 (**A**). representative images of DAPI, Annexin V-FITC and PI triple fluorescence staining showing cellular apoptosis after H_2O_2 treatment. DAPI: blue; AnnexinV: green; PI: red (**B**). Histograms reports quantification of fluorescence of DAPI, Annexin V, and PI (**C**). The bars represent ± the average ± SD of independent experiments ($n = 3$). Statistically significant difference compared to control cells: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

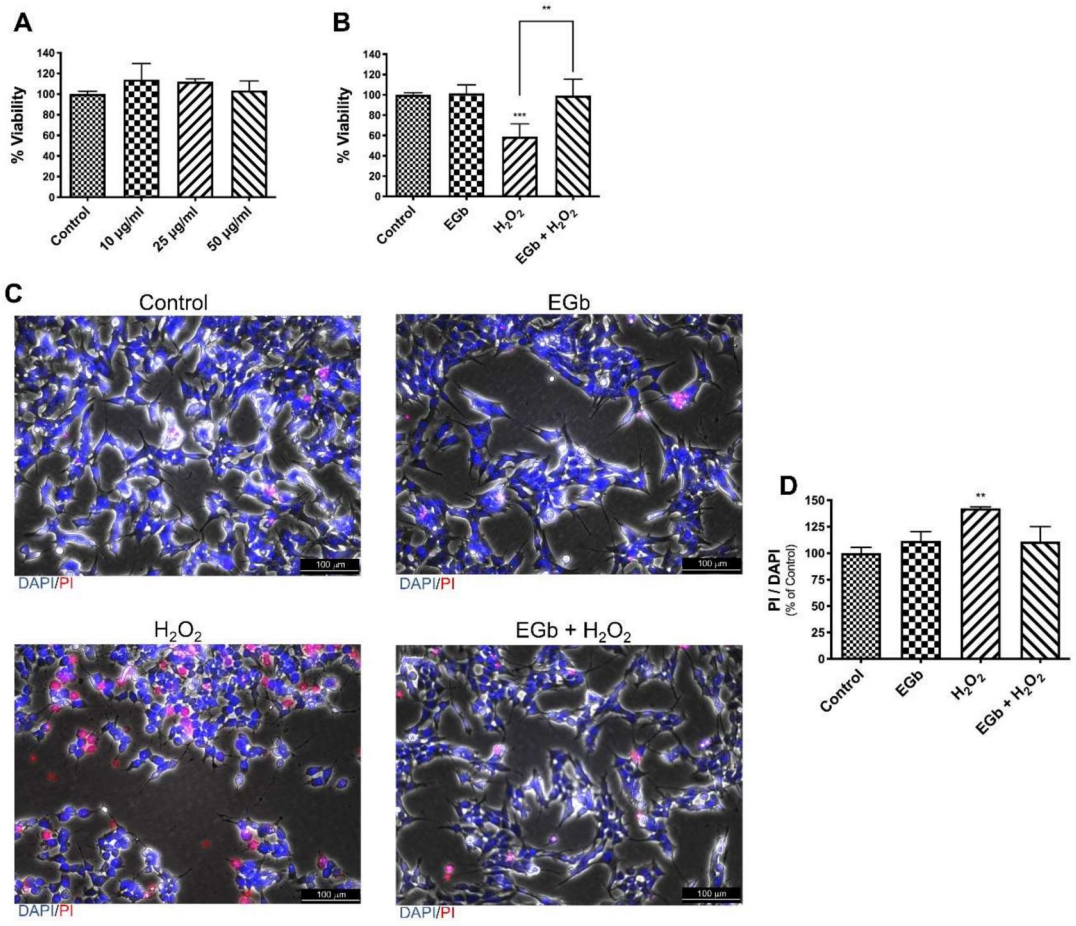

Figure 2. Effects of EGb on cell viability. Cell viability after treatments with different concentration of EGb (**A**). Cell viability after treatments with 25 mg/mL of EGb, 75 mM of H_2O_2 or a combination of them (**B**). Cells treated with DMSO were used as control. Fluorescent microscopic image of DAPI/PI stained cells; DAPI: blue; PI: red (**C**). Histogram reports quantification of fluorescence of DAPI and PI (**D**). The bars represent ± the average ± SD of independent experiments (n = 3). Statistically significant difference compared to control cells: ** $p \leq 0.01$, *** $p \leq 0.005$.

3.2. EGb Protects SK-N-BE Cells Against Oxidative Stress Induced Apoptosis

To confirm that EGb could protect cells against apoptotic cell death induced by oxidative stress, we first analysed the presence of poly(ADP-ribose) polymerase (PARP) cleavage, an hallmark of apoptosis. As expected, PARP cleavage increased after H_2O_2 treatment, although the cells were completely protected from oxidative stress-induced apoptosis in presence of EGb (Figure 3A,B). Then, to study the protective mechanism of EGb against oxidative stress-induced apoptosis, we investigated the molecular signalling pathway involved in the apoptotic cell death analysing p53 expression.

The tumor suppressor protein p53, modulating cell homeostasis, has a determinant role in cell fate.

Oxidative stress, leading to post-translational modifications of p53, allows it to regulate genes that can activate cell survival or cell death processes [21].

Gene expression analysis, by q-PCR, revealed that p53 was not modulated by oxidative stress as well as by EGb (Figure S2). It is known that increased p53-acetylation at lysine 382 (K382) promotes p53-dependent pro-apoptotic activity in cancer cells [22]. Thus, we analysed by western blot analysis whether these post-translational modifications of p53 could account for the apoptotic reduction observed in presence of EGb. Results clearly demonstrate that K382 acetyl-p53 was strongly increased following H_2O_2 insult, however p53-acetylation was inhibited in presence of EGb (Figure 3A,B).

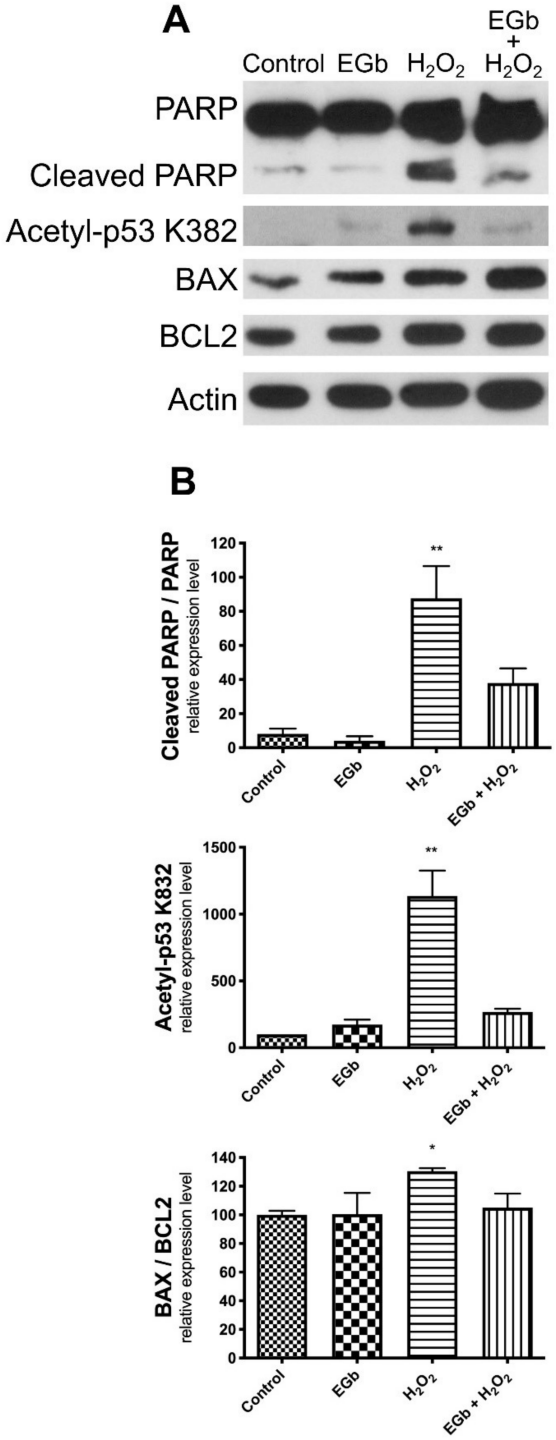

Figure 3. EGb protects SK-N-BE from apoptosis. Western blot analysis of protein expression of cleaved PARP, Acetylated-p53/K382, BAX and Bcl2 in SK-N-BE cells after treatments (**A**). expression of Acetylated-p53/K382, cleaved PARP and BAX/Bcl2 ratio normalized expression is reported in the histograms (**B**). β-Actin was used as loading control. The bars represent ± the average ± SD of independent experiments ($n = 3$). Statistically significant difference compared to control cells: * $p \leq 0.05$, ** $p \leq 0.01$. Cells treated with DMSO were used as control.

Oxidative stress activates the mitochondrial intrinsic pathway of apoptosis [23]. p53, interacting with members of the Bcl-2 family, directly participating to the activation of the intrinsic apoptosis pathway [24]. We focused our attention on the ratio between two members of the Bcl-2 family, BAX and

BCL-2, which are markers of cell susceptibility to intrinsic apoptosis. Protein expression analysis evidenced an increased BAX/Bcl-2 ratio in H_2O_2 treated SK-N-BE while pre-treatment with EGb restored a normal ratio (Figure 3A,B). These results were also confirmed by gene expression analysis, by q-PCR of the corresponding genes (Figure S1).

3.3. EGb Mitigated the H_2O_2 Induced Decrease in Mitochondrial Membrane Potential

Increased BAX/Bcl-2 ratio suggested that mitochondria are involved in apoptosis. Indeed, it is well known that, during intrinsic apoptosis, the mitochondrial membrane potential (MMP) collapses, triggering other downstream events in the apoptotic cascade. Thus, we investigated by JC-10 assay the change of MMP following H_2O_2 or EGb treatment of SK-N-BE cells. Results showed that untreated cells displayed intact, well-polarized mitochondria marked by a red punctate fluorescence. On contrary, H_2O_2 treated cells showed a reduction of the red fluorescence and an increase of the green one, indicating loss of MPM because of the progressive JC-10 dislocation from mitochondria to the cytosol. On the contrary, EGb treatment restored the fluorescence to the values of untreated cells. (Figure 4).

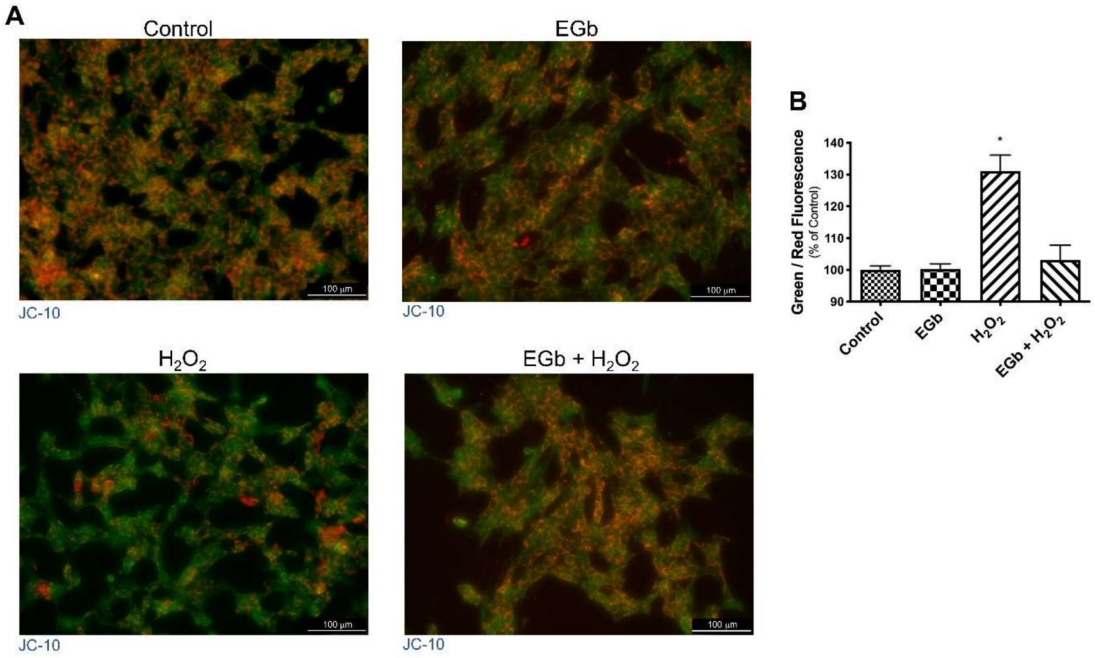

Figure 4. EGb reduce the decrease of mitochondrial membrane potential. Fluorescence analysis of mitochondria in control or EGb treated cells with or without H_2O_2 (**A**). Histogram reports quantification of fluorescence of Red (540/570 nm) and green (485/534 nm) (**B**). The bars represent ± the average ± SD of independent experiments (n = 3). Statistically significant difference compared to control cells: * $p \leq 0.05$). Cells treated with DMSO were used as control.

3.4. EGb Exhibits Intracellular Anti-Apoptotic Effect

To verify whether EGb acts as antioxidant into cells, or if it was able to directly scavenge H_2O_2 in the culture medium, SK-N-BE cells were treated for 24 h with EGb, then the culture medium was replaced and cells were challenged with H_2O_2. Results showed that that EGb determined antioxidant protection on cell viability independently by its presence in the culture medium. In fact, pre-treatment with EGb was per se sufficient to attenuate the H_2O_2-induced cell death in SK-N-BE cells (Figure 5A). Moreover, in these conditions we observed a reduced cleavage of PARP protein, a reduced amount of K382 acetyl-p53 and a reduced BAX/Bcl-2 ratio (Figure 5B,C), confirming that EGb was able to protect SNKBE cells by apoptotic cell death exerting an intracellular antioxidant action.

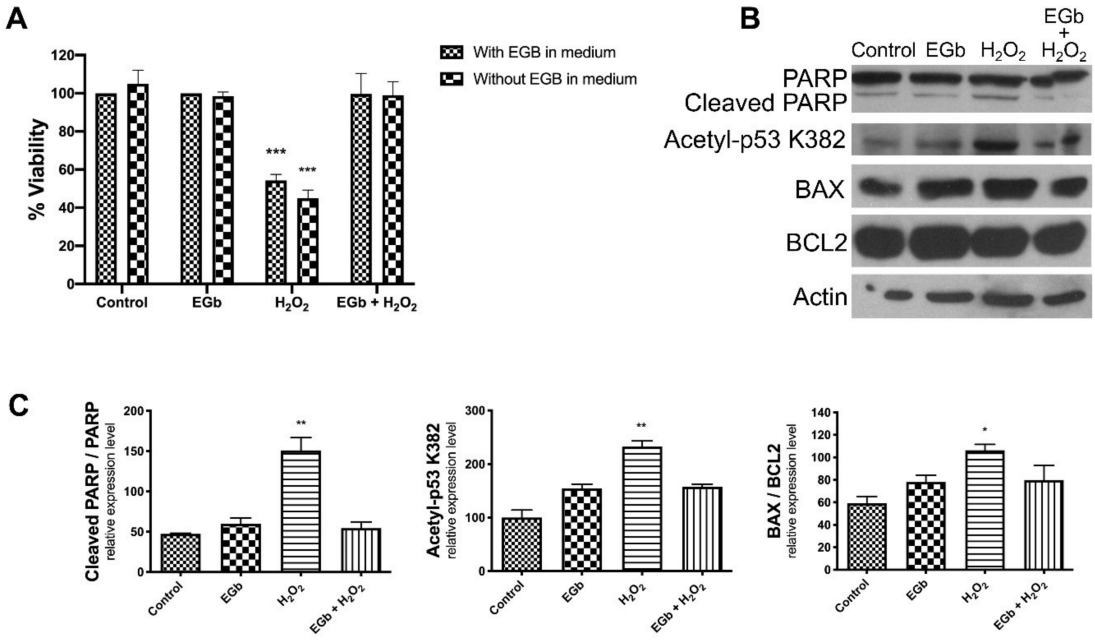

Figure 5. Intracellular effect of EGb. Cell viability analysis of SK-N-BE with or without EGb in the medium after H_2O_2 oxidative insult (**A**). Protein expression analysis of cleaved PARP, Acetylated-p53/K382, BAX and Bcl2 in SK-N-BE cells after treatments by Western blot analysis (**B**). Histograms report the expression of Acetylated-p53/K382, cleaved PARP and BAX/Bcl2 ratio normalized expression (**C**). β-Actin was used as loading control. The bars represent ± the average ± SD of independent experiments ($n = 3$). Statistically significant difference compared to control cells: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$). Cells treated with DMSO were used as control.

4. Discussion

The incidence of neurological disorders—the most dreaded maladies of older people—are expected to increase over the next few decades due to prolonged life expectancy [25]. To date, more than 1 in 10 individuals over 65 years are affected by neurodegenerative diseases and the numbers will continue to increase with age. Until now, no effective treatments have been described to cure these diseases and the costs for their management represent one of the leading medical and societal challenges faced by society [26]. For these reasonsm recent investigations have been focused to understanding their pathogenesis and to the development of novel therapeutics. *Ginkgo biloba*, a plant that has been used for thousands of years, is considered one of the more promising natural drugs. The extracts, obtained from *Ginkgo biloba* leaves, have been recently used also in clinical studies.

Most of the studies on the EGb concern its neuroprotective effects, against ageing. Standardized Ginkgo biloba extracts (EGb) have been used for treatment and prevention of different neurological disorders as Alzheimer's disease [15,16], Parkinson's disease [17,18], cerebral vascular deficit, and dementia [27,28].

Indeed, the brain is especially sensitive to the effects of ageing. This tissue being primarily composed of postmitotic cells—neurons and oligodendrocytes—is more vulnerable than proliferating cells to macromolecular damages, especially to DNA [29]. DNA damages in neurons accumulate from development throughout life. To escape this process, postmitotic neurons adopt selective mechanisms aimed to specifically repair genes actively transcribed [30].

Oxidative stress is one of the main causes of neural damage. EGb exerts neuroprotective action mainly acting as free radical–scavenger In fact, EGb is able to reduce the endogenous and the induced levels of ROS [31]. Moreover, EGb can directly upregulate antioxidant enzymes such as superoxide dismutase and catalase [32]. This activity is linked to the chemical structure of the flavonoids that

allow to not only react and directly scavenge the hydroxyl radicals, but also to inhibit the formation of new hydroxyl radicals [33]. It is well known that oxidative stress determines the activation of the apoptotic processes, thus playing a pivotal role in most of neurological diseases. EGb can act on multiple cellular pathways with the final goal to balance the existing apoptotic machinery. In fact, EGb prevents mitochondrial membrane damage reducing the release of cytochrome c from the mitochondria, upregulates the antiapoptotic protein Bcl-2-and inhibit PARP cleavage [34].

The neuroprotective effects of EGb 761 has been reported in different neuronal cell lines in which it acts by inhibiting oxidative stress induced apoptosis [35–37] or the activation of mitochondrial intrinsic apoptosis [35,36]. A recent in vivo study reported that Egb761 protected from brain injury by suppressing neuronal apoptosis [9]. Moreover, some studies reported the protective effect of Ginkgo biloba extract in people affected by neurodegenerative diseases [38,39].

In this study we analysed the protective effect of EGb on oxidative stress-induced apoptosis in SK-N-BE cells with the aim to unravel the molecular pathway in which EGb acts as antioxidant. Human neuroblastoma cell line N-type have neuronal morphology [40] and have been commonly used as model for research in neuroscience and in particular in studies related to oxidative stress and neurodegenerative diseases [41–44].

Our results demonstrated that the standardized extract EGb 761 significantly protected neuroblastoma cells from oxidative stress blocking apoptosis in a p53-dependent pathway. Interestingly, according to previous studies we found that EGB was able to inhibit p53 acetylation at lysine 382. It is known that p53 activity depends on the acetylation of specific lysines [45]. In addition, the acetylation of the C-terminal K382 lysine is crucial for p53 activation [46] since it results in the activation of PUMA promoter—a member of Bcl-2 family [47]. PUMA, promoting BAX multimerization and mitochondrial translocation, induces apoptosis [48]. Accordingly, our results show that EGb protects against mitochondrial membranes depolarization with a consequent reduction of BAX/Bcl-2 ratio. These results were supported by reduction of PARP cleavage with increased viability.

Previous studies reported that *Gingko biloba* extracts in cancer cells is able to induce apoptosis in a p53-dependent pathway by increasing the levels of p53 acetylation that, in turn, determines cell cycle arrest and apoptosis. On the contrary, our results demonstrated that the standardized extract EGb 761 significantly protected neuroblastoma cells from oxidative stress blocking apoptosis in a p53-dependent pathway. These results claim the different activity of EGb when used as neuroprotective or as anticancer drug [49].

5. Conclusions

Neurodegenerative disorders include a range of conditions that share common degenerative pathways, although they manifest with clinical differences. Increased oxidative stress has been described in almost all neurodegenerative disorders. In neurons, imbalance between the accumulatin of free radicals and antioxidant defences seems to be the link between cell death and progression of neurodegenerative diseases. Oxidative stress can trigger apoptosis in neuronal cells and excessive death of one or more populations of neurons, resulting in a neurodegenerative disease [50].

Our data suggest that EGb 761 could be considered an active antioxidant nutraceutical to be used for the prevention and treatment of neurodegenerative diseases.

Our data suggest that EGb 761, blocking the onset of p53-dependent apoptotic pathway induced by oxidative stress, could be considered as antioxidant nutraceutical to be potentially used for the prevention and treatment of neurodegenerative diseases. This hypothesis could be strengthened with a larger number of randomized clinical trials.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3921/9/4/279/s1, **Figure S1**: Cell viability after treatments with 25 µg/mL of EGb, 75 µM of H_2O_2 or a combination of them. In Egb + H_2O_2 (1) cells were treated for 24 h with EGb and then 24 h with H_2O_2. In EGb + H_2O_2 (2) cells were treated with EGb and H_2O_2 concurrent for 24 h. In EGb + H_2O_2 (3) cells were treated for 24 h with H_2O_2 and then with EGb for additional 24 h. The bars represent ± the average ± SD of independent experiments ($n = 3$). Statistically significant difference compared to control cells: ** $p \leq 0.01$, *** $p \leq 0.005$, **** $p \leq 0.001$. Cells treated with DMSO

were used as control. **Figure S2**: q-PCR analysis. Quantitative analysis of mRNA expression levels of p53, BAX and Bcl-2 in SK-N-BE cells after treatments. Histograms report the expression of p53 (**A**) and BAX/Bcl2 ratio (**B**). The bars represent ± the average ± SD of independent experiments (n = 3). Statistically significant difference compared to untreated cells: ** $p \leq 0.01$.

Author Contributions: Conceptualization, S.F. and S.C.; methodology, F.D.M. and R.C.; software O.P. and S.M.; validation F.D.M. and R.C.; formal analysis, F.D.M., R.C. and P.B.; investigation, S.C., F.D.M. and R.C.; resources G.P.; data curation F.D.M., S.F. and S.C.; writing—original draft F.D.M., S.F. and S.C.; writing—review and editing, S.F. and S.C.; visualization, O.P. and S.M.; supervision, G.P. and S.C.; project administration, O.P. and G.P.; funding acquisition, G.P. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the project PO FESR 2014–2020—Regione Campania, Asse 1—obiettivo specifico 1.2, Progetto "Sviluppo di nanotecnologie Orientate alla Rigenerazione e Ricostruzione tissutale, Implantologia e Sensoristica in Odontoiatria/oculistica (SORRISO)". Grant Number: pdt1-000410.

Acknowledgments: FDM PhD fellowship in Biology is supported by MIUR project PON "Dottorati Innovativi con caratterizzazione industriale" 2017–2018. We thank Pasquale Barba for his support in FACS analysis. We thank IGB FACS facility and Maria Rosaria Aletta for bibliographic assistance.

Conflicts of Interest: The authors confirm that there are no conflicts of interest.

References

1. Bergamini, C.M.; Gambetti, S.; Dondi, A.; Cervellati, C. Oxygen, reactive oxygen species and tissue damage. *Curr. Pharm. Des.* **2004**, *10*, 1611–1626. [CrossRef] [PubMed]
2. Behar, T.N.; Colton, C.A. Redox regulation of neuronal migration in a down syndrome model. *Free Radic. Biol. Med.* **2003**, *35*, 566–575. [CrossRef]
3. Finkel, T. Redox-dependent signal transduction. *FEBS Lett.* **2000**, *476*, 52–54. [CrossRef]
4. Martindale, J.L.; Holbrook, N.J. Cellular response to oxidative stress: Signaling for suicide and survival. *J. Cell Physiol.* **2002**, *192*, 1–15. [CrossRef]
5. Filosa, S.; Pecorelli, A.; D'Esposito, M.; Valacchi, G.; Hajek, J. Exploring the possible link between MeCP2 and oxidative stress in rett syndrome. *Free Radic. Biol. Med.* **2015**, *88*, 81–90. [CrossRef] [PubMed]
6. Dias, V.; Junn, E.; Mouradian, M.M. The role of oxidative stress in Parkinson's disease. *J. Parkinsons Dis.* **2013**, *3*, 461–491. [CrossRef]
7. Lovell, M.A.; Markesbery, W.R. Oxidative DNA damage in mild cognitive impairment and late-stage Alzheimer's disease. *Nucleic Acids Res.* **2007**, *35*, 7497–7504. [CrossRef] [PubMed]
8. Dugger, B.N.; Dickson, D.W. Pathology of neurodegenerative diseases. *Cold Spring Harb. Perspect. Biol.* **2017**, *9*, a028035. [CrossRef]
9. Yu, T.; Fan, Y.; Xu, Y.; Xu, L.; Xu, G.; Cao, F.; Jiang, H. Standardized Ginkgo biloba extract EGb 761(R) attenuates early brain injury following subarachnoid hemorrhage via suppressing neuronal apoptosis through the activation of Akt signaling. *Biomed. Pharmacother.* **2018**, *107*, 329–337. [CrossRef]
10. Chávez-Morales, R.M.; Jaramillo-Juárez, F.; Rodríguez-Vázquez, M.L.; Martínez-Saldaña, M.C.; Del Río, F.A.P.; Garfias-López, J.A. The Ginkgo biloba extract (GbE) protects the kidney from damage produced by a single and low dose of carbon tetrachloride in adult male rats. *Exp. Toxicol. Pathol.* **2017**, *69*, 430–434. [CrossRef]
11. Eckert, A.; Keil, U.; Scherping, I.; Hauptmann, S.; Muller, W.E. Stabilization of mitochondrial membrane potential and improvement of neuronal energy metabolism by Ginkgo biloba extract EGb 761. *Ann. N. Y. Acad. Sci.* **2005**, *1056*, 474–485. [CrossRef] [PubMed]
12. Chen, C.; Wei, T.; Gao, Z.; Zhao, B.; Hou, J.; Xu, H.; Xin, W.; Packer, L. Different effects of the constituents of EGb761 on apoptosis in rat cerebellar granule cells induced by hydroxyl radicals. *Biochem. Mol. Biol. Int.* **1999**, *47*, 397–405. [CrossRef] [PubMed]
13. Gohil, K.; Packer, L. Bioflavonoid-rich botanical extracts show antioxidant and gene regulatory activity. *Ann. N. Y. Acad. Sci.* **2002**, *957*, 70–77. [CrossRef] [PubMed]
14. Zhou, X.; Qi, Y.; Chen, T. Long-term pre-treatment of antioxidant Ginkgo biloba extract EGb-761 attenuates cerebral-ischemia-induced neuronal damage in aged mice. *Biomed. Pharmacother.* **2017**, *85*, 256–263. [CrossRef] [PubMed]
15. Rapp, M.; Burkart, M.; Kohlmann, T.; Bohlken, J. Similar treatment outcomes with Ginkgo biloba extract EGb 761 and donepezil in Alzheimer's dementia in very old age: A retrospective observational study. *Int. J. Clin. Pharmacol. Ther.* **2018**, *56*, 130–133. [CrossRef] [PubMed]

16. Kaur, N.; Dhiman, M.; Perez-Polo, J.R.; Mantha, A.K. Ginkgolide B revamps neuroprotective role of apurinic/apyrimidinic endonuclease 1 and mitochondrial oxidative phosphorylation against Abeta25-35 -induced neurotoxicity in human neuroblastoma cells. *J. Neurosci. Res.* **2015**, *93*, 938–947. [CrossRef]
17. Vijayakumaran, S.; Nakamura, Y.; Henley, J.M.; Pountney, D.L. Ginkgolic acid promotes autophagy-dependent clearance of intracellular alpha-synuclein aggregates. *Mol. Cell. Neurosci.* **2019**, *101*, 103416. [CrossRef]
18. Rojas, P.; Montes, P.; Rojas, C.; Serrano-Garcia, N.; Rojas-Castaneda, J.C. Effect of a phytopharmaceutical medicine, Ginko biloba extract 761, in an animal model of Parkinson's disease: Therapeutic perspectives. *Nutrition* **2012**, *28*, 1081–1088. [CrossRef]
19. Di Meo, F.; Filosa, S.; Madonna, M.; Giello, G.; Di Pardo, A.; Maglione, V.; Baldi, A.; Crispi, S. Curcumin C3 complex(R)/Bioperine(R) has antineoplastic activity in mesothelioma: An in vitro and in vivo analysis. *J. Exp. Clin. Cancer Res.* **2019**, *38*, 360. [CrossRef]
20. Crispi, S.; Calogero, R.A.; Santini, M.; Mellone, P.; Vincenzi, B.; Citro, G.; Vicidomini, G.; Fasano, S.; Meccariello, R.; Cobellis, G.; et al. Global gene expression profiling of human pleural mesotheliomas: Identification of matrix metalloproteinase 14 (MMP-14) as potential tumour target. *PLoS ONE* **2009**, *4*, e7016. [CrossRef]
21. Beyfuss, K.; Hood, D.A. A systematic review of p53 regulation of oxidative stress in skeletal muscle. *Redox Rep.* **2018**, *23*, 100–117. [CrossRef] [PubMed]
22. Reed, S.M.; Quelle, D.E. p53 Acetylation: Regulation and consequences. *Cancers* **2015**, *7*, 30–69. [CrossRef] [PubMed]
23. Redza-Dutordoir, M.; Averill-Bates, D.A. Activation of apoptosis signalling pathways by reactive oxygen species. *Biochim. Biophys. Acta* **2016**, *1863*, 2977–2992. [CrossRef] [PubMed]
24. Vaseva, A.V.; Moll, U.M. The mitochondrial p53 pathway. *Biochim. Biophys. Acta* **2009**, *1787*, 414–420. [CrossRef]
25. Wyss-Coray, T. Ageing, neurodegeneration and brain rejuvenation. *Nature* **2016**, *539*, 180–186. [CrossRef]
26. Hou, Y.; Dan, X.; Babbar, M.; Wei, Y.; Hasselbalch, S.G.; Croteau, D.L.; Bohr, V.A. Ageing as a risk factor for neurodegenerative disease. *Nat. Rev. Neurol.* **2019**, *15*, 565–581. [CrossRef]
27. Kandiah, N.; Ong, P.A.; Yuda, T.; Ng, L.L.; Mamun, K.; Merchant, R.A.; Chen, C.; Dominguez, J.; Marasigan, S.; Ampil, E.; et al. Treatment of dementia and mild cognitive impairment with or without cerebrovascular disease: Expert consensus on the use of Ginkgo biloba extract, EGb 761((R)). *CNS Neurosci. Ther.* **2019**, *25*, 288–298. [CrossRef]
28. Spiegel, R.; Kalla, R.; Mantokoudis, G.; Maire, R.; Mueller, H.; Hoerr, R.; Ihl, R. Ginkgo biloba extract EGb 761((R)) alleviates neurosensory symptoms in patients with dementia: A meta-analysis of treatment effects on tinnitus and dizziness in randomized, placebo-controlled trials. *Clin. Interv. Aging* **2018**, *13*, 1121–1127. [CrossRef]
29. Madabhushi, R.; Pan, L.; Tsai, L.H. DNA damage and its links to neurodegeneration. *Neuron* **2014**, *83*, 266–282. [CrossRef]
30. Chow, H.M.; Herrup, K. Genomic integrity and the ageing brain. *Nat. Rev. Neurosci.* **2015**, *16*, 672–684. [CrossRef]
31. Smith, J.V.; Luo, Y. Studies on molecular mechanisms of Ginkgo biloba extract. *Appl. Microbiol. Biotechnol.* **2004**, *64*, 465–472. [CrossRef] [PubMed]
32. Bridi, R.; Crossetti, F.P.; Steffen, V.M.; Henriques, A.T. The antioxidant activity of standardized extract of Ginkgo biloba (EGb 761) in rats. *Phytother. Res.* **2001**, *15*, 449–451. [CrossRef] [PubMed]
33. Zimmermann, M.; Colciaghi, F.; Cattabeni, F.; Di Luca, M. Ginkgo biloba extract: From molecular mechanisms to the treatment of Alzhelmer's disease. *Cell Mol. Biol.* **2002**, *48*, 613–623. [PubMed]
34. Smith, J.V.; Burdick, A.J.; Golik, P.; Khan, I.; Wallace, D.; Luo, Y. Anti-apoptotic properties of Ginkgo biloba extract EGb 761 in differentiated PC12 cells. *Cell Mol. Biol.* **2002**, *48*, 699–707. [PubMed]
35. Shi, C.; Zhao, L.; Zhu, B.; Li, Q.; Yew, D.T.; Yao, Z.; Xu, J. Dosage effects of EGb761 on hydrogen peroxide-induced cell death in SH-SY5Y cells. *Chem. Biol. Interact.* **2009**, *180*, 389–397. [CrossRef]
36. Jiang, X.; Nie, B.; Fu, S.; Hu, J.; Yin, L.; Lin, L.; Wang, X.; Lu, P.; Xu, X.M. EGb761 protects hydrogen peroxide-induced death of spinal cord neurons through inhibition of intracellular ROS production and modulation of apoptotic regulating genes. *J. Mol. Neurosci.* **2009**, *38*, 103–113. [CrossRef]
37. Ni, Y.; Zhao, B.; Hou, J.; Xin, W. Preventive effect of Ginkgo biloba extract on apoptosis in rat cerebellar neuronal cells induced by hydroxyl radicals. *Neurosci. Lett.* **1996**, *214*, 115–118. [CrossRef]

38. Savaskan, E.; Mueller, H.; Hoerr, R.; von Gunten, A.; Gauthier, S. Treatment effects of Ginkgo biloba extract EGb 761® on the spectrum of behavioral and psychological symptoms of dementia: Meta-analysis of randomized controlled trials. *Int. Psychogeriatr.* **2018**, *30*, 285–293. [CrossRef]
39. Gauthier, S.; Schlaefke, S. Efficacy and tolerability of Ginkgo biloba extract EGb 761® in dementia: A systematic review and meta-analysis of randomized placebo-controlled trials. *Clin. Interv. Aging* **2014**, *9*, 2065–2077. [CrossRef]
40. Corey, J.M.; Gertz, C.C.; Sutton, T.J.; Chen, Q.; Mycek, K.B.; Wang, B.S.; Martin, A.A.; Johnson, S.L.; Feldman, E.L. Patterning N-type and S-type neuroblastoma cells with Pluronic F108 and ECM proteins. *J. Biomed. Mater. Res. A* **2010**, *93*, 673–686. [CrossRef]
41. Krishna, A.; Biryukov, M.; Trefois, C.; Antony, P.M.; Hussong, R.; Lin, J.; Heinaniemi, M.; Glusman, G.; Koglsberger, S.; Boyd, O.; et al. Systems genomics evaluation of the SH-SY5Y neuroblastoma cell line as a model for Parkinson's disease. *BMC Genomics* **2014**, *15*, 1154. [CrossRef] [PubMed]
42. Maraldi, T.; Riccio, M.; Zambonin, L.; Vinceti, M.; De Pol, A.; Hakim, G. Low levels of selenium compounds are selectively toxic for a human neuron cell line through ROS/RNS increase and apoptotic process activation. *Neurotoxicology* **2011**, *32*, 180–187. [CrossRef] [PubMed]
43. Mata, I.F.; Jang, Y.; Kim, C.H.; Hanna, D.S.; Dorschner, M.O.; Samii, A.; Agarwal, P.; Roberts, J.W.; Klepitskaya, O.; Shprecher, D.R.; et al. The RAB39B p.G192R mutation causes X-linked dominant Parkinson's disease. *Mol. Neurodegener.* **2015**, *10*, 50. [CrossRef] [PubMed]
44. Testa, G.; Staurenghi, E.; Giannelli, S.; Gargiulo, S.; Guglielmotto, M.; Tabaton, M.; Tamagno, E.; Gamba, P.; Leonarduzzi, G. A silver lining for 24-hydroxycholesterol in Alzheimer's disease: The involvement of the neuroprotective enzyme sirtuin 1. *Redox Biol.* **2018**, *17*, 423–431. [CrossRef]
45. Knights, C.D.; Catania, J.; Di Giovanni, S.; Muratoglu, S.; Perez, R.; Swartzbeck, A.; Quong, A.A.; Zhang, X.; Beerman, T.; Pestell, R.G.; et al. Distinct p53 acetylation cassettes differentially influence gene-expression patterns and cell fate. *J. Cell Biol.* **2006**, *173*, 533–544. [CrossRef]
46. Olsson, A.; Manzl, C.; Strasser, A.; Villunger, A. How important are post-translational modifications in p53 for selectivity in target-gene transcription and tumour suppression? *Cell Death Differ.* **2007**, *14*, 1561–1575. [CrossRef]
47. Brochier, C.; Dennis, G.; Rivieccio, M.A.; McLaughlin, K.; Coppola, G.; Ratan, R.R.; Langley, B. Specific acetylation of p53 by HDAC inhibition prevents DNA damage-induced apoptosis in neurons. *J. Neurosci.* **2013**, *33*, 8621–8632. [CrossRef]
48. Ming, L.; Wang, P.; Bank, A.; Yu, J.; Zhang, L. PUMA Dissociates Bax and Bcl-X(L) to induce apoptosis in colon cancer cells. *J. Biol. Chem.* **2006**, *281*, 16034–16042. [CrossRef]
49. Park, H.J.; Kim, M.M. Flavonoids in Ginkgo biloba fallen leaves induce apoptosis through modulation of p53 activation in melanoma cells. *Oncol. Rep.* **2015**, *33*, 433–438. [CrossRef]
50. Mattson, M.P. Apoptosis in neurodegenerative disorders. *Nat. Rev. Mol. Cell Biol.* **2000**, *1*, 120–129. [CrossRef]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

Journal of Experimental & Clinical Cancer Research

RESEARCH Open Access

Curcumin C3 complex®/Bioperine® has antineoplastic activity in mesothelioma: an in vitro and in vivo analysis

Francesco Di Meo[1,2†], Stefania Filosa[1,3†], Michele Madonna[3], Gerarda Giello[1], Alba Di Pardo[3], Vittorio Maglione[3], Alfonso Baldi[1,4*] and Stefania Crispi[1*]

Abstract

Background: A major limitation in the treatment for malignant mesothelioma is related to serious side effects caused by chemotherapeutics and to the development of cancer-resistance. Advances in cancer therapies have been reached thanks to the introduction of alternative approaches, such as the use of phytochemicals. Curcumin-C3complex®/Bioperine® is a commercially standardized extract containing a ratio-defined mixture of three curcuminoids and piperine that greatly increase its bioavailability. Interestingly, the anticancer effect of this formulation has been described in different studies and several clinical trials have been started, but to our knowledge none refers to human mesothelioma.

Methods: Curcumin-C3complex®/Bioperine® anticancer effect was evaluated in vitro in different human mesothelioma cell lines analysing cell proliferation, colony-forming assay, wound healing assays, invasion assay and FACS analysis. In vivo anticancer properties were analysed in a mesothelioma xenograft mouse model in CD1 Nude mice.

Results: Curcumin-C3complex®/Bioperine® in vitro induced growth inhibition in all mesothelioma cell lines analysed in a dose- and time-depended manner and reduced self-renewal cell migration and cell invasive ability. Cell death was due to apoptosis. The analysis of the molecular signalling pathway suggested that intrinsic apoptotic pathway is activated by this treatment. This treatment in vivo delayed the growth of the ectopic tumours in a mesothelioma xenograft mouse model.

Conclusions: Curcumin-C3complex®/Bioperine® treatment strongly reduces in vitro tumorigenic properties of mesothelioma cells by impairing cellular self-renewal ability, proliferative cell rate and cell migration and delays tumor growth in xenograft mouse model by reducing angiogenesis and increasing apoptosis. Considering that curcumin in vivo synergizes drug effects, its administration to treatment regimen may help to enhance drug therapeutic efficacy in mesothelioma. Our results suggest that implementation of standard pharmacological therapies with novel compounds may pave the way to develop alternative approaches to mesothelioma.

