

## Effect of Mixed Lipoic Acid, Vitamin D, Phosphatidylserine and Homotaurine to Obtain a New Formulation for Brain Ageing Prevention

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### Abstract

By 2050, it is estimated that there will be two billion people aged 60 or over, of which 131 million are expected to be affected by dementia, while depression is expected to be the second-largest cause of disability worldwide in 2020. Preventing or delaying the onset of these disorders should therefore be a priority for public health systems. There is some evidence linking certain substances present in most common food supplements with a reduced risk of neuronal degeneration improving brain health. Recently, many compounds or extracts from natural products slowing aging and extending lifespan have been reported. The main goal of this study is to develop a new formulation that delay age-related diseases in human. For this reason, the effects of selected agents (such as lipoic acid, vitamin D3, phosphatidylserine and homotaurine) were assessed in order to find a new formulation able to slow down the physiological decay linked to brain ageing. Cell viability, radical oxygen species production, inflammatory marker along with some intracellular pathways have been evaluated. The results show that the new combination is highly effective to counteract the negative effects of oxidative stress and inflammation acting through some important brain markers involved in cell survival, enhancing viability of astrocytes.

**Keywords:** Healthy Ageing; Astrocytes; Intracellular Mechanism; Novel Mixed Natural Sources; Ageing Mechanisms

### Abbreviations

APP: Beta- Amyloid Precursor; CB: Cebtral®; Cz: Cognizin®; Ct: Citicoline Monosodium Salt; Co: Choline L Bitartrate Conditioned; Cu: Curcumin; Ho: Homotaurine; LA: Lipoic Acid; Pho: Phosphatidylserine; UMP: Ribocare® UMP; VD: Vitamin D3

### Introduction

Anti-ageing medicine is growing in importance in recent years, and it is expected that in the future there will be a huge increase in interest in research and expectations in prevention. There are some natural factors in modern medicine that potentially have great therapeutic potential. Recently, a wide interest has been devoted to the research of molecules that could influence the ageing process [1], both on aging of the whole body and cellular aging [2]. However, the aim of anti-ageing strategy is to improve the quality of life. [3]. According to the classical definition, ageing is a time-dependent functional decline that affects all living organisms [4], which involves several alterations that induce a gradual loss of physiological functions of tissues [5]. In particular, brain is very sensitive to damage caused by an increased oxidative stress and a failure of antioxidant defense systems [6]; in detail, the imbalance between pro-oxidants and antioxidants leads to the overproduction of the reactive oxygen species (ROS) [6]. The oxidative stress isn't the only characteristic of the ageing process. While oxidative stress plays an important role in the aging process, chronic inflammation has also been implicated as a major contributing factor for cellular senescence and it is known to be important in the etiology of many diseases since cause

