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Biological Effects of Vitamin D3 Mediated by the Interaction with VDR in Different Tissues

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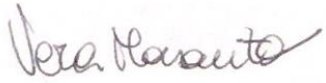

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SUMMARY

Vitamin D plays an important role in calcium homeostasis and bone metabolism, and it is able to modulate proliferation and differentiation of cells, cardiovascular function and also innate and adaptive immune function. The active form of vitamin D (vitamin D3, 1,25(OH)2D), exerts most of its effects through the vitamin D receptor (VDR). Indeed, in recent years the interest in vitamin D's studies is increased, especially due to the presence of its receptor in many tissues and organs. VDR forms a heterodimer with the retinoid X receptor (RXR) upon stimulation by 1,25(OH)2D. In turn, VDR/RXR binds to DNA sequences termed "vitamin D response elements" in target genes, regulating gene transcription. In order to exert its biological effects, VDR signaling interacts with other intracellular signaling pathways. Sometimes, vitamin D exerts its biological effects without regulating either gene expression or protein synthesis. Although the regulatory role of vitamin D in many biological processes is well understood yet, many studies indicate that the role of vitamin D is not limited to regulation of bone and mineral homeostasis, but it is also important for its extraskeletal actions. Indeed, it has become the major driving force behind the significant increase in research articles on vitamin D published over the past several decades.

For this reason, this research aimed to examine the role of vitamin D signaling in a number of extraskeletal tissues (ovarian, gastric, cardiac and brain), and to assess the feasibility of translating these findings into treatments of human diseases affecting those extracellular tissues.

It has been demonstrated that in ovarian tissues a high density of VDR is present as well and vitamin D3 acts through intracellular mechanisms similar to what observed for resveratrol, a natural antioxidant polyphenol able to exert a wide range of biological effect on several tissues. The aims of this research were to evaluate the cooperative effects of resveratrol combined with vitamin D3 on CHO-K1 cells studying cell viability, ROS production, activated pathways in *in vitro* study and in *in vivo* study to quantify vitamin D3 and resveratrol, to analyze the concentration of radical oxygen species and to study activated pathways. Moreover, the modulation of specific intracellular pathways involving ER and VDR receptors has been studied. Results show that both in *in vitro* and in *in vivo* experiments, resveratrol exerts a greater effect when administered in combination with vitamin D3. In particular, the role of vitamin D3 in maintaining and supporting the biological activity of resveratrol has been clearly observed. Moreover, resveratrol plus vitamin D3 in blood concentrations showed a biphasic absorption rate. Such results could be used as a fundamental data for the development of new therapies for gynecological conditions, such as hot-flashes.

Another widespread problem is the abnormal use of nonsteroidal anti-inflammatory drugs in order to reduce pain. There is a high risk of developing gastric and enteric damages. These patients usually receive anti-acid treatment, but a number of clinical studies provided evidence of the ineffectiveness of proton-pump inhibitors. Vitamin D, on the other hand, appears to have high preventive and therapeutic potential. Recently, it has been introduced a commercial product that, in addition to anti-acid properties of alginates, claims to possess gastroprotective properties deriving from vitamin D3 and from plant

extracts. In this research the effectiveness of vitamin D3 combined with alginates to prevent the damage induced in cultured gastric cells by Diclofenac during acidic or hyperacidic exposition have been studied measuring cell viability, radical oxygen species production along with apoptotic and survival pathways. Analyzing the effects on cardiac tissue, the study demonstrate that vitamin D3 has a cooperative effect with Q10 and L-arginine on both cardiac and endothelial cells to prevent the damage of ischemic condition. In particular, the effects of vitamin D3, Q10 and L-arginine alone or combined on cell viability, nitric oxide and reactive oxygen species productions in endothelial and cardiac cells were studied. Moreover, the involvement of PI3K/Akt and ERK/MAPK pathways leading to eNOS activation as well as the involvement of vitamin D receptor were also investigated. The same agents were tested in an animal model to verify vasodilation, nitric oxide and reactive oxygen species production. The data obtained demonstrate for the first time the beneficial and cooperative effect of stimulation with vitamin D3, Q10 and L-arginine. Indeed, in cardiac and endothelial cells, vitamin D3, Q10 and L-arginine combined were able to induce a nitric oxide production higher than the that induced by the 3 substances alone. The effects on vasodilation induced by cooperative stimulation have been confirmed in an *in vivo* model as well. The use of a combination of vitamin D3, Q10 and L-arginine to counteract increased free radical production could be a potential method to reduce myocardial injury or the effects of aging on the heart.