Keywords: Mesothelioma, Curcumin C3 complex, Intrinsic apoptosis, Tumor growth inhibition

* Correspondence: alfonsobaldi@tiscali.it; stefania.crispi@ibbr.cnr.it
†Francesco Di Meo and Stefania Filosa contributed equally to this work.
[1]Institute of Biosciences and BioResources-UOS Naples CNR, via P. Castellino, 111-, 80131 Naples, Italy
Full list of author information is available at the end of the article

© The Author(s). 2019 **Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

Background

Malignant Mesothelioma (MM) is a rare and aggressive form of cancer primarily associated with exposure to asbestos fibres that affects the mesothelium surface of the pleural cavity. Despite the rarity of this disease, MM incidence is increasing worldwide and it is estimated to peak over the next 15 years with an increase of 5.4% per year [1]. The prognosis of MM is very poor due to the long latency development (30–40 years), to the diagnosis at a very late stage and to its high chemo-resistance. To date, the standard therapeutic modalities for this type of cancer, including chemotherapy, surgery and radiotherapy have yielded unsatisfactory outcomes [2]. Thus, the development of alternative and more effective therapies is an urgent requirement.

Previously, our group has investigated the in vitro and in vivo efficacy of a piroxicam and cisplatin combined treatment in MM. This treatment determined a marked tumor growth inhibition and an extended survival both in mouse models [3] and in spontaneous MM in pets [4]. Despite the efficacy of this treatment, drug toxicity and tumor-resistance represent a serious limitation. For this reason, research is needed on novel therapeutic approaches using natural compounds with no or little cytotoxicity. In this perspective, phytochemicals represent good candidates to be used alone or associated with standard chemotherapy. Phytochemicals are bioactive plant compounds that display an adjuvant effect resulting in tumor growth inhibition and in chemoprotective action towards the healthy cells with no obvious associated side effects [5].

Among others, curcumin - normally found in the turmeric of *Curcuma longa* Linn - is a naturally occurring phytochemical that has been widely used for centuries for the treatment of several diseases [6]. The use of curcumin in cancer is based on its ability to block the proliferation of tumor cells. Curcumin modulates cell cycle regulatory proteins involved in the pathogenesis and the prognosis of several cancers, including mesothelioma [7]. More interestingly, curcumin seems to induce a selective cytotoxicity toward cancer cells blocking the expression of molecules involved in cancer growth, such as nuclear factor NFkB and thioredoxin reductase (TrxR) [8–10]. In addition, curcumin is able to overcome the multidrug resistance of cancer cells down-regulating proteins responsible for the high drug efflux in multi-drug-resistant cancer cells [11].

Increasing evidences point out a robust anti-cancer efficacy of curcumin, however more attention should be paid to the formulations used, since in most of the in vivo studies and clinical trials no-standardized curcuminoid mixtures have been used [6].

Despite its numerous applications, the pharmacological potential of curcumin is severely restricted due to its poor water solubility, photodegradation, chemical instability and rapid metabolism as well as to its poor systemic bioavailability after oral administration [12]. In order to take advantages of the beneficial effects that curcumin may have, numerous attempts have been made to increase its efficacy and bioavailability. To overcome solubility problems our group as well as others have previously investigated the bioactivity of curcumin formulations using nanocarriers for delivery and targeting. These studies indicated that curcumin efficacy is tightly linked to its bioavailability [13, 14].

Other strategies investigated the efficacy of curcumin in combination with various molecules. Among them, the most promising one is represented by the co-administration of curcumin with piperine, an alkaloid of black pepper and long pepper. Piperine significantly enhances curcumin bioavailability – up to 2000% - by preventing its metabolism through the inhibition of the glucuronidation processes [15, 16].

In this study we investigated the anticancer activity of a commercially available preparation of curcumin and piperine (C3 complex® and Bioperine®, Sabinsa Corporation, CBP). C3 complex is a standardized extract containing a ratio-defined mixture of three curcuminoids (curcumin, bisdemethoxycurcumin, and demethoxycurcumin) that recently achieved the GRAS (Generally Recognized as Safe) status.

The efficacy of CPB was evaluated in vitro in different human mesothelioma cell lines and in vivo using a xenograft mouse model of MM. Overall, our data highlighted that CBP induces apoptosis in a time-dependent manner in MM cell lines sensitive (MSTO-221H, NCI-H2452) and insensitive (Ist-Mes2) to the piroxicam and cisplatin treatment [17] and that it is able to reduce tumor growth in a mouse MM model.

Methods

Cell culture and chemicals

Human MM cell lines were maintained at 37 °C in a 5% CO_2 humidified incubator in either RPMI-1640 medium (MSTO-221H, NCI-H2452) or Dulbecco's Modified Eagle's Medium (Ist-Mes-2) supplemented with 10% fetal bovine serum (FBS), glutamine (2 mM), sodium pyruvate and antibiotics (0.02 IU/mL-1 penicillin and 0.02 mg/mL-1 streptomycin).

Curcumin C3 complex® (C3) and Bioperine® (BP) were provided from Sabinsa (Sabinsa Corporation, NJ, USA). Curcumin and Bioperine (CBP) stock solution contains 20 mM C3 complex® and 26 nM of Bioperine® in DMSO. This means that in all the experiments C3 is added to BP in the ratio 100:1 in weight (100 g of C3 for 1 g of BP).

In vitro uptake of curcumin C3 complex®

MSTO-221H cells were seeded at a density of 2×10^5 cells/well in six-well plates, and exposed to C3 with the concentration of 20 μM for 10′. For nuclear counterstain Hoechst 33342 (Invitrogen, Thermofisher, Waltham, MA, USA) was added in the culture medium. Since curcumin exhibits autofluorescence when excited at 455 nm and emits at 540 nm [18], uptake of the molecule was monitored under fluorescent microscope (DMI8, Leica, Instruments, Germany) using GFP filter with 20x magnification.

Cell proliferation assay

To evaluate the effect of curcumin or CBP on cell proliferation, approximately 1×10^4 cells/well in 48-well plates were plated and treated with 5, 10 and 20 μM for 24, 48 or 72 h. Cells were fixed with 3.7% formaldehyde for 10 min, washed with PBS and stained with 0.5% crystal violet for 10 min. A microplate reader (Cytation3 ASHI, BioTek, VT, USA) was then used to measure the absorbance at 595 nm. All the experiments were performed in triplicate. Data are expressed as the mean ± SD.

Colony formation assay

Cells were seeded at a density of 500 cells/well in six-well plates and incubated for 7 days. Then, cells were treated with 20 μM for 24 h before replacing the media. Cells were grown for additional 7 days and then colonies were stained with crystal violet and counted. Representative plates were captured using scanner (Epson Stylus Photo, PX 650). All the experiments were performed in triplicate. Data are expressed as the mean ± SD.

Wound healing assays

For wound healing assay, approximately 3×10^4 cells/well were plated in six-well plates. After overnight incubation, wounds were created using a 200 μl pipette tip. Cells were treated with 20 μM of CBP for 24, 48 or 72 h. Representative plates were photographed using phase contrast microscope (DMI8, Leica, Instruments, Germany). The gap was photographed and measured using Image J software. All the experiments were performed in triplicate. Data are expressed as the mean ± SD.

Invasion assay

The ability of cells to invade into the matrix and migrate towards was analysed in vitro using 24-well inserts with a pore size of 8 μm (Falcon, Corning NY) coated with Matrigel (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's guidelines. Approximately 2×10^4 cells were seeded in 250 μL of serum free medium with 20 μM of CBP in the upper surface of chamber; the lower chamber was filled with 750 μL of medium with 10% FBS. After 24 h of treatment, non-invasive cells remaining in upper chamber were removed by PBS washing. Invasive cells that had penetrated the Matrigel (the lower surface) were fixed with 3.7% formaldehyde for 10 min, washed with methanol for 20 min and stained with 0.5% crystal violet for 10 min, and then counted. Cells that invaded the lower surface of the filters were surveyed under a microscope at 10× magnifications, and five fields were randomly selected. Representative plates were photographed using bright field microscope (DMI8, Leica, Instruments, Germany).

All the experiments were performed in triplicate. Data are expressed as the mean ± SD.

FACS analysis

Approximately $7{,}5 \times 10^5$ cells/well were plated in 100 mm plates. After overnight incubation, cells were treated with 20 μM of CBP for 24, 48 or 72 h and stained with propidium iodide and annexin V (BD Biosciences, Franklin Lakes, NJ), according to the manufacturer's protocols. Flow cytometry was performed using a FACS-Canto TM flow cytometry system (Becton Dickinson, San Jose, CA). All the experiments were performed in triplicate. Data are expressed as the mean ± SD.

RNA extraction and q-PCR

RNA from treated or untreated cells was extracted using Trizol (Thermo Fisher Scientific, MA USA) following manufacturer's instructions. 200 ng of total RNA from each sample were retro-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Thermo Fisher Scientific, MA USA). qPCR reactions were performed by means of a 7900 HT Real Time PCR (Applied Biosystem) using gene specific primers for the following selected genes:

BAX: Forward 5′-TTTGCTTCAGGGTTTCATCCA-3′: Reverse 5′- CTCCATGTTACTGTCCAGTTCGT-3′; BCL-2: Forward 5′-GTTCCCTTTCCTTCCATCC-3′; Reverse 5′-TAGCCAGTCCAGAGGTGAG-3′; FAS: Forward 5′-CCCTCCTACCTCTGGTTCTTACG-3′; Reverse 5'TCAG TCACTTGGGCATTAACACTTT-3′; FASL: Forward 5′-CCTGAAAAAAAGGAGCTGAGGAA-3′; Reverse 5′-GGCATGGACCTTGAGTTGGA-3′; GAPDH: Forward 5′-CAAGGCTGTGGGCAAGGT-3′; Reverse 5′-GGAA GGCCATGCCAGTGA-3:

Primers were designed at exon-exon junctions using Primer express 2.0 (Applied Biosystems). Target expression level was performed as previously described [19] using GAPDH as housekeeping gene. All the experiments were performed in triplicate. Data are expressed as the mean ± SD.

Western blot

Protein extracts were prepared as previously described [20]. For each lane, 20 μg of total cell lysates were

separated in 4–15% Tris–glycine gels (Bio-Rad Laboratories, Inc., CA, USA) at 100 V. Proteins were then transferred to PVDF membranes (Biorad Laboratories, Inc., CA, USA), probed with the specific primary antibodies, followed by secondary antibodies conjugated with horseradish peroxidase according manufacturer's indications. Primary antibodies used for western blot include p53 (Cell Signaling, #2524) PARP (Cell Signaling, #9542), BCL-2 (Abcam, ab182858), BAX (Santa Cruz Biotechnology, sc-493), FAS (Abcam, ab133619) and Cytochrome *c* (Abcam, ab133504). β-Actin (Cell Signaling, #3700) was used as loading control. All the antibodies were used at working concentration indicated by manufacturers. Protein bands were detected by Clarity western ECL (Bio-Rad Laboratories, Inc., CA, USA) and quantified with ImageJ software. All the experiments were performed in triplicate. Data are expressed as the mean ± SD.

Cytochrome *c* release

To determine the cytochrome *c* release from mitochondria to cytosol, cytosolic fractions were isolated resuspending 1×10^6 cells in 100 μl of ice-cold plasma membrane permeabilization buffer (200 μg/ml digitonin, 80 mM KCl in PBS). After 5 min incubation on ice, lysates were centrifuged at 800 X g for 5 min at 4 °C, and the supernatants (cytosolic fraction) were then collected. Protein fractions were separated, transferred and probed with and Cytochrome *c* primary antibodies as described in the previous section.

Mesothelioma xenograft tumor model

Xenograft mouse model of MM was induced by dorsal injection of human mesothelioma cells (MSTO-211H) as previously described [3].

Male CD1 Nude mice were purchased from Charles River Laboratories *Italia (Calco, Italy)* and housed in the animal Facility IRRC-Neuromed in accordance with protocols approved by the IRRC-Neuromed Animal Care Review Board and by Ministry of Health. In vivo experiments were conducted according to EU directive 2010/63/EU for animal experiments.

MSTO-211H cells (2.5×10^6) were suspended in 0.2 ml serum-free DMEM medium and inoculated subcutaneously (s.c.) in the right flank of each mouse aged 5 weeks. 10 days after inoculation, when the tumours became visible, 8 mice for each experimental group were randomly assigned into control (DMSO) or treated groups (CBP or cisplatin) and treatments were administered by intra-peritoneal injection (i.p.).

To analyse anticancer properties of CBP, mice were daily treated with CBP (40 mg/kg) for 4 weeks or with Cisplatin (3.3 mg/kg) only for the first 3 days. Mice treated with vehicle alone (DMSO, daily for 4 weeks) were used as control.

Each tumor was measured weekly using a calliper; tumour's size was assessed by using the formula: (long axis × short axis × short axis)/2.

The mice were sacrificed after 4-week treatments.

Histology and immunohistochemistry

For histology, staining with hematoxylin/eosin and hematoxylin/Van Gieson were used. For immunohistochemistry, tissue sections were heated twice in a microwave oven for 5 min each at 700 W in citrate buffer (pH 6) and then processed with the standard streptavidin-biotin-immunoperoxidase method (DAKO Universal Kit, DAKO Corp., Carpinteria, CA, USA). Mouse monoclonal anti-human Ki67 (MIB-1 clone) and anti-CD31 (M0823 clone) antibodies from DAKO were used at a 1:100 dilution. Diaminobenzidine was used as the final chromogen, and hematoxylin as the nuclear counterstain. Negative control experiments for each tissue section were performed in the absence of the primary antibody. Positive controls, included in each experiment, consisted of tissue previously shown to express the antigen of interest. Two observers (S.C and A.B.), blinded to treatment conditions, evaluated the staining pattern of the proteins separately and quantitated the protein expression in each specimen by scanning the entire section and estimating the number of vessels or positive cells at the high-power-field 10 × 20. The level of concordance, expressed as the percentage of agreement between the observers, was 95%. In the remaining specimens, the score was obtained after collegial revision and agreement. The U-Mann Whitney test was used to assess relationship between ordinal data. Two-tailed *p*-value was considered significant when ≤0.05.

TUNEL assay

TUNEL reaction was performed using the peroxidase-based Apoptag kit (Oncor, Gaithersburg, MD, USA). The experiment was repeated on at least two different sections for each specimen. Fifty random fields (250X) per section were analysed (6 mm^2). The level of concordance, expressed as the percentage of agreement between the two observers (SC and AB), was 100%. The U-Mann Whitney test was used to assess relationship between ordinal data. Two-tailed *p*-value was considered significant when ≤0.05.

Statistical analysis

Analysis was performed using Graph Pad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Significance was evaluated using One-Way ANOVA with Bonferroni post hoc test for multiple comparisons or a Student's *t*-test. p-value ≤0.05 was considered statistically significant.

Results

CBP determines growth inhibition in mesothelioma cells

We have previously reported that curcumin (20 μM) only when complexed with cyclodextrin can entry into cells determining cell viability decrease in mesothelioma cells [21].

To examine the C3 bioavailability, we first analysed whether C3 was able to penetrate MSTO-221H (MSTO) cells using the same concentration. Figure 1 a shows that C3 fluorescence is widely distributed in the cells, thus indicating that this complex is able to enter into cell through the plasma membrane.

Then, in order to investigate whether BP could enhance C3 bioactivity, we treated MSTO with different concentrations of C3 alone or combined with Bioperine. CBP resulted more effective than C3 complex alone (Fig. 1 c). In addition, Bioperine alone at same concentration present in CBP did not affect cell viability (Fig. 1 b). As shown in Fig. 1 c, CBP at 20 μM is able to determine a growth inhibition increase up to 40% compared to C3 alone, indicating that BP strongly determines a C3 bioavailability enhancement. Thus, we chose to use this concentration for the subsequent experiments.

To study the CBP in vitro activity we analysed the cell viability in a time course assay. The results indicated that CBP affects cell viability in a time dependent manner both in MSTO, and in NCI-H2452 (NCI) and in Ist-Mes-2 (Mes-2 cells) (Fig. 1 d).

Subsequently, cell proliferation, cell migration and invasive ability were analysed on all three cell lines in order to evaluate the anticancer potential CBP. The results clearly showed a complete growth inhibition and the loss of self-renewal ability and proliferative potential with a failure in colony formation.

CBP was able to impair self-renewal ability and long-term proliferative potential in all the MM cell lines analysed. In fact, colony formation was almost completely inhibited after 24-h treatment with CBP (Fig. 2 a). Invasion abilities also resulted significantly reduced after CBP treatment (Fig. 2 b). Moreover, wound-healing assay showed that CBP significantly inhibited cell

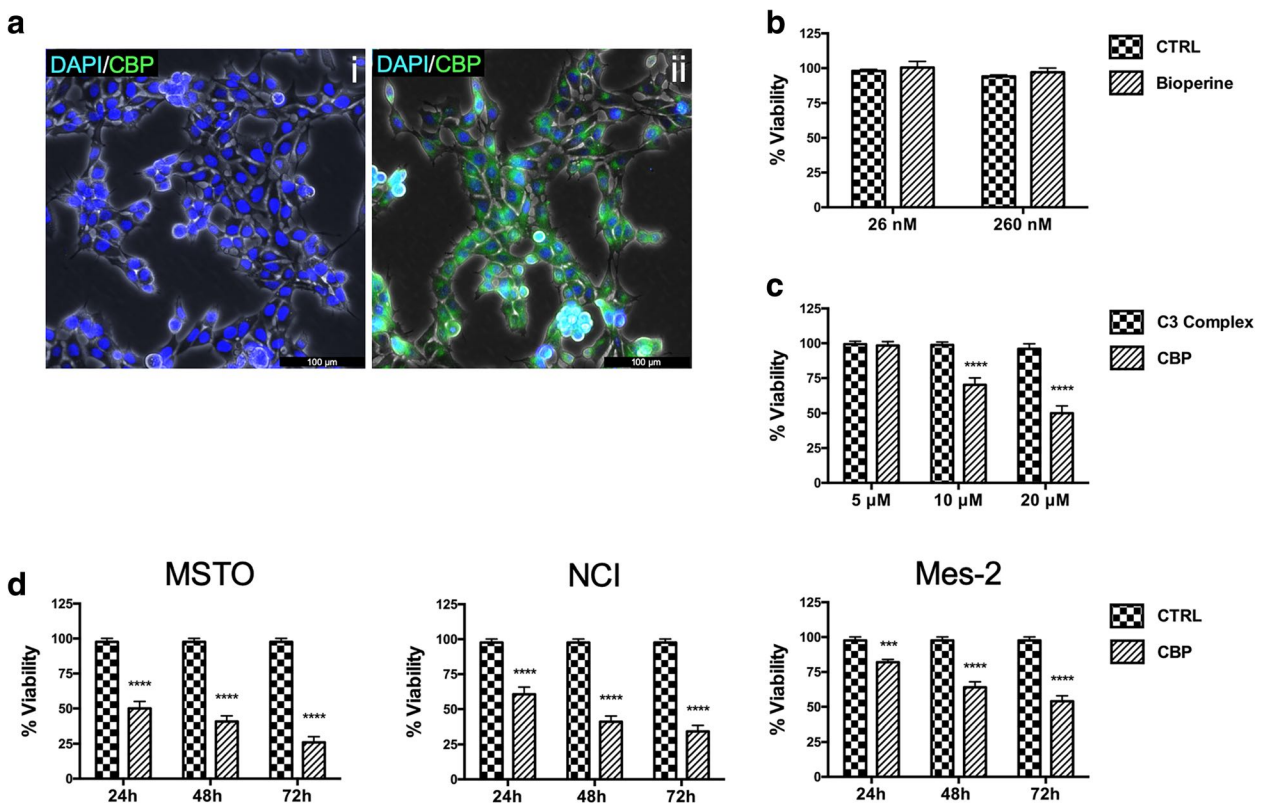

Fig. 1 CBP uptake and effects on cell viability in mesothelioma cells. **a**) Fluorescence microscope analysis of MSTO cells incubated with DAPI (i) or with DAPI/CBP (ii) for 10′. Cellular uptake was analysed by curcumin intrinsic autofluorescence using a GFP filter. DAPI was used to counterstain nuclei. (**b**) Bioperine alone does not affect MSTO cell viability even when used 10X concentrated respect to the amount of BP present in CBP used for the experiments. **c**) Curcumin and Bioperine combined treatment increases C3 Complex bioactivity determining a cell viability decrease in dose dependent manner. d) Time-course measurement of cell viability decrease in different mesothelioma cell lines treated with 20 μM CBP for the indicated time. Cell viability was determined by crystal violet assay and cell viabilities are depicted as percentages. CTRL: untreated cells used as control. The indicators represent the average ± SD of independent experiments ($n = 3$). Statistically significant difference compared to untreated cells: ***$p \leq 0.001$, ****$p \leq 0.0001$. CRTL: untreated cells

migration capacity in a time-dependent manner both in MSTO, and in NCI and in Mes-2 cells. In particular, the wound gap was about 100% after 24 h. (Fig. 2 c).

Taken together, these results indicated that CBP strongly reduces tumorigenic properties of MM cells, probably by affecting molecular pathways not involved in the response to the combined treatment with piroxicam and cisplatin since the effects are evident even in cells does not responding to this treatment [20].

CBP induces apoptosis in mesothelioma cells

To understand whether cell viability impairment was due to apoptosis, we analysed the cell cycle perturbation and the downstream signalling triggered by CBP. MSTO, NCI and Mes-2 cells were treated with CBP for 24, 48 and 72 h and untreated cells were used as control. Apoptosis was investigated by flow cytometry analysis with AnnexinV-FITC/PI. The results clearly indicate that CBP determines a growing apoptotic induction during the time, reaching about 75% at 72 h (Fig. 3). On the contrary, in the untreated cells we did not detect any apoptotic increase over the time. We observed slight modifications in apoptosis among the different mesothelioma cell lines, with MSTO showing the highest sensitivity. More interestingly, CBP is effective also in Mes-2 cells that do not undergo to apoptosis after piroxicam and cisplatin treatment [17].

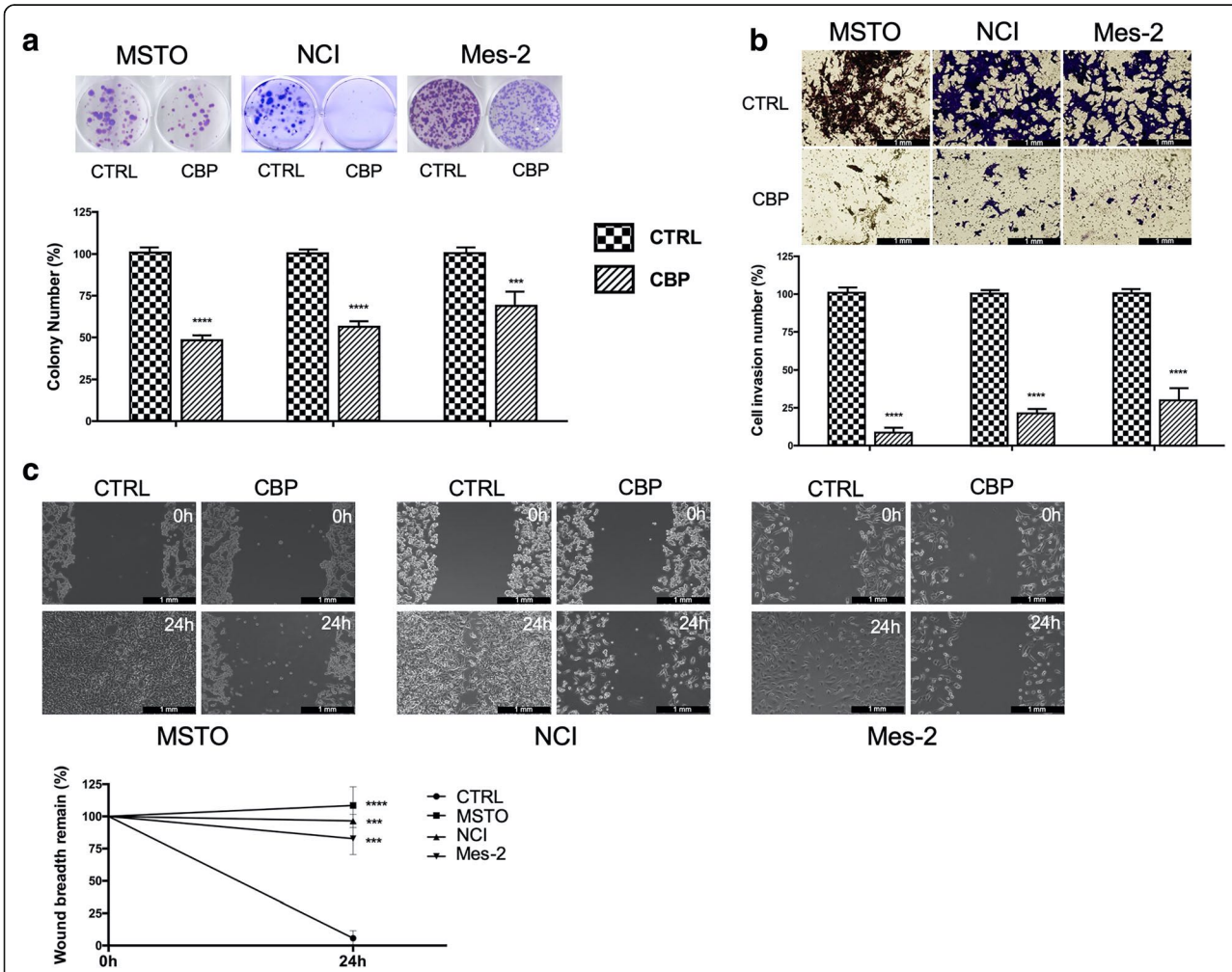

Fig. 2 CBP impairs mesothelioma tumorigenic properties. **a**) The effect of CPB on colony forming ability of MM cell lines was analysed after 7 days of culture post CPB treatment. For quantification, colonies with at least 50 cells were considered**. b**) Invasive capability of human MM cells was determined using transwell invasion assay with Matrigel after 24 h CBP treatment as described in "Methods". For each cell line five random fields in each well were counted under a microscope. Bright field images magnification: × 10. Representative micrographs shown in **a**) and **b**) were obtained after crystal violet staining. Histograms report average of colony or cell numbers respectively (% of control). c) Cell migration activity of mesothelioma cell lines was examined by wound healing assay. The wound closure rate was measured by detecting the closure distance after 24 h. Representative phase contrast images at 0 and at 24 h after CBP treatment (up) and quantification of the distance (down) are shown. Magnification of the upper panels: × 10. The indicators represent the average ± SD of independent experiments (n = 3). Statistically significant difference compared to untreated cells: $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. CTRL: untreated cells

CBP activates intrinsic apoptosis in mesothelioma cells

To investigate the molecular signalling pathway involved in the CBP-apoptotic cell death, we analysed if CBP was able to modulate p53 protein level and other apoptotic markers such as cleaved PARP, BAX and BCL2 FAS and FASL. Since untreated cells did not show modification of p53 levels or presence of PARP cleavage over the time (Additional file 1: Figure S1), we used as control samples proteins from untreated cells after 72 h culture that is the time where we observed the maximum CBP effect.

Protein analysis shows an increase of the p53 level after CBP treatment in all MM cell lines, with MSTO showing the highest increase (Fig. 4 a). In addition, the presence of PARP cleavage confirmed activation of apoptosis.

Then we focused our attention on the molecular effectors involved in the activation of the *intrinsic* [22] or the *extrinsic* [23] apoptotic pathway, by analysing the expression of key molecules responsible for the two apoptotic pathways: BAX, BCL-2 and FAS, FASL respectively.

The analysis of BAX and BCL-2 protein expression indicated that CBP induced apoptosis through intrinsic pathway (Fig. 4 a). Expression analysis by q-PCR also confirmed an increased ratio *BAX/Bcl-2* supporting the activation of intrinsic pathway (Fig. 4 b). According to these results we did not observe changes in the

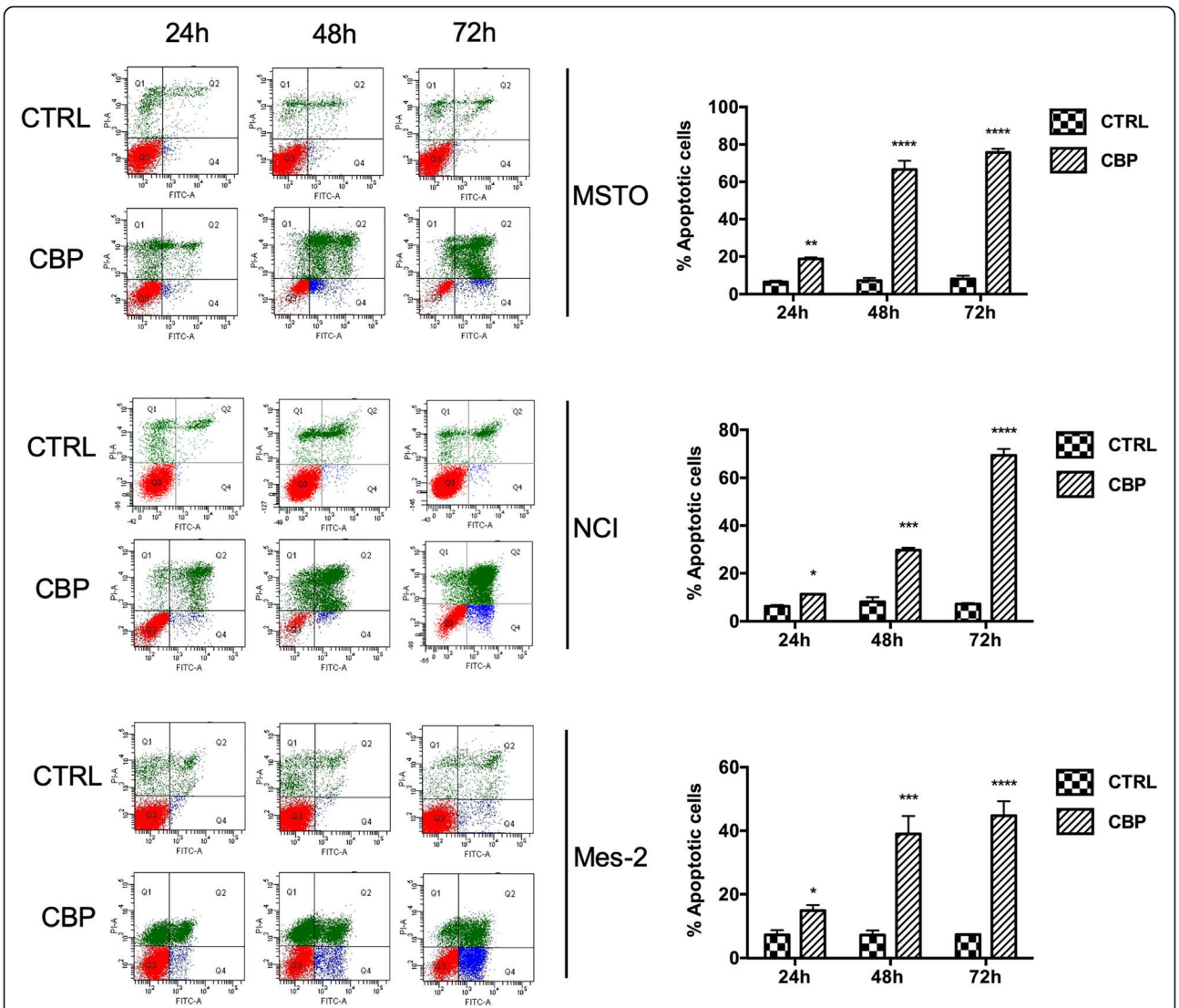

Fig. 3 CBP treatments induce apoptosis in mesothelioma cells. FACS analysis by Annexin V-FITC/PI double staining assay for the detection of apoptosis in MSTO, NCI and Mes-2 cells treated with 20 μM CPB for 24, 48 and 72 h. Q2 and Q4: early and late apoptotic cells; Q1: necrotic fraction; Q3: live cells. Histograms on the right report a data summary of the apoptotic index. Bars represent the average ± SD of independent experiments (n = 3). Statistically significant difference compared to untreated cells: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. CTRL: untreated cells

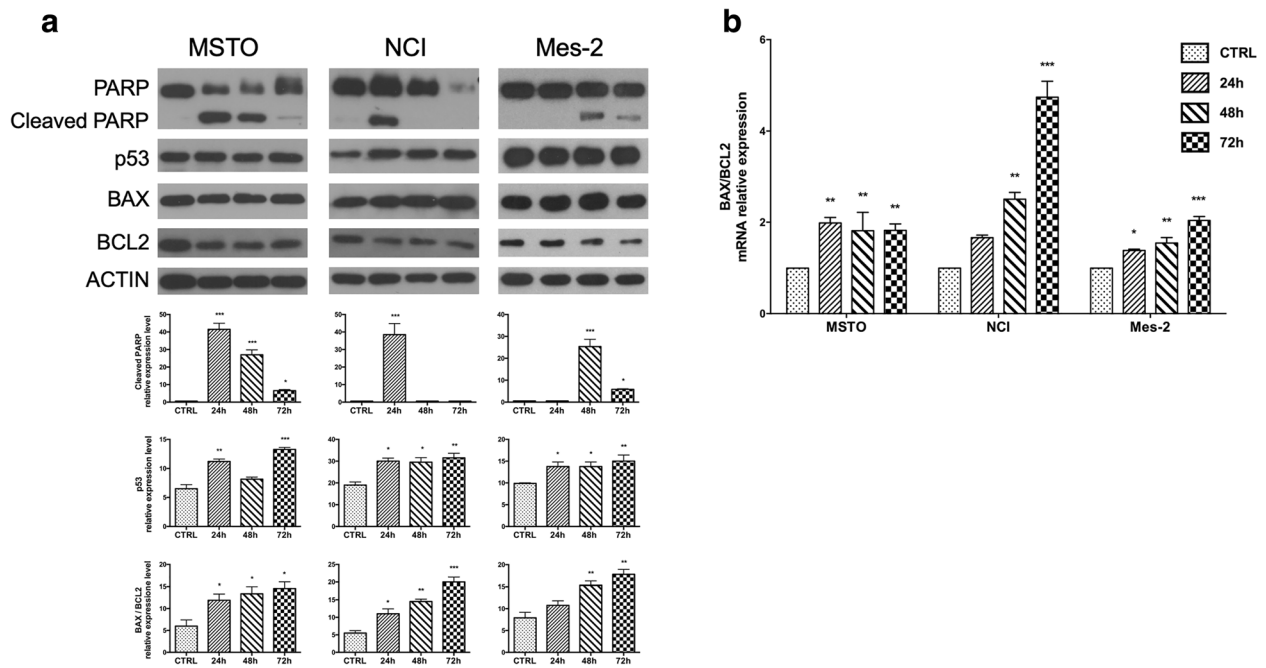

Fig. 4 CBP induces intrinsic apoptosis. **a**) Protein expression levels of p53 and apoptotic markers in MM cell lines after 20 μM CPB for 24, 48 and 72 h treatments analysed by Western blot analysis. Histograms report the expression of p53, cleaved PARP and Bax/Bcl2 ratio normalized expression. β-Actin was used as loading control. **b**) Quantitative analysis of mRNA expression levels of *Bax* and *Bcl-2* in mesothelioma cell lines after 24, 48 and 72 h of CBP treatment. The bars represent ± the average ± SD of independent experiments (n = 3). Statistically significant difference compared to untreated cells: **p ≤ 0.01, ***$p \leq 0.005$. CTRL: untreated cells after 72 h culture

expression levels of FAS protein and gene in MSTO and NCI cells, while Mes-2 cells do not express FAS protein and mRNA (Additional file 2: Figure S2). On the contrary, we did not detect any expression of *FASL* genes in MM cells [24].

Finally, to further investigate the occurrence of intrinsic apoptotic pathway, we examined the cytochrome *c* release from mitochondria following CBP treatments. As shown in Additional file 2: Figure S2, CBP induced cytochrome *c* release in MM cell lines.

These results indicated that CPB in human MM induces apoptosis through intrinsic pathway.

CBP shows anticancer activity in mesothelioma xenografts in mice

To verify the cellular and molecular in vitro results and analyse whether CBP may have beneficial effects in MM, we performed in vivo experiments using a xenograft mouse model of MM induced by dorsal injection of human mesothelioma cells (MSTO-211H).

In these experiments we analysed the effects of CBP and cisplatin (adopted as a positive control) treatments on tumour growth in comparison with treatment with vehicle (DMSO, control group). The results showed that daily administration of CBP (40 mg/kg) was able to significantly delay the growth of the ectopic tumours as compared to the controls (p-value = 0.0013), even if the efficacy of the treatment was lower in comparison with cisplatin (p-value = 0.0011) (Fig. 5 a). Histopathology analysis of ectopic tumours showed that treatment with CBP or cisplatin caused partial substitution of the tumor tissue by calcified and necrotic tissue. Tumor tissue from control samples displayed high proliferation index, as evaluated by Ki67 nuclear expression, low apoptotic index, as evaluated by TUNEL assay and high neo-angiogenesis, as detected by CD34 expression, as compared to CBP treatment (Fig. 5 b). In detail, Ki-67 expression was 25% in the controls vs. 10% in the treated tumours (p-value = 0.008), TUNEL score was 2% in the controls vs. 8% in the treated tumours (p-value = 0.0005), while the number of vessels in the control animals was 10 ± 4 and 5 ± 2 in the treated tumours (p-value = 0.0006). Interestingly, cisplatin treatment caused a similar effect on cell proliferation and angiogenesis as compared to CBP regimen, while the number of apoptotic cells was significantly higher with a TUNEL score higher than 10% (data not shown). The same CPB regimen was performed in nude mice not injected with MM cells to confirm the non-toxicity of this treatment (data not shown).

Discussion

A major limitation of the conventional treatments for cancer is related to serious side effects that chemotherapeutic drugs may cause and to the development of

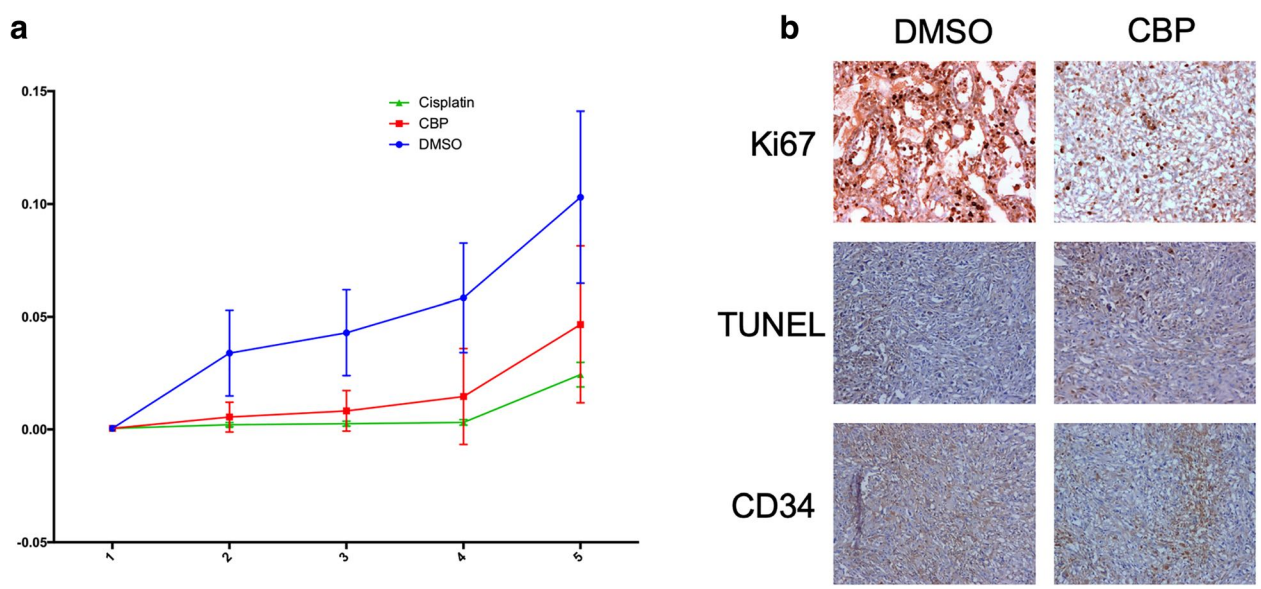

Fig. 5 In vivo anticancer properties of CBP. **a**) Antitumor effect of CBP in a xenograft model of MM. The tumor growth curves show that CBP was able to significantly delay the growth of the ectopic tumors as compared to the controls (*p*-value = 0.0013), but with lower efficacy, compared to cisplatin (p-value = 0.0011). **b**) Effects of CBP on cell proliferation apoptosis and angiogenesis as compared to the control, as determined by Ki67, TUNEL (asterisks identify apoptotic cells) and CD34 staining (original magnification × 10). CBP treatment significantly decreased cell proliferation (25% vs 10%; p-value = 0.008), increased apoptosis (8% vs 2%; p-value = 0.0005) and decreased angiogenesis (5 ± 2 vs 10 ± 4; p-value = 0.0006)

cancer resistance. Some advances in cancer therapies have been currently reached thanks to the introduction of alternative approaches, including the use of several phytochemicals, which are able to significantly reduce tumor progression and to improve healing and survival [5].