molecular deterioration such as: mitochondrial collapsing, DNA damage, protein, carbohydrate and lipid oxidation [5]. This damage can lead to early cell ageing, cell death and various chronic pathologies, like neurodegenerative disorders [7]. Nutraceutical interventions slow physiological or/and pathological progression due to their anti-oxidative, anti-inflammatory and anti-amyloidogenic properties, regulating mitochondrial stress, apoptotic factors, free radical scavenging system, and neurotrophic factors [8,9]. The aim of this study is particularly innovative, as we wanted to combine the beneficial effects of Lipoic Acid (LA) and vitamin D3 (VD) with other agents, selected among natural substances well known to have some effects on neuronal cells, in order to create a new formulation able to amplify their neuroprotective activity. In this context, the effects of different forms of choline (Cognizin<sup>®</sup>, Citicoline monosodium salt, Choline L Bitartrate conditioned), Phosphatidylserine, Homotaurine, Curcumin and Ribocare<sup>®</sup> UMP were tested on cultured astrocytes, which is a cell population mainly involved in the first stage of ageing process [10]. Choline is an organic cation that plays a critical role in the structure and function of biological membranes in all types of cells. Moreover, it plays an additional role as a precursor for the synthesis of the neurotransmitter, acetylcholine [11] in brain. Since the brain has only a limited capacity to synthesize choline *de novo*, most central nervous system (CNS) choline is derived from systemic circulation or from recycling from cerebral lipids or from food supplements [12]. Cytidine-5'-diphosphocholine (citicoline or CDP-choline) is a compound normally present in all cells throughout the body and it is an intermediate in the biosynthesis of phosphatidylcholine. It has been shown that citicoline induces neuroprotective effects in a variety of CNS injury models including cerebral ischemia [13]. In humans, citicoline is the only neuroprotectant that showed positive results in all randomized, double-blind trials and demonstrated efficacy in a meta-analysis with an overall safety similar to placebo, preventing for example fatty acids release, preservation of cardiolipin and sphingomyelin levels [13]. A decline in phosphatidylserine, a member of the membrane phospholipids, has been associated with memory impairment and deficits in mental cognitive abilities [14,15]. Due to its presence in the healthy brain, phosphatidylserine treatments were mostly proposed and investigated for its beneficial effects [16,17]. Homotaurine (3-aminopropanesulfonate), an analogue of 4-aminobutyrate ( $\gamma$ -aminobutyric acid, GABA), is a small natural aminosulfonate compound identified in different species of marine red algae and then chemically synthesized and introduced into clinical use. Indeed homotaurine may interfere with several cellular pathways, both *in vitro* and *in vivo* studies, and exert neuroprotective and neurotropic activities through different mechanisms including effects against the oxidative damage to DNA, anti-fibrillogenic activity, and antinociceptive, analgesic activities and prevents the neurotoxicity of  $\beta$ -amyloid ( $A\beta$ ) peptide by reducing amyloid aggregation [18,19]. Curcumin is the main component of *Curcuma longa* (*C. longa*), generically known as turmeric, a perennial plant that grows naturally in Southeast Asia and it is correlated to multiple beneficial effects, including its ability to act as a strong antioxidant and anti-inflammatory agent [20]. Indeed, curcumin demonstrated to have higher specificities for  $A\beta$  fibrils and adequate lipophilic properties for crossing the blood-brain barrier is a subject of current research [21,22]. Uridin has crucial role in the pyrimidine metabolism of the brain and have an additional role in the function of the CNS as signaling molecule. For example, *in vitro*, uridine was shown to amplify the phosphatide formation in the membrane and neurite outgrowth elicited by nerve growth factor (NGF) in rat pheochromocytoma (PC-12) cells. In *in vivo* experiments, UMP consumption was shown to enhance striatal dopamine levels and release. These data suggest that a uridine source may enhance some cholinergic functions, perhaps by increasing brain phosphatide levels [23]. For these reasons, it becomes interesting to evaluate the efficacy of these substances in combination with lipoic acid and vitamin D3 to obtain a new formulation able to slow down the physiological decay linked to brain ageing.

## Materials and Methods

### Astrocyte isolation

Primary mouse astrocyte cultures were isolated from both male and female C57BL/6 mouse pups, following a previously described method [24,25] in order to obtain cortical astrocytes according to National Guideline for the Use and Care of Laboratory Animals. Briefly, the cortices obtained from pups within 24h of birth were mechanically digested, let it settle for 30 minutes at room temperature and the suspension was centrifuged at 800 rpm for 5 minutes. Pellets cells were resuspended in Neuronal Basal Medium supplemented with

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5% FBS, 1% penicillin/streptomycin and 2 mM glutamine and plated in different protocols for 6 days before treatment. The cells for the experiments were plated:  $1 \times 10^4$  cells on 96-well to study cell viability by MTT test and radical oxygen species production (ROS) by colorimetric test;  $1 \times 10^6$  on 6-well to analyze the intracellular pathways activated by Western blot analysis. Before stimulations the cells were maintained in DMEM without red phenol and FBS and supplemented with 1% penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate in an incubator at 37°C, 5% CO<sub>2</sub> and 95% humidity for 3h.