As regards the research focused on vitamin D3 in brain ageing, the ability of vitamin D3 combined with lipoic acid to prevent or repair the damage caused by oxidative stress and iron accumulation, two different biological aspects involved in brain ageing and neurodegeneration, has been studied in astrocytes. Cell viability, mitochondrial membrane potential and pathways activated in oxidative condition have been investigated. In addition, catalytic iron was used to assess the protection exerted by the combination of vitamin D3 and lipoic acid in induced neurodegeneration. In these experiments, cell viability, ROS production, iron concentration and activation of intracellular pathways have been studied. In this research, the combination of vitamin D3 and lipoic acid showed beneficial effects on viability of astrocytes, since the substances are able to cross the brain barrier. In addition, vitamin D3 plus lipoic acid was able to attenuate the H_2O_2-induced apoptosis through the mitochondrial-mediated pathway. The combination was also able to counteract the adverse effects caused by iron, preventing its accumulation. All these data support the hypothesis of a cooperative activity exerted by vitamin D3 and lipoic acid in astrocytes, indicating a possible new strategy to slow down brain ageing.

Therefore, the ability of vitamin D3 and lipoic acid to counteract brain aging has resulted in the creation of a commercial product, Cebral®.

RIASSUNTO

La vitamina D svolge un ruolo fondamentale nell'omeostasi del calcio e nel metabolismo osseo, modula la proliferazione e la differenziazione cellulare, l'immunità innata ed adattativa e contribuisce a regolare la funzione cardiovascolare. La forma attiva della vitamina D (vitamina D3, 1,25 (OH) 2D), esercita la maggior parte dei suoi effetti attraverso l'interazione con il suo recettore specifico (VDR). Negli ultimi anni l'interesse nello studio della vitamina D è aumentato, vista la presenza del suo recettore in molti organi e tessuti. VDR forma un eterodimero con il recettore X retinoide (RXR) dopo il legame con la vitamina D3. A sua volta, VDR/RXR si lega alle sequenze di DNA chiamate "elementi responsivi alla vitamina D" nei geni bersaglio, regolando la trascrizione genica. Al fine di esercitare i suoi effetti biologici, la segnalazione innescata da VDR interagisce con altre vie di segnalazione intracellulari. Sebbene il suo ruolo regolatorio in molti processi biologici sia ben noto, molti studi indicano che il ruolo della vitamina D non si limita alla regolazione dell'omeostasi ossea e minerale, ma è anche importante per le sue azioni extrascheletriche. Per questo motivo, questo studio è andato ad indagare il ruolo della segnalazione della vitamina D in numerosi tessuti extrascheletrici (ovarico, gastrico, cardiaco e cerebrale) per valutare la traslazionalità di questi risultati in trattamenti di malattie che colpiscono l'uomo. È stato dimostrato che nei tessuti ovarici è presente un'alta densità di VDR e la vitamina D3 agisce attraverso meccanismi intracellulari simili a quelli osservati per il resveratrolo, un polifenolo antiossidante naturale in grado di esercitare una vasta gamma di effetti biologici su diversi tessuti. Gli obiettivi di questa ricerca sono stati valutare gli effetti cooperativi del resveratrolo combinato con la vitamina D3 su cellule CHO-K1 (cellule ovariche) studiando la vitalità cellulare, la produzione di ROS, i percorsi attivati *in vitro* ed *in vivo*, la quantificazione di vitamina D3 e resveratrolo. Inoltre, è stata studiata la modulazione di specifici pathways intracellulari che coinvolgono i recettori ER e VDR. I risultati mostrano che sia in esperimenti *in vitro*, sia *in vivo*, il resveratrolo esercita un effetto maggiore quando somministrato in combinazione con vitamina D3. In particolare, è stato chiaramente osservato il ruolo della vitamina D3 nel mantenimento e nel supporto dell'attività biologica del resveratrolo. Inoltre, il resveratrolo più la vitamina D3 hanno mostrato un tasso di assorbimento bifasico nelle concentrazioni ematiche. Tali risultati potrebbero essere utilizzati come dati fondamentali per lo sviluppo di nuove terapie per condizioni ginecologiche, come ad esempio le vampate di calore.