MM is a rare and aggressive tumor developing from the mesothelial surface of the pleural space. Clinical signs are most of the time late and unspecific. The diagnosis is difficult with routine imaging procedures and usually it is based on immunohistochemistry of pleural biopsies. Until now neither surgical nor radiation treatments and conventional chemotherapy result in favorable prognosis for MM patients.

The objective of our study was to examine the anticancer activity of CBP in mesothelioma by in vitro and in vivo analysis.

The rationale for examining the potential efficacy of CBP as a MM chemopreventive agent was based on several lines of evidence. First, recent data suggest a robust anti-cancer efficacy of curcumin [4]. Second, some studies indicated that curcumin efficacy is tightly linked to its bioavailability [13, 14] and several approaches including complexation in nanoparticles [21] or phospholipid complexes [25] have been tested. Third, piperine significantly enhances curcumin bioavailability by inhibition of its glucuronidation processes [15, 16], and by increasing its molecular uptake in the intestine, since it reduces the intestinal transit [26]. Fourth, few investigations have demonstrated a specific anti-cancer activity on MM for curcumin reporting the in vivo anticancer effect of curcumin in mesothelioma mouse or rat allograft models [27–30].

In this work we analysed the anticancer activity of curcumin in MM using a specific formulation: C3 Complex®. This is a standardised extract from dried roots and rhizomes of *Curcuma longa* containing up to 95% of three different curcuminoids that constitute the active components: curcumin (79,97%), demethoxycurcumin (17,62%) and bisdemethoxycurcumin (2,41%). C3 Complex® was used in combination (1:100) with Bioperine®.

As a first line strategy, we performed an in vitro study to evaluate the effects of C3 Complex® in three different MM cell lines: MSTO, NCI, and Mes-2. The results showed that CBP compared to C3 alone greatly impaired cell viability in MM cells in a time dependent manner. Furthermore, CBP strongly reduced tumorigenic properties in MM by impairing cellular self-renewal ability, proliferative cell rate and cell migration. We also evidenced that CBP determines cell death through a time-dependent apoptotic induction in all the MM cell lines analysed. Subsequent analysis of the expression of key signalling molecules involved in apoptosis clearly indicated that CBP through p53 pathway activates intrinsic apoptotic pathway, since we detected an increase of the BAX/BCL-2 ratio at both protein and gene level and cytochrome *c* release in all mesothelioma cell lines analysed. Indeed, unbalance between BCL-2 and BAX

favours BAX activity. BAX induces mitochondrial membrane permeabilization and the subsequent cytochrome *c* release into the cytoplasm of MM cells. This determines the onset of apoptosis that culminates in caspases machinery activation and cleavage of PARP (poly(ADP-ribose) polymerase) protein [31].

To evaluate whether CBP could have chemopreventive effects also in vivo we generated a xenograft MM mouse model. The results indicated that daily administration of CBP up to four weeks delayed tumor growth even if to a lower extent when compared to cisplatin treatment. According to this observation, immunohistochemical analysis indicated that tumor samples from CBP-treated mice showed reduction of angiogenesis and apoptosis increase.

Different studies report that natural molecules can have anticancer properties sensitizing cancer cells to standard therapy [32]. Also curcumin has been reported to act in vivo synergizing drug effects, thus enhancing the global efficacy: examples are 5-fluorouracil or FOLFOX in colorectal cancer [33–35] or cisplatin in head and neck squamous cell carcinoma and ovarian cancer [36, 37]. Our results describe for the first time the efficacy of CBP in human mesothelioma xenograft mouse model. Although numerous studies describe the anticancer or the adjuvant effects of CBP [38, 39] and several clinical trials have been started [40, 41] none of them refers to MM. To our knowledge, this is the first study analysing in vitro and in vivo the efficacy of CBP on MM.

It is important to underline that in our experimental conditions CBP was ineffective in controlling bulky neoplasms. Nevertheless, by adopting this therapeutic scheme in a dose intensity protocol, it might be possible to achieve extended tumor control, especially considering that this approach could be associated to aggressive pleurectomy. Furthermore, considering the ability of curcumin to act in vivo synergizing drug effects, it is also possible that inclusion of other drugs in the treatment regimen may help achieve an additional increase in the anti-tumor efficacy of this therapy.

Conclusions

In conclusion, our findings reveal novel, previously unappreciated anti-cancer effects of CBP in a model of MM, whose current prognosis remains very poor. Further studies aimed at delineating the exact molecular mechanisms responsible of these effects are required, since they are propaedeutic for future randomized clinical trials aimed at the evaluation of CBP as a chemotherapy agent in MM. Our results suggest that implementation of the standard pharmacological therapies with novel compounds may pave the way to develop alternative and more effective es to MM.

Additional files

Additional file 1: Figure S1. p53 and PARP are not modulated in untreated cells. Analysis of p53 (a) and PARP protein (b) expression in untreated MSTO cultured for 24, 48 and 72 h by Western blot. There is neither modulation of p53 nor presence of cleaved PARP over the time. In b) MSTO treated with 20 μM CPB for 24 h were loaded as control of PARP cleavage. Histograms report the expression of p53, cleaved PARP normalized expression. β-Actin was used as loading control. The bars represent ± the average ± SD of independent experiments ($n = 3$). Statistically significant difference compared to untreated cells: ***$p \leq 0.001$; ****$p \leq 0.0001$. CTRL: untreated cells. (PNG 371 kb)

Additional file 2: Figure. S2. CBP does not induce death receptors but induces cytochrome *c* in mesothelioma cells. Protein (a) and gene (b) expression levels of FAS in MM cell lines after 20 μM CPB for 72 h analysed by Western blot and qPCR. FAS is not modulated in MSTO and NCI cells, while no protein and gene expression was detected in Mes-2 cells. c) Western blot analysis showing cytosolic release of cytochrome *c* in MM cell lines after 20 μM CBP treatment at 72 h. Histograms report the expression of FAS or cytochrome *c* normalized expression. In western blot experiments β-Actin was used as loading control. The bars represent ± the average ± SD of independent experiments (n = 3). Statistically significant difference compared to untreated cells: ****p ≤ 0.0001. CTRL: untreated cells after 72 h culture. (PNG 750 kb)

Abbreviations

BAX: Bcl-2-associated X protein; BCL-2: B-cell lymphoma 2; CBP: C3 complex® and Bioperine®; FACS: Fluorescence activated cell sorting; FASL: Fas ligand; MM: malignant mesothelioma; PARP: (poly(ADP-ribose) polymerase) protein

Acknowledgments

FDM PhD fellowship in Biology is supported by MIUR project PON "Dottorati Innovativi con caratterizzazione industriale" 2017-2018. The work was partially supported by Sabinsa Corporation. We thank Sabinsa for providing C3 complex® and Bioperine® used in this study, and IGB FACS facility. We also thank Dr. Pasquale Barba for his support in FACS analysis, Dr. Maria Rosaria Aletta for bibliographic assistance and Dr. Maria Rosaria Chiummo for reviewing the manuscript.

Authors' contributions

FDM performed and interpreted data of the in vitro experiments; SF conceived experiments and interpreted data of in vivo experiments and contributed to revise the paper; MM performed the in vivo experiments; GG performed molecular biology experiments; ADP interpreted data on in vivo experiments; VM interpreted data on in vivo experiments; AB conceived experiments and interpreted data on in vivo experiments and drafted and revised the manuscript; SC conceived the work, interpreted the data, drafted and revised the manuscript. All authors read and approved the final manuscript.

Funding

This work was partially supported by Sabinsa Corporation to SC.

Availability of data and materials

All data generated or analysed during this study are included in this published article. The original submitted files for images are available from the corresponding author upon request.

Ethics approval and consent to participate

All animal studies were performed in accordance with approved protocols by the IRCCS Neuromed Animal Care Review Board and by Ministry of Health.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Institute of Biosciences and BioResources-UOS Naples CNR, via P. Castellino, 111-, 80131 Naples, Italy. [2]Department of Biology, University of Naples

Federico II, Complesso Universitario Monte Sant'Angelo via Cinthia, Naples, Italy. [3]IRCCS Neuromed, Localitá Camerelle, Pozzilli, IS, Italy. [4]Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, University of Campania "L. Vanvitelli", Caserta, Italy.

Received: 30 May 2019 Accepted: 8 August 2019
Published online: 16 August 2019

References

1. Sugarbaker PH. Update on the management of malignant peritoneal mesothelioma. Transl Lung Cancer Res. 2018;7(5):599–608.
2. Zervos MD, Bizekis C, Pass HI. Malignant mesothelioma 2008. Curr Opin Pulm Med. 2008;14(4):303–9.
3. Spugnini EP, Cardillo I, Verdina A, Crispi S, Saviozzi S, Calogero R, et al. Piroxicam and cisplatin in a mouse model of peritoneal mesothelioma. Clin Cancer Res. 2006;12(20 Pt 1):6133–43.
4. Spugnini EP, Crispi S, Scarabello A, Caruso G, Citro G, Baldi A. Piroxicam and intracavitary platinum-based chemotherapy for the treatment of advanced mesothelioma in pets: preliminary observations. J Exp Clin Cancer Res. 2008;27:6.
5. Piccolo MT, Menale C, Crispi S. Combined anticancer therapies: an overview of the latest applications. Anti Cancer Agents Med Chem. 2015;15(4):408–22.
6. Nelson KM, Dahlin JL, Bisson J. Graham J. Walters MA. The Essential Medicinal Chemistry of Curcumin. J Med Chem: Pauli GF; 2017.
7. Spugnini EP, Campioni M, D'Avino A, Caruso G, Citro G, Baldi A. Cell-cycle molecules in mesothelioma: an overview. J Exp Clin Cancer Res. 2007;26(4):443–9.
8. Bhattacharyya S, Mandal D, Saha B, Sen GS, Das T, Sa G. Curcumin prevents tumor-induced T cell apoptosis through stat-5a-mediated Bcl-2 induction. J Biol Chem. 2007;282(22):15954–64.
9. Bhattacharyya S, Mandal D, Sen GS, Pal S, Banerjee S, Lahiry L, et al. Tumor-induced oxidative stress perturbs nuclear factor-kappaB activity-augmenting tumor necrosis factor-alpha-mediated T-cell death: protection by curcumin. Cancer Res. 2007;67(1):362–70.
10. Fang JG, Lu J, Holmgren A. Thioredoxin reductase is irreversibly modified by curcumin - a novel molecular mechanism for its anticancer activity. J Biol Chem. 2005;280(26):25284–90.
11. Limtrakul P, Chearwae W, Shukla S, Phisalphong C, Ambudkar SV. Modulation of function of three ABC drug transporters, P-glycoprotein (ABCB1), mitoxantrone resistance protein (ABCG2) and multidrug resistance protein 1 (ABCC1) by tetrahydrocurcumin, a major metabolite of curcumin. Mol Cell Biochem. 2007;296(1–2):85–95.
12. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. Bioavailability of curcumin: problems and promises. Mol Pharm. 2007;4(6):807–18.
13. Hussain Z, Thu HE, Ng SF, Khan S, Katas H. Nanoencapsulation, an efficient and promising approach to maximize wound healing efficacy of curcumin: a review of new trends and state-of-the-art. Colloids Surf B Biointerfaces. 2017;150:223–41.
14. Mayol L, Serri C, Menale C, Crispi S, Piccolo MT, Mita L, et al. Curcumin loaded PLGA-poloxamer blend nanoparticles induce cell cycle arrest in mesothelioma cells. Eur J Pharm Biopharm. 2015;93:37–45.
15. Shoba G, Joy D, Joseph T, Majeed M, Rajendran R, Srinivas PS. Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. Planta Med. 1998;64(4):353–6.
16. Vecchione R, Quagliariello V, Calabria D, Calcagno V, De Luca E, Iaffaioli RV, et al. Curcumin bioavailability from oil in water nano-emulsions: in vitro and in vivo study on the dimensional, compositional and interactional dependence. J Control Release. 2016;233:88–100.
17. Baldi A, Piccolo MT, Boccellino MR, Donizetti A, Cardillo I, La Porta R, et al. Apoptosis Induced by Piroxicam plus Cisplatin Combined Treatment Is Triggered by p21 in Mesothelioma. Plos One. 2011;6(8):e23569.
18. Minear S, O'Donnell AF, Ballew A, Giaever G, Nislow C, Stearns T, et al. Curcumin inhibits growth of Saccharomyces cerevisiae through iron chelation. Eukaryot Cell. 2011;10(11):1574–81.
19. Crispi S, Calogero RA, Santini M, Mellone P, Vincenzi B, Citro G, et al. Global gene expression profiling of human pleural mesotheliomas: identification of matrix metalloproteinase 14 (MMP-14) as potential tumour target. PLoS One. 2009;4(9):e7016.
20. Baldi A, Piccolo MT, Boccellino MR, Donizetti A, Cardillo I, La Porta R, et al. Apoptosis induced by piroxicam plus cisplatin combined treatment is triggered by p21 in mesothelioma. PLoS One. 2011;6(8):e23569.
21. Serri C, Argiro M, Piras L, Mita DG, Saija A, Mita L, et al. Nano-precipitated curcumin loaded particles: effect of carrier size and drug complexation with (2-hydroxypropyl)-beta-cyclodextrin on their biological performances. Int J Pharm. 2017;520(1–2):21–8.
22. Edlich F. BCL-2 proteins and apoptosis: recent insights and unknowns. Biochem Biophys Res Commun. 2018;500(1):26–34.
23. Waring P, Mullbacher A. Cell death induced by the Fas/Fas ligand pathway and its role in pathology. Immunol Cell Biol. 1999;77(4):312–7.
24. Kavurma MM, Khachigian LM. Signaling and transcriptional control of Fas ligand gene expression. Cell Death Differ. 2003;10(1):36–44.
25. Appendino G, Belcaro G, Cornelli U, Luzzi R, Togni S, Dugall M, et al. Potential role of curcumin phytosome (Meriva) in controlling the evolution of diabetic microangiopathy. A pilot study. Panminerva Med. 2011;53(3 Suppl 1):43–9.
26. Bajad S, Shulaev V. LC-MS-based metabolomics. Methods Mol Biol. 2011;708: 213–28.
27. Masuelli L, Benvenuto M, Di Stefano E, Mattera R, Fantini M, De Feudis G, et al. Curcumin blocks autophagy and activates apoptosis of malignant mesothelioma cell lines and increases the survival of mice intraperitoneally transplanted with a malignant mesothelioma cell line. Oncotarget. 2017;8(21):34405–22.
28. Pouliquen DL, Nawrocki-Raby B, Nader J, Blandin S, Robard M, Birembaut P, et al. Evaluation of intracavitary administration of curcumin for the treatment of sarcomatoid mesothelioma. Oncotarget. 2017;8(34):57552–73.
29. Wang Y, Rishi AK, Wu W, Polin L, Sharma S, Levi E, et al. Curcumin suppresses growth of mesothelioma cells in vitro and in vivo, in part, by stimulating apoptosis. Mol Cell Biochem. 2011;357(1–2):83–94.
30. Zhang C, Hao Y, Wu L, Dong X, Jiang N, Cong B, et al. Curcumin induces apoptosis and inhibits angiogenesis in murine malignant mesothelioma. Int J Oncol. 2018;53(6):2531–41.
31. Fischer U, Janicke RU, Schulze-Osthoff K. Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ. 2003;10(1): 76–100.
32. Nuvoli B, Santoro R, Catalani S, Battistelli S, Benedetti S, Canestrari F, et al. CELLFOOD induces apoptosis in human mesothelioma and colorectal cancer cells by modulating p53, c-myc and pAkt signaling pathways. J Exp Clin Cancer Res. 2014;33:24.
33. Howells LM, Sale S, Sriramareddy SN, Irving GR, Jones DJ, Ottley CJ, et al. Curcumin ameliorates oxaliplatin-induced chemoresistance in HCT116 colorectal cancer cells in vitro and in vivo. Int J Cancer. 2011;129(2):476–86.
34. Toden S, Okugawa Y, Jascur T, Wodarz D, Komarova NL, Buhrmann C, et al. Curcumin mediates chemosensitization to 5-fluorouracil through miRNA-induced suppression of epithelial-to-mesenchymal transition in chemoresistant colorectal cancer. Carcinogenesis. 2015;36(3):355–67.
35. Zhang P, Lai ZL, Chen HF, Zhang M, Wang A, Jia T, et al. Curcumin synergizes with 5-fluorouracil by impairing AMPK/ULK1-dependent autophagy, AKT activity and enhancing apoptosis in colon cancer cells with tumor growth inhibition in xenograft mice. J Exp Clin Cancer Res. 2017;36(1):190.
36. Fetoni AR, Paciello F, Mezzogori D, Rolesi R, Eramo SL, Paludetti G, et al. Molecular targets for anticancer redox chemotherapy and cisplatin-induced ototoxicity: the role of curcumin on pSTAT3 and Nrf-2 signalling. Br J Cancer. 2015;113(10):1434–44.
37. He M, Wang D, Zou D, Wang C, Lopes-Bastos B, Jiang WG, et al. Re-purposing of curcumin as an anti-metastatic agent for the treatment of epithelial ovarian cancer: in vitro model using cancer stem cell enriched ovarian cancer spheroids. Oncotarget. 2016;7(52):86374–87.
38. James MI, Iwuji C, Irving G, Karmokar A, Higgins JA, Griffin-Teal N, et al. Curcumin inhibits cancer stem cell phenotypes in ex vivo models of colorectal liver metastases, and is clinically safe and tolerable in combination with FOLFOX chemotherapy. Cancer Lett. 2015;364(2):135–41.
39. Li Y, Zhang T. Targeting cancer stem cells by curcumin and clinical applications. Cancer Lett. 2014;346(2):197–205.
40. Irving GR, Howells LM, Sale S, Kralj-Hans I, Atkin WS, Clark SK, et al. Prolonged biologically active colonic tissue levels of curcumin achieved after oral administration--a clinical pilot study including assessment of patient acceptability. Cancer Prev Res (Phila). 2013;6(2):119–28.
41. Irving GR, Iwuji CO, Morgan B, Berry DP, Steward WP, Thomas A, et al. Combining curcumin (C3-complex, Sabinsa) with standard care FOLFOX chemotherapy in patients with inoperable colorectal cancer (CUFOX): study protocol for a randomised control trial. Trials. 2015;16:110.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

International Journal of
Molecular Sciences

MDPI

Review

Bioactive Polyphenols and Neuromodulation: Molecular Mechanisms in Neurodegeneration

Francesco Di Meo [1,2,†], Anna Valentino [3,†], Orsolina Petillo [4], Gianfranco Peluso [4], Stefania Filosa [1,5,*,‡] and Stefania Crispi [1,6,*,‡]

1 Institute of Biosciences and BioResources-UOS Naples CNR, Via P. Castellino, 111, 80131 Naples, Italy; francesco.dimeo.90@gmail.com
2 Department of Biology, University of Naples Federico II, Complesso Universitario Monte Sant'Angelo Via Cinthia, 80126 Naples, Italy
3 Elleva Pharma s.r.l., Via P. Castellino 111, 80131 Naples, Italy; anna.valentino@ellevapharma.com
4 Institute on Terrestrial Ecosystems (IRET) CNR, Via P. Castellino 111, 80131 Naples, Italy; orsolina.petillo@cnr.it (O.P.); gianfranco.peluso@cnr.it (G.P.)
5 IRCCS Neuromed, Localitá Camerelle, 86077 Pozzilli (IS), Italy
6 Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Napoli, Italy
* Correspondence: stefania.filosa@ibbr.cnr.it (S.F.); stefania.crispi@ibbr.cnr.it (S.C.)
† These authors contributed equally to this work.
‡ These authors contributed equally to this work.

Received: 3 March 2020; Accepted: 3 April 2020; Published: 7 April 2020

Abstract: The interest in dietary polyphenols in recent years has greatly increased due to their antioxidant bioactivity with preventive properties against chronic diseases. Polyphenols, by modulating different cellular functions, play an important role in neuroprotection and are able to neutralize the effects of oxidative stress, inflammation, and apoptosis. Interestingly, all these mechanisms are involved in neurodegeneration. Although polyphenols display differences in their effectiveness due to interindividual variability, recent studies indicated that bioactive polyphenols in food and beverages promote health and prevent age-related cognitive decline. Polyphenols have a poor bioavailability and their digestion by gut microbiota produces active metabolites. In fact, dietary bioactive polyphenols need to be modified by microbiota present in the intestine before being absorbed, and to exert health preventive effects by interacting with cellular signalling pathways. This literature review includes an evaluation of the literature in English up to December 2019 in PubMed and Web of Science databases. A total of 307 studies, consisting of research reports, review articles and articles were examined and 146 were included. The review highlights the role of bioactive polyphenols in neurodegeneration, with a particular emphasis on the cellular and molecular mechanisms that are modulated by polyphenols involved in protection from oxidative stress and apoptosis prevention.

Keywords: polyphenols; neuroprotection; apoptosis prevention; oxidative stress; gut microbiota

1. Introduction

An improvement in socio-economic conditions, especially in developed countries, results in an increase in elderly people. This determines a corresponding enlargement of the pathologies linked to brain aging, such as cognitive and neurodegenerative diseases. The potential mechanisms underpinning brain neurodegeneration have not yet been completely elucidated; nevertheless, oxidative stress and inflammation are considered the main effectors of brain decline.

Nowadays, there is a growing interest in dietary polyphenol nutrients since different epidemiological studies have suggested that diets rich in plant-derived phytochemicals and, in particular, polyphenols, are beneficial to human health [1]

Among phytochemicals, polyphenols are the major group of compounds produced by plants as secondary metabolites that protect plants against reactive oxygen species (ROS), ultraviolet radiation (UV), pathogens, parasites and plant predators. They act as natural antioxidants thanks to their metal-chelating and free radical scavenger properties. Bioactive polyphenols have been reported to prevent the age-related cognitive decline typical of neurodegenerative diseases. Dietary bioactive polyphenols can also modulate cognitive deficits and synaptic plasticity and promote neurogenesis [2,3].

Neurodegenerative diseases are characterized by the progressive loss of function of a specific population of neurons and neural stem cells that results in sensory and motor deficits and cognitive impairment. Different signaling pathways are involved in neurodegeneration, including oxidative stress, inflammation and apoptosis. Bioactive polyphenols are able to counteract these processes by directly scavenging free radical species inhibiting 'pro-oxidant' enzymes, activating anti-oxidant enzymes [4] or intervening in apoptotic pathways [5].

Polyphenols act by preventing the DNA damage triggered by hydrogen peroxide (H_2O_2) and by transition metals such as copper and iron. Iron-mediated oxidative DNA damage by hydroxyl radical ($^{\bullet}OH$) is the primary cause of cell death under oxidative stress conditions for both prokaryotes and eukaryotes [6]. Polyphenols can also directly scavenge ROS or inhibit the expression of molecules sensitive to oxidative stress such as nuclear factor-kB (Nf-κB) and activator protein-1 (AP-1) [7].

The molecular mechanism of neuroprotection involves the regulation of the mitochondrial apoptosis cascade, which is finely tuned by the imbalance by between B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax). Phytochemical neuroprotection can be achieved by introducing anti-apoptotic Bcl-2, thus preventing apoptosis [8].

Bioactive polyphenols can exert neuromodulatory effects, activating different intracellular signaling pathways that are crucial for neuroprotection. The PI3K/Akt (Phosphoinositide 3-kinases/Akt) pathway prevents apoptosis by upregulating the expression of Bcl-2; the PKC-ERK1/2 (Protein kinase C/ Extracellular signal-regulated protein kinases 1 and 2) pathway decreases Bcl-2 (B-cell lymphoma 2), Bcl-w (Bcl-2-Like Protein 2) and Bcl-xL (B-cell lymphoma-extra large), which are other anti-apoptotic proteins; Akt-ERK1/2 inhibits the pro-apoptotic activity of Bad (BCL2 associated agonist of cell death) and Bim (Bcl-2-interacting mediator) and activates caspases 9 and 3 [9,10].

The neuroprotective effects of polyphenols can be carried out through synthesis of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) or glial cell line-derived neurotrophic factor (GDNF). They can exert neurotrophic activity by binding the cognate receptors and activating the downstream neuroprotective pathways [11].

The beneficial health effects of bioactive phenolic compounds mainly depend on their bioavailability and absorption rate. These characteristics can be modulated through interaction with other dietary nutrients and through the action of enzymes present in the small intestine and in the liver. Polyphenols are usually recognized as xenobiotics by the human body. After ingestion, their absorption through the gut barrier can be increased following specific biotransformation and conjugation such as methylation, sulfation and glucuronidation [12].

Polyphenols are effective in neuroprotection in a different way—directly, by affecting brain functions and indirectly, by modulating gut microbiota composition and the metabolites produced. Both actions determine the production of neurotransmitters and neuropeptides that are able to influence the brain functions.

In this review, we discuss the different cellular and molecular mechanisms by which dietary bioactive polyphenols exert neuroprotective functions. The review evaluates the main publications in the field, providing a comprehensive analysis of the efficacy of bioactive polyphenols.

2. Methodology

We performed a literature review, searching within the PubMed and Web of Science database. As keywords, we used the terms: oxidative stress, antioxidant, polyphenols, neurodegeneration, neuroprotection, Alzheimer's disease, Parkinson's disease, and Huntington's disease. Research reports, review articles and articles in English published up to December 2019 were selected and evaluated. In addition, we examined the citations therein and included them when appropriate. In total, we examined 307 references and included 146 of them in the present review.

3. Oxidative Stress and Polyphenols

Oxidative stress occurs when ROS accumulate in the cells for excessive production or insufficient neutralization. ROS are a contradiction for the cells. They are produced in normal metabolism as part of several physiologic processes and, when present in physiologic concentrations, they control redox homeostasis in the cell. However, an imbalance between their production and the ability of the cell to enforce antioxidant defense mechanisms may affect cellular structure and functional integrity, resulting in cell damage and leading to necrotic or apoptotic cell death [13–15].

ROS cause severe molecular damage in the major cell components such as protein oxidation, lipid oxidation, DNA oxidation, and glycoxidation. They can be divided into free radicals and nonradicals. Free radicals are the molecular species containing at least one unpaired electron in the shell around the atomic nucleus.

Among free radicals, H_2O_2 is one of the most important ROS with a physiological significance. H_2O_2 is formed in the cells in a reaction catalyzed by the superoxide dismutase enzyme (SOD) and also, at relatively low concentrations, it can penetrate the biological membranes and cause severe damage to cellular macromolecules. ROS increase with exposure to the environment. To counterbalance the effect of oxidants, cells have evolved an intricate network of defense mechanisms. Indeed, depending on the intensity and duration of oxidative stress, the cell response can be different, ranging from cell proliferation to cell cycle arrest or cell death by apoptosis or necrosis [16].

The adverse effects of ROS can be inactivated by the action of antioxidants [17]. They are defined as compounds able to inhibit the oxidation of any molecule, even when present at very low concentration [18]. By inhibiting or quenching free radical reactions, antioxidants delay or block cellular damage.

It is widely accepted that neurodegenerative development is associated with oxidative stress that determines severe injury in the cell. In fact, oxidative stress can result in deep biological damage. An imbalance in redox regulation determines the overproduction of ROS with the induction of progressive cellular damage, which is fundamental in neurodegenerative diseases. The production of excessive ROS in neuronal tissue is mainly due to the activity of excitatory amino acids and neurotransmitters [19]. Thus, neuronal cells need antioxidants to scavenge and prevent the formation of ROS.

The body protects itself from ROS by using enzymatic antioxidant mechanisms [20]. The antioxidant enzymes reduce the levels of lipid hydroperoxide and H_2O_2; thus, they are important in preventing lipid peroxidation and maintaining the structure and function of cell membranes.

The nonenzymatic antioxidants are the natural antioxidants present in plants. Among them, polyphenols are the most powerful antioxidants able to balance the cellular ROS by activating antioxidant signaling pathways [21,22]. Different studies indicate that dietary antioxidants consumed daily exert a protective role [23].

Plants produce nonenzymatic antioxidants such as vitamins that act to interrupt free radical chain reactions. Vitamin C is present in all plant cells and it has intracellular and extracellular antioxidant capacity. Vitamin E is present exclusively in plastids. It represents the principal defense against oxidative membrane damage, being concentrated in the hydrophobic bilayer of the cell membrane. Vitamin E avoids lipid peroxidation, thus protecting polyunsaturated fatty acids (PUFA) from reactive oxygen damage [24].

Antioxidant plant polyphenols belong to different groups: phenolic acids, phenolic diterpenes, volatile oils, flavonoids and stilbene (Figure 1) [25]. All act as ROS scavengers, or induce antioxidant enzymes [26].

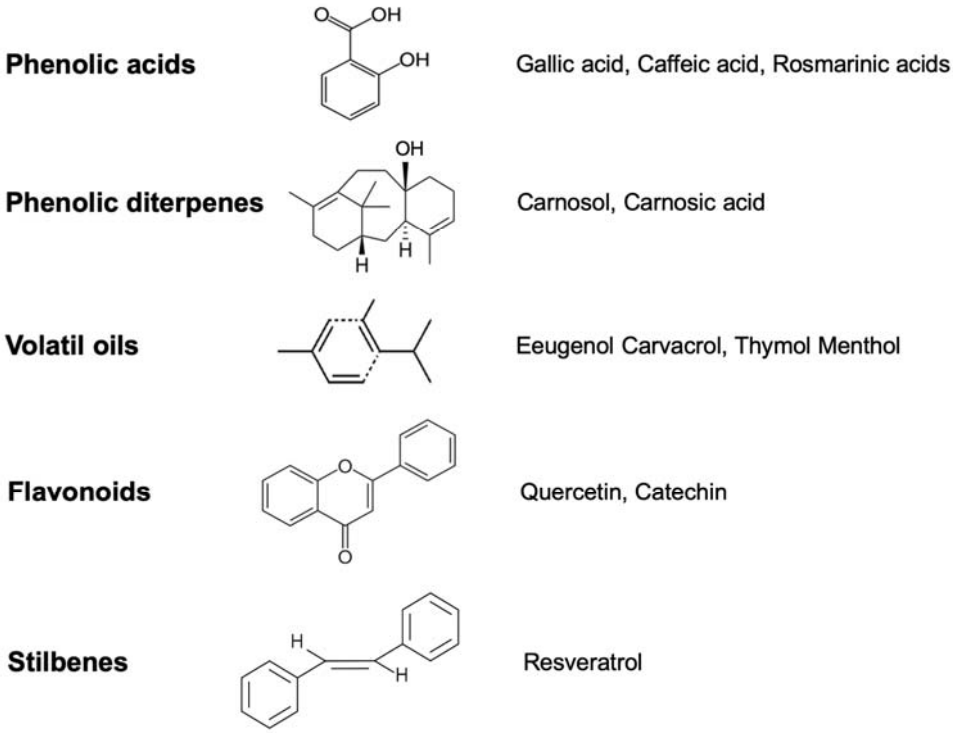

Figure 1. Typical representatives of antioxidant polyphenol classes with their basic chemical structure.

Phenolic acids exert their antioxidant action, trapping free radicals [27]. The antioxidant mechanism of phenolic diterpenes is related to the scavenging activities of lipid free radicals and to the inhibition of low-density lipoprotein oxidation [28]. Volatile oils, as well as phenolic compounds, act as antioxidants by reacting with peroxyl radicals [29]. Flavonoids are the most abundant class of plant polyphenols, due to their metal-chelating and free radical scavenger properties [30]. On the contrary, stilbenes scavenge hydroxyl radicals.

Other non-flavonoid polyphenols present in foods have antioxidant properties. Among them, curcumin, from turmeric Curcuma longa, prevents lipid peroxidation by scavenging H_2O_2 and hydroxyl radicals. Curcumin can also act as an antioxidant, reducing ferric ion (Fe^{3+}) and chelating ferrous ion (Fe^{2+}) [31].

4. Molecular Pathways Involved in Neuroprotection of Polyphenols

Although cells are equipped with a high variety of antioxidants, some tissues and organs are much more vulnerable than others to oxidative stress, probably because of their elevated consumption of oxygen and the consequent generation of large amounts of ROS. Several observations suggest that the brain is particularly vulnerable to oxidative stress [32] and oxidative stress has been implicated in the pathogenesis of many clinical conditions and in the process of aging.

Oxidative stress has been found to be increased in several human age-related degenerative diseases, including genetically-linked neurodegenerative diseases, like Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) [33]. However, whether oxidative stress is a primary cause or a downstream consequence of these neuropathological conditions or, more in general, of the aging process is still an open question [34].

Neurons are particularly sensitive to oxidative stress because, being postmitotic cells, they will not be replaced. Moreover, the ability of cells to respond to oxidative protein damage also seems to decline with age [35]. Since the antioxidant systems are overwhelmed in pathological conditions, the use of natural molecules like polyphenols can be viewed as a novel antioxidant therapeutic strategy to reduce neuronal ROS and ameliorate the neurodegenerative process.

Polyphenols, due to their ability to modulate multiple cellular processes including redox balance, have invaluable potential as antioxidant agents.

Polyphenols such as resveratrol from grapes and wine, curcumin from tumeric, and epigallocatechin-3-gallate from green tea (EGCG), are able to protect against neurodegenerative diseases by activating the protein kinases signaling molecular pathways such as Keap1/Nrf-2/ARE, the major protective pathway against endogenous and exogenous ROS [36,37]. The interaction between Keap1 and bioactive molecules leads to the disruption of the Keap1/Nrf2 complex, allowing Nrf2 to translocate to the nucleus where it binds adenylate and uridylate (AU)-rich elements (AREs) and triggers the expression of antioxidant proteins such as heme oxygenase-1 [38].

Polyphenols also stimulate neurotrophic receptor factors with a pivotal role in the maintenance of neuronal health. Examples are BDNF, which is involved in learning and memory [39], NGF, which is crucial for the survival of brain neurons [40], and GDNF, which regulates cell survival and synaptic plasticity [41]. Several studies reported that different flavonoids, including resveratrol, are able to enhance the expression of these factors. Quercetin and genistein were shown to stimulate NGF-induced neurite outgrowth [42,43]. Resveratrol is able to induce the release of BDNF and GDNF, thus protecting cells from neurotoxicity [44,45].

The neuroprotective role of polyphenols is carried out by activating the pathways that, in cells, regulate transcription, translation, proliferation, growth and survival, such as PI3K/Akt, PKC-ERK1/2, Akt-ERK1/2 and MAPK (Mitogen-activated protein kinase). Their binding to Trk receptors (tyrosine receptor kinases) activates the protein kinase cascades and, finally, cAMP (3′,5′-cyclic adenosine monophosphate)response element-binding protein (CREB), increasing the expression of Bcl-2, Bcl-xL and of neurotrophic factors [46]. In addition, this binding promotes receptor dimerization and autophosphorylation by activating the downstream signaling cascades and promoting the survival of motor neurons, hippocampal neuronal cells, and spinal ganglion neurons [47].

A flavone derivative has been reported to act as a TrkB agonist and to activate the downstream signaling pathway [48]. More recently, resveratrol was shown to exert neuroprotection through its interaction with Trk receptors [49].

In the brain, the anti-inflammatory action of polyphenols leads to neuroprotective effects. In fact, polyphenols inhibit the release of cytokines from activated glia and they also downregulate pro-inflammatory transcription factors such as NF-κB [50]. For example, quercetin determines a strong neuroprotective effect, repressing NF-κB [51]; catechin, after stress injury, increases cell survival by downregulating the NF-κB and MAP kinase pathways [52].

The molecular mechanisms modulated by polyphenols are summarized in Figure 2.

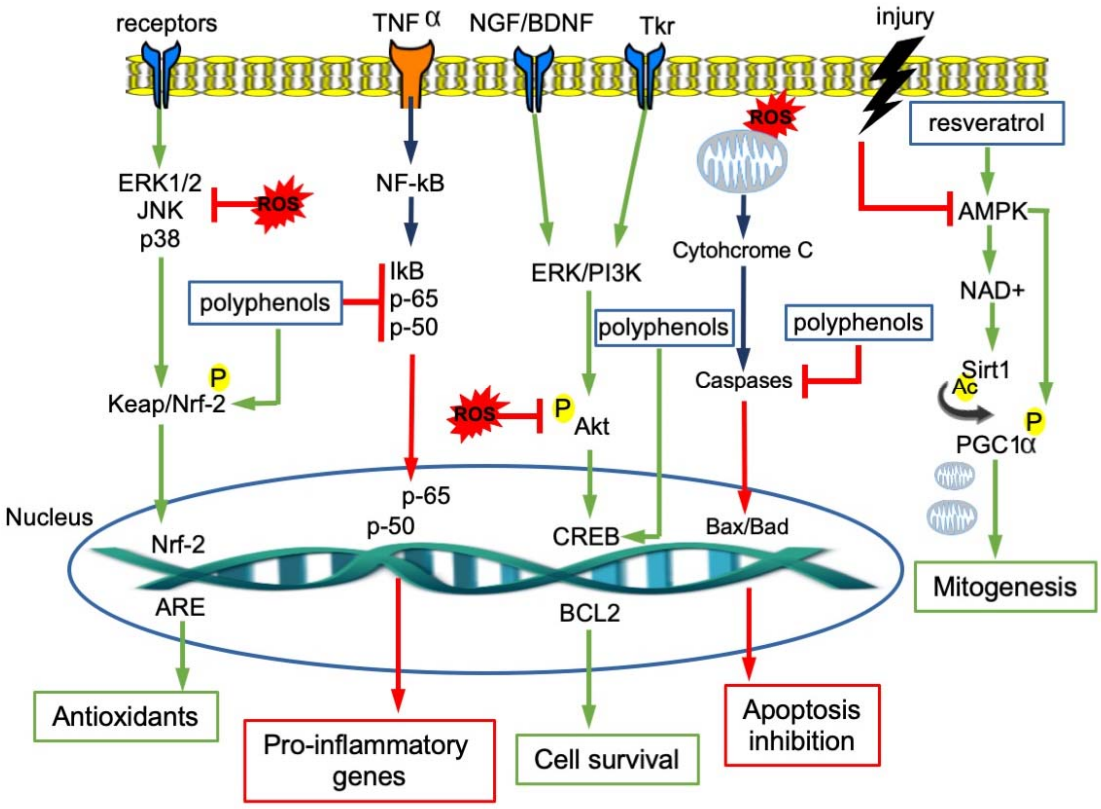

Figure 2. Intracellular signaling pathways involved in neuroprotection and modulated by polyphenols.

5. Effect of Polyphenols on Neurodegenerative Disorders

Despite the variability in the clinical picture of neurodegenerative disorders, these diseases share common molecular traits. Inflammation and oxidative stress are responsible for the disruption of the functions of the neurovascular units in AD, PD, HD and dementia. ROS are able to interact with different neuronal signaling pathways, such as protein kinase and lipid kinase signaling cascades [53,54] (Table 1).

Table 1. Signaling pathways activated by polyphenols in neurodegenerative diseases.

Polyphenol	Signaling Pathway	References
Resveratrol	SIRT1/PGC-1 PI3K/Akt	[52,55]
Curcumin	AMPK/NF-kB PI3K/Akt/GSK-3β	[56–58]
Quercetin	MAPK/AKT/ PI3K ERK/CREB	[59]
Catechins (EGCG)	PKC/MAPK/PI3K/Akt MEK/ERK1/2	[60–62]

5.1. Alzheimer's Disease

AD is the most frequent form of dementia in the elderly population [63], with progressive neurodegeneration. It is characterized by the deposition of Amyloid β (Aβ) peptides as Aβ plaques and intracellular neurofibrillary tangles [64,65] that ultimately lead to a gradual deterioration in brain structure and to the loss of intellectual function [66].

The presence of amyloid beta (Aβ) peptides in Alzheimer's disease confers oxidative insult on neurons and glial cells, leading to a change in synaptic plasticity [67]. The neuronal cytotoxicity in

AD seems to be imputable to N-methyl- D-aspartic acid (NMDA) receptor activation coupled with ROS production [68]. This mechanism, through the PKC/MAPK pathways, leads to the release of arachidonic acid, involved in AD neuron apoptosis. Aβ peptides and ROS promote neurotoxicity in AD, inactivating PI3k/Akt pathways. The Akt inactivation regulates various pro-apoptotic mediators [56]. Several studies indicated that a diet rich in polyphenols inhibits the above-mentioned pathways [69]. Curcumin displays anti-amyloidogenic properties, preventing the neurodegenerative process in AD through the inhibition of MAPK and PI-3K pathways [70]. In addition, curcumin is able to block the BDNF decrease in rats inoculated with Aβ peptide, modulating the Akt/GSK-3β signaling pathway, thus determining a cognitive improvement [71]. Recently, it has been reported in an AD mouse model that curcumin reduced Aβ production through the inhibition of β-secretase (BACE-1), the enzyme that is responsible for the proteolytic processing of the amyloid precursor protein [72].