### Experimental protocol

The cells were used to study a new formulation, named Cebra<sup>®</sup>, analyzing different composition in order to obtain a main formula able to act on astrocytes. This study has been divided into three parts. In the first set of experiments the effects of single agents of the formulation have been assessed in order to obtain the best combination to add to lipoic and vitamin D3 to create Cebra<sup>®</sup>. In particular, the role of 100 µM Cognizin<sup>®</sup> (Kyowa Hakko Bio.CO, Japan; Cz), 100 µM Citicoline monosodium salt (Kyowa Hakko Bio.CO, Japan; Ct), 100 µM Choline L Bitartrate conditioned (Barentz Service SPA, Italy; Co) [19], 5 µg/ml Phosphatidylserine SHARP PS (Frutarom Health, USA; Pho) [17], 1 mM Homotaurine (Barentz Service SPA, Italy; Ho) [26], 5 µM Curcumin BMC-95<sup>®</sup> DC (Arjuna Natural Ltd, USA; Cu) [27] and 1.5% Ribocare<sup>®</sup> UMP (Prosol SPA, Italy; UMP) [23] were analyzed, in physiological conditions, evaluating cell viability and comparing data to the combination between lipoic acid (50 µM; LA) and vitamin D3 (100 nM; VD) [25]. In the second set of experiments, different formulations composed of the single agents investigated before were verify on cell viability and ROS production in order to obtain the best composition (composed by 50 µM lipoic acid, 100 nM vitamin D3, 5 µg/ml Phosphatidylserine, 1 mM homotaurine) to use in successive experiments. This final composition, able to induce the most evident beneficial effects on astrocytes, was then used to prepare a commercial product called Cebra<sup>®</sup> (Laborest Italia srl, Milan, Italy; CB) to be used as a dietary supplement to slow down brain ageing. This dietary supplement is composed of lipoic acid (600 mg), vitamin D3 (1600 UI), Phosphatidylserine (40 mg), Homotaurine (50 mg) and supplemented with group B vitamins. In the third set of experiments, the effects of CB on the main intracellular pathways involved on brain ageing were investigated by Western blot comparing to the other formulation tested before. All substances were dissolved according to solubility information reported in manufacturer's instructions, directly in the DMEM without red phenol and FBS but supplemented with 1% penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate (white medium).

### MTT test

Cell viability was measured by MTT-based *In Vitro* Toxicology Assay Kit (Sigma-Aldrich) performed on a 96 well-plate as previous described [28]. Briefly, at the end of stimulations, cells were incubated with 1% MTT dye and after 2h at 37°C in an incubator, the purple formazan crystals were dissolved in equal volume of MTT Solubilization Solution. The absorbance was measured at 570 nm with correction at 690 nm, through a spectrometer (VICTOR X4, multilabel plate reader) and cell viability calculated by comparing results to control.

### ROS production

ROS production was measured as a rate of superoxide anion release produced by astrocytes after stimulations [29]. After treatment, 100 µL of cytochrome C and 100 µL of superoxide dismutase were added for 30 min in an incubator in all samples (all substances were from Sigma-Aldrich). The absorbance was measured at 550 nm by a spectrometer (VICTOR X4, multilabel plate reader) and O<sub>2</sub> was expressed as mean ± SD of nanomoles per reduced cytochrome C per microgram of protein compared to the control on percentage (%).

### NF-κB transcription factor assay kit

Enzyme-Linked Immunosorbent Assay (ELISA) was used to analyze the effect of CB on NF-κB DNA binding activity, following the manufacturer's instruction (Cayman Chemical, Michigan, United States) [30]. Nuclear extracts were prepared using a nuclear extraction protocol composed by two lysis buffers: Hypotonic Buffer (20 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl<sub>2</sub> all purchased from Sigma-Aldrich) to remove the cytoplasmic fraction and Cell Extraction Buffer (10 mM Tris pH7.4, 100 mM NaCl, 1 mM EDTA, 1 mM NaF, 20mM Na<sub>2</sub>PO<sub>4</sub>,

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1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate; supplemented before the use with 2 mM sodium orthovanadate, 1 mM PMSF, 1:100 protease inhibitor cocktail. All purchased from Sigma-Aldrich) to obtain only the nuclear extracts. NF- $\kappa$ B contained in a nuclear extract, binds specifically to the NF- $\kappa$ B response element, detected by addition of specific primary antibody directed against it. A secondary antibody conjugated to HRP is added to provide a sensitive colorimetric measured by a spectrometer (VICTOR X4, multilabel plate reader) at 450 nm and the concentration of NF- $\kappa$ B was calculated by comparing results to the standard curve [31].