Un altro problema diffuso è l'utilizzo anormale di farmaci antinfiammatori non steroidei al fine di ridurre il dolore che porta però ad un alto rischio di sviluppare danni gastrici ed enterici. Questi pazienti di solito ricevono un trattamento anti-acido, ma una serie di studi clinici ha fornito prove dell'inefficacia degli inibitori di pompa protonica. La vitamina D, d'altra parte, sembra avere un alto potenziale preventivo e terapeutico. Recentemente, è stato introdotto un prodotto commerciale che, oltre alle proprietà anti-acide degli alginati, afferma di possedere proprietà gastroprotettive derivanti dalla vitamina D3 e dagli estratti vegetali. In questa ricerca, l'efficacia della vitamina D3 combinata con gli alginati è stata studiata analizzando la vitalità cellulare, la produzione di specie reattive dell'ossigeno

insieme a percorsi apoptotici e di sopravvivenza per prevenire il danno indotto da Diclofenac in cellule gastriche in condizioni di acidità o iperacidità.

Analizzando gli effetti anche sul tessuto cardiaco, lo studio dimostra che la vitamina D3 ha un effetto cooperativo con Q10 e L-arginina su cellule cardiache (cellule H9c2) ed endoteliali (cellule PAE) per prevenire il danno in condizioni ischemiche. In particolare, sono stati studiati gli effetti di vitamina D3, Q10 e L-arginina da soli e combinati sulla vitalità cellulare, sull'ossido nitrico e sulla produzione di specie reattive dell'ossigeno in cellule endoteliali e cardiache. Inoltre, sono stati studiati anche il coinvolgimento dei percorsi PI3K/Akt ed ERK/MAPK che portano all'attivazione di eNOS ed il coinvolgimento del recettore della vitamina D. Gli stessi agenti sono stati testati in un modello animale per verificare la produzione di specie reattive dell'ossigeno e di ossido nitrico. I dati ottenuti dimostrano per la prima volta l'effetto benefico e cooperativo della stimolazione con vitamina D3, Q10 e L-arginina. In effetti, nelle cellule cardiache ed endoteliali, vitamina D3, Q10 e L-arginina combinati sono stati in grado di indurre una produzione di ossido nitrico superiore a quella indotta dalle 3 sostanze singolarmente. Gli effetti sulla vasodilatazione indotti dalla stimolazione cooperativa sono stati confermati anche in un modello *in vivo*. L'uso della combinazione di vitamina D3, Q10 e L-arginina potrebbe essere un potenziale metodo per ridurre le lesioni del miocardio o gli effetti dell'invecchiamento sul cuore.

Per quanto riguarda la parte dello studio incentrato sulla vitamina D3 nell'invecchiamento cerebrale, è stata studiata la capacità della vitamina D3 combinata con l'acido lipoico di prevenire o riparare i danni causati dallo stress ossidativo e dall'accumulo di ferro, due diversi aspetti biologici coinvolti nell'invecchiamento cerebrale e nella neurodegenerazione astrocitaria. Sono state studiate la vitalità cellulare, il potenziale di membrana mitocondriale ed i pathways molecolari attivati in condizioni di stress ossidativo. Inoltre, il ferro catalitico è stato utilizzato per valutare la protezione esercitata dalla combinazione di vitamina D3 ed acido lipoico nella neurodegenerazione indotta. In questi esperimenti, sono stati studiati la vitalità cellulare, la produzione di ROS, la concentrazione di ferro e l'attivazione di pathways intracellulari. In questo studio, la combinazione di vitamina D3 e acido lipoico ha mostrato effetti benefici sulla vitalità degli astrociti. Inoltre, la vitamina D3 e l'acido lipoico sono stati in grado di attenuare l'apoptosi indotta da H_2O_2 attraverso la via mediata dai mitocondri. La combinazione è stata anche in grado di contrastare gli effetti negativi causati dal ferro, impedendone l'accumulo. Tutti questi dati supportano l'ipotesi di un'attività cooperativa esercitata dalla vitamina D3 e dall'acido lipoico negli astrociti, indicando una possibile nuova strategia per rallentare l'invecchiamento cerebrale. Pertanto, la capacità della vitamina D3 e dell'acido lipoico di contrastare l'invecchiamento cerebrale ha portato alla creazione di un prodotto commerciale, Cebral®.