The antioxidant activity of resveratrol protects the memory decline in AD. In cells, it has been reported that resveratrol suppresses Aβ-induced ROS generation and apoptosis [73]. Another study reported that resveratrol exerts a neuroprotective role through its modulation of the PI3K/Akt signaling pathway [74]. SIRT1 (Sirtuin 1) in brain has been shown to be protective against neurodegeneration by deacetylating several transcription factors involved in neuronal protection and stress resistance [75]. Dietary resveratrol protects against Aβ formation and oxidative stress by modulating SIRT1 expression [76,77]. In particular, this ability is linked to the deacetylation of PGC-1α, to the presence of active proliferator-activated receptor-γ (PPAR-γ), and to the protection against mitochondrial damage by Bcl-2 upregulation [55]. EGCG was described as active towards AD in animal studies, being able to significantly reduce the cognitive decline and Aβ peptides, and to upregulate proteins related to synaptic plasticity [78]. EGCG prevents neuronal apoptosis from neurotoxic processes inhibiting BACE-1 [79]. In addition, EGCG significantly improves neuronal survival and hippocampal neurogenesis by activating the PI3K/Akt signaling pathway and inhibiting the MAPK pathway [80]. In vitro and in vivo studies reported the neuroprotective role of quercetin against Aβ toxicity, showing that this polyphenol determined cell viability increase with a reduction in neuronal oxidative stress [81]. Quercetin seems to inhibit the Aβ plaque aggregation and the formation of neurofibrillary tangles probably increasing the levels of apolipoprotein E, that has a key role in the clearance of Aβ [82]. A study performed in a triple transgenic mouse model of AD showed that quercetin, after three months treatment, was able to reduce the amount of the β-amyloid fibers with improvement of cognitive performances [83]. More recently, it was shown that a quercetin-enriched diet affected the latency in APP/PS1 mice only when administered at early stage. The effect was due to the inhibition of amyloidogenic processing through the reduction in the BACE-1 enzyme [84]. Finally, quercetin treatments in Drosophila models of AD indicated that this flavonoid could restore Aβ-induced perturbation by acting on cell cycle signaling pathways and DNA replication [85].

5.2. Parkinson's Disease

PD is a chronic and long-term degenerative disorder, characterized by the loss of dopaminergic neurons in the substantia nigra, which determines clinical motor deficits such as rigidity, bradykinesia and tremors [57].

Oxidative stress remains the strongest leading theory to explain the progressive loss of dopaminergic neurons in substantia nigra in PD patients [58]. Microglia produce high levels of ROS through NADPH oxidase, which induces the PKC delta and ERK1/2 pathways' activation of genes involved in apoptosis [59]. The neuroprotective effect of polyphenols, has been linked to their free radical scavenging and anti-inflammatory properties, both in cellular and animal models [60,61]. They are able to reduce neurotoxicity by interacting with protein aggregates such as α-synuclein [62].

Different studies using in vitro and in vivo models of toxin- induced PD indicated that curcumin reduces oxidative stress with a reduction in apoptosis through the Akt/Nrf2 signaling pathway, since an increase in antioxidant enzyme activity via Nrf2 transcription was detected [86–88]. Several experiments also reported that curcumin in PD is able to counteract the decrease in the enzyme

tyrosine hydroxylase—the rate-limiting enzyme involved in dopamine synthesis—which has been suggested to be causative in the onset of PD [89]. Curcumin exerts neuroprotective effects in PD by inhibiting oxidative stress through the decrease in ROS, TNF-α and IL-6 and the concomitant increase in Glutathione (GSH) levels [90]. On the other hand, in PD, curcumin not only acts as an antioxidant, but also as anti-inflammatory, reducing the production of TNF-α and Interleukin-6 (IL-6). The beneficial effect of curcumin in the pathophysiology of PD can be also linked to the ability to decrease toll-like receptor 4 (TLR4) and its downstream effectors (NF-κB, IRF3 (Interferon Regulatory Factor 3) and MyD88 (Myeloid differentiation primary response 88) [91].

Resveratrol seems to be a direct modulator in PD-affected pathways. Numerous in vitro studies have demonstrated that this molecule is able to prevent the rotenone-induced autophagic dysfunction by promoting the degradation of α-synuclein [92]. Other studies showed that resveratrol has a protective effect acting on the AKT/GSK-3β signaling pathway [93] or by inhibiting apoptosis through the upregulation of antioxidant enzymes [94]. In addition, resveratrol neurotrophic effects are accomplished through CREB activation in the hippocampus and amygdala neurons, thus reducing oxidative damage induced by neurotoxins [11]. The antioxidant effects of quercetin in PD have not been fully elucidated. Some studies reported that quercetin protected neurons in a rotenone-induced rat model of PD in a dose-dependent manner, upregulating mitochondrial complex-I activity. This molecular mechanism strongly suggests that quercetin has the ability to repair defective mitochondrial electron transport, a hallmark of PD. In addition, quercetin decreases glutathione levels and increases catalase and superoxide dismutase [95]. EGCG in PD exerts neuroprotective effects through AMPK activation, which positively regulates the mitochondrial biogenesis needed for dopaminergic neuronal survival. EGCG in vivo has been reported to preserve the loss of dopaminergic neurons by inhibiting neuronal nitric oxide synthase [96]. EGCG was also shown to prevent striatal dopamine depletion and dopaminergic neuron loss in substantia nigra [97].

5.3. Huntington's Disease and Vascular Dementia

In addition to AD and PD, two other neurodegenerative disorders, Huntington's disease and dementia, are characterized by a total compromise in cognition. The role of polyphenols in prevention/treatment is not so extensively studied in these two neurodegenerative diseases.

HD is a dominantly inherited neurodegenerative disorder characterized by progressive striatal and cortical neurodegeneration with associated motor and cognitive defects. The disease-causing mutation is an expansion of a CAG trinucleotide repeat (>36 repeats) encoding a polyglutamine stretch in the N-terminal region of the huntingtin protein, a ubiquitous protein whose function is still unclear [98]. Mutated Huntington is expressed not only in the brain neurons, but also in the enteric neurons [99,100]. HD has also been associated with mitochondrial dysfunction and oxidative stress, as possible disease mechanisms [101,102]. HD cellular models displayed a deregulation in mitochondrial membrane potential and respiration, implicating a decline in mitochondrial function. It has been reported that resveratrol in HD increases the transcription of genes associated to mitochondrial function [103].

To date, only a few studies have analyzed the effects of curcumin in HD. It has been described that curcumin treatment in a rat model of HD reduced mitochondrial damage and exerted antioxidant effects by activating the Nrf2 pathway [104]. A different study using *Drosophila melanogaster* as an HD model showed that curcumin protects against neurodegeneration, suppressing polyglutamine cytotoxicity and cell death [105]. The neuroprotective role of curcumin has been recently reported in an HD transgenic animal model. This study evidenced that curcumin protected the brain from neuropathological and phenotypic complications associated to the disease [106]. It has been reported that resveratrol in HD increases the transcription of genes associated to mitochondrial function [103]. Resveratrol in HD seems to modulate SIRT. Resveratrol in mouse models of HD has been shown to strongly increase transcription of mitochondrial genes and to enhance mitochondrial function. This activity determines an improvement of motor function in HD transgenic mice [103]. Quercetin was able to reduce mitochondrial oxidative stress in HD. This effect leads to an increase in motor skills and

coordination, as reported in a drug-induced HD model. Indeed, a reduction in the neuro-inflammatory response and an increased number of astrocytes and decreased microglial proliferation were observed in core lesions [107,108].

Vascular dementia arises from chronic vascular damages in the brain. Cerebral ischemia, increasing ROS production, induces neuronal injury accompanied by a progressive decline in memory and cognitive function [109]. In dementia, curcumin, resveratrol and catechins act as free radical scavengers, as well as natural anti-inflammatory agents, by suppressing the TNF-mediated NF-κB activation. In addition, they act to upregulate endogenous antioxidant enzymes and downregulate enzymes involved in the production of ROS [110]. Curcumin was shown to restore memory deficit in an induced mouse model of memory impairment, thanks to its antioxidant action and to the improvement of cerebral circulation [111]. Resveratrol was neuroprotective against vascular dementia by reducing cell death in the hippocampus and preventing the loss of reference memory [112]. In a rat model of vascular dementia, resveratrol was able to restore the cognitive deficits, to reverse oxidative stress levels and BDNF depletion [113]. In a mouse model of dementia, treatment with quercetin restored cognitive deficit and energy metabolism by directly scavenging superoxide, hydroxyl radicals and by inhibiting various oxidases [114]. A summary of the effects of polyphenols in neurodegenerative diseases is reported in Table 2.

Table 2. Summary of the effects of polyphenol treatment from in vitro and in vivo studies.

Pathology	Type of Study	Polyphenols (dose)	Time	Effect	References
Alzheimer's disease	mice	grape extract (5–20 μM)	5 months	Inhibition of Aβ oligomerization	[3]
Parkinson's disease	neuroblastoma cell line	caffeic acid (10–100 μM)	1 hour	Prevention of apoptotic cell death	[9]
Neurodegenerative disorders	neonatal mouse cerebellum cells	curcumin (1–50 μM)	24 hours	Enhancement and repair of neural plasticity	[37]
Alzheimer's disease	rats	curcumin (50–200 mg/kg)	7 days	Improvement of cognitive deficits	[71,72]
CNS disorders	mice	Resveratrol (20 mg/kg)	7 days	Regulation of pathway involved in CNS disorder and aging	[73]
Alzheimer's disease	mice	Resveratrol (24 mg/kg)	45 days	Anti-oxidant effect against beta-amyloid plaque formation	[76,77]
Alzheimer's disease	mice	ECGC (50 mg/kg)	6 months	Reduction in A-β deposition	[78]
Alzheimer's disease	human brain microvascular endothelial cells	Quercetin (0.3–30 μmol/L)	24 hours	Increase in cell viability and of antioxidant activity	[81]
Parkinson's disease	primary rat mesencephalic cells	Catechin, quercetin (40 μM)	48 hours	Protective effect on DA neurons under oxidative stress	[61,70]
Parkinson's disease	rodent model	Curcumin 30 mg/kg)	4 days	Neuroprotective actions (anti-inflammatory and anti-oxidative)	[88,91,115]
Parkinson- like disease	dopaminergic-like cells	Resveratrol (50–200 μM)	12 hours	Neuroprotective effects by inhibiting apoptosis caused by oxidative stress	[92]
Parkinson's disease	rats	Resveratrol (20 mg/kg)	21 days	Prevention of neuronal death	[94,95]
Parkinson's disease	rats	Quercetin (25–75 mg/kg)	4 days	Neuroprotective effect observed in neurotoxin-induced Parkinsonism	[95]

Table 2. *Cont.*

Pathology	Type of Study	Polyphenols (dose)	Time	Effect	References
Parkinson's disease	mice	ECGC (20 mg/kg)	5 days	Preventive effects on NOS	[96,97]
Huntington's disease	mice	ECGC (1 mg/kg)	28 days	Improvement of gene transcription associated to mitochondrial function	[103]
Huntington's disease	rats	Curcumin (40 mg/kg)	7 days	Amelioration of mitochondrial dysfunctions	[104,105]
Huntington's disease	mice	Curcumin (20–40 mg/kg)	7 days	Alleviation of debilitating symptoms associated with the disease	[106]
Huntington's disease	rats	Quercetin (25–50 mg/kg)	4 days	Potential use for inflammatory damages	[107,108]
Memory and cerebral blood flow	mice	Curcumin (10–50 mg/kg)	21 days	Beneficial effects of oxidative stress associated with neurodegenerative disorders	[111]
Dementia	rats	Resveratrol (10–20 mg/kg)	4 days	Neurorestorative effects	[113]
Memory dysfunction	mice	Quercetin (2.5–10 mg/kg)	21 days	Protective toward off dementia and neurodegenerative disorders	[114]

6. Bioactivity of Polyphenols and Gut Microbiota Interplay

Most of the polyphenols from plants ingested in the diet undergo intestinal transformation before being absorbed by enterocytes and colonocytes. The modifications can be either by enzymes produced by the enterocytes themselves or by the microbiota present in the intestine (Figure 3).

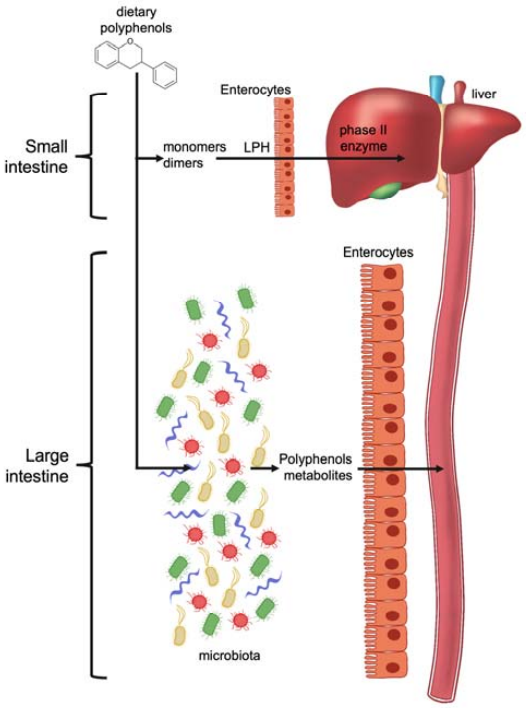

Figure 3. Dietary polyphenol metabolism in small and large intestine. In the small intestine, low molecular weight polyphenols, monomers or dimers, can be absorbed directly or after phase II reaction metabolic conversion. In the large intestine, high molecular weight and conjugated polyphenols are absorbed after transformation processes by enzymes produced by bacteria.

The bioavailability of native polyphenols is very low after recruitment at circulating levels. Polyphenols, to be effective, must be transformed into potentially more bioactive compounds, such as low-molecular weight metabolites [116].

Gut microbiota is essential in transforming numerous compounds that reach the colon, thanks to the capacity of microorganisms to produce a huge and varied range of enzymes.

Enzymatic conjugation processes are needed to reduce the potential toxic effects of polyphenols. These modifications result in the formation of polyphenol metabolites that show new biological activities [117].

The two main factors determining the modification of dietary polyphenols in the gastrointestinal tract are the chemical polyphenols' structure and the individual variety of gut microbiota composition. In fact, the structural subfamily and its scaffold enable only some transformations, thus reducing the kind (class, species) of final bioactive compounds that are produced. Some modifications of polyphenols can occur through enzymes produced by the majority of bacteria, while others require enzymes produced by specific bacterial species. The presence or absence of these bacteria in the individual's microbiota will cause different biotransformation of dietary polyphenols. This means that beneficial effects for consumers depend on the dietary polyphenols and on the individual's microbiota composition.

Polyphenols are molecules with high complexity and for this reason they generally reach the large intestine without modifications. The most complex oligomers are not absorbed in the small intestine, but are processed in the colon by microbiota [118].

EGCG, for example, is transformed in aglycones and gallate, which is further decarboxylated into pyrogallol. In vitro pyrogallol drastically inhibits monocyte migration by reducing levels of inflammatory macrophage differentiation. In addition, pyrogallol increases the phosphorylation of PI3K-AKT and AMPK and reduces caspase levels. In this way, it inhibits monocyte-associated cell death [119]. Pyrogallol is also a GPR35 agonist. GPR35 is an orphan G protein-coupled receptor and is associated to inflammation, cardiovascular diseases, metabolic disorders, Parkinson's disease and other neuronal disorders. GPR35 agonists, as catechol-O-methyl transferase inhibitors, are commonly used for the treatment of Parkinson's disease [120]. Pyrogallol can also block aggregate formation in α-synuclein [121]. The presence of aberrant soluble oligomeric conformations of α-synuclein may contribute to PD pathogenesis. Another example is curcumin that is modified in the colon tract by a specific enzyme produced by *E. coli*. This bacterium converts curcumin in its active metabolite, tetrahydrocurcumin, in a two-step reduction reaction. This metabolite shows both greater in vitro and in vivo antioxidant activity than curcumin [122]. Resveratrol bioavailability is greatly increased by gut microbiota metabolism. Resveratrol in plants is present as a glycosidic form of piceid, that is a stilbenoid glucoside. Two different bacteria—*Bifidobacteria infantis* and *Lactobacillus acidophilus*—convert piceid into resveratrol [123]. Then, resveratrol is further metabolized to obtain the active aglycone form by *Slackia equolifaciens* and *Adlercreutzia equolifaciens* [124].

Bacteria in the gut can cleave the ring structure of several flavonoids into short-chain fatty acids (SCFAs) like hydroxyphenylacetic and hydroxyphenylpropionic acids, as well as into acetate and butyrate [125]. Some polyphenols, like quercetin, are not modified in SCFAs; nonetheless, they can enhance the production of SCFAs, especially butyric acid [126].

Dietary polyphenols show neuroprotective potential, but their selective permeability across the blood–brain barrier (BBB) limits their bioavailability, thus limiting their protective efficacy. The BBB is a dynamic interface that regulates molecular interactions between the blood and the neuronal tissue, having an essential role in providing nutrients and other molecules and regulating the access of compounds to the brain. After intestinal absorption, some polyphenol metabolites can reach concentrations in the bloodstream that can exert effects in vivo. However, the effective brain uptake of these polyphenols' metabolites, and their possible direct neuroprotective potential, is still controversial, given that the exact mechanisms by which they may permeate the BBB are not completely understood. Nerveless, polyphenol microbial metabolites largely showed greater

permeability through gut and blood–brain barriers compared to their parent compounds. For example, 5-(hydroxyphenyl)-γ-valerolactone-*O*-sulfate, a secondary microbial metabolite of the flavan-3-ols, is able to reach the brain and cross the BBB in in silico, in vitro and in vivo models [127].

Microbial metabolites derived from dietary lignans, a class of polyphenols, like equol and enterolactone, passively cross both the gut and BBB barriers and show protective ability against inflammation in microglia [128]. A recent study demonstrates that microbial polyphenol metabolites could be transported across the BBB endothelium [129]. In vitro experiments showed that some polyphenols' metabolites cross BBB by transmembrane diffusion and their lipophilicity can determine greater or minor uptake [130]. Nevertheless, it is not clear whether the primary route by which polyphenols' metabolites cross the BBB happens by simple diffusion or by carrier-mediated transport.

Gut microbiota and polyphenols influence each other. Gut microbiota transforms polyphenols, improving bioavailability and health effects. At the same time, dietary polyphenols regulate microbiota composition, favoring the growth of some bacteria and avoiding the growth of pathogens. Specifically, dietary polyphenols have shown the ability to modulate gut microbiota composition and function, interfering with bacterial quorum sensing, membrane permeability, as well as sensitizing bacteria to xenobiotics.

Specific polyphenols modulate microbiota community composition, modifying the ratio of bacteroides/firmicutes, the most frequent bacteria genera present in the distal gut [131]. For example, resveratrol presented a significant antibacterial activity towards clinically important bacteria, such as *Salmonella enterica, Enterococcus faecalis*, and *Escherichia coli* [132]. The effects of polyphenols on gut microbiota have been shown in human studies. Dietary polyphenols' mechanism of action is different in Gram-positive and Gram-negative bacteria due to changes in cell membrane structure. Polyphenols bind bacterial cell membranes in a concentration-dependent manner; thus, they modify their membrane and alter their growth. Catechins reacted with the dissolved oxygen in aqueous solution, resulting in the generation of hydrogen peroxide. H_2O_2 modifies the permeability of the microbial cell membrane, thus sensitizing the bacteria to the effects of antibiotics [133].

Thus, different polyphenols can change the composition of the microbiota (Table 3), and different bacterial populations possess different enzymes that can change the metabolism of dietary polyphenols in different ways. For example, the intake of catechins, which occur in green tea and black tea, considerably boosted the growth and development of members of *E. coli*. As mentioned above, *E. coli* is able to modify curcumin in its active metabolite, tetrahydrocurcumin, which has to be anti-inflammatory and neuroprotective. So, polyphenols can modify the composition of gut microbiota which, in turn, produce secondary metabolites that have neuroprotective effects.

Table 3. Summary of the main findings of clinical trial studies related to the effects of polyphenols on gut microbiota by increase (+) or decrease (−) of specific strains.

Polyphenols		Bacteria	References
Catechin and epicatechin	+	*Clostridium coccoides–Eubacterium rectale*	[134]
	+	*E. coli*	
	−	*Clostridium histolyticum*	
Proanthocyanidin	+	*Bifidobacteria*	[135]
Pomegranate extract	+	*Odonbacter*	[136]
	+	*Faecalibacterium*	
	+	*Butyricicoccus*	
	+	*Butyricimonas*	
	+	*Bacteroides*	
	−	*Parvimonas*	
	−	*Metanobrevibacter*	
	−	*Metanosphaera*	
Cocoa flavonols	+	*Bifidobacterium*	[137]
	−	*Lactobacillus*	
	−	*Clostridia*	

Table 3. *Cont.*

Polyphenols		Bacteria	References
Red wine	+	*Enterococcus*	[138]
	+	*Prevotella*	
	+	*Bacteroides*	
	+	*Bifidobacterium*	
	+	*Enterococcus*	
	+	*Bacteroides uniformis*	
	+	*Eggerthella lenta*	
	+	*Blautia coccoides*	
Orange juice	+	*Mogibacteriaceae*	[139]
	+	*Tissierellaceae*	
	+	*Veillonellaceae*	
	+	*Odoribacteraceae*	
	+	*Ruminococcaceae*	

However, gut microbiota can have a direct influence on brain function and alterations of the microbiota composition have been found in some neurodegenerative diseases, including PD and AD [140,141]. In addition, microbiota can exert neuroprotective effects by producing neurotransmitters and neuropeptides [142]. It has been shown that Streptococcus, Escherichia and Enterococcus spp. produce serotonin [143,144] and that Bifidobacterium infantis can modulate central serotonin transmission by increasing plasma tryptophan levels [145]. Meanwhile, different Lactobacillus and Bifidobacterium species are able to produce γ-aminobutyric acid (GABA), the principal inhibitory neurotransmitter in the central nervous system [146]. Therefore, gut microbiota can directly or indirectly contribute to neuroprotection.

7. Conclusions

The continuous increase in life expectancy is inversely correlated with quality of life during aging. During life there is, particularly in the brain, an accumulation of damage and a decrease in all of the mechanisms needed for cell repair. These processes can induce cell death.

Aging is one of the leading "risk factors" in the development of neurodegenerative processes. Indeed, neurodegenerative diseases are caused by nervous system dysfunction resulting from neuronal cell failure or cell death.

Neurodegenerative diseases increase with age and are becoming a big challenge for modern societies. Actually, neurodegenerative disorders in developed regions will have a strong increasing impact on medical and socio-economic conditions, since the population is becoming older. Therefore, it is essential to find strategies that help to prevent cognitive decline and improve the life quality of people living with dementia.

For thousands of years, it has been well known that food and health are related to each other. Indeed, even Hippocrates (400 BC), emphasized the importance of nutrition to prevent or cure diseases. In more recent years, different epidemiological studies have confirmed this thought, evidencing a strong link between the consumption of polyphenols and neurocognitive protection and suggesting that a polyphenol-rich diet can be an effective strategy to improve cognitive function in elderly populations.

Polyphenols are bioactive compounds contained in in food and beverages that are able to modulate the metabolic process, thus promoting health and preventing the age-related decline of cognitive, motor and sensory activities. Being antioxidants, polyphenols are able to counteract the oxidative damage accumulated in the cells. Moreover, they modulate different cellular signaling pathways, protecting cells from stress injury. Therefore, understanding the mechanisms by which polyphenols act at a molecular level is crucial in order to use them as dietary supplements to prevent neurodegenerative disorders.

Although polyphenols are abundant in fruits and vegetables and their intake can be increased in the diet, their bioavailability is also influenced by their chemical structure. Unlike in vitro, polyphenols in vivo have limited availability and they need to be metabolized rapidly in order to overcome biological

barriers. Thus, gut microbiota has a key role in the production of specific bioactive polyphenols' metabolites that are responsible for these health effects. Moreover, it is emerging that polyphenols can also modulate the bacterial composition of the gut microbiota, thus influencing the production of specific metabolites that act in modulating brain functions.

There is a growing attention being paid to food diversity, to the consumption of food rich in antioxidants and to developing novel food rich in the different bioactive nutrient groups. To date, the analysis of the impact of these products on neurodegeneration is limited, thus there is a need for an increasing number of studies to better evaluate the dose–effect relationship.

In conclusion, polyphenols and their metabolites are essential compounds with multiple biological activities. Their efficacy as antioxidants and their capacity to modulate pro-survival or anti-apoptotic signaling pathways are essential in preventing and slowing down neurodegenerative disorders. Moreover, considering that they are safe and have a very low toxicity, it is easy to test their efficacy in pre-clinical and clinical studies for the treatment of neurodegenerative diseases. Finally, different polyphenols, or their synthetic derivatives, have been patented as drugs against various human diseases in recent years.

Author Contributions: All authors actively participated in the review design, preparation and organization of the manuscript. They also extensively discussed the topics presented, giving positive criticism about the work. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the project PO FESR 2014-2020–Regione Campania, Asse 1–obiettivo specifico 1.2, Progetto "Sviluppo di nanotecnologie Orientate alla Rigenerazione e Ricostruzione tissutale, Implantologia e Sensoristica in Odontoiatria/oculistica (SORRISO)" (Grant Number: pdt1-000410) amd by CNR-DISBA project NutrAge.

Acknowledgments: FDM's PhD fellowship in Biology is supported by MIUR project PON "Dottorati Innovativi con caratterizzazione industriale" 2017–2018. We thank Chiara Nobile and Valentina Brasiello for their editing assistance. We thank Maria Rosaria Aletta for her bibliographic assistance and Maria Rosaria Chiummo for her help in reviewing the manuscript.

Conflicts of Interest: The authors confirm that there are no conflicts of interest.

References

1. Pandey, K.B.; Rizvi, S.I. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid. Med. Cell. Longev.* **2009**, *2*, 270–278. [CrossRef] [PubMed]
2. Fernandez-Fernandez, L.; Comes, G.; Bolea, I.; Valente, T.; Ruiz, J.; Murtra, P.; Ramirez, B.; Angles, N.; Reguant, J.; Morello, J.; et al. LMN diet, rich in polyphenols and polyunsaturated fatty acids, improves mouse cognitive decline associated with aging and Alzheimer's disease. *Behav. Brain Res.* **2012**, *228*, 261–271. [CrossRef] [PubMed]
3. Liu, P.; Kemper, L.; Wang, J.; Zahs, K.; Ashe, K.; Pasinetti, G. Grape seed polyphenolic extract specifically decreases Aβ* 56 in the brains of Tg2576 mice. *J. Alzheimers Dis.* **2011**, *26*, 657–666. [CrossRef] [PubMed]
4. Sandoval-Acuña, C.; Ferreira, J.; Speisky, H. Polyphenols and mitochondria: An update on their increasingly emerging ROS-scavenging independent actions. *Arch. Biochem. Biophys.* **2014**, *559*, 75–90. [CrossRef] [PubMed]
5. Smolensky, D.; Rhodes, D.; McVey, D.S.; Fawver, Z.; Perumal, R.; Herald, T.; Noronha, L. High-polyphenol sorghum bran extract inhibits cancer cell growth through ROS induction, cell cycle arrest, and apoptosis. *J. Med. Food* **2018**, *21*, 990–998. [CrossRef] [PubMed]
6. Mello-Filho, A.C.; Meneghini, R. Iron is the intracellular metal involved in the production of DNA damage by oxygen radicals. *Mutat. Res.* **1991**, *251*, 109–113. [CrossRef]
7. Gupta, S.; Hastak, K.; Afaq, F.; Ahmad, N.; Mukhtar, H. Essential role of caspases in epigallocatechin-3-gallate-mediated inhibition of nuclear factor kappa B and induction of apoptosis. *Oncogene* **2004**, *23*, 2507–2522. [CrossRef]
8. Kinarivala, N.; Patel, R.; Boustany, R.M.; Al-Ahmad, A.; Trippier, P.C. Discovery of aromatic carbamates that confer neuroprotective activity by enhancing autophagy and inducing the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2). *J. Med. Chem.* **2017**, *60*, 9739–9756. [CrossRef]

9. Nakaso, K.; Ito, S.; Nakashima, K. Caffeine activates the PI3K/Akt pathway and prevents apoptotic cell death in a Parkinson's disease model of SH-SY5Y cells. *Neurosci. Lett.* **2008**, *432*, 146–150. [CrossRef]
10. Vauzour, D.; Vafeiadou, K.; Rice-Evans, C.; Williams, R.J.; Spencer, J.P. Activation of pro-survival Akt and ERK1/2 signalling pathways underlie the anti-apoptotic effects of flavanones in cortical neurons. *J. Neurochem.* **2007**, *103*, 1355–1367. [CrossRef]
11. Moosavi, F.; Hosseini, R.; Saso, L.; Firuzi, O. Modulation of neurotrophic signaling pathways by polyphenols. *Drug Des. Dev.* **2016**, *10*, 23–42.
12. Di Meo, F.; Donato, S.; Di Pardo, A.; Maglione, V.; Filosa, S.; Crispi, S. New therapeutic drugs from bioactive natural molecules: The role of gut microbiota metabolism in neurodegenerative diseases. *Curr. Drug Metab.* **2018**, *19*, 478–489. [CrossRef] [PubMed]
13. Filosa, S.; Fico, A.; Paglialunga, F.; Balestrieri, M.; Crooke, A.; Verde, P.; Abrescia, P.; Bautista, J.M.; Martini, G. Failure to increase glucose consumption through the pentose-phosphate pathway results in the death of glucose-6-phosphate dehydrogenase gene-deleted mouse embryonic stem cells subjected to oxidative stress. *Biochem. J.* **2003**, *370*, 935–943. [CrossRef] [PubMed]
14. Fico, A.; Paglialunga, F.; Cigliano, L.; Abrescia, P.; Verde, P.; Martini, G.; Iaccarino, I.; Filosa, S. Glucose-6 -phosphate dehydrogenase plays a crucial role in protection from redox-stress-induced apoptosis. *Cell Death Differ.* **2004**, *11*, 823–831. [CrossRef] [PubMed]
15. Redza-Dutordoir, M.; Averill-Bates, D.A. Activation of apoptosis signalling pathways by reactive oxygen species. *Biochim. Biophys. Acta* **2016**, *1863*, 2977–2992. [CrossRef]
16. Martindale, J.L.; Holbrook, N.J. Cellular response to oxidative stress: Signaling for suicide and survival. *J. Cell Physiol.* **2002**, *192*, 1–15. [CrossRef]
17. Young, I.S.; Woodside, J.V. Antioxidants in health and disease. *J. Clin. Pathol.* **2001**, *54*, 176–186. [CrossRef] [PubMed]
18. Halliwell, B. Antioxidant characterization. Methodology and mechanism. *Biochem. Pharm.* **1995**, *49*, 1341–1348. [CrossRef]
19. Ward, R.J.; Dexter, D.T.; Crichton, R.R. Ageing, neuroinflammation and neurodegeneration. *Front. Biosci.* **2015**, *7*, 189–204. [CrossRef]
20. Matés, J.M. Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. *Toxicology* **2000**, *153*, 83–104. [CrossRef]
21. Qin, S.; Hou, D.X. Multiple regulations of Keap1/Nrf2 system by dietary phytochemicals. *Mol. Nutr. Food Res.* **2016**, *60*, 1731–1755. [CrossRef] [PubMed]
22. Zhang, Y.J.; Gan, R.Y.; Li, S.; Zhou, Y.; Li, A.N.; Xu, D.P.; Li, H.B. Antioxidant phytochemicals for the prevention and treatment of chronic diseases. *Molecules* **2015**, *20*, 21138–21156. [CrossRef] [PubMed]
23. Pisoschi, A.M.; Pop, A. The role of antioxidants in the chemistry of oxidative stress: A review. *Eur. J. Med. Chem.* **2015**, *97*, 55–74. [CrossRef] [PubMed]
24. Kurutas, E.B. The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: Current state. *Nutr. J.* **2016**, *15*, 71. [CrossRef]
25. Shan, B.; Cai, Y.Z.; Sun, M.; Corke, H. Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. *J. Agric. Food Chem.* **2005**, *53*, 7749–7759. [CrossRef]
26. Lü, J.M.; Lin, P.H.; Yao, Q.; Chen, C. Chemical and molecular mechanisms of antioxidants: Experimental approaches and model systems. *J. Cell Mol. Med.* **2010**, *14*, 840–860. [CrossRef]
27. Gheldof, N.; Engeseth, N.J. Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of in vitro lipoprotein oxidation in human serum samples. *J. Agric. Food Chem.* **2002**, *50*, 3050–3055. [CrossRef]
28. Del Bano, M.J.; Lorente, J.; Castillo, J.; Benavente-Garcia, O.; del Rio, J.A.; Ortuno, A.; Quirin, K.W.; Gerard, D. Phenolic diterpenes, flavones, and rosmarinic acid distribution during the development of leaves, flowers, stems, and roots of *Rosmarinus officinalis*. *Antioxid. Act. J. Agric. Food Chem.* **2003**, *51*, 4247–4253. [CrossRef]
29. Amorati, R.; Foti, M.C.; Valgimigli, L. Antioxidant activity of essential oils. *J. Agric. Food Chem.* **2013**, *61*, 10835–10847. [CrossRef]
30. Tungmunnithum, D.; Thongboonyou, A.; Pholboon, A.; Yangsabai, A. Flavonoids and other phenolic compounds from medicinal plants for pharmaceutical and medical aspects: An overview. *Medicines* **2018**, *5*, 93. [CrossRef]

31. Ak, T.; Gülçin, I. Antioxidant and radical scavenging properties of curcumin. *Chem. Biol. Interact.* **2008**, *174*, 27–37. [CrossRef] [PubMed]
32. Foley, T.D. Reductive reprogramming: A not-so-radical hypothesis of neurodegeneration linking redox perturbations to neuroinflammation and excitotoxicity. *Cell Mol. Neurobiol.* **2019**, *39*, 577–590. [CrossRef]
33. Cui, K.; Luo, X.; Xu, K.; Ven Murthy, M.R. Role of oxidative stress in neurodegeneration: Recent developments in assay methods for oxidative stress and nutraceutical antioxidants. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **2004**, *28*, 771–799. [CrossRef]
34. Andersen, J.K. Oxidative stress in neurodegeneration: Cause or consequence? *Nat. Med.* **2004**, *10*, S18–S25. [CrossRef]
35. Mattson, M.P.; Magnus, T. Ageing and neuronal vulnerability. *Nat. Rev. Neurosci.* **2006**, *7*, 278–294. [CrossRef] [PubMed]
36. Lim, H.J.; Lee, K.S.; Lee, S.; Park, J.H.; Choi, H.E.; Go, S.H.; Kwak, H.J.; Park, H.Y. 15d-PGJ2 stimulates HO-1 expression through p38 MAP kinase and Nrf-2 pathway in rat vascular smooth muscle cells. *Toxicol. Appl. Pharm.* **2007**, *223*, 20–27. [CrossRef] [PubMed]
37. Kim, S.J.; Son, T.G.; Park, H.R.; Park, M.; Kim, M.S.; Kim, H.S.; Chung, H.Y.; Mattson, M.P.; Lee, J. Curcumin stimulates proliferation of embryonic neural progenitor cells and neurogenesis in the adult hippocampus. *J. Biol. Chem.* **2008**, *283*, 14497–14505. [CrossRef] [PubMed]
38. Bhakkiyalakshmi, E.; Dineshkumar, K.; Karthik, S.; Sireesh, D.; Hopper, W.; Paulmurugan, R.; Ramkumar, K.M. Pterostilbene-mediated Nrf2 activation: Mechanistic insights on Keap1: Nrf2 interface. *Bioorg. Med. Chem.* **2016**, *24*, 3378–3386. [CrossRef]
39. Lu, B.; Chow, A. Neurotrophins and hippocampal synaptic transmission and plasticity. *J. Neurosci. Res.* **1999**, *58*, 76–87. [CrossRef]
40. Chao, M.V.; Rajagopal, R.; Lee, F.S. Neurotrophin signalling in health and disease. *Clin. Sci.* **2006**, *110*, 167–173. [CrossRef]
41. Sariola, H.; Saarma, M. Novel functions and signalling pathways for GDNF. *J. Cell Sci.* **2003**, *116*, 3855–3862. [CrossRef]
42. Nakajima, K.; Niisato, N.; Marunaka, Y. Quercetin stimulates NGF-induced neurite outgrowth in PC12 cells via activation of Na(+)/K(+)/2Cl(−) cotransporter. *Cell Physiol. Biochem.* **2011**, *28*, 147–156. [CrossRef]
43. Nakajima, K.; Niisato, N.; Marunaka, Y. Genistein enhances the NGF-induced neurite outgrowth. *Biomed. Res.* **2011**, *32*, 351–356. [CrossRef]
44. Zhang, F.; Wang, Y.Y.; Liu, H.; Lu, Y.F.; Wu, Q.; Liu, J.; Shi, J.S. Resveratrol produces neurotrophic effects on cultured dopaminergic neurons through prompting astroglial BDNF and GDNF release. *Evid. Based Complement. Altern. Med.* **2012**, *2012*, 937605. [CrossRef]
45. Yuan, H.; Zhang, J.; Liu, H.; Li, Z. The protective effects of resveratrol on Schwann cells with toxicity induced by ethanol in vitro. *Neurochem. Int.* **2013**, *63*, 146–153. [CrossRef]
46. Huang, E.J.; Reichardt, L.F. TRK receptors: Roles in neuronal signal transduction. *Annu. Rev. Biochem.* **2003**, *72*, 609–642. [CrossRef]
47. Liu, C.; Chan, C.B.; Ye, K. 7,8-dihydroxyflavone, a small molecular TrkB agonist, is useful for treating various BDNF-implicated human disorders. *Transl. Neurodegener.* **2016**, *5*, 2. [CrossRef]
48. Jang, S.W.; Liu, X.; Yepes, M.; Shepherd, K.R.; Miller, G.W.; Liu, Y.; Wilson, W.D.; Xiao, G.; Blanchi, B.; Sun, Y.E.; et al. A selective TrkB agonist with potent neurotrophic activities by 7,8-dihydroxyflavone. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 2687–2692. [CrossRef]
49. Guo, Z.; Liu, Y.; Cheng, M. Resveratrol protects bupivacaine-induced neuro-apoptosis in dorsal root ganglion neurons via activation on tropomyosin receptor kinase A. *Biomed. Pharm.* **2018**, *103*, 1545–1551. [CrossRef]
50. Spencer, J.P.; Vafeiadou, K.; Williams, R.J.; Vauzour, D. Neuroinflammation: Modulation by flavonoids and mechanisms of action. *Mol. Asp. Med.* **2012**, *33*, 83–97. [CrossRef]
51. Si, T.L.; Liu, Q.; Ren, Y.F.; Li, H.; Xu, X.Y.; Li, E.H.; Pan, S.Y.; Zhang, J.L.; Wang, K.X. Enhanced anti-inflammatory effects of DHA and quercetin in lipopolysaccharide-induced RAW264.7 macrophages by inhibiting NF-κB and MAPK activation. *Mol. Med. Rep.* **2016**, *14*, 499–508. [CrossRef] [PubMed]
52. Baur, J.A.; Pearson, K.J.; Price, N.L.; Jamieson, H.A.; Lerin, C.; Kalra, A.; Prabhu, V.V.; Allard, J.S.; Lopez-Lluch, G.; Lewis, K.; et al. Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* **2006**, *444*, 337–342. [CrossRef]