### Cell lysis and western blot

After treatments, astrocytes were washed with ice-PBS 1x and lysed in ice Ripa Buffer (50 mM Hepes, 150 mM NaCl, 0.1% SDS, 1% TRITON 100x, 1% deoxycholate acid, 10% glycerol, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM NaF; all purchased from Sigma-Aldrich) supplemented with 2 mM sodium orthovanadate (Sigma-Aldrich), 1 mM phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich) and 1:100 mix Protease Inhibitor Cocktail (Sigma-Aldrich). 35  $\mu$ g proteins of each samples were resolved into 8% and 15% SDS-PAGE gels, and polyvinylidene difluoride (PVDF) membranes (GE Healthcare) were incubated overnight at 4°C with specific primary antibody: anti-Phospho-Tau (1:1000, Thermofischer, Italy), anti-beta amyloid precursor (APP) (1:250, Santa Cruz, CA, United States), anti-sirt1 (1:1000, Sigma-Aldrich, Missouri, United States). Protein expression was normalized to the specific total protein, if possible, and verified through  $\beta$ -actin detection (1:5000; Sigma-Aldrich) and expressed as a mean  $\pm$  SD (% vs control).

### Statistical analysis

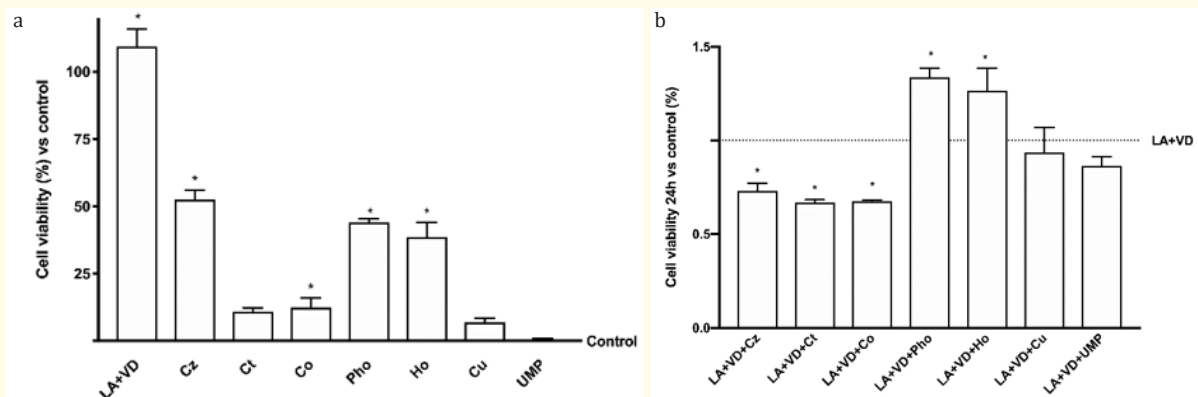
The results obtained from each experimental protocol are expressed as means  $\pm$  SD of at least four independent experiments performed on four technical replicates. One-way ANOVA followed by Bonferroni post hoc test was used for statistical analysis, and pairwise differences compared by Mann-Whitney U tests followed by Welch's test. p values < 0.05 were considered statistically significant.

## Results and Discussion

In our previous study, we demonstrated the positive effects of the combination between LA and VD during brain ageing. In particular, we hypothesized that this combination can slow down the aging processes through a decrease of ROS production and an increase of cell viability in neuronal cells [25]. The most recent findings available in literature concerning LA [25,32-34] support the hypothesis of amplifying the beneficial effects of combined in order to create a new formulation able to act on different aspects involved in brain ageing.