1. Introduction

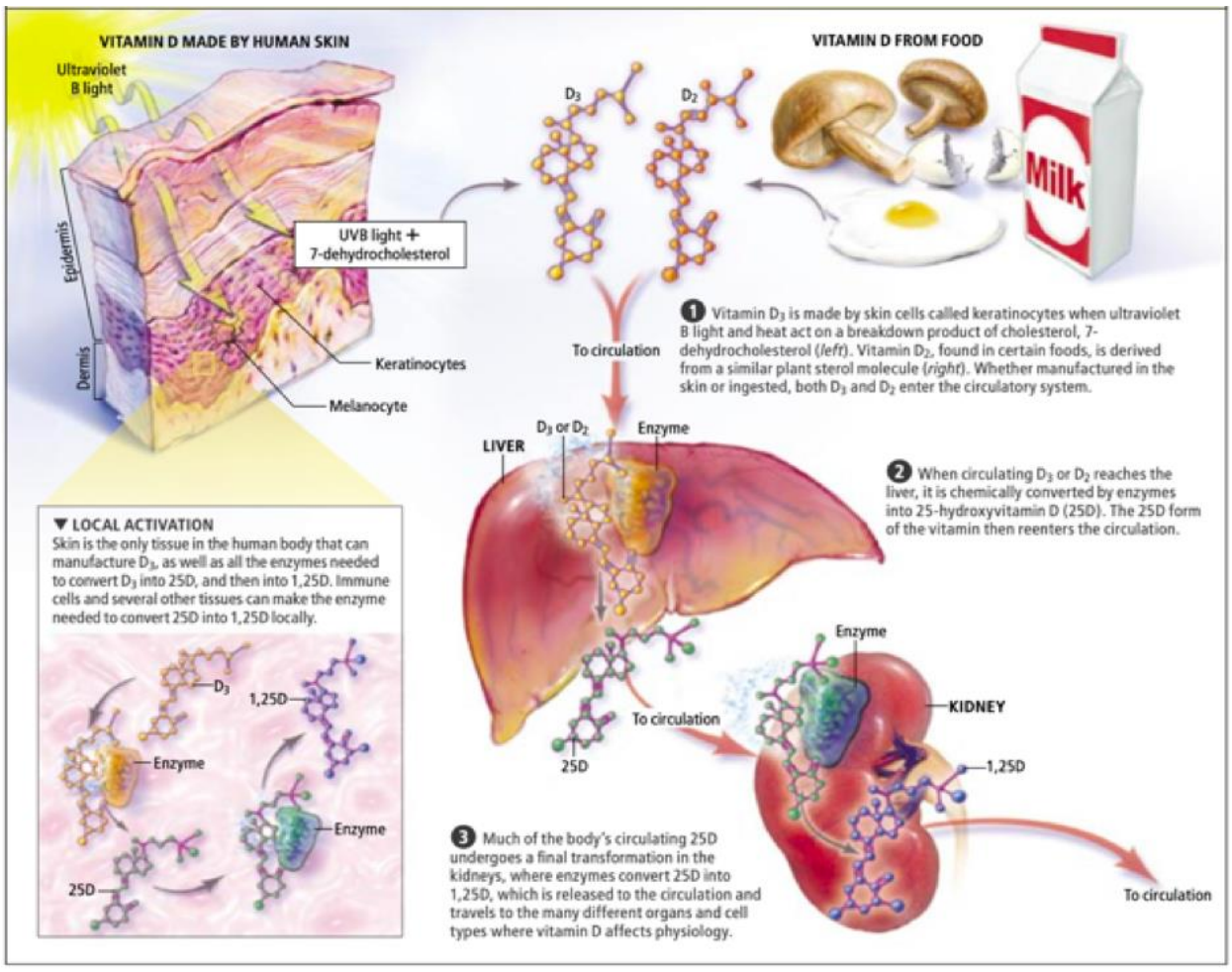
Vitamin D refers to a group of fat-soluble corticosteroids hormone responsible for enhancing intestinal absorption of calcium, iron, magnesium, phosphate and zinc. In humans, the most important compounds in this group are vitamin D3 (also known as cholecalciferol) and vitamin D2 (ergocalciferol) [1].

They are presents naturally in very few foods, for example in fish (vitamin D3) and in some plants (mainly vitamin D2) and for this reason a number of foods have been fortified by the food industries.