53. Zhu, X.; Raina, A.K.; Lee, H.G.; Casadesus, G.; Smith, M.A.; Perry, G. Oxidative stress signalling in Alzheimer's disease. *Brain Res.* **2004**, *1000*, 32–39. [CrossRef]
54. Di Pardo, A.; Amico, E.; Scalabrì, F.; Pepe, G.; Castaldo, S.; Elifani, F.; Capocci, L.; De Sanctis, C.; Comerci, L.; Pompeo, F.; et al. Impairment of blood-brain barrier is an early event in R6/2 mouse model of Huntington's disease. *Sci. Rep.* **2017**, *7*, 41316. [CrossRef]
55. Knutson, M.D.; Leeuwenburgh, C. Resveratrol and novel potent activators of SIRT1: Effects on aging and age-related diseases. *Nutr. Rev.* **2008**, *66*, 591–596. [CrossRef]
56. Polivka, J.; Janku, F. Molecular targets for cancer therapy in the PI3K/AKT/mTOR pathway. *Pharmacol. Therap.* **2014**, *142*, 164–175. [CrossRef]
57. Alexander, G.E. Biology of Parkinson's disease: Pathogenesis and pathophysiology of a multisystem neurodegenerative disorder. *Dialogues Clin. Neurosci.* **2004**, *6*, 259–280.
58. Miller, R.L.; James-Kracke, M.; Sun, G.Y.; Sun, A.Y. Oxidative and inflammatory pathways in Parkinson's disease. *Neurochem. Res.* **2009**, *34*, 55–65. [CrossRef]
59. Miller, R.L.; Sun, G.Y.; Sun, A.Y. Cytotoxicity of paraquat in microglial cells: Involvement of PKCδ- and ERK1/2-dependent NADPH oxidase. *Brain Res.* **2007**, *1167*, 129–139. [CrossRef]
60. Weinreb, O.; Mandel, S.; Amit, T.; Youdim, M.B. Neurological mechanisms of green tea polyphenols in Alzheimer's and Parkinson's diseases. *J. Nutr. Biochem.* **2004**, *15*, 506–516. [CrossRef]
61. Mercer, L.D.; Kelly, B.L.; Horne, M.K.; Beart, P.M. Dietary polyphenols protect dopamine neurons from oxidative insults and apoptosis: Investigations in primary rat mesencephalic cultures. *Biochem. Pharm.* **2005**, *69*, 339–345. [CrossRef] [PubMed]
62. Masuda, M.; Suzuki, N.; Taniguchi, S.; Oikawa, T.; Nonaka, T.; Iwatsubo, T.; Hisanaga, S.; Goedert, M.; Hasegawa, M. Small molecule inhibitors of alpha-synuclein filament assembly. *Biochemistry* **2006**, *45*, 6085–6094. [CrossRef] [PubMed]
63. Andrieu, S.; Coley, N.; Lovestone, S.; Aisen, P.S.; Vellas, B. Prevention of sporadic Alzheimer's disease: Lessons learned from clinical trials and future directions. *Lancet Neurol.* **2015**, *14*, 926–944. [CrossRef]
64. Haass, C.; Selkoe, D.J. Soluble protein oligomers in neurodegeneration: Lessons from the Alzheimer's amyloid beta-peptide. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 101–112. [CrossRef]
65. Querfurth, H.W.; LaFerla, F.M. Alzheimer's disease. *N. Engl. J. Med.* **2010**, *362*, 329–344. [CrossRef]
66. Selkoe, D.J. Alzheimer's disease: Genes, proteins, and therapy. *Physiol. Rev.* **2001**, *81*, 741–766. [CrossRef]
67. Selkoe, D.J. Alzheimer's disease results from the cerebral accumulation and cytotoxicity of amyloid beta-protein. *J. Alzheimers Dis.* **2001**, *3*, 75–80. [CrossRef]
68. Snyder, E.M.; Nong, Y.; Almeida, C.G.; Paul, S.; Moran, T.; Choi, E.Y.; Nairn, A.C.; Salter, M.W.; Lombroso, P.J.; Gouras, G.K.; et al. Regulation of NMDA receptor trafficking by amyloid-beta. *Nat. Neurosci.* **2005**, *8*, 1051–1058. [CrossRef]
69. Flanagan, E.; Müller, M.; Hornberger, M.; Vauzour, D. Impact of flavonoids on cellular and molecular mechanisms underlying age-related cognitive decline and neurodegeneration. *Curr. Nutr. Rep.* **2018**, *7*, 49–57. [CrossRef]
70. Wang, R.; Li, Y.H.; Xu, Y.; Li, Y.B.; Wu, H.L.; Guo, H.; Zhang, J.Z.; Zhang, J.J.; Pan, X.Y.; Li, X.J. Curcumin produces neuroprotective effects via activating brain-derived neurotrophic factor/TrkB-dependent MAPK and PI-3K cascades in rodent cortical neurons. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **2010**, *34*, 147–153. [CrossRef]
71. Zhang, L.; Fang, Y.; Xu, Y.; Lian, Y.; Xie, N.; Wu, T.; Zhang, H.; Sun, L.; Zhang, R.; Wang, Z. Curcumin improves amyloid β-peptide (1–42) induced spatial memory deficits through BDNF-ERK signaling pathway. *PLoS ONE* **2015**, *10*, e0131525. [CrossRef]
72. Zheng, K.; Dai, X.; Xiao, N.; Wu, X.; Wei, Z.; Fang, W.; Zhu, Y.; Zhang, J.; Chen, X. Curcumin ameliorates memory decline via inhibiting BACE1 expression and β-amyloid pathology in 5 × FAD transgenic mice. *Mol. Neurobiol.* **2017**, *54*, 1967–1977. [CrossRef]
73. Zhao, Y.N.; Li, W.F.; Li, F.; Zhang, Z.; Dai, Y.D.; Xu, A.L.; Qi, C.; Gao, J.M.; Gao, J. Resveratrol improves learning and memory in normally aged mice through microRNA-CREB pathway. *Biochem. Biophys. Res. Commun.* **2013**, *435*, 597–602. [CrossRef]
74. Fu, Z.; Yang, J.; Wei, Y.; Li, J. Effects of piceatannol and pterostilbene against β-amyloid-induced apoptosis on the PI3K/Akt/Bad signaling pathway in PC12 cells. *Food Funct.* **2016**, *7*, 1014–1023. [CrossRef]

75. Donmez, G.; Outeiro, T.F. SIRT1 and SIRT2: Emerging targets in neurodegeneration. *EMBO Mol. Med.* **2013**, *5*, 344–352. [CrossRef]
76. Karuppagounder, S.S.; Pinto, J.T.; Xu, H.; Chen, H.L.; Beal, M.F.; Gibson, G.E. Dietary supplementation with resveratrol reduces plaque pathology in a transgenic model of Alzheimer's disease. *Neurochem. Int.* **2009**, *54*, 111–118. [CrossRef]
77. Wang, R.; Zhang, Y.; Li, J.; Zhang, C. Resveratrol ameliorates spatial learning memory impairment induced by Aβ. *Neuroscience* **2017**, *344*, 39–47. [CrossRef]
78. Rezai-Zadeh, K.; Arendash, G.W.; Hou, H.; Fernandez, F.; Jensen, M.; Runfeldt, M.; Shytle, R.D.; Tan, J. Green tea epigallocatechin-3-gallate (EGCG) reduces beta-amyloid mediated cognitive impairment and modulates tau pathology in Alzheimer's transgenic mice. *Brain Res.* **2008**, *1214*, 177–187. [CrossRef]
79. Evin, G.; Barakat, A.; Masters, C.L. BACE: Therapeutic target and potential biomarker for Alzheimer's disease. *Int. J. Biochem. Cell Biol.* **2010**, *42*, 1923–1926. [CrossRef]
80. Ortiz-López, L.; Márquez-Valadez, B.; Gómez-Sánchez, A.; Silva-Lucero, M.D.; Torres-Pérez, M.; Téllez-Ballesteros, R.I.; Ichwan, M.; Meraz-Ríos, M.A.; Kempermann, G.; Ramírez-Rodríguez, G.B. Green tea compound epigallo-catechin-3-gallate (EGCG) increases neuronal survival in adult hippocampal neurogenesis in vivo and in vitro. *Neuroscience* **2016**, *322*, 208–220. [CrossRef]
81. Li, Y.; Zhou, S.; Li, J.; Sun, Y.; Hasimu, H.; Liu, R.; Zhang, T. Quercetin protects human brain microvascular endothelial cells from fibrillar β-amyloid1–40-induced toxicity. *Acta Pharm. Sin. B* **2015**, *5*, 47–54. [CrossRef]
82. Zhang, X.; Hu, J.; Zhong, L.; Wang, N.; Yang, L.; Liu, C.C.; Li, H.; Wang, X.; Zhou, Y.; Zhang, Y.; et al. Quercetin stabilizes apolipoprotein E and reduces brain Aβ levels in amyloid model mice. *Neuropharmacology* **2016**, *108*, 179–192. [CrossRef]
83. Sabogal-Guáqueta, A.M.; Muñoz-Manco, J.I.; Ramírez-Pineda, J.R.; Lamprea-Rodriguez, M.; Osorio, E.; Cardona-Gómez, G.P. The flavonoid quercetin ameliorates Alzheimer's disease pathology and protects cognitive and emotional function in aged triple transgenic Alzheimer's disease model mice. *Neuropharmacology* **2015**, *93*, 134–145. [CrossRef]
84. Lu, Y.; Liu, Q.; Yu, Q. Quercetin enrich diet during the early-middle not middle-late stage of Alzheimer's disease ameliorates cognitive dysfunction. *Am. J. Transl. Res.* **2018**, *10*, 1237–1246.
85. Kong, Y.; Li, K.; Fu, T.; Wan, C.; Zhang, D.; Song, H.; Zhang, Y.; Liu, N.; Gan, Z.; Yuan, L. Quercetin ameliorates Aβ toxicity in *Drosophila* AD model by modulating cell cycle-related protein expression. *Oncotarget* **2016**, *7*, 67716–67731. [CrossRef]
86. Cui, Q.; Li, X.; Zhu, H. Curcumin ameliorates dopaminergic neuronal oxidative damage via activation of the Akt/Nrf2 pathway. *Mol. Med. Rep.* **2016**, *13*, 1381–1388. [CrossRef]
87. Wu, J.; Li, Q.; Wang, X.; Yu, S.; Li, L.; Wu, X.; Chen, Y.; Zhao, J.; Zhao, Y. Neuroprotection by curcumin in ischemic brain injury involves the Akt/Nrf2 pathway. *PLoS ONE* **2013**, *8*, e59843. [CrossRef]
88. Ramkumar, M.; Rajasankar, S.; Swaminathan Johnson, W.M.; Prabu, K.; Venkatesh Gobi, V. Demethoxycurcumin ameliorates rotenone-induced toxicity in rats. *Front. Biosci.* **2019**, *11*, 1–11.
89. Dauer, W.; Przedborski, S. Parkinson's disease: Mechanisms and models. *Neuron* **2003**, *39*, 889–909. [CrossRef]
90. Wang, Y.L.; Ju, B.; Zhang, Y.Z.; Yin, H.L.; Liu, Y.J.; Wang, S.S.; Zeng, Z.L.; Yang, X.P.; Wang, H.T.; Li, J.F. Protective Effect of Curcumin Against Oxidative Stress-Induced Injury in Rats with Parkinson's Disease Through the Wnt/ β-Catenin Signaling Pathway. *Cell. Physiol. Biochem.* **2017**, *43*, 2226–2241. [CrossRef]
91. Yu, S.; Wang, X.; He, X.; Wang, Y.; Gao, S.; Ren, L.; Shi, Y. Curcumin exerts anti-inflammatory and antioxidative properties in 1-methyl-4-phenylpyridinium ion (MPP(+))-stimulated mesencephalic astrocytes by interference with TLR4 and downstream signaling pathway. *Cell Stress Chaperones* **2016**, *21*, 697–705. [CrossRef]
92. Potdar, S.; Parmar, M.S.; Ray, S.D.; Cavanaugh, J.E. Protective effects of the resveratrol analog piceid in dopaminergic SH-SY5Y cells. *Arch. Toxicol.* **2018**, *92*, 669–677. [CrossRef]
93. Zeng, W.; Zhang, W.; Lu, F.; Gao, L.; Gao, G. Resveratrol attenuates MPP. *Neurosci. Lett.* **2017**, *637*, 50–56. [CrossRef]
94. Gaballah, H.H.; Zakaria, S.S.; Elbatsh, M.M.; Tahoon, N.M. Modulatory effects of resveratrol on endoplasmic reticulum stress-associated apoptosis and oxido-inflammatory markers in a rat model of rotenone-induced Parkinson's disease. *Chem. Biol. Interact.* **2016**, *251*, 10–16. [CrossRef]

95. Karuppagounder, S.S.; Madathil, S.K.; Pandey, M.; Haobam, R.; Rajamma, U.; Mohanakumar, K.P. Quercetin up-regulates mitochondrial complex-I activity to protect against programmed cell death in rotenone model of Parkinson's disease in rats. *Neuroscience* **2013**, *236*, 136–148. [CrossRef]
96. Choi, J.Y.; Park, C.S.; Kim, D.J.; Cho, M.H.; Jin, B.K.; Pie, J.E.; Chung, W.G. Prevention of nitric oxide-mediated 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease in mice by tea phenolic epigallocatechin 3-gallate. *Neurotoxicology* **2002**, *23*, 367–374. [CrossRef]
97. Levites, Y.; Weinreb, O.; Maor, G.; Youdim, M.B.; Mandel, S. Green tea polyphenol(−)-epigallocatechin-3-gallate prevents *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dopaminergic neurodegeneration. *J. Neurochem.* **2001**, *78*, 1073–1082. [CrossRef]
98. Walker, F.O. Huntington's Disease. *Semin. Neurol.* **2007**, *27*, 143–150. [CrossRef]
99. Strong, T.V.; Tagle, D.A.; Valdes, J.M.; Elmer, L.W.; Boehm, K.; Swaroop, M.; Kaatz, K.W.; Collins, F.S.; Albin, R.L. Widespread expression of the human and rat Huntington's disease gene in brain and nonneural tissues. *Nat. Genet.* **1993**, *5*, 259–265. [CrossRef]
100. Sciacca, S.; Favellato, M.; Madonna, M.; Metro, D.; Marano, M.; Squitieri, F. Early enteric neuron dysfunction in mouse and human Huntington's disease. *Parkinsonism Relat. Disord.* **2017**, *34*, 73–74. [CrossRef]
101. Siddiqui, A.; Rivera-Sánchez, S.; Castro, M.e.R.; Acevedo-Torres, K.; Rane, A.; Torres-Ramos, C.A.; Nicholls, D.G.; Andersen, J.K.; Ayala-Torres, S. Mitochondrial DNA damage is associated with reduced mitochondrial bioenergetics in Huntington's disease. *Free Radic. Biol. Med.* **2012**, *53*, 1478–1488. [CrossRef]
102. Xun, Z.; Rivera-Sánchez, S.; Ayala-Peña, S.; Lim, J.; Budworth, H.; Skoda, E.M.; Robbins, P.D.; Niedernhofer, L.J.; Wipf, P.; McMurray, C.T. Targeting of XJB-5–131 to mitochondria suppresses oxidative DNA damage and motor decline in a mouse model of Huntington's disease. *Cell Rep.* **2012**, *2*, 1137–1142. [CrossRef]
103. Naia, L.; Rosenstock, T.R.; Oliveira, A.M.; Oliveira-Sousa, S.I.; Caldeira, G.L.; Carmo, C.; Laço, M.N.; Hayden, M.R.; Oliveira, C.R.; Rego, A.C. Comparative mitochondrial-based protective effects of resveratrol and nicotinamide in Huntington's disease models. *Mol. Neurobiol.* **2017**, *54*, 5385–5399. [CrossRef]
104. Sandhir, R.; Yadav, A.; Mehrotra, A.; Sunkaria, A.; Singh, A.; Sharma, S. Curcumin nanoparticles attenuate neurochemical and neurobehavioral deficits in experimental model of Huntington's disease. *Neuromol. Med.* **2014**, *16*, 106–118. [CrossRef]
105. Chongtham, A.; Agrawal, N. Curcumin modulates cell death and is protective in Huntington's disease model. *Sci. Rep.* **2016**, *6*, 18736. [CrossRef]
106. Elifani, F.; Amico, E.; Pepe, G.; Capocci, L.; Castaldo, S.; Rosa, P.; Montano, E.; Pollice, A.; Madonna, M.; Filosa, S.; et al. Curcumin dietary supplementation ameliorates disease phenotype in an animal model of Huntington's disease. *Hum. Mol. Genet.* **2019**, *28*, 4012–4021. [CrossRef]
107. Sandhir, R.; Mehrotra, A. Quercetin supplementation is effective in improving mitochondrial dysfunctions induced by 3-nitropropionic acid: Implications in Huntington's disease. *Biochim. Biophys. Acta* **2013**, *1832*, 421–430. [CrossRef]
108. Chakraborty, J.; Singh, R.; Dutta, D.; Naskar, A.; Rajamma, U.; Mohanakumar, K.P. Quercetin improves behavioral deficiencies, restores astrocytes and microglia, and reduces serotonin metabolism in 3-nitropropionic acid-induced rat model of Huntington's disease. *CNS Neurosci.* **2014**, *20*, 10–19. [CrossRef]
109. Jellinger, K.A. The enigma of vascular cognitive disorder and vascular dementia. *Acta Neuropathol.* **2007**, *113*, 349–388. [CrossRef]
110. Molino, S.; Dossena, M.; Buonocore, D.; Ferrari, F.; Venturini, L.; Ricevuti, G.; Verri, M. Polyphenols in dementia: From molecular basis to clinical trials. *Life Sci.* **2016**, *161*, 69–77. [CrossRef]
111. Awasthi, H.; Tota, S.; Hanif, K.; Nath, C.; Shukla, R. Protective effect of curcumin against intracerebral streptozotocin induced impairment in memory and cerebral blood flow. *Life Sci.* **2010**, *86*, 87–94. [CrossRef]
112. Anastácio, J.R.; Netto, C.A.; Castro, C.C.; Sanches, E.F.; Ferreira, D.C.; Noschang, C.; Krolow, R.; Dalmaz, C.; Pagnussat, A. Resveratrol treatment has neuroprotective effects and prevents cognitive impairment after chronic cerebral hypoperfusion. *Neurol. Res.* **2014**, *36*, 627–633. [CrossRef]
113. Shen, D.; Tian, X.; Sang, W.; Song, R. Effect of melatonin and resveratrol against memory impairment and hippocampal damage in a rat model of vascular dementia. *Neuroimmunomodulation* **2016**, *23*, 318–331. [CrossRef]

114. Tota, S.; Awasthi, H.; Kamat, P.K.; Nath, C.; Hanif, K. Protective effect of quercetin against intracerebral streptozotocin induced reduction in cerebral blood flow and impairment of memory in mice. *Behav. Brain Res.* **2010**, *209*, 73–79. [CrossRef]
115. Jayaraj, R.; Elangovan, N.; Manigandan, K.; Singh, S.; Shukla, S. CNB-001 a novel curcumin derivative, guards dopamine neurons in MPTP model of Parkinson's disease. *BioMed Res. Int.* **2014**. [CrossRef]
116. Selma, M.V.; Espín, J.C.; Tomás-Barberán, F.A. Interaction between phenolics and gut microbiota: Role in human health. *J. Agric. Food Chem.* **2009**, *57*, 6485–6501. [CrossRef]
117. Aura, A. Microbial metabolism of dietary phenolic compounds in the colon. *Phytochem. Rev.* **2008**, *7*, 407–429. [CrossRef]
118. Deprez, S.; Mila, I.; Huneau, J.F.; Tome, D.; Scalbert, A. Transport of proanthocyanidin dimer, trimer, and polymer across monolayers of human intestinal epithelial Caco-2 cells. *Antioxid. Redox. Signal.* **2001**, *3*, 957–967. [CrossRef]
119. Kang, S.M.; Lee, S.H.; Heo, S.J.; Kim, K.N.; Jeon, Y.J. Evaluation of antioxidant properties of a new compound, pyrogallol-phloroglucinol-6,6′-bieckol isolated from brown algae, *Ecklonia cava*. *Nutr. Res. Pract.* **2011**, *5*, 495–502. [CrossRef]
120. Deng, H.; Fang, Y. The three catecholics benserazide, catechol and pyrogallol are GPR35 agonists. *Pharmaceuticals* **2013**, *6*, 500–509. [CrossRef]
121. Di Giovanni, S.; Eleuteri, S.; Paleologou, K.E.; Yin, G.; Zweckstetter, M.; Carrupt, P.A.; Lashuel, H.A. Entacapone and tolcapone, two catechol O-methyltransferase inhibitors, block fibril formation of alpha-synuclein and beta-amyloid and protect against amyloid-induced toxicity. *J. Biol. Chem.* **2010**, *285*, 14941–14954. [CrossRef]
122. Di Meo, F.; Margarucci, S.; Galderisi, U.; Crispi, S.; Peluso, G. Curcumin, gut microbiota, and neuroprotection. *Nutrients* **2019**, *11*, 2426. [CrossRef] [PubMed]
123. Basholli-Salihu, M.; Schuster, R.; Mulla, D.; Praznik, W.; Viernstein, H.; Mueller, M. Bioconversion of piceid to resveratrol by selected probiotic cell extracts. *Bioprocess. Biosyst. Eng.* **2016**, *39*, 1879–1885. [CrossRef] [PubMed]
124. Bode, L.M.; Bunzel, D.; Huch, M.; Cho, G.S.; Ruhland, D.; Bunzel, M.; Bub, A.; Franz, C.M.; Kulling, S.E. In vivo and in vitro metabolism of trans-resveratrol by human gut microbiota. *Am. J. Clin. Nutr.* **2013**, *97*, 295–309. [CrossRef]
125. Blaut, M.; Schoefer, L.; Braune, A. Transformation of flavonoids by intestinal microorganisms. *Int. J. Vitam. Nutr. Res.* **2003**, *73*, 79–87. [CrossRef]
126. Zheng, C.J.; Liu, R.; Xue, B.; Luo, J.; Gao, L.; Wang, Y.; Ou, S.; Li, S.; Peng, X. Impact and consequences of polyphenols and fructooligosaccharide interplay on gut microbiota in rats. *Food Funct.* **2017**, *8*, 1925–1932. [CrossRef]
127. Angelino, D.; Carregosa, D.; Domenech-Coca, C.; Savi, M.; Figueira, I.; Brindani, N.; Jang, S.; Lakshman, S.; Molokin, A.; Urban, J.F.; et al. 5-(hydroxyphenyl)-γ-valerolactone-sulfate, a key microbial metabolite of flavan-3-ols, is able to reach the brain: Evidence from different in. *Nutrients* **2019**, *11*, 2678. [CrossRef]
128. Johnson, S.L.; Kirk, R.D.; DaSilva, N.A.; Ma, H.; Seeram, N.P.; Bertin, M.J. Polyphenol microbial metabolites exhibit gut and blood-brain barrier permeability and protect murine microglia against LPS-induced inflammation. *Metabolites* **2019**, *9*, 78. [CrossRef]
129. Figueira, I.; Garcia, G.; Pimpão, R.C.; Terrasso, A.P.; Costa, I.; Almeida, A.F.; Tavares, L.; Pais, T.F.; Pinto, P.; Ventura, M.R.; et al. Polyphenols journey through blood-brain barrier towards neuronal protection. *Sci. Rep.* **2017**, *7*, 11456. [CrossRef]
130. Youdim, K.A.; Qaiser, M.Z.; Begley, D.J.; Rice-Evans, C.A.; Abbott, N.J. Flavonoid permeability across an in situ model of the blood-brain barrier. *Free Radic. Biol. Med.* **2004**, *36*, 592–604. [CrossRef]
131. Filosa, S.; Di Meo, F.; Crispi, S. Polyphenols-gut microbiota interplay and brain neuromodulation. *Neural. Regen. Res.* **2018**, *13*, 2055–2059.
132. Bird, J.K.; Raederstorff, D.; Weber, P.; Steinert, R.E. Cardiovascular and antiobesity effects of resveratrol mediated through the gut microbiota. *Adv. Nutr.* **2017**, *8*, 839–849. [CrossRef]
133. Arakawa, H.; Maeda, M.; Okubo, S.; Shimamura, T. Role of hydrogen peroxide in bactericidal action of catechin. *Biol. Pharm. Bull.* **2004**, *27*, 277–281. [CrossRef]
134. Tzounis, X.; Vulevic, J.; Kuhnle, G.G.; George, T.; Leonczak, J.; Gibson, G.R.; Kwik-Uribe, C.; Spencer, J.P. Flavanol monomer-induced changes to the human faecal microflora. *Br. J. Nutr.* **2008**, *99*, 782–792. [CrossRef]

135. Yamakoshi, J.; Tokutake, S.; Kikuchi, M.; Kubota, Y.; Konishi, H.; Mitsuoka, T. Effect of Proanthocyanidin-Rich Extract from Grape Seeds on Human Fecal Flora and Fecal Odor. *Microb. Ecol. Health Dis.* **2001**, *13*, 25–31. [CrossRef]
136. García-Mediavilla, M.V.; Sánchez-Campos, S.; Tuñón, M.J. Fruit polyphenols, immunity and inflammation. *Br. J. Nutr.* **2010**, *104*, S15–S27.
137. Tzounis, X.; Rodriguez-Mateos, A.; Vulevic, J.; Gibson, G.R.; Kwik-Uribe, C.; Spencer, J.P. Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study. *Am. J. Clin. Nutr.* **2011**, *93*, 62–72. [CrossRef]
138. Queipo-Ortuño, M.I.; Boto-Ordóñez, M.; Murri, M.; Gomez-Zumaquero, J.M.; Clemente-Postigo, M.; Estruch, R.; Cardona Diaz, F.; Andrés-Lacueva, C.; Tinahones, F.J. Influence of red wine polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers. *Am. J. Clin. Nutr.* **2012**, *95*, 1323–1334. [CrossRef]
139. Brasili, E.; Hassimotto, N.M.A.; Del Chierico, F.; Marini, F.; Quagliariello, A.; Sciubba, F.; Miccheli, A.; Putignani, L.; Lajolo, F. Daily consumption of orange juice from *Citrus sinensis* L. Osbeck cv. Cara Cara and cv. Bahia differently affects gut microbiota profiling as unveiled by an integrated meta-omics approach. *J. Agric. Food Chem.* **2019**, *67*, 1381–1391. [CrossRef]
140. Gerhardt, S.; Mohajeri, M.H. Changes of colonic bacterial composition in Parkinson's disease and other neurodegenerative diseases. *Nutrients* **2018**, *10*, 708. [CrossRef]
141. Sun, M.F.; Shen, Y.Q. Dysbiosis of gut microbiota and microbial metabolites in Parkinson's disease. *Ageing Res. Rev.* **2018**, *45*, 53–61. [CrossRef]
142. Hsiao, E.Y.; McBride, S.W.; Hsien, S.; Sharon, G.; Hyde, E.R.; McCue, T.; Codelli, J.A.; Chow, J.; Reisman, S.E.; Petrosino, J.F.; et al. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* **2013**, *155*, 1451–1463. [CrossRef]
143. Lyte, M. Probiotics function mechanistically as delivery vehicles for neuroactive compounds: Microbial endocrinology in the design and use of probiotics. *Bioessays* **2011**, *33*, 574–581. [CrossRef]
144. Nzakizwanayo, J.; Dedi, C.; Standen, G.; Macfarlane, W.M.; Patel, B.A.; Jones, B.V. *Escherichia coli* Nissle 1917 enhances bioavailability of serotonin in gut tissues through modulation of synthesis and clearance. *Sci. Rep.* **2015**, *5*, 17324. [CrossRef]
145. O'Mahony, S.M.; Clarke, G.; Borre, Y.E.; Dinan, T.G.; Cryan, J.F. Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. *Behav. Brain Res.* **2015**, *277*, 32–48. [CrossRef]
146. Yunes, R.A.; Poluektova, E.U.; Dyachkova, M.S.; Klimina, K.M.; Kovtun, A.S.; Averina, O.V.; Orlova, V.S.; Danilenko, V.N. GABA production and structure of *gad*B/*gad*C genes in *Lactobacillus* and *Bifidobacterium* strains from human microbiota. *Anaerobe* **2016**, *42*, 197–204. [CrossRef]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Review

Curcumin, Gut Microbiota, and Neuroprotection

Francesco Di Meo [1,2], Sabrina Margarucci [3], Umberto Galderisi [4], Stefania Crispi [1,2,*] and Gianfranco Peluso [3,*]

1 Institute of Biosciences and BioResources-UOS Naples CNR, Via P. Castellino, 80100 Naples, Italy; francesco.dimeo.90@gmail.com
2 Department of Biology, University of Naples Federico II, Complesso Universitario Monte Sant'Angelo via Cinthia, 80100 Naples, Italy
3 Institute of Research on Terrestrial Ecosystems, 05010 Porano TR, Italy; sabrina.margarucci@cnr.it
4 Department of Experimental Medicine, University of Campania "Luigi Vanvitelli", Via Santa Maria di Costantinopoli, 80100 Naples, Italy; umberto.galderisi@unicampania.it
* Correspondence: stefania.crispi@cnr.it (S.C.); gianfranco.peluso@cnr.it (G.P.)

Received: 31 August 2019; Accepted: 4 October 2019; Published: 11 October 2019

Abstract: Curcumin, a nontoxic, naturally occurring polyphenol, has been recently proposed for the management of neurodegenerative and neurological diseases. However, a discrepancy exists between the well-documented pharmacological activities that curcumin seems to possess in vivo and its poor aqueous solubility, bioavailability, and pharmacokinetic profiles that should limit any therapeutic effect. Thus, it is possible that curcumin could exert direct regulative effects primarily in the gastrointestinal tract, where high concentrations of curcumin are present after oral administration. Indeed, a new working hypothesis that could explain the neuroprotective role of curcumin despite its limited availability is that curcumin acts indirectly on the central nervous system by influencing the "microbiota–gut–brain axis", a complex bidirectional system in which the microbiome and its composition represent a factor which preserves and determines brain "health". Interestingly, curcumin and its metabolites might provide benefit by restoring dysbiosis of gut microbiome. Conversely, curcumin is subject to bacterial enzymatic modifications, forming pharmacologically more active metabolites than curcumin. These mutual interactions allow to keep proper individual physiologic functions and play a key role in neuroprotection.

Keywords: curcumin; gut microbioma; polyphenols; bioactivity; metabolism; neuroprotection

1. Introduction

Over the last decades, the interest in herbal medicines has greatly increased since they can exert preventive effects against chronic and degenerative diseases including cancer. In addition, these molecules play an important role in neuroprotection by modulating different cellular functions. Different studies have shown that curcumin, like others dietary polyphenols, is able to counteract the effects of toxic damage in different tissues [1–3].

Polyphenols are a large class of compounds (curcumin, lignans, lignins, stilbenes, flavonoids, coumarins, cinnamic acid, benzoic acid, etc.) which possess at least one aromatic ring structure with one or more hydroxyl groups. They are present in most vegetables and secondary metabolites and derive from the shikimic acid pathway. Contrasting findings on the bioavailability of polyphenols created doubts about their usefulness as beneficial antioxidant compounds. Currently, there are findings showing that polyphenols can exert their biological effects following chemical modifications performed by gut microbiota. Indeed, enzymes of the gut microbiota can perform deglycosylation, dihydroxylation, and ademethylation of polyphenols that result in small catabolic products, which

may be easily absorbed during intestinal transit. These catabolites may fall in two classes: some have higher biological activities compared with "parental" compound, and others lose biological activity [4].

Among polyphenols, curcumin has received great attention by researchers in the last years. Curcumin is normally found in the turmeric of *Curcuma longa* Linn. This plant belongs to the Zingiberaceae family and it is native from South Asia. Turmeric has been used in Asia from ancient times in traditional medicine and nowadays it is widely used in food, cosmetic, and pharmaceutical industries [5].

Turmeric contains essential oils such as zingiberene [6] and coloring agents that are known as curcuminoids [7]. Curcumin is a polyphenol that represents the most important curcuminoid isolated from the rhizome of the plants. It has been used for centuries as a herbal remedy for the treatment of several diseases in East Asia [8–10] due to its safety and intrinsic nontoxicity to humans, even at high doses [11].

The general interest in its therapeutic efficacy arises from the different biological and pharmacological effects. Curcumin possesses antioxidant [12,13] and anti-inflammatory properties [14,15], and it has shown to exert beneficial effects against several types of cancers [16–18].

It should also be highlighted that an increasing number of clinical trials based on curcumin administration have been published or are currently in progress, therefore demonstrating the expanding interest of the scientific community on the therapeutic potential of curcumin [8,19].

However, the pharmacological potential of curcumin is widely restricted because of its poor water solubility, chemical instability, and rapid metabolism. In addition, curcumin bioavailability is very low after oral administration [20,21]. In this regard, numerous attempts have been made to increase its efficacy and bioavailability. To overcome solubility problems, different curcumin formulations using nanocarriers have been tested [22–24]. Other strategies developed to increase curcumin bioavailability are based on combined administration of curcumin with other molecules. For example, the co-administration of curcumin with piperine, an alkaloid of black pepper and long pepper, significantly enhances curcumin bioavailability since piperine prevents curcumin metabolism through the inhibition of the glucuronidation processes [25,26].

The puzzling between the poor bioavailability and the large variety of pharmacological activities of curcumin can be solved by taking into account the reciprocal interactions between curcumin and the gut microbiota.

Gut microbiota is one of the most densely and dynamic populated microbial ecosystems that contribute to individual health. Gut microbiota composition changes with age and it is strictly related to diet. Different unbalanced diets determine alterations in the gut microbiota composition, resulting in modification of gut permeability and in gut low-grade inflammation [27].

Like other dietary polyphenols, curcumin bioactivity is related not only to the absorption rate, but also to its metabolism that is due to the digestion from intestinal microbes. Biological activity of the derivative metabolites may differ from the native curcumin. In addition, specific biological properties often depend on bioactive metabolites produced by gut microbiota digestion [28]. In this regard, it is worthy to note that curcumin, after administration, accumulates in the gut where, after microbial digestion, it can be transformed into biologically active metabolites [29–31].

In this review, we discuss the curcumin–gut microbiota interplay that allows transforming dietary curcumin into bioactive derivatives and how these molecules can exert neuroprotective functions.

2. Curcumin Metabolism

The bioavailability of curcumin, as with other polyphenols, is generally poor and after oral dosing, its blood levels are extremely low.

The oral bioavailability of curcumin is low due to a relatively low intestinal absorption and the rapid metabolism in the liver, followed by elimination through the gall bladder. An oral dose of 0.1 g/kg administered to mice yielded a peak plasma concentration of free curcumin that was only 2.25 μg/mL [32]. In a clinical trial with an oral dose of 3.6 g of curcumin, a plasma level as low as

11.1 nmol L^{-1} was detected an hour after oral dosing [33]. Also, after ingestion of high doses of curcumin, 8 g per day via oral administration, the plasmatic level of free curcumin was negligible [34].

Curcumin is used in different dosage depending on the disease. Recent in vitro studies report that curcumin is effective in reducing oxidative stress (OS) and in preventing neurodegeneration when used at a concentration ranging from 5–50 μM [35,36] and at a dose from 50 to 200 mg/Kg/day in vivo [37–39]. In clinical trials, curcumin is effective on oxidative stress and inflammation at a dosage from 90 to 2000 mg/day [40–42], while in neurodegenerative diseases, prevention is evident at a dosage of 500–2000 mg [43].

Turmeric contains about 2%–9% of curcuminoids. Commercial turmeric extracts contain approximately 70%–75% curcumin, 20% demethoxycurcumin, and 5% bisdemethoxycurcumin. Chemically, curcumin is a β-diketone α- β-unsaturated ferulic acid. Curcumin shows a keto–enol tautomerism, and the balance between the two forms depends on the polarity and pH of the solvent with the keto and enol forms existing in given proportions. Once dissolved, the enol form predominates (Figure 1). Curcumin is soluble in ethanol, acetic acid, dichloromethane, chloroform, methanol, ethyl acetate, dimethyl sulfoxide, and acetone, while it is insoluble in water [44].

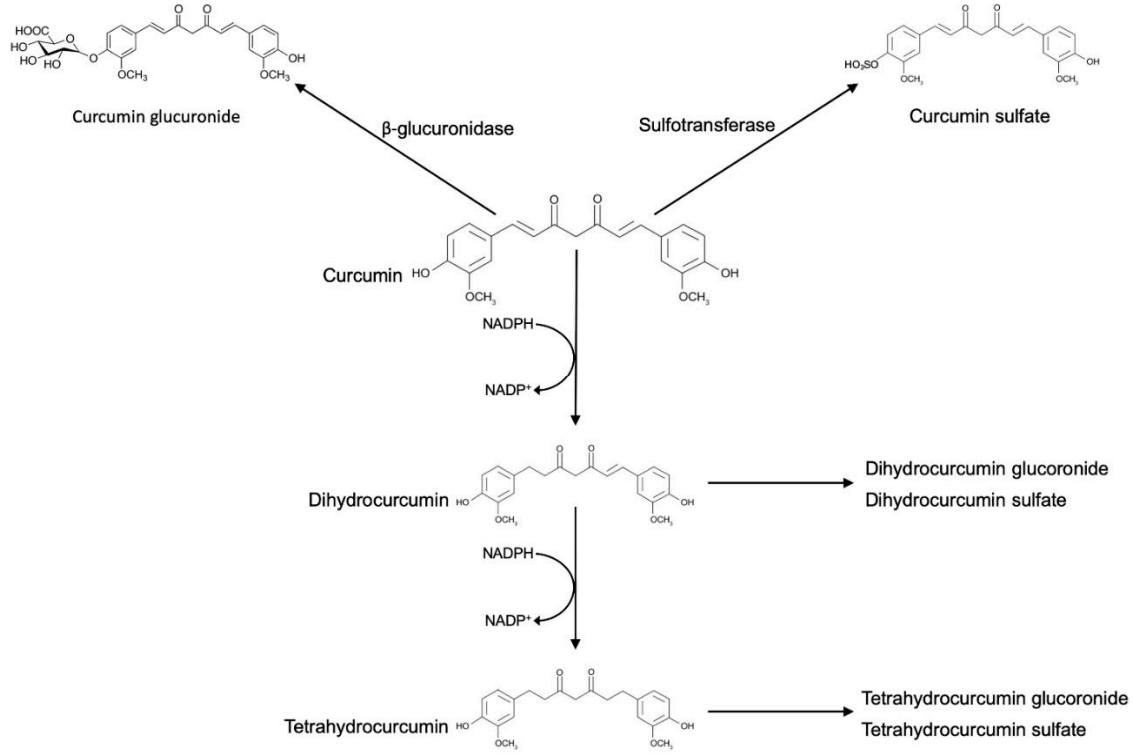

Figure 1. Reductive and conjugative metabolism of curcumin. Glucuronidation and sulfation are the predominating pathways of conjugation. Curcumin is also reduced by endogenous reductase systems in a stepwise manner and subsequently, curcumin metabolites are glucuronidated and sulfurated.

The bioavailability of curcumin has been studied extensively in mouse and humans. After oral administration of high doses, curcumin is poorly absorbed from the gastrointestinal tract, with peak blood levels rapidly reached within one hour after dosing [45]. In rats, after oral administration, plasma curcumin is mainly found in the form of glucuronide conjugates, while just a small amount of the unmodified form is detected. In humans, after curcumin oral administration, glucuronide conjugates and sulfate conjugates are detected in blood, while intact curcumin is barely detected [46].

Ingested curcumin first passes through the stomach, where the absorption of polyphenols is practically absent. Due to its resistance to low pH, curcumin reaches the large intestine without any chemical or structural modifications. In the large intestine, curcumin may be modified by phase I

enzymes, a class of enzymes that introduces reactive and polar groups into their substrates. Phase I reactions often produce active metabolites. One of the most common enzymes of this group is cytochrome P450 that catalyzes a substrate hydroxylation. Unexpectedly, curcumin appears not to be metabolized by cytochrome P450, because no products of demethylation or hydroxylation were detected after incubation of curcumin with rat liver microsomes [47]. The phase I metabolism of curcumin includes the successive reduction of the four double bonds. The enzymes responsible for the metabolic reduction have been found to reside in the cytosol of the enterocytes and include the alcohol dehydrogenases [48]. It has been demonstrated that phase I metabolism yielded three metabolites, namely, tetrahydrocurcumin (M1), hexahydrocurcumin (M2), and octahydrocurcumin (M3). Then, curcumin and the phase I metabolites were subject to conjugation via phase II metabolism to yield their corresponding glucuronide and sulfate O-conjugated metabolites.

The result of these reactions is the increase of molecular weight and the production of less active metabolites than their substrates. One of the most important enzymes of this group is the glutathione S-transferase. In vitro and in vivo, curcumin and its reductive metabolites appear to be easily conjugated [32]. Among the reported conjugates, monoglucuronides, monosulfates, and mixed glucuronide/sulfates are included. Glucuronidation is the predominating pathway of conjugation, and the glucuronide of curcumin is usually found as the major metabolite of curcumin in body fluids, organs, and cells [32].

Commercial curcumin also contains demethoxycurcumin and bisdemethoxycurcumin, and these molecules undergo reductive metabolism very similar to curcumin, with the hexahydro product as the major metabolite and much smaller amounts of the octa-, tetra-, and dihydro products.

3. Biotransformation of Curcumin by Gut Microbiota

The transformation of curcumin does not occur only by enzymes produced by the enterocytes or by hepatocytes, but also by enzymes produced by the gut microbiota. Gut microbiota can be described as a biological reactor because of its own formidable metabolic functions, like the transformation of numerous compounds that reach the colon. This activity is made possible through the capacity of microorganisms for producing a huge and varied range of enzymes. In particular, curcumin intestinal transformations include several steps and different classes of microbial enzymes.