### Cell viability of astrocytes treated with the substances alone

In order to select the more useful compounds to add to the combination of VD and LA, the effects of the single agents (100  $\mu$ M Cz, 100  $\mu$ M Ct, 100  $\mu$ M Co, 5  $\mu$ g/ml Pho, 1 mM Ho, 5  $\mu$ M Cu, 1.5% UMP) were tested on cell viability of astrocytes by MTT at 24h of stimulations. As shown in figure 1A, the combination of 50  $\mu$ M LA and 100 nM VD confirmed its beneficial effect on cell viability compared to control (p < 0.05); among the other substances selected, only 100  $\mu$ M Cz, 100  $\mu$ M Co, 5  $\mu$ g/ml Pho and 1 mM Ho seems to have a positive effect on cell viability compared to control (p < 0.05) and between only these, Cz, Pho and Ho have a similar effects supporting the hypothesis of their use in combination with LA+VD. However, the importance of the activity of single agents may be observed also in combination with LA+VD in order to exclude any inhibitory effects. Indeed, some preclinical and clinical studies indicate that a strong neuroprotection can be achieved by administration of multiple agents with overlapping and complementary function vs that achieved by individual agents [35]. We therefore hypothesized that treatment with a cocktail of the above agents may exert higher neuroprotection than what observed with any other individually administered agent. As reported in figure 1B, Pho and Ho added to LA+VD confirmed their beneficial effects compared to LA+VD (p < 0.05), indicating the possible synergistic effects and supporting the hypothesis about a possible new strategy to slow down ageing. In addition, among these formulations, LA+VD+Pho seemed the one with the greater effect compared to other compositions (about 6% vs LA+VD+Ho). These data contribute to hypothesize the final formulation, that is composed of LA+VD+Pho plus other molecules. Moreover, the combinations of LA+VD with Cu or UMP were able to increase cell viability similarly to what observed with LA+VD only, indicating an effect on cell viability. For this reason, Pho, Ho, Cu and UMP were maintained in successive experiments.