Vitamin D3 and vitamin D2 are inactive in many biological systems and have to undergo a series of metabolic transformations before exerting effects in target tissues [2-4]. Vitamin D is also popularly known as “sunshine vitamin” because in the skin sun exposure produces vitamin D. Indeed, the prohormone vitamin D3 is produced in the skin through ultraviolet irradiation of 7-dehydrocholesterol, than to make it biologically active the prohormone vitamin D is transported in the blood by the vitamin D binding protein (DBP) to the liver, where it is metabolized to 25-hydroxyvitamin [5]. Vitamin D is hydroxylated at C-25 by cytochrome P450 vitamin D 25-hydroxylases in the liver, forming 25-hydroxyvitamin D (25(OH)D3). This is the major circulating metabolite of vitamin D in plasma, and its measurements are used to provide an index of vitamin D nutritional status [3, 4]

However, 25(OH)D3 itself is metabolically inactive and must be modified before function [4]. On the next metabolic step, 25(OH)D3 is transported by DBP to the kidney, where in the proximal convoluted tubule of the nephron at the position C1 is hydroxylated, resulting in the hormonally active form of vitamin D 1,25-dihydroxyvitamin D3 (1,25(OH)2D3), which is responsible for most of the biological actions of vitamin D [5]. As a result of 1- and 25-hydroxylation, the prohormone vitamin D is being structurally transformed into an active hormone.

The best known target organs or tissues for 1,25(OH)2D3 are the intestine to stimulate absorption of calcium and phosphate and the bone to cause release of calcium and phosphate. Beside 1,25(OH)2D3, the kidney can also produce 24,25 dihydroxyvitamin D3 (24,25(OH)2D3), a relatively inactive metabolite comparing to 1,25 (OH)2D3. The metabolite 24,25 (OH)2D3 is produced by 24 hydroxyvitamin D3 24 hydroxylase from both substrates: 25(OH)D3 and 1,25 (OH)2D3 [5, 6]. This enzyme 24(OH)D3 24 hydroxylase limits the amount of 1,25(OH)2D3 in target tissues by accelerating the catabolism of 1,25(OH)2D3 and by decreasing the amount of 25(OH)D3 available to 1 hydroxylation [5]. Vitamin D and its nuclear receptor (VDRs) affect the expression of many genes acting as a transcription factor [7] but also non-genomic effects of vitamin D have also been described [8-15].



1.1 Seasonal Variation of Vitamin D Levels

Seasonal variation of vitamin D is a well-documented phenomenon [16]. The endogenous production of vitamin D in winter is drastically reduced because of the reduced exposure to UVB radiation, while in summer, exposure to UVB radiation is adequate for vitamin D synthesis by the skin [17].

Studies have also shown that this seasonal variation might depend on latitude, since it has been found that vitamin D production is greater on latitudes close to the equator [18].

In northern latitudes, production of vitamin D is problematic during the winter and there is an expected seasonal variation in vitamin D status.

The use of sun blocking agents, that is a ubiquitous issue at all latitudes, reduced the endogenous vitamin D production. SPF 8 will block 95% of UVB light while SFP 15 will block 98%, leading to decreased production of vitamin D3 from its precursor 7-dehydrocholesterol. Further, vitamin D is a fat soluble vitamin with a half-life of approximately 15 days [19]. However, vitamin D deficiency has even been reported in sunny regions [20]. This deficiency has been linked to many factors. For instance, skin pigmentation has a strong effect on vitamin D status, since it reduces the UVB radiation that effectively reaches the skin [21]. In the same way, sunscreen use decreases vitamin D production [22]. Another factor capable of influencing the metabolism of vitamin D is obesity. It has been proposed that this is due to fatty tissue uptake of vitamin D, reducing its bioavailability. BMI has been negatively associated with vitamin D levels and greater prevalence of deficiency [23-25]. Regarding age, it has been proposed that vitamin D deficiency in the elderly can be attributed to a decrease in the skin capacity to produce vitamin D due to ageing, from a lack of exposure to sunlight, or from a deficient dietary intake [26].

1.2 Vitamin D Deficiency

A surprisingly high prevalence of vitamin D insufficiency has been recently reported world-wide regardless of their insolation rate [27-30]. In Canada, 30–50 % of children and adults are vitamin D deficient and need vitamin D supplementation [31]. Similar data have been reported in Australia, Brazil, Middle East, Mongolia, Africa and New Zealand documenting a high risk for vitamin D deficiency in adults and children [31-33]. Cross-sectional studies of vitamin D status in adolescents have found deficiency in 17–47% with an increased risk in black and Hispanic teenagers [31-33].