Thus, the composition of the microbiota will cause different biotransformation of dietary curcumin.

Accordingly, the beneficial effects for consumers depend not only on the polyphenols taken from the diet, but also on the type of microbial population of the individual.

Several enteric bacteria capable of modifying curcumin have been identified.

Curcumin can be modified in the colon tract by a specific microorganism, *Escherichia coli*. The enzyme NADPH-dependent curcumin/dihydrocurcumin reductase (CurA) converts curcumin first into the intermediate, dihhydrocurcumin, and then in the final product, tetrahydrocurcumin, with a two-step reduction that is NADPH-dependent. In the first step of reaction, dihhydrocurcumin is generated from curcumin by reductive destruction of the chromophoric diarylheptatrienone chain. NADPH is an indispensable cofactor for the reduction of curcumin in dihydrocurcumin by CurA. In the second step, dihydrocurcumin is converted in tetrahydrocurcumin with the same mechanism [49].

CurA showed sequence similarity with some enzymes of the medium-chain dehydrogenase/reductase superfamily, which contains many different families including the alcohol dehydrogenases family, implicated in curcumin reduction.

Again, the firmicute *Blautia* sp. (MRG-PMF1), another human intestinal bacteria strain, is involved in curcumin metabolism. This bacterium produces two derivatives, demethylcurcumin and bisdemethylcurcumin by demethylation reaction [50]. Other bacteria capable of modifying curcumin have been identified. *Escherichia fergusonii* (ATCC 35469) and two *E. coli* strains (ATCC 8739 and DH10B) produce dihydrocurcumin, tetrahydrocurcumin, and ferulic acid [51]. Other microorganisms such as *Bifidobacteria longum* BB536, *Bifidobacteria pseudocatenulaum* G4, *Escherichia coli* K-12, *Enterococcus*

faecalis JCM 5803, *Lactobacillus acidophilus*, and *Lactobacillus casei* are all biologically relevant bacterial strains capable of degrading curcumin [52].

It has been reported that microbial metabolism of curcumin by *Pichia anomala* or by a bacterial strain of *Bacillus megaterium* DCMB-002 yielded new metabolites through different metabolic processes including hydroxylation, demethylation, reduction, and demethoxylation.

Recently, new curcumin metabolites produced by fecal bacteria have been identified. An analysis performed by using ultra-performance liquid chromatography coupled with quadrupole time of flight mass identified a total of 23 metabolites and discovered different novel human gut microbiota curcumin metabolic pathways, by demethylation, reduction, hydroxylation, and acetylation, or the combination of these [53].

Finally, bacteria from colon may deconjugate glucuronide and sulfate O-conjugated inactive metabolites produced by phase II enzymes and reconvert them to the corresponding phase I active metabolites [48].

Interestingly, there is evidence that curcumin metabolites display a similar or superior potency to curcumin [35]. Indeed, tetrahydrocurcumin possesses superiority over curcumin as a free-radical quencher and it was shown to have therapeutic effects in neurodegenerative diseases. These effects could be due, at least in part, to the inhibition of prominent cytokines' release, including interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), or by inhibition of NF-κB activation.

4. Curcumin Effects on Gut Microbiota

Human mucosal surfaces are connected with a diverse microbial community composed primarily by bacteria, but also by viruses, fungi, archaea, and protozoa [54]. The gastrointestinal tract is inhabited by a complex and abundant microbial community, with 100 trillion bacteria, about 10–100 times more than the quantity of eukaryotic cells [55].

In the intestine, curcumin, after oral or intraperitoneal administration, can exert a regulative effect on the gut microbiota community, affecting microbial richness, diversity, and composition., This effect should be involved in curcumin pharmacological activity [56] (Figure 2).

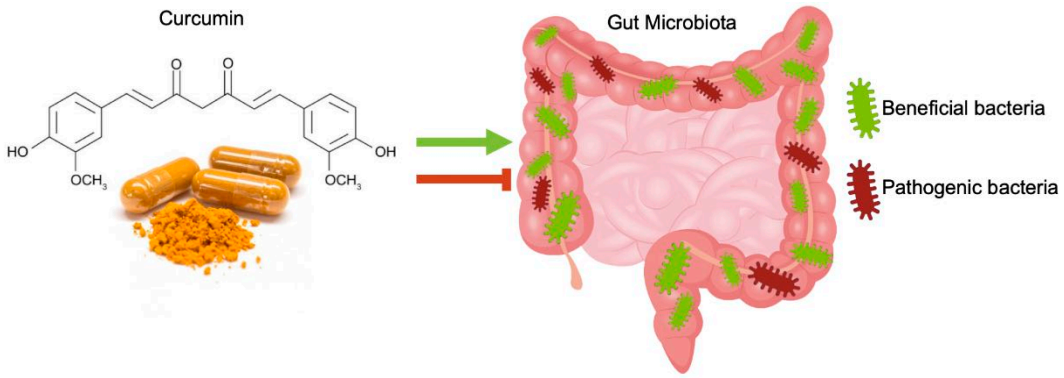

Figure 2. Schematic illustration of the interactions between curcumin and gut microbiota. The administration of curcumin considerably changed the ratio between beneficial and pathogenic bacteria in a gut microbiota community, favoring the growth of beneficial bacteria and limiting the growth of pathogenic ones.

The administration of curcumin considerably changed the ratio between beneficial and pathogenic bacteria by increasing the abundance of *Bifidobacteria*, *Lactobacilli*, and reducing the loads of *Prevotellaceae*, *Coriobacterales*, *Enterobacteria*, and *Enterococci* [57].

Oral administration of curcumin tends to decrease the microbial richness and diversity in mice [58] and reduce the abundance of several representative bacterial families in gut microbial communities, such as *Prevotellaceae, Bacteroidaceae, and Rikenellaceae*, often associated to the onset of systemic diseases [56,59,60].

This study reported that curcumin administration induced significant weight loss in ovariectomized rats, increasing the number of species of seven different bacterial genus: *Serratia*, *Anaerotruncus*, *Shewanella*, *Pseudomonas*, *Papillibacter*, *Exiguobacterium*, and *Helicobacter*. This study suggests that curcumin can partially reverse changes in the composition of gut microbiota in rats caused by estrogen deficiency induced by ovariectomy [61].

A new pilot work evaluating the effects of curcumin on gut microbiota showed that in the placebo group, there was an overall reduction of bacterial species up to 15%. On the contrary, curcumin-treated subjects showed an increase of bacterial species up of 69%. Subjects responsive to the treatment had uniform increases in *Clostridium* spp., *Bacteroides* spp., *Citrobacter* spp., *Cronobacter* spp., *Enterobacter* spp., *Enterococcus* spp., *Klebsiella* spp., *Parabacteroides* spp., and *Pseudomonas* spp., as well as reduced relative abundance in several *Blautia* spp. and most *Ruminococcus* spp. [62].

Thus, curcumin can change the composition of the microbiota, regulating not only the bacterial populations of the gut, but also the ability of intestinal bacterial strains to produce more active compounds from curcumin itself.

5. The Direct Neuroprotective Role of Curcumin and Curcumin Metabolites Produced by Enteric Bacteria

Among the different biological effects exerted by curcumin, the antioxidant one is considered the most interesting in terms of prevention and treatment of neurodegenerative diseases.

Neurodegenerative diseases are characterized by progressive loss of function of a specific population of neurons that determines neural deficit and cognitive impairment. The mechanisms responsible of neurodegeneration have not yet been completely elucidated; however oxidative stress and inflammation are considered the main effectors [63–65].

Oxidative stress is generated in the cell when there is imbalance between the amount of reactive oxygen species (ROS) produced and the cellular defense mechanisms that neutralize them.

High levels of ROS irreversibly damage all the cells, but neuronal cells are particularly sensitive also to relatively low levels of ROS. ROS, in fact, are the main factor determining brain ageing and they are involved in the onset and progression of neurodegenerative diseases [66,67].

Curcumin exerts a neuroprotective role by directly or indirectly scavenging free radical species. In fact, curcumin increases significantly the Superoxide dismutase (SOD) activity [68]. SOD, one of major antioxidant enzymes, is able to dismutate superoxide into hydrogen peroxide and oxygen.

Curcumin also shows indirect antioxidant action by elevating catalase plasma activity [69]. Catalases, a class of enzymes able to catalyze decomposition of hydrogen peroxide to water and molecular oxygen, belong to the antioxidant defense system of the cell and protect the cell from oxidative damage by reactive oxygen species.

Different studies indicated that curcumin favors brain health by modulating specific pathways such as: the PI3K/Akt (Phosphatidylinositol 3-kinase/Protein Kinase B) pathway, AMP (AMP-activated protein) kinase pathway, MAPK (Mitogen-Activated Protein Kinase)/Akt pathways [70–72] and Akt/Nrf2 (Nuclear factor-E2-related factor 2) pathway [70].

AMPK regulates energy metabolism and plays an important role in the maintenance of cellular homeostasis. This pathway, once activated, protects neuronal damage by decreasing ROS species—ER-associated (Endoplasmic Reticulum) [73].

In neurons, MAPK/Akt and PI3K/Akt represent the major oxidative stress-sensitive signal transduction pathways that regulate cell growth and cell death [74].

Finally, Nrf2 is a key regulator of antioxidant defense during oxidative stress. Activation of Nrf2 upregulates in the neurons defensive antioxidant mechanisms through upregulation of the genes involved in ROS metabolism [75]. When phosphorylated, Akt facilitates Nrf2 nuclear translocation, positively promoting its function [70].

Nitric oxide (NO) is a signaling molecule that shows a key role in inflammation. In physiological conditions, NO has an anti-inflammatory effect. Several studies in humans indicated that curcumin

supplementation improves vascular endothelial function in healthy people by increasing NO bioavailability and reducing oxidative stress [41,76].

Thus, curcumin neuroprotective activity seems to be related to the ability of the natural compound to inhibit, directly or indirectly, OS in neurons.

Again, tetrahydrocurcumin is the most studied bacterium-modified curcumin derivative in neuroprotection. It is known that tetrahydrocurcumin reduces oxidative stress and the number of apoptotic neurons, activates autophagy, and inhibits the mitochondrial apoptotic pathway after traumatic brain injury [77]. This metabolite was reported to be protective in vitro against Aβ-oligomer-induced toxicity [78], to modulate in vivo the neuroinflammation, to reduce the level of ROS triggered by β-amyloid fibers, to decrease the mitochondrial membrane potential, and to inhibit caspase activation [79]. In brain injury, tetrahydrocurcumin avoids neuronal cell apoptosis and improves neurobehavioral function by upregulating the Nrf2 pathway [39].

Tetrahydrocurcumin treatment has been reported to be effective also in Parkinson's disease (PD). A study carried out in a mouse model of PD showed that tetrahydrocurcumin was able to increase dopamine levels and to inhibit the activity of monoamine oxidase, the enzyme that determines the increase of neuronal OS levels through the degradation of neurotransmitters [80,81]. While these results clearly indicate that tetrahydrocurcumin may prevent neurodegeneration [78,82], little is known about the neuroprotective efficacy of others curcumin by-products, such as bisdemethoxycurcumin and demethoxycurcumin and octahydrocurcumin.

Demethoxycurcumin has been reported to exert neuroprotective effects in neuronal cells by glutathione increase and ROS decrease [83,84].

Octahydrocurcumin also represents a very promising antioxidant molecule able to enhance the expression of antioxidant protein through Nrf2 pathway activation [85].

Bisdemethoxycurcumin was reported to show the highest affinity for Aβ-containing plaques in cortical Alzheimer's Disease brain tissue in comparison with other curcuminoids [86]. Indeed, several in vitro and in vivo experiments suggest that curcumin can also operate by preventing the formation of extra- or intracellular aggregates present in many neurodegenerative disorders.

For prevention and treatment of Alzheimer's disease, curcumin has been shown to effectively maintain the normal structure and function of cerebral vessels, mitochondria, and synapses, thus reducing risk factors for a variety of chronic diseases and decreasing the risk of Alzheimer's disease onset [43].

The effect of curcumin on Alzheimer's disease is related to the modulation of multiple signaling pathways, to its anti-amyloid and metal iron-chelating properties, antioxidation, and its anti-inflammatory activities [87,88].

However, further studies are needed to identify other curcumin metabolites therapeutically active in neuroprotection.

6. Curcumin and Gut Microbiota

As detailed before, the paradox of poor bioavailability of curcumin and the wide range of health effects of curcumin can be explained by considering the reciprocal influence existing between curcumin and gut microbiota. Curcumin in the gut favors the growth of beneficial bacteria strains such as Bifidobacteria and Lactobacilli, with reduction of pathogenic strains [56,89]. In addition, curcumin treatment has been found to decrease the microbial richness and diversity, with a specific reduction of species found as cancer-related [90].

Several studies reported that curcumin actively reduces intestinal inflammation by modulating different molecular pathways. Thus, it is possible that curcumin, by modulating the homeostasis of the gut–brain axis, could also determine neuroprotective beneficial [91].

Indeed, dysbiosis is able to induce neuroinflammation, leading to an increased amyloidogenesis or to Lewy bodies accumulation, and to augment specific micro-RNA, down-regulating the triggering receptor expressed in microglial/myeloid cells-2 (TREM2)-mediated amyloid phagocytosis, to reduce,

in case of a decrease in butyrate-producing bacteria within the microbiome, the availability of butyrate, an important metabolite known to promote neuron survival [92–94].

In particular, gut dysbiosis has been postulated to trigger the onset of some neurodegenerative diseases [95]. Several reports suggest that compromised gut microbiota dysbiosis may represent an important co-factor in Alzheimer's disease (AD) [96,97]. In fact, different bacterial species are able to produce or aggravate the production of Aβ plaques. Gut dysbiosis, priming the innate immune system by microbiota, determines a neuroinflammatory response that causes misfolding of neuronal amyloid-β and α-synuclein [98].

Gastrointestinal dysfunctions, can be considered as early biomarkers in PD since they are consistently associated with the disease and may precede the classical motor manifestations by decades [99]. In addition, these dysfunctions make the etiology of PD more complicated. It is known that in PD patients, the impairment of the epithelial barrier has been associated with a decrease of Prevotellaceae. These strains are the main producers of mucin, a protein that protects the epithelium from pathogens [100].

More interestingly, in PD patients, there is a decrease of butyrate, a histone deacetylase inhibitor which protects dopaminergic neurons from degeneration by upregulating the neurotrophic factors, such as BDNF (Brain Derived Neurotrophic Factor) and GDNF (Glial Cell Line-derived Neurotrophic Factor) [101].

Studies in humans and in Huntington Disease (HD) animal models also reveal gastrointestinal dysfunctions, which may contribute to the worsening of the disease. The consistent loss of body weight—the most important non-neurological complication of HD and a direct consequence of gastrointestinal defects—has been associated with an impaired gut mobility and malabsorption [102]. Gut microbiota could have a role in HD since presymptomatic HD subjects display serum metabolomic shifts that suggest changes in gut microbial-derived metabolites [103].

Efforts to modulate gut microbiome in cases of neurodegeneration are limited and involve the use of antibiotics, fecal microbiota transplant, prebiotic/probiotic supplementation, and dietary interventions. In this context, curcumin can represent a potential therapeutic option against neurodegeneration since it exerts beneficial effects on gut microbiome, without any apparent toxicity, restoring the dysbiosis within patients suffering from neurodegenerative diseases and maintaining a proper microbiota–gut–brain axis.

These suggestions may be promising to unravel new therapeutic strategies for neurodegenerative diseases. It is known that neurodegeneration determines an imbalance in gut microbiota metabolism that results in changes of endocrine signaling in the host [104]. Further analysis on the microbiota composition and administration of specific curcumin neuroactive metabolites will be helpful in the identification of novel targeted treatments active in neuroprotection.

7. Conclusions

Curcumin represents one the most studied herbal remedies. It is generally considered the main component of turmeric responsible for the different pharmacological activities. However, curcumin is characterized by low systemic bioavailability and rapid metabolism. To address its pharmacological and therapeutic advantages, it is fundamental to consider curcumin interplay with gut microbiota that might pave the way to fill the gap between the low bioavailability and the wide health effects.

In fact, gut microbiota impact on curcumin metabolism, providing active metabolites. On the other hand, curcumin can influence gut microbiota composition, allowing the growth of strains needed to maintain correct host physiologic functions. This is the case of neurodegenerative diseases in which often a gut dysbiosis precedes the onset of the clinical signs.

Curcumin metabolism can be different among individuals, since everyone has his/her own microbiota compositions. Thus, the beneficial effects can be different due to the individual different bacterial content. Analysis of gut microbiota changes in health and diseases in the presence of curcumin will allow to identify bacterial strains in curcumin conversion.

The results summarized in the review suggest that curcumin alone can exert a neuroprotective function by affecting different neuropathological pathways. The role of microbiota in enhancing these positive effects could be related both to the production of metabolites more active and with better pharmacokinetics and to the modification of microbiota composition, with a prevalence of the healthy gut bacteria, like *Bifidobacteria* and *Lactobacilli*.

Additional studies, especially in humans will be necessary to unravel in depth the modification of microbiota composition achieved by curcumin. This will lead to an understanding of strategies needed for delivering health benefits by microbiota modulation and represents the first step to considering novel therapeutic applications of curcumin, gut microbiota-based.

Modification of microbiota and its metabolites will provide a new consideration for novel therapeutic intervention in neurodegenerative diseases.

Author Contributions: All authors actively participated to the review design, preparation, and organization. They also extensively discussed about the topics presented, giving a positive criticism to the work.

Funding: This research was funded by PO FESR 2014–2020—Regione Campania, Asse 1—obiettivo specifico 1.2, Progetto "Sviluppo di nanotecnologie Orientate alla Rigenerazione e Ricostruzione tissutale, Implantologia e Sensoristica in Odontoiatria/oculistica (SORRISO)". Grant Number: pdt1-000410; POR CAMPANIA FESR 2014/2020 DECRETO N. 651 - ALBITECH: Tecnologie abilitanti per la sintesi eco-sostenibile di nuovi materiali per la restaurativa.

Acknowledgments: FDM PhD fellowship in Biology is supported by MIUR project PON "Dottorati Innovativi con caratterizzazione industriale" 2017–2018. This work was supported by Regione Campania Research and Innovation project.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Pandey, K.B.; Rizvi, S.I. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid. Med. Cell. Longev.* **2009**, *2*, 270–278. [CrossRef]
2. Del Rio, D.; Rodriguez-Mateos, A.; Spencer, J.P.; Tognolini, M.; Borges, G.; Crozier, A. Dietary (poly) phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid. Redox Signal.* **2013**, *18*, 1818–1892. [CrossRef]
3. Di Meo, F.; Aversano, R.; Diretto, G.; Demurtas, O.; Villano, C.; Cozzolino, S.; Filosa, S.; Carputo, D.; Crispi, S. Anti-cancer activity of grape seed semi-polar extracts in human mesothelioma cell lines. *J. Funct. Foods* **2019**, *61*, 103515. [CrossRef]
4. van Duynhoven, J.; Vaughan, E.E.; Jacobs, D.M.; Kemperman, R.A.; van Velzen, E.J.; Gross, G.; Roger, L.C.; Possemiers, S.; Smilde, A.K.; Doré, J.; et al. Metabolic fate of polyphenols in the human superorganism. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 4531–4538. [CrossRef]
5. Ayati, Z.; Ramezani, M.; Amiri, M.S.; Moghadam, A.T.; Rahimi, H.; Abdollahzade, A.; Sahebkar, A.; Emami, S.A. Ethnobotany, phytochemistry and traditional uses of curcuma spp. and pharmacological profile of two important species (C. longa and C. zedoaria): A review. *Curr. Pharm. Des.* **2019**, *25*, 871–935. [CrossRef] [PubMed]
6. Qin, N.Y.; Yang, F.Q.; Wang, Y.T.; Li, S.P. Quantitative determination of eight components in rhizome (Jianghuang) and tuberous root (Yujin) of Curcuma longa using pressurized liquid extraction and gas chromatography-mass spectrometry. *J. Pharm. Biomed. Anal.* **2007**, *43*, 486–492. [CrossRef] [PubMed]
7. Ruby, A.J.; Kuttan, G.; Babu, K.D.; Rajasekharan, K.N.; Kuttan, R. Anti-tumour and antioxidant activity of natural curcuminoids. *Cancer Lett.* **1995**, *94*, 79–83. [CrossRef]
8. Hatcher, H.; Planalp, R.; Cho, J.; Torti, F.M.; Torti, S.V. Curcumin: From ancient medicine to current clinical trials. *Cell. Mol. Life Sci.* **2008**, *65*, 1631–1652. [CrossRef]
9. Prasad, S.; Gupta, S.C.; Tyagi, A.K.; Aggarwal, B.B. Curcumin, a component of golden spice: From bedside to bench and back. *Biotechnol. Adv.* **2014**, *32*, 1053–1064. [CrossRef]
10. Nelson, K.M.; Dahlin, J.L.; Bisson, J.; Graham, J.; Pauli, G.F.; Walters, M.A. The Essential Medicinal Chemistry of Curcumin. *J. Med. Chem.* **2017**, *60*, 1620–1637. [CrossRef]

11. Lao, C.D.; Ruffin, M.T.; Normolle, D.; Heath, D.D.; Murray, S.I.; Bailey, J.M.; Boggs, M.E.; Crowell, J.; Rock, C.L.; Brenner, D.E. Dose escalation of a curcuminoid formulation. *BMC Complement. Altern. Med.* **2006**, *6*, 10. [CrossRef] [PubMed]
12. Mansuri, M.L.; Parihar, P.; Solanki, I.; Parihar, M.S. Flavonoids in modulation of cell survival signalling pathways. *Genes Nutr.* **2014**, *9*, 400. [CrossRef] [PubMed]
13. Esatbeyoglu, T.; Huebbe, P.; Ernst, I.M.; Chin, D.; Wagner, A.E.; Rimbach, G. Curcumin—from molecule to biological function. *Angew. Chem. Int. Ed. Engl.* **2012**, *51*, 5308–5332. [CrossRef] [PubMed]
14. Jacob, J.N.; Badyal, D.K.; Bala, S.; Toloue, M. Evaluation of the in vivo anti-inflammatory and analgesic and in vitro anti-cancer activities of curcumin and its derivatives. *Nat. Prod. Commun.* **2013**, *8*, 359–362. [CrossRef] [PubMed]
15. Gupta, S.; Sung, B.; Kim, J.; Prasad, S.; Li, S.; Aggarwal, B. Multitargeting by turmeric, the golden spice: From kitchen to clinic. *Mol. Nutr. Food Res.* **2013**, *57*, 1510–1528. [CrossRef] [PubMed]
16. Tong, W.; Wang, Q.; Sun, D.; Suo, J. Curcumin suppresses colon cancer cell invasion via AMPK-induced inhibition of NF-κB, uPA activator and MMP9. *Oncol. Lett.* **2016**, *12*, 4139–4146. [CrossRef] [PubMed]
17. Di Meo, F.; Filosa, S.; Madonna, M.; Giello, G.; Di Pardo, A.; Maglione, V.; Baldi, A.; Crispi, S. Curcumin C3 complex®/Bioperine® has antineoplastic activity in mesothelioma: An in vitro and in vivo analysis. *J. Exp. Clin. Cancer Res.* **2019**, *38*, 360. [CrossRef]
18. Shehzad, A.; Lee, J.; Lee, Y.S. Curcumin in various cancers. *Biofactors* **2013**, *39*, 56–68. [CrossRef]
19. Irving, G.R.; Iwuji, C.O.; Morgan, B.; Berry, D.P.; Steward, W.P.; Thomas, A.; Brown, K.; Howells, L.M. Combining curcumin (C3-complex, Sabinsa) with standard care FOLFOX chemotherapy in patients with inoperable colorectal cancer (CUFOX): Study protocol for a randomised control trial. *Trials* **2015**, *16*, 110. [CrossRef]
20. Aggarwal, B.B.; Sung, B. Pharmacological basis for the role of curcumin in chronic diseases: An age-old spice with modern targets. *Trends Pharmacol. Sci.* **2009**, *30*, 85–94. [CrossRef]
21. Kumar, A.; Ahuja, A.; Ali, J.; Baboota, S. Conundrum and Therapeutic Potential of Curcumin in Drug Delivery. *Crit. Rev. Ther. Drug Carrier Syst.* **2010**, *27*, 279–312. [CrossRef] [PubMed]
22. Mayol, L.; Serri, C.; Menale, C.; Crispi, S.; Piccolo, M.T.; Mita, L.; Giarra, S.; Forte, M.; Saija, A.; Biondi, M.; et al. Curcumin loaded PLGA-poloxamer blend nanoparticles induce cell cycle arrest in mesothelioma cells. *Eur. J. Pharm. Biopharm.* **2015**, *93*, 37–45. [CrossRef] [PubMed]
23. Mirzaei, H.; Shakeri, A.; Rashidi, B.; Jalili, A.; Banikazemi, Z.; Sahebkar, A. Phytosomal curcumin: A review of pharmacokinetic, experimental and clinical studies. *Biomed. Pharmacother.* **2017**, *85*, 102–112. [CrossRef] [PubMed]
24. Serri, C.; Argirò, M.; Piras, L.; Mita, D.G.; Saija, A.; Mita, L.; Forte, M.; Giarra, S.; Biondi, M.; Crispi, S.; et al. Nano-precipitated curcumin loaded particles: Effect of carrier size and drug complexation with (2-hydroxypropyl)-β-cyclodextrin on their biological performances. *Int. J. Pharm.* **2017**, *520*, 21–28. [CrossRef]
25. Shoba, G.; Joy, D.; Joseph, T.; Majeed, M.; Rajendran, R.; Srinivas, P.S. Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. *Planta Med.* **1998**, *64*, 353–356. [CrossRef]
26. Vecchione, R.; Quagliariello, V.; Calabria, D.; Calcagno, V.; De Luca, E.; Iaffaioli, R.V.; Netti, P.A. Curcumin bioavailability from oil in water nano-emulsions: In vitro and in vivo study on the dimensional, compositional and interactional dependence. *J. Control Release.* **2016**, *233*, 88–100. [CrossRef]
27. Turnbaugh, P.J.; Hamady, M.; Yatsunenko, T.; Cantarel, B.L.; Duncan, A.; Ley, R.E.; Sogin, M.L.; Jones, W.J.; Roe, B.A.; Affourtit, J.P.; et al. A core gut microbiome in obese and lean twins. *Nature* **2009**, *457*, 480–484. [CrossRef]
28. Carmody, R.N.; Turnbaugh, P.J. Host-microbial interactions in the metabolism of therapeutic and diet-derived xenobiotics. *J. Clin. Investig.* **2014**, *124*, 4173–4181. [CrossRef]
29. Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Remesy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81*, 230S–242S. [CrossRef]
30. Strong, T.V.; Tagle, D.A.; Valdes, J.M.; Elmer, L.W.; Boehm, K.; Swaroop, M.; Kaatz, K.W.; Collins, F.S.; Albin, R.L. Widespread expression of the human and rat Huntington's disease gene in brain and nonneural tissues. *Nat. Genet.* **1993**, *5*, 259–265. [CrossRef]
31. Williamson, G.; Clifford, M. Role of the small intestine, colon and microbiota in determining the metabolic fate of polyphenols. *Biochem. Pharmacol.* **2017**, *139*, 24–39. [CrossRef] [PubMed]

32. Pan, M.H.; Huang, T.M.; Lin, J.K. Biotransformation of curcumin through reduction and glucuronidation in mice. *Drug Metab. Dispos.* **1999**, *27*, 486–494. [PubMed]
33. Sharma, R.A.; Euden, S.A.; Platton, S.L.; Cooke, D.N.; Shafayat, A.; Hewitt, H.R.; Marczylo, T.H.; Morgan, B.; Hemingway, D.; Plummer, S.M.; et al. Phase I clinical trial of oral curcumin: Biomarkers of systemic activity and compliance. *Clin. Cancer Res.* **2004**, *10*, 6847–6854. [CrossRef] [PubMed]
34. Dhillon, N.; Aggarwal, B.B.; Newman, R.A.; Wolff, R.A.; Kunnumakkara, A.B.; Abbruzzese, J.L.; Ng, C.S.; Badmaev, V.; Kurzrock, R. Phase II trial of curcumin in patients with advanced pancreatic cancer. *Clin. Cancer Res.* **2008**, *14*, 4491–4499. [CrossRef]
35. Edwards, R.L.; Luis, P.B.; Varuzza, P.V.; Joseph, A.I.; Presley, S.H.; Chaturvedi, R.; Schneider, C. The anti-inflammatory activity of curcumin is mediated by its oxidative metabolites. *J. Biol. Chem.* **2017**, *292*, 21243–21252. [CrossRef]
36. Wang, X.; Gao, J.; Wang, Y.; Zhao, B.; Zhang, Y.; Han, F.; Zheng, Z.; Hu, D. Curcumin pretreatment prevents hydrogen peroxide-induced oxidative stress through enhanced mitochondrial function and deactivation of Akt/Erk signaling pathways in rat bone marrow mesenchymal stem cells. *Mol. Cell. Biochem.* **2018**, *443*, 37–45. [CrossRef]
37. Da Silva Morrone, M.; Schnorr, C.E.; Behr, G.A.; Gasparotto, J.; Bortolin, R.C.; Moresco, K.S.; Bittencourt, L.; Zanotto-Filho, A.; Gelain, D.P.; Moreira, J.C. Oral administration of curcumin relieves behavioral alterations and oxidative stress in the frontal cortex, hippocampus, and striatum of ovariectomized Wistar rats. *J. Nutr. Biochem.* **2016**, *32*, 181–188. [CrossRef]
38. Maithilikarpagaselvi, N.; Sridhar, M.G.; Swaminathan, R.P.; Zachariah, B. Curcumin prevents inflammatory response, oxidative stress and insulin resistance in high fructose fed male Wistar rats: Potential role of serine kinases. *Chem. Biol. Interact.* **2016**, *244*, 187–194. [CrossRef]
39. Wei, G.; Chen, B.; Lin, Q.; Li, Y.; Luo, L.; He, H.; Fu, H. Tetrahydrocurcumin Provides Neuroprotection in Experimental Traumatic Brain Injury and the Nrf2 Signaling Pathway as a Potential Mechanism. *Neuroimmunomodulation* **2017**, *24*, 348–355. [CrossRef]
40. Takahashi, M.; Suzuki, K.; Kim, H.K.; Otsuka, Y.; Imaizumi, A.; Miyashita, M.; Sakamoto, S. Effects of curcumin supplementation on exercise-induced oxidative stress in humans. *Int. J. Sports Med.* **2014**, *35*, 469–475. [CrossRef]
41. Santos-Parker, J.R.; Strahler, T.R.; Bassett, C.J.; Bispham, N.Z.; Chonchol, M.B.; Seals, D.R. Curcumin supplementation improves vascular endothelial function in healthy middle-aged and older adults by increasing nitric oxide bioavailability and reducing oxidative stress. *Aging (Albany NY)* **2017**, *9*, 187–208. [CrossRef] [PubMed]
42. Sukardi, R.; Sastroasmoro, S.; Siregar, N.C.; Djer, M.M.; Suyatna, F.D.; Sadikin, M.; Ibrahim, N.; Rahayuningsih, S.E.; Witarto, A.B. The role of curcumin as an inhibitor of oxidative stress caused by ischaemia re-perfusion injury in tetralogy of Fallot patients undergoing corrective surgery. *Cardiol. Young* **2016**, *26*, 431–438. [CrossRef] [PubMed]
43. Chen, M.; Du, Z.Y.; Zheng, X.; Li, D.L.; Zhou, R.P.; Zhang, K. Use of curcumin in diagnosis, prevention, and treatment of Alzheimer's disease. *Neural Regen. Res.* **2018**, *13*, 742–752. [CrossRef] [PubMed]
44. Purpura, M.; Lowery, R.P.; Wilson, J.M.; Mannan, H.; Münch, G.; Razmovski-Naumovski, V. Analysis of different innovative formulations of curcumin for improved relative oral bioavailability in human subjects. *Eur. J. Nutr.* **2018**, *57*, 929–938. [CrossRef]
45. Liu, W.; Zhai, Y.; Heng, X.; Che, F.Y.; Chen, W.; Sun, D.; Zhai, G. Oral bioavailability of curcumin: Problems and advancements. *J. Drug Target.* **2016**, *24*, 694–702. [CrossRef]
46. Tsuda, T. Curcumin as a functional food-derived factor: Degradation products, metabolites, bioactivity, and future perspectives. *Food Funct.* **2018**, *9*, 705–714. [CrossRef]
47. Hoehle, S.I.; Pfeiffer, E.; Sólyom, A.M.; Metzler, M. Metabolism of curcuminoids in tissue slices and subcellular fractions from rat liver. *J. Agric. Food Chem.* **2006**, *54*, 756–764. [CrossRef]
48. Metzler, M.; Pfeiffer, E.; Schulz, S.I.; Dempe, J. Curcumin uptake and metabolism. *Biofactors* **2013**, *39*, 14–20. [CrossRef]
49. Hassaninasab, A.; Hashimoto, Y.; Tomita-Yokotani, K.; Kobayashi, M. Discovery of the curcumin metabolic pathway involving a unique enzyme in an intestinal microorganism. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 6615–6620. [CrossRef]

50. Burapan, S.; Kim, M.; Han, J. Curcuminoid demethylation as an alternative metabolism by human intestinal microbiota. *J. Agric. Food Chem.* **2017**, *65*, 3305–3310. [CrossRef]
51. Tan, S.; Rupasinghe, T.W.; Tull, D.L.; Boughton, B.; Oliver, C.; McSweeny, C.; Gras, S.L.; Augustin, M.A. Degradation of curcuminoids by in vitro pure culture fermentation. *J. Agric. Food Chem.* **2014**, *62*, 11005–11015. [CrossRef] [PubMed]
52. Jazayeri, S.D. Survival of Bifidobacteria and other selected intestinal bacteria in TPY medium supplemented with Curcumin as assessed in vitro. *Int. J. Probiotics Prebiotics* **2009**, *4*, 15–22.
53. Lou, Y.; Zheng, J.; Hu, H.; Lee, J.; Zeng, S. Application of ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry to identify curcumin metabolites produced by human intestinal bacteria. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2015**, *985*, 38–47. [CrossRef] [PubMed]
54. Sekirov, I.; Russell, S.L.; Antunes, L.C.; Finlay, B.B. Gut microbiota in health and disease. *Physiol. Rev.* **2010**, *90*, 859–904. [CrossRef]
55. Qin, J.; Li, R.; Raes, J.; Arumugam, M.; Burgdorf, K.S.; Manichanh, C.; Nielsen, T.; Pons, N.; Levenez, F.; Yamada, T.; et al. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* **2010**, *464*, 59–65. [CrossRef]
56. Shen, L.; Liu, L.; Ji, H.F. Regulative effects of curcumin spice administration on gut microbiota and its pharmacological implications. *Food Nutr. Res.* **2017**, *61*, 1361780. [CrossRef]
57. Zam, W. Gut microbiota as a prospective therapeutic target for curcumin: A review of mutual influence. *J. Nutr. Metab.* **2018**, *2018*, 1367984. [CrossRef]
58. Feng, W.; Wang, H.; Zhang, P.; Gao, C.; Tao, J.; Ge, Z.; Zhu, D.; Bi, Y. Modulation of gut microbiota contributes to curcumin-mediated attenuation of hepatic steatosis in rats. *Biochim. Biophys. Acta Gen. Subj.* **2017**, *1861*, 1801–1812. [CrossRef]
59. Le Chatelier, E.; Nielsen, T.; Qin, J.; Prifti, E.; Hildebrand, F.; Falony, G.; Almeida, M.; Arumugam, M.; Batto, J.M.; Kennedy, S.; et al. Richness of human gut microbiome correlates with metabolic markers. *Nature* **2013**, *500*, 541–546. [CrossRef]
60. Cotillard, A.; Kennedy, S.P.; Kong, L.C.; Prifti, E.; Pons, N.; Le Chatelier, E.; Almeida, M.; Quinquis, B.; Levenez, F.; Galleron, N.; et al. Dietary intervention impact on gut microbial gene richness. *Nature* **2013**, *500*, 585–588. [CrossRef]
61. Zhang, Z.; Chen, Y.; Xiang, L.; Wang, Z.; Xiao, G.G.; Hu, J. Effect of curcumin on the diversity of gut microbiota in ovariectomized rats. *Nutrients* **2017**, *9*, 1146. [CrossRef] [PubMed]
62. Peterson, C.T.; Vaughn, A.R.; Sharma, V.; Chopra, D.; Mills, P.J.; Peterson, S.N.; Sivamani, R.K. Effects of turmeric and curcumin dietary supplementation on human gut microbiota: A double-blind, randomized, placebo-controlled pilot study. *J. Evid. Based Integr. Med.* **2018**, *23*, 2515690X18790725. [CrossRef] [PubMed]
63. Bors, W.; Heller, W.; Michel, C.; Saran, M. Flavonoids as antioxidants: Determination of radical-scavenging efficiencies. *Methods Enzymol.* **1990**, *186*, 343–355. [PubMed]
64. Amić, D.; Davidović-Amić, D.; Beslo, D.; Rastija, V.; Lucić, B.; Trinajstić, N. SAR and QSAR of the antioxidant activity of flavonoids. *Curr. Med. Chem.* **2007**, *14*, 827–845. [CrossRef]
65. Smolensky, D.; Rhodes, D.; McVey, D.S.; Fawver, Z.; Perumal, R.; Herald, T.; Noronha, L. High-polyphenol sorghum bran extract inhibits cancer cell growth through ROS induction, cell cycle arrest, and apoptosis. *J. Med. Food* **2018**, *21*, 990–998. [CrossRef]
66. Campos, P.B.; Paulsen, B.S.; Rehen, S.K. Accelerating neuronal aging in in vitro model brain disorders: A focus on reactive oxygen species. *Front. Aging Neurosci.* **2014**, *6*, 292. [CrossRef]
67. Gu, F.; Chauhan, V.; Chauhan, A. Glutathione redox imbalance in brain disorders. *Curr. Opin. Clin. Nutr. Metab. Care* **2015**, *18*, 89–95. [CrossRef]
68. Samarghandian, S.; Azimi-Nezhad, M.; Farkhondeh, T.; Samini, F. Anti-oxidative effects of curcumin on immobilization-induced oxidative stress in rat brain, liver and kidney. *Biomed. Pharmacother.* **2017**, *87*, 223–229. [CrossRef]
69. DiSilvestro, R.A.; Joseph, E.; Zhao, S.; Bomser, J. Diverse effects of a low dose supplement of lipidated curcumin in healthy middle aged people. *Nutr. J.* **2012**, *11*, 79. [CrossRef]
70. Wu, J.; Li, Q.; Wang, X.; Yu, S.; Li, L.; Wu, X.; Chen, Y.; Zhao, J.; Zhao, Y. Neuroprotection by curcumin in ischemic brain injury involves the Akt/Nrf2 pathway. *PLoS ONE* **2013**, *8*, e59843. [CrossRef]