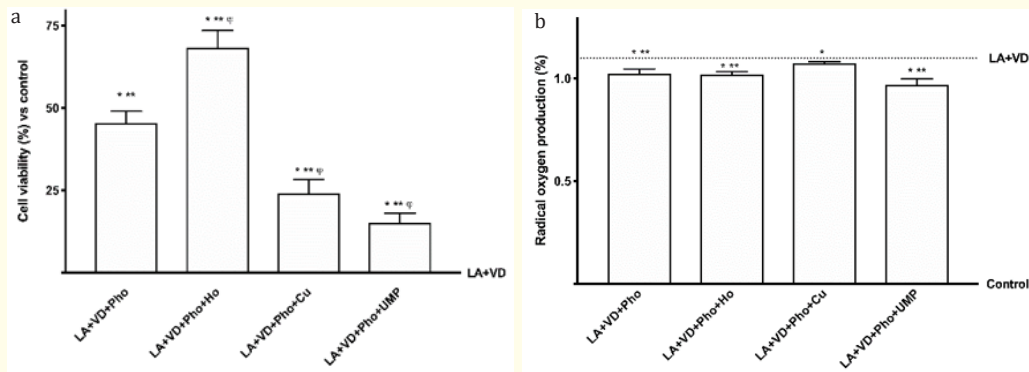


**Figure 1:** Cell viability of astrocytes treated with different substances. In panel (a) cell viability measured after the single treatments on astrocytes at 24h. LA+VD: lipoic acid + vitamin D3; Cz: Cognizin®; Ct: Citicoline monosodium salt; Co: Choline L Bitartrate conditioned; Cu: Curcumin; Ho: Homotaurine; Pho: Phosphatidylserine; UMP: Ribocare® UMP. Data are expressed as means ± SD (%) of four independent experiments normalized to control values (0% line). \*  $p < 0.05$  vs control. In (b) analysis of cell viability after stimulation for 24h of LA+VD plus the single agents. LA+VD: lipoic acid + vitamin D3; LA+VD+Cz: lipoic acid + vitamin D3+Cognizin®; LA+VD+Ct: lipoic acid + vitamin D3+Citicoline monosodium salt; LA+VD+Co: lipoic acid + vitamin D3+Choline L Bitartrate conditioned; LA+VD+Cu: lipoic acid + vitamin D3+curcumin; LA+VD+Ho: lipoic acid + vitamin D3+homotaurine; LA+VD+Pho: lipoic acid + vitamin D3+Phosphatidylserine; LA+VD+UMP: lipoic acid + vitamin D3+ Ribocare® UMP. Data are expressed as means ± SD (%) of four independent experiments normalized to control values (0% line) and illustrated normalized to LA+VD (dashed line). All combinations are  $p < 0.05$  vs control; \*  $p < 0.05$  vs LA+VD.

### Cell viability and ROS production of the different combinations added to LA+VD+Pho

The clinical evidence shows that nutrients used in combination, can significantly counteract mitochondrial-based neurodegeneration. The combination of endogenous antioxidants and exogenous orthomolecular dietary supplements offers real potential to prevent neurodegenerative states [36]. As well known, combined LA and Pho induce clinically benefits in brain. Particularly, they are able to enhance brain growth factor receptors involved in brain decline [36]. At the same time, some commercial food supplements contain VD combined with Pho in order to maintain the cognitive performance. Based on these considerations and on previous data, some additional experiments were carried out to analyze the effects on cell viability and ROS production using astrocytes of LA+VD+Pho together with Ho, Cu and UMP in order to set a main formulation. As shown in figure 2, experiments were performed in astrocytes in order to investigate the potential ability of Ho, Cu and UMP added to LA+VD+Pho to influence viability. Exposure to LA+VD+Pho plus Ho, Cu or UMP were able to induce an increase in viability ( $p < 0.05$ ) compared to LA+VD alone and adding Ho, rather than Cu or UMP the resulting combination, LA+VD+Pho+Ho, was able to induce a greater effect on cell viability (about 50%,  $p < 0.05$ ) compared to LA+VD+Pho. This increase was higher than the subsequent addition (Cu and UMP), indicating a main influence of the combination LA+VD+Pho+Ho compared to the one with CU and UMP. Since the main theory at the basis of brain ageing regards the oxidative imbalance [37], additional experiments on ROS production were performed (Figure 2B). The combinations LA+VD+Pho, LA+VD+Pho+Ho and LA+VD+Pho+UMP were able to induce a significant reduction ( $p < 0.05$ ) on ROS production compared to LA+VD confirming the anti-oxidant property. Furthermore, among the last 3 formulations, no significant differences have been observed supporting the hypothesis of their safety during use. For these reason LA+VD+Pho, LA+VD+Pho+Ho and LA+VD+Pho+UMP were maintained in successive experiments.

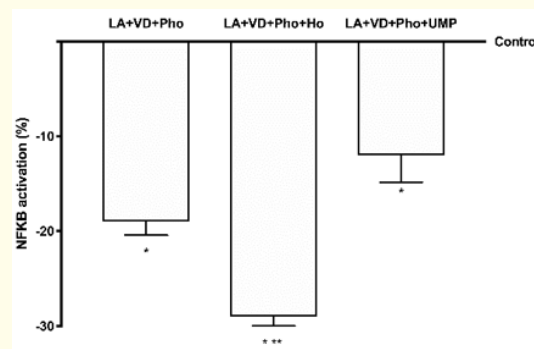




**Figure 2:** Effects of LA+VD+Pho combined with Ho, Cu, UMP. In panel (a) cell viability and in panel (b) ROS production measured after stimulations for 24h during. The abbreviations are the same reported in Fig.1. Data are expressed as means  $\pm$  SD (%) of four independent experiments normalized to control values in (b) and illustrated normalized to LA+VD in (a). \*  $p < 0.05$  vs control; \*\*  $p < 0.05$  vs LA+VD;  $\phi$   $p < 0.05$  vs LA+VD+Pho.