Vitamin D insufficiency is defined as levels <30 ng/ml (80 nmol/L), whereas levels below 20 ng/ml (50 nmol/L) and 10 ng/mL (25 nmol/L) represent deficiency and severe deficiency, respectively (Table 1) [34]. Levels between 40 and 60 ng/mL are the preferred range, while vitamin D intoxication usually does not occur until 25(OH)D3 reaches levels higher that 150 ng/ml [31].

25[OH] Level (ng/mL)	25[OH]D Level (nmoL/L)	Laboratory Diagnosis
25[OH] Level (ng/mL)	25[OH]D Level (nmoL/L)	Laboratory Diagnosis
<20	<50	Deficiency
20–32	50–80	Insufficiency
54–90	135–225	Normal in sunny countries
>100	>250	Excess
>150	>325	Intoxication

Table 1: Blood levels of vitamin D.

1.3 The Genomic Mechanism of Action of Vitamin D

All genomic actions of vitamin D are mediated by VDR. VDR is a transcription factor and a member of the steroid hormone nuclear receptor family, related to the retinoic acid receptors and as most of the receptors has a DNA-binding domain (C-Domain), a ligand-binding domain (E-domain) and finally activating domain (F-domain) [2]. It has been proved that a single

receptor mediates all of the functions of vitamin D. This receptor is a 427 amino acid peptide and acts through vitamin D-responsive elements (VDREs), a complexes required for its genomic activity which are placed on the start site of the target gene [2]. These complexes can be both gene and cell specific, cells research enabling the selectivity of vitamin D3 action from cell type to cell type [4]. VDR was proved to be found in almost all tissues and cells. It has been established that the diverse biological actions of 1,25-dihydroxyvitamin D3 are initiated through precise changes in gene expression, which are mediated by an intracellular VDR. Activation of the VDR through direct interaction with 1,25(OH)2D3 prompts the receptor's rapid binding to regulatory regions of target genes, where it acts to nucleate the formation of large protein complexes whose functional activities are essential for directed changes in transcription. When VDR interacts with the ligand, the repressor is no longer able to bind the receptor and the receptor changes conformation, forming heterodimer at the VDREs [2, 35]. At the same time, it binds several other proteins required in the transcription complex and acquires an activator [2, 35]. Once the complex is formed, DNA bends, biochemical processes take place, and transcription is either initiated or suppressed depending on the gene [2, 35, 36]. These responses are tissue-specific and range from highly complex actions essential for homeostatic control of mineral metabolism to focal actions that control the growth, differentiation and functional activity of numerous cell types including those of the immune system, skin, the pancreas, and bone.

1.4 The Nongenomic Mechanism

Vitamin D3 is also able to activate very rapid nongenomic mechanisms [4], lasting from seconds to 10 minutes [37]. This mechanism involves signal transduction pathways including activation of adenylyl cyclase-cAMP-protein kinase A and phospholipase C-diacylglycerol-inositol (1,4,5)-trisphosphate-protein kinase C signal transduction pathways [38]. Particularly,

the second messengers Raf (rapidly accelerated fibrosarcoma)/MAPK play an important role because they may engage in cross-talk with the nucleus to modulate gene expression [37]. This firstly identified nongenomic activation is associated with the rapid stimulation of intestinal calcium transport called “transcaltachia” [39]. Successively, this effect was identified in the chondrocytes of the bone growth plate [39] and in keratinocytes of the skin [40]. Identification of the receptor for vitamin D3 has focused on the VDR itself albeit in a different configuration to identify agonists able to induce nongenomic effects [41]. Interaction between vitamin D3 and membrane-associated rapid response steroid binding protein (MARRS) has been studied as well. These receptors are located in the membrane within caveolae/lipid rafts [42] where they are poised to activate kinases, phosphatases, and ion channels.