71. Li, Y.; Li, J.; Li, S.; Wang, X.; Liu, B.; Fu, Q.; Ma, S. Curcumin attenuates glutamate neurotoxicity in the hippocampus by suppression of ER stress-associated TXNIP/NLRP3 inflammasome activation in a manner dependent on AMPK. *Toxicol. Appl. Pharmacol.* **2015**, *286*, 53–63. [CrossRef] [PubMed]
72. Fu, Z.; Yang, J.; Wei, Y.; Li, J. Effects of piceatannol and pterostilbene against β-amyloid-induced apoptosis on the PI3K/Akt/Bad signaling pathway in PC12 cells. *Food Funct.* **2016**, *7*, 1014–1023. [CrossRef] [PubMed]
73. Wu, N.; Zheng, B.; Shaywitz, A.; Dagon, Y.; Tower, C.; Bellinger, G.; Shen, C.H.; Wen, J.; Asara, J.; McGraw, T.E.; et al. AMPK-dependent degradation of TXNIP upon energy stress leads to enhanced glucose uptake via GLUT1. *Mol. Cell* **2013**, *49*, 1167–1175. [CrossRef] [PubMed]
74. Dudek, H.; Datta, S.R.; Franke, T.F.; Birnbaum, M.J.; Yao, R.; Cooper, G.M.; Segal, R.A.; Kaplan, D.R.; Greenberg, M.E. Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* **1997**, *275*, 661–665. [CrossRef] [PubMed]
75. Shah, Z.A.; Li, R.C.; Thimmulappa, R.K.; Kensler, T.W.; Yamamoto, M.; Biswal, S.; Doré, S. Role of reactive oxygen species in modulation of Nrf2 following ischemic reperfusion injury. *Neuroscience* **2007**, *147*, 53–59. [CrossRef]
76. Maiti, P.; Dunbar, G.L. Use of curcumin, a natural polyphenol for targeting molecular pathways in treating age-related neurodegenerative diseases. *Int. J. Mol. Sci.* **2018**, *19*, 1637. [CrossRef]
77. Gao, Y.; Zhuang, Z.; Gao, S.; Li, X.; Zhang, Z.; Ye, Z.; Li, L.; Tang, C.; Zhou, M.; Han, X.; et al. Tetrahydrocurcumin reduces oxidative stress-induced apoptosis via the mitochondrial apoptotic pathway by modulating autophagy in rats after traumatic brain injury. *Am. J. Transl. Res.* **2017**, *9*, 887–899.
78. Mishra, S.; Mishra, M.; Seth, P.; Sharma, S. Tetrahydrocurcumin confers protection against amyloid beta induced toxicity. *Neuroreport* **2011**, *22*, 23–27. [CrossRef]
79. Begum, A.N.; Jones, M.R.; Lim, G.P.; Morihara, T.; Kim, P.; Heath, D.D.; Rock, C.L.; Pruitt, M.A.; Yang, F.; Hudspeth, B.; et al. Curcumin structure-function, bioavailability, and efficacy in models of neuroinflammation and Alzheimer's disease. *J. Pharmacol. Exp. Ther.* **2008**, *326*, 196–208. [CrossRef]
80. Rajeswari, A.; Sabesan, M. Inhibition of monoamine oxidase-B by the polyphenolic compound, curcumin and its metabolite tetrahydrocurcumin, in a model of Parkinson's disease induced by MPTP neurodegeneration in mice. *Inflammopharmacology* **2008**, *16*, 96–99. [CrossRef]
81. Singh, C.; Bortolato, M.; Bali, N.; Godar, S.; Scott, A.; Chen, K.; Thompson, R.; Shih, J. Cognitive abnormalities and hippocampal alterations in monoamine oxidase A and B knockout mice. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 12816–12821. [CrossRef] [PubMed]
82. Wu, J.C.; Tsai, M.L.; Lai, C.S.; Wang, Y.J.; Ho, C.T.; Pan, M.H. Chemopreventative effects of tetrahydrocurcumin on human diseases. *Food Funct.* **2014**, *5*, 12–17. [CrossRef] [PubMed]
83. Zeng, Y.; Qiu, F.; Liu, Y.; Qu, G.; Yao, X. Isolation and identification of phase 1 metabolites of demethoxycurcumin in rats. *Drug Metab. Dispos.* **2007**, *35*, 1564–1573. [CrossRef] [PubMed]
84. Khanna, S.; Park, H.A.; Sen, C.K.; Golakoti, T.; Sengupta, K.; Venkateswarlu, S.; Roy, S. Neuroprotective and antiinflammatory properties of a novel demethylated curcuminoid. *Antioxid. Redox Signal.* **2009**, *11*, 449–468. [CrossRef]
85. Luo, D.D.; Chen, J.F.; Liu, J.J.; Xie, J.H.; Zhang, Z.B.; Gu, J.Y.; Zhuo, J.Y.; Huang, S.; Su, Z.R.; Sun, Z.H. Tetrahydrocurcumin and octahydrocurcumin, the primary and final hydrogenated metabolites of curcumin, possess superior hepatic-protective effect against acetaminophen-induced liver injury: Role of CYP2E1 and Keap1-Nrf2 pathway. *Food Chem. Toxicol.* **2019**, *123*, 349–362. [CrossRef]
86. Veldman, E.R.; Jia, Z.; Halldin, C.; Svedberg, M.M. Amyloid binding properties of curcumin analogues in Alzheimer's disease postmortem brain tissue. *Neurosci. Lett.* **2016**, *630*, 183–188. [CrossRef]
87. Pinkaew, D.; Changtam, C.; Tocharus, C.; Thummayot, S.; Suksamrarn, A.; Tocharus, J. Di-O-demethylcurcumin protects SK-N-SH cells against mitochondrial and endoplasmic reticulum-mediated apoptotic cell death induced by Aβ25-35. *Neurochem. Int.* **2015**, *80*, 110–119. [CrossRef]
88. Wu, A.; Noble, E.E.; Tyagi, E.; Ying, Z.; Zhuang, Y.; Gomez-Pinilla, F. Curcumin boosts DHA in the brain: Implications for the prevention of anxiety disorders. *Biochim. Biophys. Acta* **2015**, *1852*, 951–961. [CrossRef]
89. McFadden, R.M.; Larmonier, C.B.; Shehab, K.W.; Midura-Kiela, M.; Ramalingam, R.; Harrison, C.A.; Besselsen, D.G.; Chase, J.H.; Caporaso, J.G.; Jobin, C.; et al. The Role of Curcumin in Modulating Colonic Microbiota During Colitis and Colon Cancer Prevention. *Inflamm. Bowel Dis.* **2015**, *21*, 2483–2494. [CrossRef]

90. Rashmi, R.; Santhosh Kumar, T.R.; Karunagaran, D. Human colon cancer cells differ in their sensitivity to curcumin-induced apoptosis and heat shock protects them by inhibiting the release of apoptosis-inducing factor and caspases. *FEBS Lett.* **2003**, *538*, 19–24. [CrossRef]
91. Di Meo, F.; Donato, S.; Di Pardo, A.; Maglione, V.; Filosa, S.; Crispi, S. New Therapeutic Drugs from Bioactive Natural Molecules: The Role of Gut Microbiota Metabolism in Neurodegenerative Diseases. *Curr. Drug Metab.* **2018**, *19*, 478–489. [CrossRef] [PubMed]
92. Zhao, Y.; Lukiw, W.J. Microbiome-generated amyloid and potential impact on amyloidogenesis in Alzheimer's disease (AD). *J. Nat. Sci.* **2015**, *1*, e138. [PubMed]
93. Fang, Z.; Lu, W.; Zhao, J.; Zhang, H.; Qian, L.; Wang, Q.; Chen, W. Probiotics modulate the gut microbiota composition and immune responses in patients with atopic dermatitis: A pilot study. *Eur. J. Nutr.* **2019**. [CrossRef] [PubMed]
94. Alexandrov, P.; Zhao, Y.; Jones, B.; Bhattacharjee, S.; Lukiw, W. Expression of the phagocytosis-essential protein TREM2 is down-regulated by an aluminum-induced miRNA-34a in a murine microglial cell line. *J. Inorg. Biochem.* **2013**, *128*, 267–269. [CrossRef] [PubMed]
95. Ilieva, H.; Maragakis, N.J. Motoneuron disease: Basic science. *Adv. Neurobiol.* **2017**, *15*, 163–190. [CrossRef]
96. Harach, T.; Marungruang, N.; Duthilleul, N.; Cheatham, V.; Mc Coy, K.D.; Frisoni, G.; Neher, J.J.; Fåk, F.; Jucker, M.; Lasser, T.; et al. Reduction of Abeta amyloid pathology in APPPS1 transgenic mice in the absence of gut microbiota. *Sci. Rep.* **2017**, *7*, 41802. [CrossRef]
97. Wu, J.H.; Guo, Z.; Kumar, S.; Lapuerta, P. Incidence of serious upper and lower gastrointestinal events in older adults with and without Alzheimer's disease. *J. Am. Geriatr. Soc.* **2011**, *59*, 2053–2061. [CrossRef]
98. Friedland, R.P. Mechanisms of molecular mimicry involving the microbiota in neurodegeneration. *J. Alzheimers Dis.* **2015**, *45*, 349–362. [CrossRef]
99. Poirier, A.A.; Aubé, B.; Côté, M.; Morin, N.; Di Paolo, T.; Soulet, D. Gastrointestinal dysfunctions in Parkinson's disease: Symptoms and treatments. *Parkinsons Dis.* **2016**, *2016*, 6762528. [CrossRef]
100. Scheperjans, F.; Aho, V.; Pereira, P.A.; Koskinen, K.; Paulin, L.; Pekkonen, E.; Haapaniemi, E.; Kaakkola, S.; Eerola-Rautio, J.; Pohja, M.; et al. Gut microbiota are related to Parkinson's disease and clinical phenotype. *Mov. Disord.* **2015**, *30*, 350–358. [CrossRef]
101. Wu, X.; Chen, P.S.; Dallas, S.; Wilson, B.; Block, M.L.; Wang, C.C.; Kinyamu, H.; Lu, N.; Gao, X.; Leng, Y.; et al. Histone deacetylase inhibitors up-regulate astrocyte GDNF and BDNF gene transcription and protect dopaminergic neurons. *Int. J. Neuropsychopharmacol.* **2008**, *11*, 1123–1134. [CrossRef] [PubMed]
102. van der Burg, J.M.; Winqvist, A.; Aziz, N.A.; Maat-Schieman, M.L.; Roos, R.A.; Bates, G.P.; Brundin, P.; Björkqvist, M.; Wierup, N. Gastrointestinal dysfunction contributes to weight loss in Huntington's disease mice. *Neurobiol. Dis.* **2011**, *44*, 1–8. [CrossRef] [PubMed]
103. Rosas, H.D.; Doros, G.; Bhasin, S.; Thomas, B.; Gevorkian, S.; Malarick, K.; Matson, W.; Hersch, S.M. A systems-level "misunderstanding": The plasma metabolome in Huntington's disease. *Ann. Clin. Transl. Neurol.* **2015**, *2*, 756–768. [CrossRef] [PubMed]
104. Muegge, B.; Kuczynski, J.; Knights, D.; Clemente, J.; Gonzalez, A.; Fontana, L.; Henrissat, B.; Knight, R.; Gordon, J. Diet Drives Convergence in Gut Microbiome Functions Across Mammalian Phylogeny and Within Humans. *Science* **2011**, *332*, 970–974. [CrossRef] [PubMed]

© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

[Downloaded free from http://www.nrronline.org on Wednesday, February 10, 2021, IP: 140.164.74.178]

REVIEW

Polyphenols-gut microbiota interplay and brain neuromodulation

Stefania Filosa[1, 2], **Francesco Di Meo**[1], **Stefania Crispi**[1, *]

1 Institute of Biosciences and Bioresources, National Research Council, *via* P. Castellino, Naples, Italy
2 Istituto di Ricovero e Cura a Carattere Scientifico Neuromed, Pozzilli, Italy

Funding: *This work was supported by Italian Ministry of Health "Ricerca Corrente" (to SF).*

Abstract

Increasing evidence suggests that food ingested polyphenols can have beneficial effects in neuronal protection acting against oxidative stress and inflammatory injury. Moreover, polyphenols have been reported to promote cognitive functions. Biotransformation of polyphenols is needed to obtain metabolites active in brain and it occurs through their processing by gut microbiota. Polyphenols metabolites could directly act as neurotransmitters crossing the blood-brain barrier or indirectly by modulating the cerebrovascular system. The microbiota-gut-brain axis is considered a neuroendocrine system that acts bidirectionally and plays an important role in stress responses. The metabolites produced by microbiota metabolism can modulate gut bacterial composition and brain biochemistry acting as neurotransmitters in the central nervous system. Gut microbiota composition can be influenced by dietary ingestion of natural bioactive molecules such as probiotics, prebiotics and polyphenol. Microbiota composition can be altered by dietary changes and gastrointestinal dysfunctions are observed in neurodegenerative diseases. In addition, several pieces of evidence support the idea that alterations in gut microbiota and enteric neuroimmune system could contribute to onset and progression of these age-related disorders. The impact of polyphenols on microbiota composition strengthens the idea that maintaining a healthy microbiome by modulating diet is essential for having a healthy brain across the lifespan. Moreover, it is emerging that they could be used as novel therapeutics to prevent brain from neurodegeneration.

Key Words: *polyphenols; gut-microbiota; gut-brain axis; metagenomic; neurodegeneration; neurotransmitters; prebiotics; probiotics*

Correspondence to:
Stefania Crispi, PhD,
stefania.crispi@ibbr.cnr.it.

orcid:
0000-0002-7566-1554
(Stefania Crispi)

doi: *10.4103/1673-5374.241429*

Received: *2018-06-25*
Accepted: *2018-08-07*

Introduction

Neurodegenerative disorders are increasing with the ageing of human population. In addition, environmental stressors such as oxidants determine progressive neurons degeneration affecting cognitive functions.

Diet and lifestyle play an important role in the onset and progression of age-related disorders, and there is a growing interest on the bioactivity of polyphenol-rich foods. Indeed, polyphenols are able to protect neurons against injury, suppressing neuroinflammation, thus promoting memory, learning, and cognitive functions (Pandey and Rizvi, 2009).

Recent evidences suggest that polyphenols exert beneficial effects acting through multiple pathways involved in oxidative/inflammatory stress signalling and leading to the expression of antioxidant enzymes neurotrophic factors, and cytoprotective proteins. All these processes act to maintain brain homeostasis.

The mechanisms by which polyphenols act on cognitive functions have not been fully elucidated. It has been reported that polyphenols carry out brain protection through direct and indirect actions. Direct actions depend on their ability to cross the blood-brain barrier. In fact, some polyphenol metabolites can modulate directly neuronal receptors (Youdim et al., 2004). Moreover a recent study using different neuronal systems reported that metabolites from dietary polyphenols exert neuroprotective effects after reaching the brain by crossing blood-brain barrier (Figueira et al., 2017).

Polyphenols indirect actions involve mechanisms that improve the peripheral cerebrovascular health. Several studies in humans indicated that dietary polyphenols improve vasodilatatory response and increase levels of circulating nitric oxide (NO) species that are essential in the control of vascular tone; vasodilation and blood flow in the body and in cerebral circulation (Bondonno et al., 2012; Laranjinha et al., 2012).

Bioavailability of polyphenols is related to their chemical structure. The majority of dietary polyphenols are present as esters, glycosides or polymers that cannot be absorbed in this native form. After ingestion, to gives rise to beneficial effects at human body, polyphenols must become bioactive. This is realized through gut intestinal transformations that produce bioavailable and active phenolic metabolites.

Polyphenols are usually hydrolysed by intestinal enzymes or by gut microbiota. These modifications produce metabolites completely different from those present in food. In this form they reach blood, tissues and brain where exert biological activities.

In this review we briefly discuss the protective effects that polyphenols-gut microbiota interaction exert on the onset and progression of neurodegenerative diseases.

Polyphenols and Gut Microbiota

Polyphenols are a class of organic chemicals characterized by the presence of multiples phenol structural units. They

[Downloaded free from http://www.nrronline.org on Wednesday, February 10, 2021, IP: 140.164.74.178]

are produced by plants as secondary metabolites as defence against exogenous stresses. Specifically, polyphenols protect plants against reactive oxygen species (ROS), ultraviolet radiation (UV), pathogens, parasites and plant predators. In humans, the antioxidant properties of polyphenols are effective in different diseases and in cancer (Zhang, 2015). These molecules act as natural antioxidants thanks to the metal-chelating and free radical scavenger properties. The scavenging activity is linked to their structure, since compounds with similar structures exhibit similar antioxidant activity. In general, the antioxidant activity increase is directly linked to the number of hydroxyl groups and indirectly to glycosylation (Di Meo et al., 2018).

Polyphenols bioactivity is related to their 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. Interestingly, the properties of polyphenols depend from bioactive metabolites produced when they are metabolised by microbiota (Carmody and Turnbaugh, 2014).

In particular, polyphenols are characterized by low bioavailability, and after ingestion they are recognized as xenobiotics. The majority of them, due to the structural complexity, reach the large intestine without modifications. On the contrary, small polyphenols can be directly adsorbed in the small intestine. Therefore, complex polyphenols are converted into low-molecular-weight metabolites by the gut microbial community and become readily absorbable (Aura, 2008).

Dietary polyphenols biotransformations such as deglycosylations can be carried out by different gut microbial species. On the contrary, other reactions such as the generation of urolithins or (S)-equol require the presence of specific bacteria strains (Marin et al., 2015).

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 (**Figure 1**). In fact, polyphenols can modify microbiota composition and functions by acting on their growth or metabolism (Cardona et al., 2013). Polyphenols metabolism can be different among individuals since everyone has own microbiota compositions. Thus, similar daily intake of polyphenols can have different effect on health in people with a different bacterial content.

Specific polyphenols are able to inhibit/increase the growth of specific bacteria resulting in changes of gut microbial composition. For example the ingestion of different polyphenols result in different ratio between the healthy gut bacteria, *Bacteroides* and *Firmicutes* favouring the growth of *Bacteroides* that possess a higher number of glycan-degrading enzymes (Rastmanesh, 2011).

Polyphenols metabolites produced by gut microbiota are well absorbed in the intestine and are able to persist in the plasma for a longer time. 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 hydrophilic properties.

Due to their "prebiotic-like" effect, polyphenols can also modify gut microbiota composition. *In vitro* and *in vivo* studies showed that different polyphenols can modulate the growth of specific bacterial strains. Indeed, polyphenols can increase beneficial strains as *Bifidobacteria Lactobacilli*, reducing the number of pathogens, such as *C. perfringens* and *C. histolyticum* (Duenas et al., 2015).

The Gut-Brain Axis

In humans, the gut microbiota is one of the most densely populated microbial ecosystems. Gut microbiota contributes to healthy state and several diseases can be linked to dysbiosis. Microbial interactions are important in infections, being able to block pathogens activities. Furthermore, microbiota composition is tightly linked to diet.

The connection between microbiota present in the gastrointestinal tract and the central nervous system, the gut-brain axis, plays an important role in stress response and it is widely recognized as neuroendocrine system. Communication between the gut and the brain involves multiple overlapping pathways: the enteric nervous system, the neuroimmune and the neuroendocrine systems. By acting on the nervous, endocrine and immune system, gut microbiota can influence brain functions. In addition, microbiota, being able to produce both neurotransmitters and neuropeptide, can directly influence the brain functions by acting on neuroactive metabolites production (Cryan and Dinan, 2012).

The gut microbiota can synthesise neurotransmitters or regulate their levels acting on their precursors. This is the case of *Bifidobacterium infantis* that influence central serotonin transmission by increasing plasma tryptophan levels (O'Mahony et al., 2015).

On the contrary, different *Lactobacillus* and *Bifidobacterium* species are able to produce γ-aminobutyric acid (GABA) (Yunes et al., 2016) while *Streptococcus*, *Escherichia* and *Enterococcus spp*. produce serotonin (Lyte, 2011; Nzakizwanayo et al., 2015).

All these neurotransmitters crossing the mucosal layer of the intestines, reach the brain where they could mediate different physiological events.

Large-scale metagenomic sequencing analyses have allowed understanding the functional relationship existing between functional components of the microbiome, microbial metabolism and metabolites production. These studies have indicated the existence of a high inter-individual diversity in microbiota composition and a strong association between unbalance in composition or stability and disease states (hmpdacc.org; human-microbiome.org).

Alteration in the homeostasis of gut-brain axis has been associated also to neurological disorders and neurodegen-

[Downloaded free from http://www.nrronline.org on Wednesday, February 10, 2021, IP: 140.164.74.178]

erative diseases (Zhu et al., 2017). Moreover, it is emerging that the regulation of microbiota composition can be realized using natural bioactive molecules such as polyphenols derived by plants, suggesting that polyphenols could be used to restore the altered brain functions that characterize neurodegenerative diseases.

The homeostasis of the gut microbiota can be modified by changing dietary ingestion of probiotics and prebiotics. Probiotics are live organisms that can exert health benefits to the host, while prebiotics are food ingredients able to induce in the host the growth beneficial microorganisms.

The use of probiotics and prebiotics can be viewed as novel strategy to change the gut microbiota composition thus modulating the gut-brain axis (Petschow et al., 2013).

Several studies both in humans and animals showed that these molecules could increase the levels of neurotransmitters allowing modulating mood and cognition (Sudo et al., 2004; Savignac et al., 2013; Alherz et al., 2017).

These findings suggest that communication between gut microbiota and brain is flexible and they also contribute to the identification of specific targets to be used in patients with altered stress responses associated with gut dysbiosis.

Microbiota and Neurodegeneration

Neural aging is characterized by progressive loss of function that involves central and peripheral neurons and neural stem cells. This degenerative process leads to neurodegeneration and can be considered the principal cause of cognitive impairment and sensory and motor deficits during aging.

It has been described that the onset of neurodegeneration starts earlier than the symptoms appears. At this time neurons have already been destroyed and this can explain why therapeutic approaches have little or no effect.

Gut microbiota can interact with central nervous system through different mechanisms and pathways such as the neurosystems implicated in stress and stress-related disorders (sympathetic and parasympathetic branches of the autonomic nervous system, neuroendocrine and neuroimmune systems). The communication between these pathways occurs through vagus nerve, cell wall, metabolites, and through neurotransmitters and brain neurotrophic factors. Gut microbiota are able to synthetize neurotransmitters thus microbiota homeostasis can impact on complex neurodegenerative disorders. Example are short-chain fatty acids (SCFAs), tryptophan, GABA and brain-derived neurotrophic factor (BDNF) (Rieder et al., 2017).

SCFAs represent the most abundant product of bacterial fermentation and have neuroactive properties. They modulate the release of serotonin as well as peptide YY, an important neuropeptide acting at multiple levels of the gut brain axis. Microbial-derived SCFAs acting on the production of secondary peptides represent a mechanism through which the gut microbiota influences human behaviour. In fact, it has been reported that systemic administration of the SCFA butyrate determines an antidepressant-like behavioural response (Jiang et al., 2018). In addition a recent study reported that SCFAs are active in Alzheimer's disease by inhibiting the generation of beta-amyloid peptides (Ho et al., 2018).

Tryptophan is an essential amino acid a precursor of many biologically active agents, including the synthesis of serotonin in the central nervous system. Serotonin, a neurotransmitter active both in the central nervous system and in the gut, plays an important role maintaining mood and cognition (Szapacs et al., 2004). Alterations in the levels of serotonin can be associated to the onset of gastrointestinal and mood disorders, and tryptophan dysregulation is linked to many disorders both in the brain and in the gastrointestinal tract. The direct regulation of tryptophan on serotonin by microbiota has been demonstrated in germ free animals in which that increased levels of circulating tryptophan and decreased levels of serotonin were restored after bacteria colonization of these animals (Clarke et al., 2013).

GABA, is the main inhibitory neurotransmitter that regulates neuronal excitability. It is produced from glutamate metabolism by different bacteria species, as demonstrated by *in vitro* studies. Dysfunction of the GABA system has been implicated in the pathophysiology of several chronic neurological diseases. GABA treatments were shown to decrease cytotoxicity induced by beta-amyloid fibers (Sun et al., 2012). GABA system represents an important mechanism through which bacteria can modulate brain chemistry.

BDNF is a neurotrophin widely expressed in the central nervous system with neuroprotective functions. BDNF play an important role in the growth and plasticity of synapses as well as in the survival and differentiation of neurons. Levels of BDNF are decreased in the cerebral cortex in Alzheimer's disease and several clinical trials aims to use BDNF as therapeutics for different neurodegenerative diseases (Nagahara and Tuszynski, 2011). Neurological mood diseases have been reported to be linked to BDNF levels and they have been associated with the gut-brain axis. It has been reported that microbiota fecal transplants in pathogen free mice increased the levels of hippocampal BDNF (O'Sullivan et al., 2011).

Several pre-clinical and clinical studies have indicated that microbiota, maintaining physiological homeostasis, can significantly interfere with human for brain functions and cognitive systems studies (Savignac et al., 2013).

Aged healthy people do not show changes in the microbiota diversity. Studies in centenarians have stressed the importance of microbial diversity in maintaining health with age (Biagi et al., 2016). On the contrary, dysbiosis have been observed in neurodevelopmental diseases as autism and in neurodegenerative including Alzheimer's disease, Huntington's disease and Parkinson's disease (Di Meo et al., 2018).

The role of the gut microbiome in the pathogenesis of chronic neurodevelopmental and neurodegenerative disorders such as Alzheimer's and Parkinson's diseases is beginning to emerge.

[Downloaded free from http://www.nrronline.org on Wednesday, February 10, 2021, IP: 140.164.74.178]

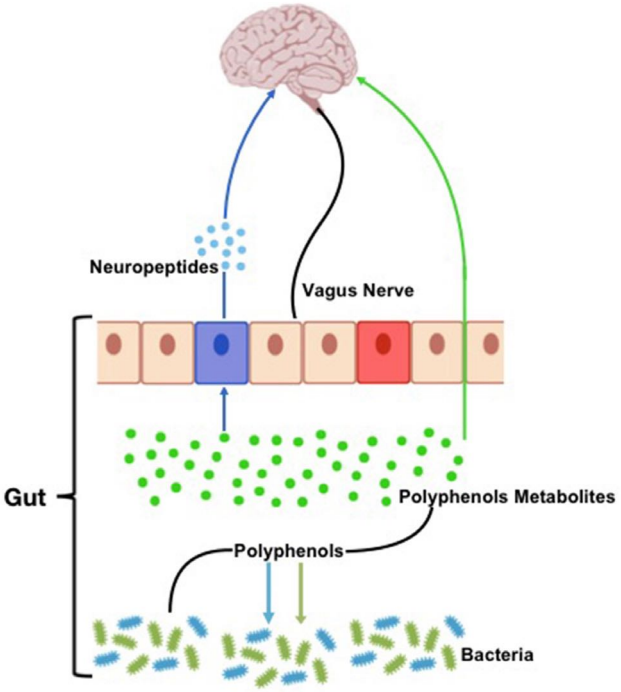

Figure 1 Schematic representation of the gut microbiota-brain interaction.
Gut bacteria metabolism produce neurotransmitters and bioactive metabolites from polyphenols. These molecules reach the brain crossing the intestinal barrier.

Studies regarding the associations between gut microbiome and neurodevelopmental or neurological disorders have been performed mainly in animal models (Bercik et al., 2011; Sampson et al., 2016; Brandscheid et al., 2017). And very few studies have been reported in humans.

Studies performed in early childhood showed that in autism there are gut microbiota perturbations. In particular *Proteobacteria* and *Bacteroidetes* are strongly increased while *Firmicutes* and *Bifidobacteria* are less abundance respect to healthy controls (Finegold et al., 2012). A recent small study in Alzheimer's disease patients described a reduction of gut microbiota diversity respect to the healthy controls. In particular in the Alzheimer group it was described a decrease of *Firmicutes* and *Actinobacteria* and increase of *Bacteroidetes* (Vogt et al., 2017). In Parkinson's disease it has been reported that the disease might begin in the gut and than spread to brain *via* the gut-brain axis. Indeed microbiota analysis in Parkinson's disease patients showed a decrease of *Prevotella* strains and increase of *Enterobacteria* (Scheperjans et al., 2015).

Considering the protective role of neurotransmitters produced by gut microbiota metabolism in neuroplasticity and neurological disorders, it will be fundamental to examine in depth the associations and the conditions by which the levels of these factors are influenced or regulated by microbiota.

Additional studies are needed to better understand if gut microbiota changes are central in the pathophysiology of neurodegenerative diseases or if they represent epiphenomena.

However, the association between gut microbiota and brain disorders suggests that a healthy microbiota may be one of the keys to longevity.

Conclusion

Emerging evidence indicates that changes in the composition of the microbiota may contribute to the onset of neurodegenerative disorders that increase with age.

Adults undergo to dramatic changes in microbiota composition following diet modifications. Furthermore, ageing is related to specific changes in the microbiota diversity that result in health outcomes in the elderly. Since microbiota can significantly interfere with human cognitive system, the understanding of the microbiota composition in healthy people is fundamental for maintaining brain and mental health during all over the life.

Gut microbial composition can be modulated by polyphenols. It is becoming clear that dietary polyphenols through their metabolites contribute to the maintenance of gut health by the modulation of the gut microbial balance. Polyphenols act by enhancing the growth of beneficial bacteria and inhibiting the growth of pathogens, thus exert prebiotic-like effects.

Regulation of microbiota composition using polyphenols, or other probiotics and prebiotics, may help to restore gut equilibrium and to set up new therapeutic intervention in neuropathologies. Since brain dysfunctions are associated with dysbiosis of the gut microbiota, it is possible that a rebalance in the microbiota composition may result in a partial or complete reversion of these diseases.

Finally, a better understanding of the interplay between polyphenols and gut microbiota will provide more insight into the health effects of polyphenols and open new possibilities to develop microbiota-based therapies for treating neuronal disorders.

Acknowledgments: *We thank Dr. Maria Rosaria Aletta (National Research Council, Napoli, Italy) for bibliographic support.*
Author contributions: *Manuscript conception: SC; manuscript writing and revising: SF, FDM and SC; literature collection and figure design: FDM.*
Financial support: *This work was supported by Italian Ministry of Health "Ricerca Corrente" (to SF).*
Conflicts of interest: *None declared.*
Copyright license agreement: *The Copyright License Agreement has been signed by all authors before publication.*
Plagiarism check: *Checked twice by iThenticate.*
Peer review: *Externally peer reviewed.*
Open access statement: *This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.*

References

Alherz F, Alherz M, Almusawi H (2017) NMDAR hypofunction and somatostatin-expressing GABAergic interneurons and receptors: A newly identified correlation and its effects in schizophrenia. Schizophr Res Cogn 8:1-6.

[Downloaded free from http://www.nrronline.org on Wednesday, February 10, 2021, IP: 140.164.74.178]

Aura AM (2008) Microbial metabolism of dietary phenolic compounds in the colon. Phytochem Rev 7:407-429.
Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J, Deng YK, Blennerhassett P, Macri J, McCoy KD, Verdu EF, Collins SM (2011) The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology 141: 599-609, 609.e1-3.
Biagi E, Franceschi C, Rampelli S, Severgnini M, Ostan R, Turroni S, Consolandi C, Quercia S, Scurti M, Monti D, Capri M, Brigidi P, Candela M (2016) Gut microbiota and extreme longevity. Curr Biol 26:1480-1485.
Bondonno CP, Yang X, Croft KD, Considine MJ, Ward NC, Rich L, Puddey IB, Swinny E, Mubarak A, Hodgson JM (2012) Flavonoid-rich apples and nitrate-rich spinach augment nitric oxide status and improve endothelial function in healthy men and women: a randomized controlled trial. Free Radic Biol Med 52:95-102.
Brandscheid C, Schuck F, Reinhardt S, Schafer KH, Pietrzik CU, Grimm M, Hartmann T, Schwiertz A, Endres K (2017) Altered gut microbiome composition and tryptic activity of the 5xFAD Alzheimer's mouse model. J Alzheimers Dis 56:775-788.
Cardona F, Andres-Lacueva C, Tulipani S, Tinahones FJ, Queipo-Ortuno MI (2013) Benefits of polyphenols on gut microbiota and implications in human health. J Nutr Biochem 24:1415-1422.
Carmody RN, Turnbaugh PJ (2014) Host-microbial interactions in the metabolism of therapeutic and diet-derived xenobiotics. J Clin Invest 124:4173-4181.
Clarke G, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F, Dinan TG, Cryan JF (2013) The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. Mol Psychiatr 18:666-673.
Cryan JF, Dinan TG (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci 13:701-712.
Di Meo F, Donato S, Di Pardo A, Maglione V, Filosa S, Crispi S (2018) New therapeutic drugs from bioactive natural molecules: the role of gut microbiota metabolism in neurodegenerative diseases. Curr Drug Metab 19:478-489.
Duenas M, Munoz-Gonzalez I, Cueva C, Jimenez-Giron A, Sanchez-Patan F, Santos-Buelga C, Moreno-Arribas MV, Bartolome B (2015) A survey of modulation of gut microbiota by dietary polyphenols. Biomed Res Int 2015:850902.
Figueira I, Garcia G, Pimpao RC, Terrasso AP, Costa I, Almeida AF, Tavares L, Pais TF, Pinto P, Ventura MR, Filipe A, McDougall GJ, Stewart D, Kim KS, Palmela I, Brites D, Brito MA, Brito C, Santos CN (2017) Polyphenols journey through blood-brain barrier towards neuronal protection. Sci Rep 7:11456.
Finegold SM, Downes J, Summanen PH (2012) Microbiology of regressive autism. Anaerobe 18:260-262.
Ho L, Ono K, Tsuji M, Mazzola P, Singh R, Pasinetti GM (2018) Protective roles of intestinal microbiota derived short chain fatty acids in Alzheimer's disease-type beta-amyloid neuropathological mechanisms. Expert Rev Neurother 18:83-90.
Jiang HY, Zhang X, Yu ZH, Zhang Z, Deng M, Zhao JH, Ruan B (2018) Altered gut microbiota profile in patients with generalized anxiety disorder. J Psychiatr Res 104:130-136.
Laranjinha J, Santos RM, Lourenco CF, Ledo A, Barbosa RM (2012) Nitric oxide signaling in the brain: translation of dynamics into respiration control and neurovascular coupling. Ann N Y Acad Sci 1259:10-18.
Lyte M (2011) Probiotics function mechanistically as delivery vehicles for neuroactive compounds: Microbial endocrinology in the design and use of probiotics. Bioessays 33:574-581.
Marin L, Miguelez EM, Villar CJ, Lombo F (2015) Bioavailability of dietary polyphenols and gut microbiota metabolism: antimicrobial properties. Biomed Res Int 2015:905215.
Nagahara AH, Tuszynski MH (2011) Potential therapeutic uses of BDNF in neurological and psychiatric disorders. Nat Rev Drug Discov 10:209-219.
Nzakizwanayo J, Dedi C, Standen G, Macfarlane WM, Patel BA, Jones BV (2015) Escherichia coli Nissle 1917 enhances bioavailability of serotonin in gut tissues through modulation of synthesis and clearance. Sci Rep 5:17324.
O'Mahony SM, Clarke G, Borre YE, Dinan TG, Cryan JF (2015) Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. Behav Brain Res 277:32-48.
O'Sullivan E, Barrett E, Grenham S, Fitzgerald P, Stanton C, Ross RP, Quigley EM, Cryan JF, Dinan TG (2011) BDNF expression in the hippocampus of maternally separated rats: does Bifidobacterium breve 6330 alter BDNF levels? Benef Microbes 2:199-207.
Pandey KB, Rizvi SI (2009) Plant polyphenols as dietary antioxidants in human health and disease. Oxid Med Cell Longev 2:270-278.
Petschow B, Dore J, Hibberd P, Dinan T, Reid G, Blaser M, Cani PD, Degnan FH, Foster J, Gibson G, Hutton J, Klaenhammer TR, Ley R, Nieuwdorp M, Pot B, Relman D, Serazin A, Sanders ME (2013) Probiotics, prebiotics, and the host microbiome: the science of translation. Ann N Y Acad Sci 1306:1-17.
Rastmanesh R (2011) High polyphenol, low probiotic diet for weight loss because of intestinal microbiota interaction. Chem Biol Interact 189:1-8.
Rieder R, Wisniewski PJ, Alderman BL, Campbell SC (2017) Microbes and mental health: A review. Brain Behav Immun 66:9-17.
Sampson TR, Debelius JW, Thron T, Janssen S, Shastri GG, Ilhan ZE, Challis C, Schretter CE, Rocha S, Gradinaru V, Chesselet MF, Keshavarzian A, Shannon KM, Krajmalnik-Brown R, Wittung-Stafshede P, Knight R, Mazmanian SK (2016) Gut microbiota regulate motor deficits and neuroinflammation in a model of Parkinson's disease. Cell 167:1469-1480. e1412.
Savignac HM, Corona G, Mills H, Chen L, Spencer JP, Tzortzis G, Burnet PW (2013) Prebiotic feeding elevates central brain derived neurotrophic factor, N-methyl-D-aspartate receptor subunits and D-serine. Neurochem Int 63:756-764.
Scheperjans F, Aho V, Pereira PA, Koskinen K, Paulin L, Pekkonen E, Haapaniemi E, Kaakkola S, Eerola-Rautio J, Pohja M, Kinnunen E, Murros K, Auvinen P (2015) Gut microbiota are related to Parkinson's disease and clinical phenotype. Mov Disord 30:350-358.
Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, Kubo C, Koga Y (2004) Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. J Physiol 558:263-275.
Sun X, Meng X, Zhang J, Li Y, Wang L, Qin X, Sui N, Zhang Y (2012) GABA attenuates amyloid toxicity by downregulating its endocytosis and improves cognitive impairment. J Alzheimers Dis 31:635-649.
Szapacs ME, Mathews TA, Tessarollo L, Ernest Lyons W, Mamounas LA, Andrews AM (2004) Exploring the relationship between serotonin and brain-derived neurotrophic factor: analysis of BDNF protein and extraneuronal 5-HT in mice with reduced serotonin transporter or BDNF expression. J Neurosci Methods 140:81-92.
Vogt NM, Kerby RL, Dill-McFarland KA, Harding SJ, Merluzzi AP, Johnson SC, Carlsson CM, Asthana S, Zetterberg H, Blennow K, Bendlin BB, Rey FE (2017) Gut microbiome alterations in Alzheimer's disease. Sci Rep 7:13537.
Youdim KA, Shukitt-Hale B, Joseph JA (2004) Flavonoids and the brain: Interactions at the blood-brain barrier and their physiological effects on the central nervous system. Free Radical Bio Med 37:1683-1693.
Yunes RA, Poluektova EU, Dyachkova MS, Klimina KM, Kovtun AS, Averina OV, Orlova VS, Danilenko VN (2016) GABA production and structure of gadB/gadC genes in Lactobacillus and Bifidobacterium strains from human microbiota. Anaerobe 42:197-204.
Zhang PY (2015) Polyphenols in health and disease. Cell Biochem Biophys 73:649-664.
Zhu X, Han Y, Du J, Liu R, Jin K, Yi W (2017) Microbiota-gut-brain axis and the central nervous system. Oncotarget 8:53829-53838.

C-Editor: Zhao M, Yu J; T-Editor: Liu XL

Send Orders for Reprints to reprints@benthamscience.ae

Current Drug Metabolism, 2018, *19*, 478-489

REVIEW ARTICLE

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

Francesco Di Meo[1], Stella Donato[1,2], Alba Di Pardo[2], Vittorio Maglione[2], Stefania Filosa[1,2,*] and Stefania Crispi[1,*]

[1]*Institute of Biosciences and BioResources-UOS Naples CNR, via P. Castellino, 111- 80131 Naples, Italy;* [2]*IRCCS Neuromed, Pozzilli (IS), Italy*

ARTICLE HISTORY

Received: August 28, 2017
Revised: December 15, 2017
Accepted: February 18, 2018

DOI:
10.2174/1389200219666180404094147

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.

Method: 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].

*Address correspondence to these authors at the Institute of Biosciences and BioResources, UOS Naples, CNR via P. Castellino, 111- 80131 Naples Italy; Tel: +39081 6132717; E-mail: stefania.filosa@cnr.it
Gene Expression & Molecular Genetics Laboratory, Institute of Biosciences and BioResources, UOS Naples, CNR via P. Castellino, 111- 80131 Naples Italy; Tel: +39081 6132622; E-mail: stefania.crispi@ibbr.cnr.it

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].

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

Current Drug Metabolism

 © 2018 Bentham Science Publishers

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.

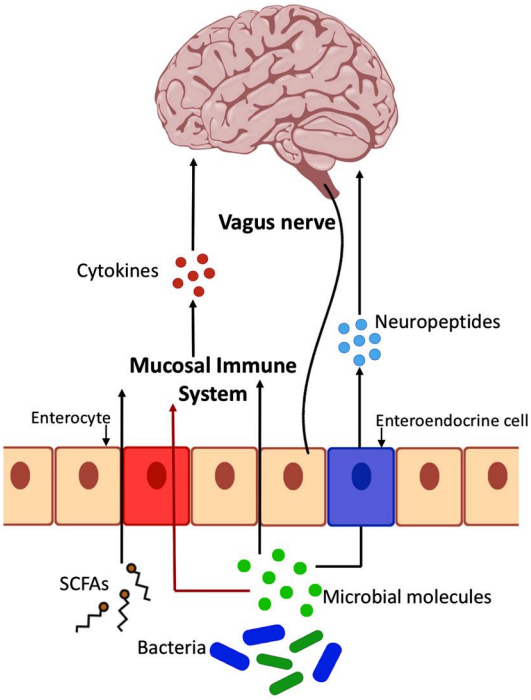

Fig. (1). Schematic representation of the microbiota-gut-brain axis. Microbial metabolites can influence brain and host behaviour. The connection between gut microbiota and CNS is mediated by vagus nerve.

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	

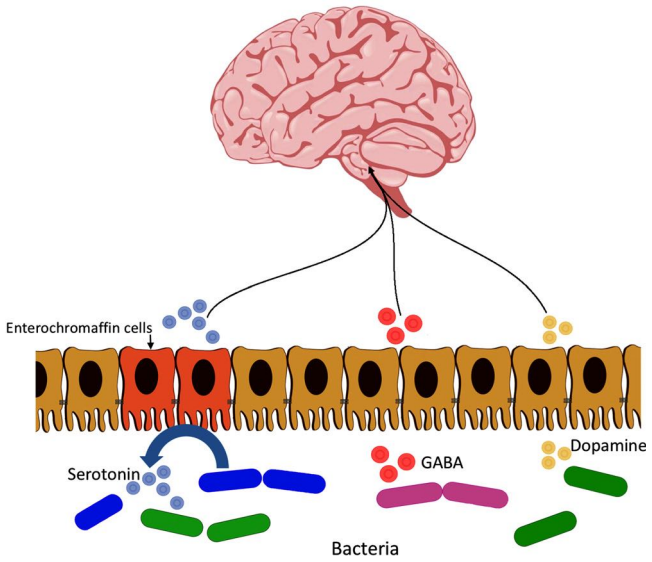

Fig. (2). Schematic representation of the neurotransmitters produced by gut microbiota. Microbiota itself can release neurotransmitters that pass the intestinal barrier and reach the brain.