### Analysis of inflammatory markers and intracellular pathways

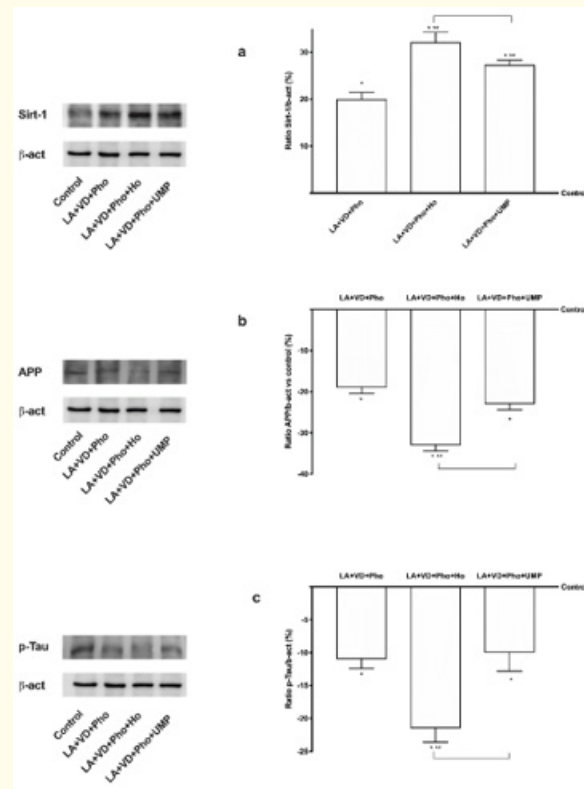
One of the hallmarks of aging is the increased oxidative stress and inflammation [38]. Indeed, the term ‘inflamm-aging’ has been recently coined to describe the heightened inflammatory status that is characteristic of ageing [39]. Notably, high levels of inflammation in the elderly are associated with neurobehavioral complications. For instance, a recent cross-sectional study investigating the association between inflammatory markers and executive functioning in an elderly population indicated an age-associated increase in the serum expression of C-reactive protein (CRP), IL-6 and IL-10. Elevated levels of these cytokines were inversely associated with executive performance on neuropsychological tests [40]. Heightened inflammatory signaling in aging is evident in the peripheral system and the CNS alike. For instance, microarray studies of the adult and aged human prefrontal cortex reported age-dependent increased expression of inflammation-associated genes, such as, NFKB, Toll-like-receptor (TLR)-4, IL-1R, and GFAP [41]. These findings collectively indicate an age-associated increase in inflammatory signaling and oxidative stress in ageing brain. Based on these findings, NFKB activity was investigated during stimulation with LA+VD+Pho, LA+VD+Pho+Ho and LA+VD+Pho+UMP on astrocytes. As illustrated in figure 3, all formulation induced a significant decreased of NFKB activity ( $p < 0.05$ ) compared to control, indicating an anti-inflammatory activity of all formulations; between them the main effect was observed in presence of LA+VD+Pho+Ho ( $p < 0.05$ ) which may be a possible better choice to prepare CB.



**Figure 3:** NFKB activity measured on astrocytes treated with LA+VD+Pho combined with Ho or UMP. The other abbreviations are the same reported in figure 2. Data are expressed as means  $\pm$  SD (%) of four independent experiments normalized to control values. \*  $p < 0.05$  vs control; \*\*  $p < 0.05$  vs LA+VD+Pho.