1.5 Physiological Effects of Vitamin D

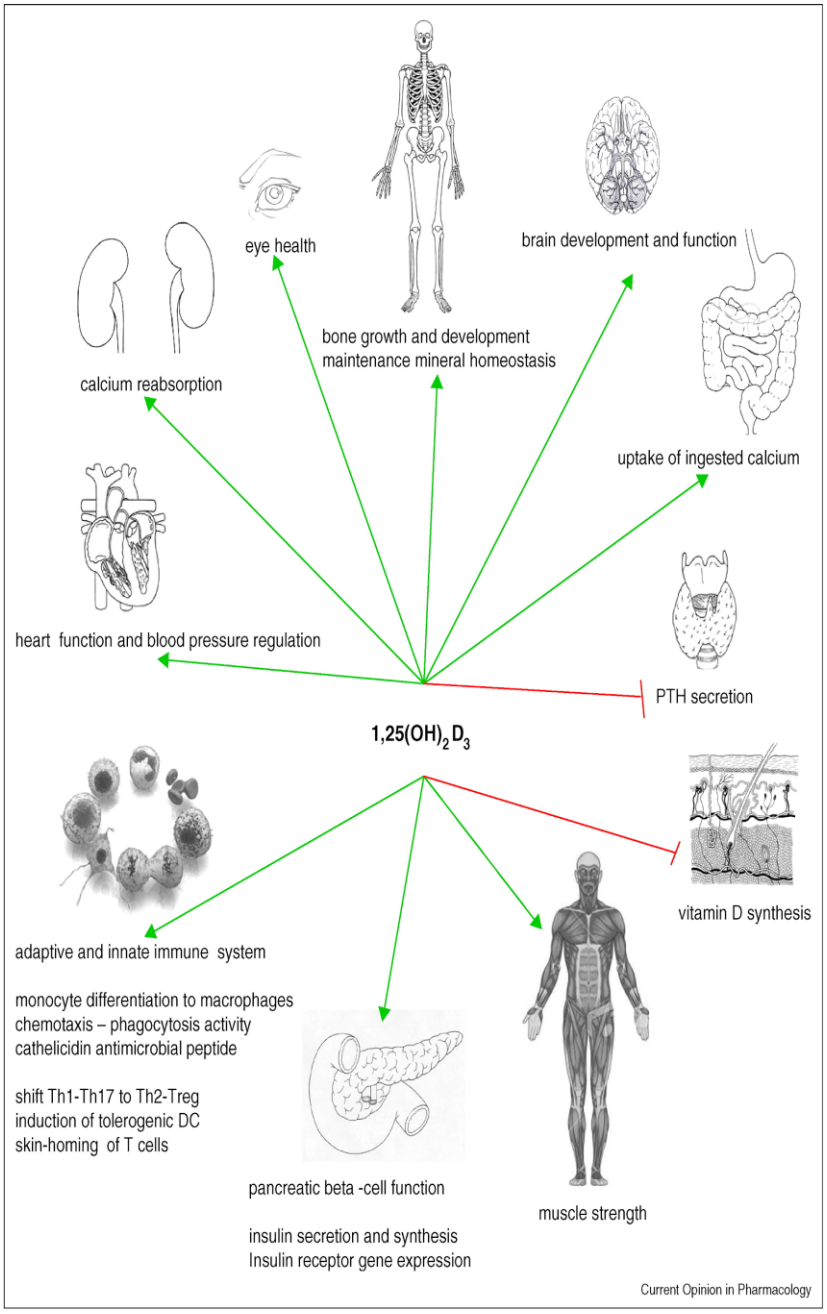
Vitamin D is involved in mineral metabolism, bone growth and it also stimulates absorption of phosphate and magnesium ions; indeed, its main effect is to facilitate intestinal absorption of calcium. In cases when there is a lack of vitamin D, dietary calcium is not absorbed efficiently. The best studied of these calcium transporters is calbindin, an intracellular protein that carries calcium across the intestinal epithelial cell [43].

There is evidence in the literature on the effects of vitamin D on bone tissue: it induces the expression of osteocalcin and suppresses synthesis of type I collagen and in cell cultures, vitamin D stimulates differentiation of osteoclasts. The crucial effect of vitamin D on bone is to provide the proper balance of calcium and phosphorus to support mineralization [43].

Some experiments, using cultured cells, have demonstrated that vitamin D has potent effects on the growth and differentiation of many types of cells. These findings suggest that vitamin D has physiologic effects much broader than that of a role in mineral homeostasis and bone

function. This is an active area of research and a much better understanding of this area will likely be available in the near future [43].

Of interest, VDRs are not only present in different tissues, such as bone, skin, intestine and kidneys, but also non-classical organs like brain, eyes, heart, pancreatic islets (β-cells), immune cells, muscle, adipose tissue, thyroid, parathyroid and adrenal glands [44].



Importantly, many of these non-classical tissues also express vitamin D-activating enzymes, hence, allowing these non-classical actions to occur via local activation of vitamin D.

Recently, a crucial role for vitamin D also in digestive system health has been described [45]. Vitamin D links to several target tissues in the digestive system, in the oral region, in the salivary glands, in the stomach and in the small and large intestine [46].