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-β (Aβ) peptides as Aβ 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 Aβ 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-β and α-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 hy-

perpermeability 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 α-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.

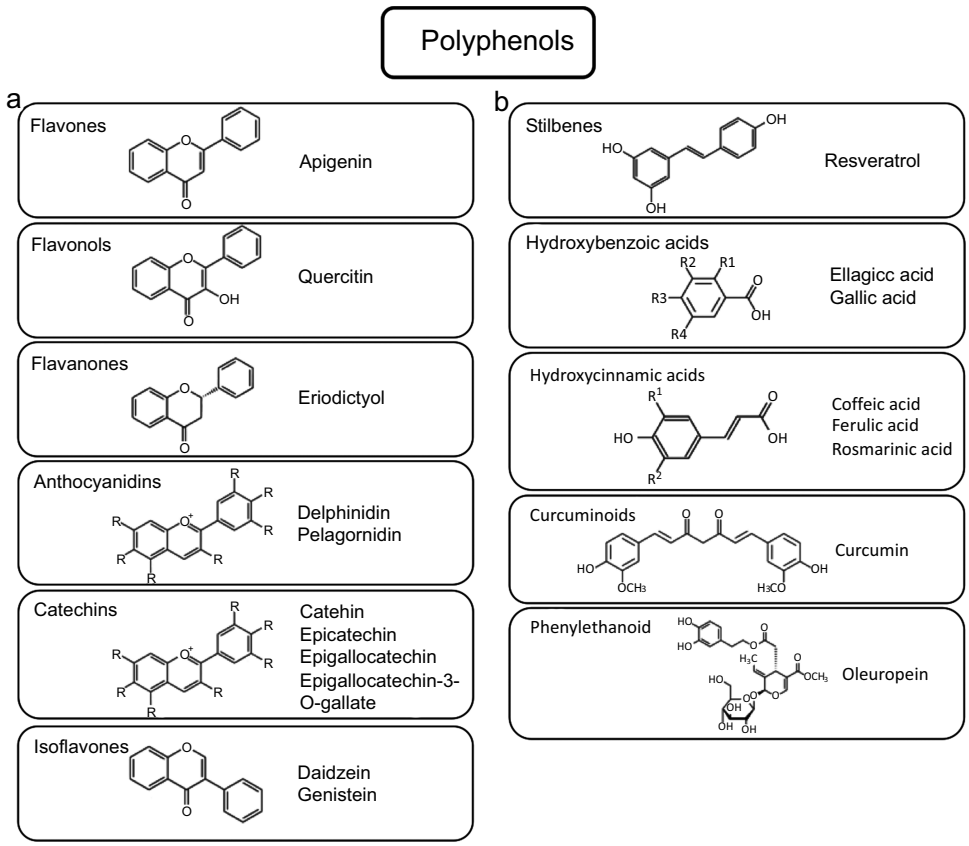

Fig. (3). Structures of polyphenols. Polyphenols are classified by the number of phenol rings in flavonoids (**a**) and non-flavonoids (**b**). The figure shows chemical structures and some typical representatives.

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.

1.6. 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

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

majority 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.

ACKNOWLEDGEMENTS

We thank Dr. Mariarosaria Aletta for bibliographic support and Dr. Maria Rosaria Chiummo for help in editing the manuscript. This work was supported by PONPE_00060_7, PONPE_00060_3, PON03PE_00106_1 and by Italian Ministry of Health "Ricerca Corrente".

REFERENCES

[1] Li, M.; Gu, D.; Xu, N.; Lei, F.; Du, L.; Zhang, Y.; Xie, W. Gut carbohydrate metabolism instead of fat metabolism regulated by gut microbes mediates high-fat diet-induced obesity. *Benef. Microbes.,* **2014**, *5*, 335-344.

[2] Turnbaugh, P. J.; Hamady, M.; Yatsunenko, T.; Cantarel, B. L.; Duncan, A.; Ley, R. E.; Sogin, M. L.; Jones, W. J.; Roe, B. A.; Affourtit, J. P.; Egholm, M.; Henrissat, B.; Heath, A. C.; Knight, R.; Gordon, J. I. A core gut microbiome in obese and lean twins. *Nature,* **2009**, *457*, 480-484.

[3] Arnold, J. W.; Roach, J.; Azcarate-Peril, M. A. Emerging technologies for gut microbiome research. *Trends Microbiol.,* **2016**, *24*, 887-901.

[4] Clemente, J. C.; Ursell, L. K.; Parfrey, L. W.; Knight, R. The impact of the gut microbiota on human health: An integrative view. *Cell,* **2012**, *148*, 1258-1270.

[5] Spor, A.; Koren, O.; Ley, R. Unravelling the effects of the environment and host genotype on the gut microbiome. *Nat. Rev. Microbiol.,* **2011**, *9*, 279-290.

[6] Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. *Nature,* **2012**, *486*, 207-214.

[7] Petersen, C.; Round, J. L. Defining dysbiosis and its influence on host immunity and disease. *Cell Microbiol.,* **2014**, *16*, 1024-1033.

[8] Larraufie, P.; de Wouters, T.; Potocki-Veronese, G.; Blottiere, H. M.; Dore, J. Functional metagenomics to decipher food-microbe-host crosstalk. *Proc. Nutr. Soc.,* **2015**, *74*, 1-4.

[9] Schroeckh, V.; Scherlach, K.; Nutzmann, H. W.; Shelest, E.; Schmidt-Heck, W.; Schuemann, J.; Martin, K.; Hertweck, C.; Brakhage, A. A. Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus Nidulans*. *Proc. Natl. Acad. Sci. USA,* **2009**, *106*, 14558-14563.

[10] Tralau, T.; Sowada, J.; Luch, A. Insights on the human microbiome and its xenobiotic metabolism: What is known about its effects on human physiology? *Expert. Opin. Drug. Metab. Toxicol.,* **2015**, *11*, 411-425.

[11] Bercik, P.; Verdu, E. F.; Foster, J. A.; Macri, J.; Potter, M.; Huang, X. X.; Malinowski, P.; Jackson, W.; Blennerhassett, P.; Neufeld, K. A.; Lu, J.; Khan, W. I.; Corthesy-Theulaz, I.; Cherbut, C.; Bergonzelli, G. E.; Collins, S. M. Chronic gastrointestinal inflammation induces anxiety-like behavior and alters central nervous system biochemistry in mice. *Gastroenterology,* **2010**, *139*, 2102-2409.

[12] Mayer, E. A. Gut feelings: The emerging biology of gut-brain communication. *Nat. Rev. Neurosci.,* **2011**, *12*, 453-466.

[13] Goldstein, A. M.; Hofstra, R. M.; Burns, A. J. Building a brain in the gut: Development of the enteric nervous system. *Clin. Genet.,* **2013**, *83*, 307-316.

[14] Laperuta, C.; Spizzichino, L.; D'Adamo, P.; Monfregola, J.; Maiorino, A.; D'Eustacchio, A.; Ventruto, V.; Neri, G.; D'Urso, M.; Chiurazzi, P.; Ursini, M. V.; Miano, M. G. MRX87 family with Aristaless X dup24bp mutation and implication for polyAlanine expansions. *BMC Med. Genet.,* **2007**, *8*, 25.

[15] Poeta, L.; Fusco, F.; Drongitis, D.; Shoubridge, C.; Manganelli, G.; Filosa, S.; Paciolla, M.; Courtney, M.; Collombat, P.; Lioi, M. B.; Gecz, J.; Ursini, M. V.; Miano, M. G. A regulatory path associated with X-linked intellectual disability and epilepsy links KDM5C to the polyalanine expansions in ARX. *Am. J. Hum. Genet.,* **2013**, *92*, 114-125.

[16] Coman, D.; Fullston, T.; Shoubridge, C.; Leventer, R.; Wong, F.; Nazaretian, S.; Simpson, I.; Gecz, J.; McGillivray, G. X-linked lissencephaly with absent corpus callosum and abnormal genitalia: An evolving multisystem syndrome with severe congenital intestinal diarrhea disease. *Child Neurol. Open,* **2017**, *4*, 2329048.

[17] Gargiulo, A.; Auricchio, R.; Barone, M. V.; Cotugno, G.; Reardon, W.; Milla, P. J.; Ballabio, A.; Ciccodicola, A.; Auricchio, A. Filamin A is mutated in X-linked chronic idiopathic intestinal pseudo-obstruction with central nervous system involvement. *Am. J. Hum. Genet.,* **2007**, *80*, 751-758.

[18] Cryan, J. F.; Dinan, T. G. Mind-altering microorganisms: The impact of the gut microbiota on brain and behaviour. *Nat. Rev. Neurosci.,* **2012**, *13*, 701-712.

[19] Forsythe, P.; Bienenstock, J.; Kunze, W. A. Vagal pathways for microbiome-brain-gut axis communication. *Adv. Exp. Med. Biol.,* **2014**, *817*, 115-133.

[20] Bravo, J. A.; Forsythe, P.; Chew, M. V.; Escaravage, E.; Savignac, H. M.; Dinan, T. G.; Bienenstock, J.; Cryan, J. F. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc. Natl. Acad. Sci. USA,* **2011**, *108*, 16050-16055.

[21] O'Mahony, S. M.; Clarke, G.; Borre, Y. E.; Dinan, T. G.; Cryan, J. F. Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. *Behav. Brain. Res.,* **2015**, *277*, 32-48.

[22] Barrett, E.; Ross, R. P.; O'Toole, P. W.; Fitzgerald, G. F.; Stanton, C. gamma-Aminobutyric acid production by culturable bacteria from the human intestine. *J. Appl. Microbiol.,* **2012**, *113*, 411-417.

[23] Reigstad, C. S.; Salmonson, C. E.; Rainey, J. F.; Szurszewski, J. H.; Linden, D. R.; Sonnenburg, J. L.; Farrugia, G.; Kashyap, P. C. Gut microbes promote colonic serotonin production through an effect of short-chain fatty acids on enterochromaffin cells. *Faseb J.,* **2015**, *29*, 1395-1403.

[24] Savignac, H. M.; Corona, G.; Mills, H.; Chen, L.; Spencer, J. P.; Tzortzis, G.; Burnet, P. W. Prebiotic feeding elevates central brain derived neurotrophic factor, N-methyl-D-aspartate receptor subunits and D-serine. *Neurochem. Int.,* **2013**, *63*, 756-764.

[25] Tillisch, K.; Labus, J.; Kilpatrick, L.; Jiang, Z.; Stains, J.; Ebrat, B.; Guyonnet, D.; Legrain-Raspaud, S.; Trotin, B.; Naliboff, B.; Mayer, E. A. Consumption of fermented milk product with probiotic modulates brain activity. *Gastroenterology,* **2013**, *144*, 1394-1401.

[26] Brglez Mojzer, E.; Knez Hrncic, M.; Skerget, M.; Knez, Z.; Bren, U. Polyphenols: Extraction methods, antioxidative action, bioavailability and anticarcinogenic effects. *Molecules,* **2016**, *21*(7), pii: E901.

[27] Scalbert, A.; Morand, C.; Manach, C.; Remesy, C. Absorption and metabolism of polyphenols in the gut and impact on health. *Biomed. Pharmacother.,* **2002**, *56*, 276-282.

[28] Sanchez-Carbente, M. R.; Castro-Obregon, S.; Covarrubias, L.; Narvaez, V. Motoneuronal death during spinal cord development is mediated by oxidative stress. *Cell Death Differ.,* **2005**, *12*, 279-291.

[29] Hugon, P.; Lagier, J. C.; Colson, P.; Bittar, F.; Raoult, D. Repertoire of human gut microbes. *Microb. Pathog.,* **2017**, *106*, 103-112.

[30] Bengmark, S. Gut microbiota, immune development and function. *Pharmacol. Res.,* **2013**, *69*, 87-113.

[31] Messaoudi, M.; Lalonde, R.; Violle, N.; Javelot, H.; Desor, D.; Nejdi, A.; Bisson, J. F.; Rougeot, C.; Pichelin, M.; Cazaubiel, M.; Cazaubiel, J. M. Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects. *Brit. J. Nutr.,* **2011**, *105*, 755-764.

[32] Desbonnet, L.; Garrett, L.; Clarke, G.; Kiely, B.; Cryan, J. F.; Dinan, T. G. Effects of the probiotic *Bifidobacterium infantis* in the maternal separation model of depression. *Neuroscience,* **2010**, *170*, 1179-1188.

[33] Iyer, L. M.; Aravind, L.; Coon, S. L.; Klein, D. C.; Koonin, E. V. Evolution of cell-cell signaling in animals: Did late horizontal gene transfer from bacteria have a role? *Trends Genet.,* **2004**, *20*, 292-299.

[34] Lyte, M. Probiotics function mechanistically as delivery vehicles for neuroactive compounds: Microbial endocrinology in the design and use of probiotics. *Bioessays,* **2011**, *33*, 574-581.

[35] Lyte, M. Microbial endocrinology in the microbiome-gut-brain axis: How bacterial production and utilization of neurochemicals influence behavior. *PLoS Pathog.* **2013**, *9*(11): e1003726.

[36] Ozogul, F. Production of biogenic amines by *Morganella morganii, Klebsiella pneumoniae* and *Hafnia alvei* using a rapid HPLC method. *Eur. Food Res. Technol.,* **2004**, *219*, 465-469.

[37] Tiso, M.; Schechter, A. N. Nitrate reduction to nitrite, nitric oxide and ammonia by gut bacteria under physiological conditions. *Plos One,* **2015**, *10*(5): e0127490

[38] Grenham, S.; Clarke, G.; Cryan, J. F.; Dinan, T. G. Brain-gut-microbe communication in health and disease. *Front Physiol.,* **2011**, *2*, 94.

[39] Schwarcz, R.; Bruno, J. P.; Muchowski, P. J.; Wu, H. Q. Kynurenines in the mammalian brain: when physiology meets pathology. *Nat. Rev. Neurosci.,* **2012**, *13*, 465-477.

[40] Cryan, J. F.; Leonard, B. E. 5-HT1A and beyond: The role of serotonin and its receptors in depression and the antidepressant response. *Hum. Psychopharm. Clin.,* **2000**, *15*, 113-135.

[41] Tack, J.; Broekaert, D.; Fischler, B.; Van Oudenhove, L.; Gevers, A. M.; Janssens, J. A controlled crossover study of the selective serotonin reuptake inhibitor citalopram in irritable bowel syndrome. *Gut,* **2006**, *55*, 1095-1103.

[42] Clarke, G.; Grenham, S.; Scully, P.; Fitzgerald, P.; Moloney, R. D.; Shanahan, F.; Dinan, T. G.; Cryan, J. F. The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol. Psychiatr.,* **2013**, *18*, 666-673.

[43] Stone, T. W.; Stoy, N.; Darlington, L. G. An expanding range of targets for kynurenine metabolites of tryptophan. *Trends Pharmacol. Sci.,* **2013**, *34*, 136-143.

[44] Moloney, G. M.; O'Leary, O. F.; Salvo-Romero, E.; Desbonnet, L.; Shanahan, F.; Dinan, T. G.; Clarke, G.; Cryan, J. F. Microbial regulation of hippocampal miRNA expression: Implications for transcription of kynurenine pathway enzymes. *Behav. Brain Res.,* **2017**, *334*, 50-54.

[45] Higuchi, T.; Hayashi, H.; Abe, K. Exchange of glutamate and gamma-aminobutyrate in a Lactobacillus strain. *J Bacteriol.,* **1997**, *179*, 3362-3364.

[46] Guthrie, G. D.; Nicholson-Guthrie, C. S. gamma-Aminobutyric acid uptake by a bacterial system with neurotransmitter binding characteristics. *Proc. Natl. Acad. Sci. USA,* **1989**, *86*, 7378-7381.

[47] Nakajima, K.; Tooyama, I.; Kuriyama, K.; Kimura, H. Immunohistochemical demonstration of GABAB receptors in the rat gastrointestinal tract. *Neurochem. Res.,* **1996**, *21*, 211-215.

[48] Hughes, D. T.; Sperandio, V. Inter-kingdom signalling: communication between bacteria and their hosts. *Nat. Rev. Microbiol.,* **2008**, *6*, 111-120.

[49] Macfarlane, G. T.; Macfarlane, S. Bacteria, colonic fermentation, and gastrointestinal health. *J AOAC Int.,* **2012**, *95*, 50-60.

[50] Ganapathy, V.; Thangaraju, M.; Prasad, P. D.; Martin, P. M.; Singh, N. Transporters and receptors for short-chain fatty acids as the molecular link between colonic bacteria and the host. *Curr. Opin. Pharmacol.,* **2013**, *13*, 869-874.

[51] Mawe, G. M.; Hoffman, J. M. Serotonin signalling in the gut-functions, dysfunctions and therapeutic targets. *Nat. Rev. Gastroenterol. Hepatol.,* **2013**, *10*, 473-486.

[52] Holzer, P.; Reichmann, F.; Farzi, A. Neuropeptide Y, peptide YY and pancreatic polypeptide in the gut-brain axis. *Neuropeptides,* **2012**, *46*, 261-274.

[53] Vijay, N.; Morris, M. E. Role of monocarboxylate transporters in drug delivery to the brain. *Curr. Pharm. Design.,* **2014**, *20*, 1487-1498.

[54] Schroeder, F. A.; Lin, C. L.; Crusio, W. E.; Akbarian, S. Antidepressant-like effects of the histone deacetylase inhibitor, sodium butyrate, in the mouse. *Biol. Psychiat.,* **2007**, *62*, 55-64.

[55] Ley, R. E.; Hamady, M.; Lozupone, C.; Turnbaugh, P. J.; Ramey, R. R.; Bircher, J. S.; Schlegel, M. L.; Tucker, T. A.; Schrenzel, M. D.; Knight, R.; Gordon, J. I. Evolution of mammals and their gut microbes. *Science,* **2008**, *320*, 1647-1651.

[56] Ilieva, H.; Maragakis, N. J. Motoneuron disease: Basic science. *Adv. Neurobiol.,* **2017**, *15*, 163-190.

[57] Haass, C.; Selkoe, D. J. Soluble protein oligomers in neurodegeneration: Lessons from the Alzheimer's amyloid beta-peptide. *Nat. Rev. Mol. Cell Biol.,* **2007**, *8*, 101-112.

[58] Walker, F. O. Huntington's Disease. *Semin. Neurol.,* **2007**, *27*, 143-150.

[59] Goldman, J. G.; Postuma, R. Premotor and nonmotor features of Parkinson's disease. *Curr. Opin. Neurol.,* **2014**, *27*, 434-441.

[60] Biagi, E.; Nylund, L.; Candela, M.; Ostan, R.; Bucci, L.; Pini, E.; Nikkila, J.; Monti, D.; Satokari, R.; Franceschi, C.; Brigidi, P.; De Vos, W. Through ageing, and beyond: Gut microbiota and inflammatory status in seniors and centenarians. *PLoS One,* **2010**, *5*(5), e10667.

[61] Claesson, M. J.; Cusack, S.; O'Sullivan, O.; Greene-Diniz, R.; de Weerd, H.; Flannery, E.; Marchesi, J. R.; Falush, D.; Dinan, T.; Fitzgerald, G.; Stanton, C.; van Sinderen, D.; O'Connor, M.; Harnedy, N.; O'Connor, K.; Henry, C.; O'Mahony, D.; Fitzgerald, A. P.; Shanahan, F.; Twomey, C.; Hill, C.; Ross, R. P.; O'Toole, P. W. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. *Proc. Natl. Acad. Sci. USA,* **2011**, *108* (Suppl 1), 4586-4591.

[62] Brown, R. H.; Al-Chalabi, A. Amyotrophic lateral sclerosis. *N. Engl. J. Med.,* **2017**, *377*, 162-172.

[63] Toepfer, M.; Folwaczny, C.; Klauser, A.; Riepl, R. L.; Muller-Felber, W.; Pongratz, D. Gastrointestinal dysfunction in amyotrophic lateral sclerosis. *Amyotroph. Lateral Scler. Other Motor Neuron. Disord.,* **1999**, *1*, 15-19.

[64] Wu, S.; Yi, J.; Zhang, Y. G.; Zhou, J.; Sun, J. Leaky intestine and impaired microbiome in an amyotrophic lateral sclerosis mouse model. *Physiol. Rep.,* **2015**, *3*(4), pii: e12356.

[65] Zhang, Y. G.; Wu, S. P.; Yi, J. X.; Xia, Y. L.; Jin, D. P.; Zhou, J. S.; Sun, J. Target Intestinal Microbiota to Alleviate Disease Progression in Amyotrophic Lateral Sclerosis. *Clin. Ther.,* **2017**, *39*, 322-336.

[66] Zhang, R.; Miller, R. G.; Gascon, R.; Champion, S.; Katz, J.; Lancero, M.; Narvaez, A.; Honrada, R.; Ruvalcaba, D.; McGrath, M. S. Circulating endotoxin and systemic immune activation in sporadic amyotrophic lateral sclerosis (sALS). *J. Neuroimmunol.,* **2009**, *206*, 121-124.

[67] Andrieu, S.; Coley, N.; Lovestone, S.; Aisen, P. S.; Vellas, B. Prevention of sporadic Alzheimer's disease: lessons learned from clinical trials and future directions. *Lancet Neurol.,* **2015**, *14*, 926-944.

[68] Querfurth, H. W.; LaFerla, F. M. Alzheimer's disease. *N. Engl. J. Med.,* **2010**, *362*, 329-344.

[69] Selkoe, D. J. Alzheimer's disease: Genes, proteins, and therapy. *Physiol. Rev.,* **2001**, *81*, 741-766.

[70] Clarke, J. R.; Silva, N. M. L. E.; Figueiredo, C. P.; Frozza, R. L.; Ledo, J. H.; Beckman, D.; Katashima, C. K.; Razolli, D.; Carvalho, B. M.; Frazao, R.; Silveira, M. A.; Ribeiro, F. C.; Bomfim, T. R.; Neves, F. S.; Klein, W. L.; Medeiros, R.; LaFerla, F. M.; Carvalheira, J. B.; Saad, M. J.; Munoz, D. P.; Velloso, L. A.; Ferreira, S. T.; De Felice, F. G. Alzheimer-associated A beta oligomers impact the central nervous system to induce peripheral metabolic deregulation. *Embo. Mol. Med.,* **2015**, *7*, 190-210.

[71] Karri, S.; Acosta-Martinez, V.; Coimbatore, G. Effect of dihydrotestosterone on gastrointestinal tract of male Alzheimer's disease transgenic mice. *Indian J. Exp. Biol.,* **2010**, *48*, 453-465.

[72] Harach, T.; Marungruang, N.; Duthilleul, N.; Cheatham, V.; Mc Coy, K. D.; Frisoni, G.; Neher, J. J.; Fak, F.; Jucker, M.; Lasser, T.; Bolmont, T. Reduction of Abeta amyloid pathology in APPPS1 transgenic mice in the absence of gut microbiota. *Sci. Rep.,* **2017**, *7*, 41802.

[73] Brandscheid, C.; Schuck, F.; Reinhardt, S.; Schafer, K. H.; Pietrzik, C. U.; Grimm, M.; Hartmann, T.; Schwiertz, A.; Endres, K. Altered gut microbiome composition and tryptic activity of the 5xFAD Alzheimer's mouse model. *J. Alzheimers Dis.,* **2017**, *56*, 775-788.

[74] Wu, J. H.; Guo, Z. C.; Kumar, S.; Lapuerta, P. Incidence of serious upper and lower gastrointestinal events in older adults with and without Alzheimer's disease. *J. Am. Geriatr. Soc.,* **2011**, *59*, 2053-2061.

[75] Friedland, R. P. Mechanisms of molecular mimicry involving the microbiota in neurodegeneration. *J. Alzheimers Dis.,* **2015**, *45*, 349-362.

[76] Mielcarek, M. Huntington's disease is a multi-system disorder. *Rare Dis.,* **2015**, *3*(1), e1058464.

[77] Strong, T. V.; Tagle, D. A.; Valdes, J. M.; Elmer, L. W.; Boehm, K.; Swaroop, M.; Kaatz, K. W.; Collins, F. S.; Albin, R. L. Widespread expression of the human and rat Huntingtons-disease gene in brain and nonneural tissues. *Nat. Genet.,* **1993**, *5*, 259-265.

[78] Sciacca, S.; Favellato, M.; Madonna, M.; Metro, D.; Marano, M.; Squitieri, F. Early enteric neuron dysfunction in mouse and human Huntington disease. *Parkinsonism Relat. Disord.,* **2017**, *34*, 73-74.

[79] van der Burg, J. M. M.; Winqvist, A.; Aziz, N. A.; Maat-Schieman, M. L. C.; Roos, R. A. C.; Bates, G. P.; Brundin, P.; Bjorkqvist, M.; Wierup, N. Gastrointestinal dysfunction contributes to weight loss in Huntington's disease mice. *Neurobiol. Dis.,* **2011**, *44*, 1-8.

[80] Rosas, H. D.; Doros, G.; Bhasin, S.; Thomas, B.; Gevorkian, S.; Malarick, K.; Matson, W.; Hersch, S. M. A systems-level "misunderstanding": The plasma metabolome in Huntington's disease. *Ann. Clin. Transl. Neur.,* **2015**, *2*, 756-768.

[81] Beitz, J. M. Parkinson's disease: A review. *Front Biosci (Schol. Ed),* **2014**, *6*, 65-74.

[82] Poirier, A. A.; Aube, B.; Cote, M.; Morin, N.; Di Paolo, T.; Soulet, D. Gastrointestinal dysfunctions in Parkinson's disease: Symptoms and treatments. *Parkinsons Dis.,* **2016**, 2016, 6762528.

[83] Sampson, T. R.; Debelius, J. W.; Thron, T.; Janssen, S.; Shastri, G. G.; Ilhan, Z. E.; Challis, C.; Schretter, C. E.; Rocha, S.; Gradinaru, V.; Chesselet, M. F.; Keshavarzian, A.; Shannon, K. M.; Krajmalnik-Brown, R.; Wittung-Stafshede, P.; Knight, R.; Mazmanian, S. K. Gut microbiota regulate motor deficits and neuroinflammation in a model of Parkinson's disease. *Cell,* **2016**, *167*, 1469-1480.

[84] Parashar, A.; Udayabanu, M. Gut microbiota: Implications in Parkinson's disease. *Parkinsonism Relat Disord.,* **2017**, *38*, 1-7.

[85] Scheperjans, F.; Aho, V.; Pereira, P. A. B.; Koskinen, K.; Paulin, L.; Pekkonen, E.; Haapaniemi, E.; Kaakkola, S.; Eerola-Rautio, J.; Pohja, M.; Kinnunen, E.; Murros, K.; Auvinen, P. Gut microbiota are related to Parkinson's disease and clinical phenotype. *Movement Disord.,* **2015**, *30*, 350-358.

[86] Keshavarzian, A.; Green, S. J.; Engen, P. A.; Voigt, R. M.; Naqib, A.; Forsyth, C. B.; Mutlu, E.; Shannon, K. M. Colonic Bacterial

Composition in Parkinson's Disease. *Movement Disord.,* **2015**, *30*, 1351-1360.

[87] Wu, X. F.; Chen, P. S.; Dallas, S.; Wilson, B.; Block, M. L.; Wang, C. C.; Kinyamu, H.; Lu, N.; Gao, X.; Leng, Y.; Chuang, D. M.; Zhang, W. Q.; Lu, R. B.; Hong, J. S. Histone deacetylase inhibitors up-regulate astrocyte GDNF and BDNF gene transcription and protect dopaminergic neurons. *Int. J. Neuropsychoph.,* **2008**, *11*, 1123-1134.

[88] Guseo, A. The Parkinson puzzle. *Orv. Hetil.,* **2012**, *153*, 2060-2069.

[89] Forsythe, P.; Sudo, N.; Dinan, T.; Taylor, V. H.; Bienenstock, J. Mood and gut feelings. *Brain Behav. Immun.,* **2010**, *24*, 9-16.

[90] O'Mahony, L.; McCarthy, J.; Kelly, P.; Hurley, G.; Luo, F. Y.; Chen, K. S.; O'Sullivan, G. C.; Kiely, B.; Collins, J. K.; Shanahan, F.; Quigley, E. M. M. Lactobacillus and Bifidobacterium in irritable bowel syndrome: Symptom responses and relationship to cytokine profiles. *Gastroenterology,* **2005**, *128*, 541-551.

[91] O'Mahony, S. M.; Hyland, N. P.; Dinan, T. G.; Cryan, J. F. Maternal separation as a model of brain-gut axis dysfunction. *Psychopharmacology,* **2011**, *214*, 71-88.

[92] Hsiao, E. Y.; McBride, S. W.; Hsien, S.; Sharon, G.; Hyde, E. R.; McCue, T.; Codelli, J. A.; Chow, J.; Reisman, S. E.; Petrosino, J. F.; Patterson, P. H.; Mazmanian, S. K. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell,* **2013**, *155*, 1451-1463.

[93] Gareau, M. G.; Wine, E.; Rodrigues, D. M.; Cho, J. H.; Whary, M. T.; Philpott, D. J.; MacQueen, G.; Sherman, P. M. Bacterial infection causes stress-induced memory dysfunction in mice. *Gut,* **2011**, *60*, 307-317.

[94] Benton, D.; Williams, C.; Brown, A. Impact of consuming a milk drink containing a probiotic on mood and cognition. *Eur. J. Clin. Nutr.,* **2007**, *61*, 355-361.

[95] Gibson, G. R.; Roberfroid, M. B. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr.,* **1995**, *125*, 1401-1412.

[96] Petschow, B.; Dore, J.; Hibberd, P.; Dinan, T.; Reid, G.; Blaser, M.; Cani, P. D.; Degnan, F. H.; Foster, J.; Gibson, G.; Hutton, J.; Klaenhammer, T. R.; Ley, R.; Nieuwdorp, M.; Pot, B.; Relman, D.; Serazin, A.; Sanders, M. E. Probiotics, prebiotics, and the host microbiome: the science of translation. *Ann. NY Acad. Sci.,* **2013**, *1306*, 1-17.

[97] Sudo, N.; Chida, Y.; Aiba, Y.; Sonoda, J.; Oyama, N.; Yu, X. N.; Kubo, C.; Koga, Y. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *J. Physiol.,* **2004**, *558*, 263-275.

[98] Alherz, F.; Alherz, M.; Almusawi, H. NMDAR hypofunction and somatostatin-expressing GABAergic interneurons and receptors: A newly identified correlation and its effects in schizophrenia. *Schizophr. Res. Cogn.,* **2017**, *8*, 1-6.

[99] Mannie, Z. N.; Harmer, C. J.; Cowen, P. J. Increased waking salivary cortisol levels in young people at familial risk of depression. *Am. J. Psychiat.,* **2007**, *164*, 617-621.

[100] Nelson, E. D.; Ramberg, J. E.; Best, T.; Sinnott, R. A. Neurologic effects of exogenous saccharides: A review of controlled human, animal, and *in vitro* studies. *Nutr. Neurosci.,* **2012**, *15*, 149-162.

[101] Smriga, M.; Chen, J.; Zhang, J.T.; Narui, T.; Shibata, S.; Hirano, E. Isolichenan, an alpha-glucan isolated from lichen *Cetrariella islandica*, repairs impaired learning behaviors and facilitates hippocampal synaptic plasticity. *Proc. Jpn. Acad., Ser. B,* **1999**, *75,* 219-223.

[102] Dinan, T. G.; Stanton, C.; Cryan, J. F. Psychobiotics: A novel class of psychotropic. *Biol. Psychiatry.,* **2013**, *74*, 720-726.

[103] Kumar, S.; Pandey, A. K. Chemistry and biological activities of flavonoids: An overview. *ScientificWorldJournal,* **2013**, *2013*, 162750.

[104] Bravo, L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.,* **1998**, *56*, 317-333.

[105] Pasinetti, G. Lifestyle factors and Alzheimer's disease: Experimental approaches and clinical implication. *Alzheimers Dement.,* **2007**, *3*, S198.

[106] Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.,* **2000**, *130*, 2073S-2085S.

[107] Kitagawa, S. Inhibitory effects of polyphenols on p-glycoprotein-mediated transport. *Biol. Pharm. Bull.,* **2006**, *29*, 1-6.

[108] Campos-Bedolla, P.; Walter, F. R.; Veszelka, S.; Deli, M. A. Role of the blood-brain barrier in the nutrition of the central nervous system. *Arch. Med. Res.,* **2014**, *45*, 610-638.

[109] Thannickal, V. J.; Fanburg, B. L. Reactive oxygen species in cell signaling. *Am. J. Physiol. Lung Cell Mol. Physiol.,* **2000**, *279*, L1005-1028.

[110] Thorp, H. H. The importance of being r: greater oxidative stability of RNA compared with DNA. *Chem. Biol.,* **2000**, *7*, R33-R36.

[111] Gotz, M. E.; Kunig, G.; Riederer, P.; Youdim, M. B. Oxidative stress: free radical production in neural degeneration. *Pharmacol. Ther.,* **1994**, *63*, 37-122.

[112] Barnham, K. J.; Masters, C. L.; Bush, A. I. Neurodegenerative diseases and oxidative stress. *Nat. Rev. Drug Discov.,* **2004**, *3*, 205-214.

[113] Uttara, B.; Singh, A. V.; Zamboni, P.; Mahajan, R. T. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Curr. Neuropharmacol.,* **2009**, *7*, 65-74.

[114] Forstermann, U.; Sessa, W. C. Nitric oxide synthases: regulation and function. *Eur. Heart J.,* **2012**, *33*, 829-837.

[115] Halliwell, B. Oxidative stress and neurodegeneration: where are we now? *J. Neurochem.,* **2006**, *97*, 1634-1658.

[116] Sun, G. Y.; Xia, J.; Draczynska-Lusiak, B.; Simonyi, A.; Sun, A. Y. Grape polyphenols protect neurodegenerative changes induced by chronic ethanol administration. *Neuroreport,* **1999**, *10*, 93-96.

[117] Ono, K.; Naiki, H.; Yamada, M. The development of preventives and therapeutics for Alzheimer's disease that inhibit the formation of beta-amyloid fibrils (fA beta), as well as destabilize preformed fA beta. *Curr. Pharm. Design.,* **2006**, *12*, 4357-4375.

[118] Schraufstatter, E.; Bernt, H. Antibacterial action of curcumin and related compounds. *Nature,* **1949**, *164*, 456.

[119] Jiang, J.; Wang, W.; Sun, Y. J.; Hu, M.; Li, F.; Zhu, D. Y. Neuroprotective effect of curcumin on focal cerebral ischemic rats by preventing blood-brain barrier damage. *Eur. J. Pharmacol.,* **2007**, *561*, 54-62.

[120] Yang, F.; Lim, G. P.; Begum, A. N.; Ubeda, O. J.; Simmons, M. R.; Ambegaokar, S. S.; Chen, P. P.; Kayed, R.; Glabe, C. G.; Frautschy, S. A.; Cole, G. M. Curcumin inhibits formation of amyloid beta oligomers and fibrils, binds plaques, and reduces amyloid *in vivo*. *J. Biol. Chem.,* **2005**, *280*, 5892-5901.

[121] Ono, K.; Yamada, M. Antioxidant compounds have potent anti-fibrillogenic and fibril-destabilizing effects for alpha-synuclein fibrils in vitro. *J. Neurochem.,* **2006**, *97*, 105-115.

[122] Mandel, S.; Weinreb, O.; Amit, T.; Youdim, M. B. Cell signaling pathways in the neuroprotective actions of the green tea polyphenol (-)-epigallocatechin-3-gallate: implications for neurodegenerative diseases. *J. Neurochem.,* **2004**, *88*, 1555-1569.

[123] Sutherland, B. A.; Shaw, O. M.; Clarkson, A. N.; Jackson, D. N.; Sammut, I. A.; Appleton, I. Neuroprotective effects of (-)-epigallocatechin gallate following hypoxia-ischemia-induced brain damage: Novel mechanisms of action. *FASEB J.,* **2005**, *19*, 258-260.

[124] Rezai-Zadeh, K.; Shytle, D.; Sun, N.; Mori, T.; Hou, H.; Jeanniton, D.; Ehrhart, J.; Townsend, K.; Zeng, J.; Morgan, D.; Hardy, J.; Town, T.; Tan, J. Green tea Epigallocatechin-3-gallate (EGCG) modulates amyloid precursor protein cleavage and reduces cerebral amyloidosis in Alzheimer transgenic mice. *J. Neurosci.,* **2005**, *25*, 8807-8814.

[125] Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L. Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.,* **2004**, *79*, 727-747.

[126] Aura, A. M. Microbial metabolism of dietary phenolic compounds in the colon. *Phytochem. Rev.,* **2008**, *7*, 407-429.

[127] Ley, R. E.; Lozupone, C. A.; Hamady, M.; Knight, R.; Gordon, J. I. Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat. Rev. Microbiol.,* **2008**, *6*, 776-788.

[128] Bosscher, D.; Breynaert, A.; Pieters, L.; Hermans, N. Food-based strategies to modulate the composition of the intestinal microbiota and their associated health effects. *J. Physiol. Pharmacol.,* **2009**, *60*, 5-11.

[129] Selma, M. V.; Espin, J. C.; Tomas-Barberan, F. A. Interaction between phenolics and gut microbiota: Role in human health. *J. Agr. Food Chem.,* **2009**, *57*, 6485-6501.

[130] Griffiths, L. A.; Barrow, A. Metabolism of flavonoids compounds in germ-free rats. *Biochem. J.,* **1972**, *130*, 1161-1162.

[131] Rechner, A. R.; Smith, M. A.; Kuhnle, G.; Gibson, G. R.; Debnam, E. S.; Srai, S. K. S.; Moore, K. P.; Rice-Evans, C. A. Colonic metabolism of dietary polyphenols: Influence of structure on microbial fermentation products. *Free Radical Biol. Med.,* **2004**, *36*, 212-225.

[132] Schoefer, L.; Mohan, R.; Schwiertz, A.; Braune, A.; Blaut, M. Anaerobic degradation of flavonoids by Clostridium orbiscindens. *Appl. Environ. Microb.,* **2003**, *69*, 5849-5854.

[133] Coldham, N. G.; Darby, C.; Hows, M.; King, L. J.; Zhang, A. Q.; Sauer, M. J. Comparative metabolism of genistin by human and rat gut microflora: Detection and identification of the end-products of metabolism. *Xenobiotica,* **2002**, *32*, 45-62.

[134] Jandhyala, S. M.; Talukdar, R.; Subramanyam, C.; Vuyyuru, H.; Sasikala, M.; Nageshwar Reddy, D. Role of the normal gut microbiota. *World J. Gastroenterol.,* **2015**, *21*, 8787-8803.

[135] Duenas, M.; Munoz-Gonzalez, I.; Cueva, C.; Jimenez-Giron, A.; Sanchez-Patan, F.; Santos-Buelga, C.; Moreno-Arribas, M. V.; Bartolome, B. A survey of modulation of gut microbiota by dietary polyphenols. *Biomed. Res. Int.,* **2015**, *2015*, 850902.

[136] Parkar, S. G.; Stevenson, D. E.; Skinner, M. A. The potential influence of fruit polyphenols on colonic microflora and human gut health. *Int. J. Food Microbiol.,* **2008**, *128*, 416-416.

[137] Lee, H. C.; Jenner, A. M.; Low, C. S.; Lee, Y. K. Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. *Res. Microbiol.,* **2006**, *157*, 876-884.

[138] Carmody, R. N.; Turnbaugh, P. J. Host-microbial interactions in the metabolism of therapeutic and diet-derived xenobiotics. *J. Clin. Invest.,* **2014**, *124*, 4173-4181.

[139] Tillisch, K.; Labus, J.; Kilpatrick, L.; Jiang, Z.; Stains, J.; Ebrat, B.; Guyonnet, D.; Legrain-Raspaud, S.; Trotin, B.; Naliboff, B.; Mayer, E. A. Consumption of fermented milk product with probiotic modulates brain activity. *Gastroenterology,* **2013**, *144*, 1394-U1136.

[140] Van Oudenhove, L.; McKie, S.; Lassman, D.; Uddin, B.; Paine, P.; Coen, S.; Gregory, L.; Tack, J.; Aziz, Q. Fatty acid-induced gutbrainsignaling attenuates neural and behavioral effects of sad emotionin humans. *J. Clin. Inves.,* **2011**, *121*, 3094-3099.