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Moreover, additional experiments are necessary to investigate some molecular mechanism involved to slow down brain ageing. In particular, Sirt1, APP (amyloid-beta precursor) and Tau protein expressions were investigated by Western blot. Sirt1 is responsible for protection from neuronal, and specifically axonal, degeneration [42] suggesting that the activation of Sirt1 might be important to prevent brain ageing caused by oxidative stress and inflammation, since its overexpression has been shown to increase antioxidant defense enzymes and inhibit NF-κB activation [43]. The activity of Sirt1 has also been linked to chronic neurodegenerative diseases, since is essential in maintaining normal learning, memory and synaptic plasticity [44]. Indeed, in Alzheimer’s disease patients, a decrease in Sirt1 is present in the parietal cortex, which is closely associated with the accumulation of APP and tau protein and cognitive impairment [45]. As reported in figure 4, the results obtained by the tested formulations followed the intracellular pathways reported; indeed Sirt1 (Figure 4A) expression was significantly increased by all stimulations ( $p < 0.05$ ) compared to control, but in presence of LA+VD+Pho+Ho a greater effect was observed ( $p < 0.05$ ) compared to LA+VD+Pho (about 60%) and LA+VD+Pho+UMP (about 19%), indicating a better influence of this composition. APP and Tau expressions had a similar profile: between the formulations LA+VD+Pho+Ho had a main effect on both proteins ( $p < 0.05$ ) compared to LA+VD+Pho (about 1.7 and 2 times, respectively) and to LA+VD+Pho+UMP (about 1.5 and 2.1 times, respectively). All these results confirmed a previous hypothesis about the choice of CB composition; the better formulation which had greater effects on all analyzed parameters was composed of LA+VD+Pho+Ho.



**Figure 4:** Western blot and densitometric analysis after treatments with LA+VD+Pho combined with Ho or UMP. In (a) Sirt 1, in (b) APP and in (c) Tau expressions analysed through Western blot (an example on the left) and densitometric analysis (on the right) of astrocytes treated for 24h. The abbreviations are the same used in figure 3. All results are expressed as means ± SD (%) normalized to control values of four independent experiments. \*  $p < 0.05$  vs control; \*\*  $p < 0.05$  vs LA+VD+Pho; arrows indicate  $p < 0.05$  between the presence of Ho and UMP.

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Recent findings led to the use of homotaurine as new pharmacological intervention since the very early stages of brain imbalance. This substance is well known for its neuroprotective properties, as shown in a number of *in vitro* and *in vivo* experimental models [46]. The therapeutic effectiveness of homotaurine has been investigated in several clinical trials as well, in particular in the treatment of cognitive impairment. For this reason, is actually used in common clinical practice [46]. Furthermore, great attention has recently been focused on UMP due to its rapid absorption. A dose of UMP up to 2000 mg, raises plasma uridine levels by 3 folds for 5-6 hours in humans. Moreover, circulating uridine crosses the blood-brain barrier via an adenosine transporter. Thus, dietary UMP can easily increase brain levels of uridine and subsequently, brain levels of UTP [23]. For this reason, the importance of the combination LA+VD+Pho+Ho on APP is more interesting compared to homotaurine and UMP alone in order to create a new strategy to slow down brain ageing. Indeed, LA+VD+Pho+Ho induced a significant decreased ( $p<0.05$ ) on APP expression compared both to Ho and UMP alone (about 5 times and 6 times, respectively; data not shown).

### Conclusion

The population of aged individuals is constantly increasing worldwide and this has significant health and socio-economic implications. Increase in life span, however, is met with a new challenge, namely the extension of health span of the aging individuals. In other words, slowing aging in itself is not sufficient unless paralleled with healthy aging. Although aging is not a disease, it is a significant risk factor for functional decline, affective impairments, exaggerated response to illnesses, dementia, and overall vulnerability for diseases [47,48]. Conversely, life events associated with psychological stress and injury can also lead to accelerated aging-associated impairments and dementia [49]. There is some evidence linking certain dietary patterns, particularly the Mediterranean diet, with a reduced risk of dementia and depression. Specific dietary components have also been investigated in relation to brain health, with emerging evidence supporting protective roles for example of n-3 PUFA, polyphenols, vitamin D and B-vitamins [50]. In this context, the study about the new formulation is more important to prevent or slow down ageing acting on oxidative stress and inflammatory network. This work reports for the first time, the strategy to study a new formulation named Cebreal<sup>®</sup>, which contains selected components able to act on these mechanisms in astrocytes which are the cells that the brain loses during ageing.

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### Conflict of Interest

The authors declare no competing financial interests.

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## Effect of Mixed Lipoic Acid, Vitamin D, Phosphatidylserine and Homotaurine to Obtain a New Formulation for Brain Ageing Prevention

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