In gastric mucosa, vitamin D appears to have high preventive and therapeutic potentials [47] and it is able to regulate endocrine and paracrine secretion of gastrin with secondary effects, for instance, on parietal cell HCl and pepsinogen secreting chief cells [46].

A growing body of data suggests that both vitamin D and VDR, play an important role also in the regulation of cardiovascular homeostasis and suggest a protective effect for vitamin D against cardiovascular disease [48-51]. Vitamin D3 deficiency is significantly associated with cardiomyopathy and increased risk of cardiovascular disease with consequent mortality [4].

The Intermountain Heart Collaborative Study revealed that vitamin D3 blood levels below 15 ng/mL compared to vitamin D3 above 30 ng/mL are associated with significant increases in the prevalence of type 2 diabetes mellitus, hypertension, hyperlipidemia and peripheral vascular disease, coronary artery disease, myocardial infarction, heart failure, and stroke, as well as with incident death, heart failure, coronary artery disease/myocardial infarction and stroke [51]. The ability of this hormonal system to inhibit the renin-angiotensin system [52], control blood pressure, inhibit cellular proliferation and hypertrophy, reduce fibrosis and suppress immune function suggests a variety of plausible mechanisms that could contribute to these palliative effects. However, the cardioprotective “hypothesis” has not been without controversy and results to date have been inconclusive.

Some, but not all, observational studies in humans provide support for these experimental findings, raising the possibility that vitamin D or its analogs might prove useful therapeutically in the prevention or treatment of cardiovascular disease. For example, VDR knockout mice

have elevated circulating levels of renin and angiotensin II and develop hypertension [52]. A similar phenotype occurs in mice lacking the 1α-hydroxylase gene [53]. In mice, injection of 1,25(OH)2D3 analogs suppresses renin production *in vivo* and negatively regulates the expression of the angiotensinogen gene [54-56].

Vitamin D is also important in brain homeostasis; the role of vitamin D in the brain development and function of neurons was suggested when the first evidence of 1α-hydroxylase, an enzyme responsible of an active form of vitamin D and VDR, were found to be present in the brain [57]. The specific mechanisms that mediate the neuroprotective effect of vitamin D are still unclear; however, vitamin D may act in many pathways including antioxidant pathways, neuronal calcium regulation, immunomodulation and glutamatergic systems [58-61].

A meta-analysis examining the association between vitamin D3 status and the risk of cerebrovascular events, including more than 1200 stroke cases, found that the pooled relative risk for stroke was 52% higher when comparing 25(OH)D levels ≤12.4 ng/mL with 25(OH)D levels >18.8 ng/mL [62]. As concerned to cerebral effects, the observed widespread distribution of 1α-hydroxylase and the nuclear VDR in both neurons and glial cells suggest that vitamin D3 may have autocrine and paracrine properties in the brain [63, 64]. VDR is highly expressed in multiple brain regions [65] in animal [66] and human [67] brain, particularly in the pontine-midbrain area, cerebellum, thalamus, hypothalamus, basal ganglia, hippocampus, olfactory system, and the temporal, orbital and cingulate areas of brain cortex [68]. Mounting evidence indicates that vitamin D3 and its receptors play an important role in the brain, ranging from neuroprotection to immunomodulation [69], cells proliferation and differentiation [70], and plays an important role both in developing [71] and adult brain [65]. Vitamin D3 can exert these effects since it is able to cross the blood-brain barrier and can bind to VDR within the brain [72, 73].

Moreover, vitamin D3 protects neurons against NMDA, glutamate, 6-hydroxydopamine and reactive oxygen species [74, 75]. It has been hypothesized that vitamin D3 exerts its neuroprotective effects via the modulation of neuronal Ca^{2+} homeostasis, in particular through the downregulation of the L-type voltage-sensitive Ca^{2+} channel in hippocampal neurons against excitotoxic insults [65], accompanied by an increase in VDR density. Vitamin D3 is able to inhibit proinflammatory cytokine and NOS [76] typically increased during various insults or disorders, such as ischemia and reperfusion, Alzheimer's disease, Parkinson's disease, multiple sclerosis and experimental autoimmune encephalomyelitis. Early vitamin D3 deficiency may be considered as a risk factor for a number of neurological disorders including schizophrenia, autism [73, 77], multiple sclerosis, Parkinson's disease and stroke [62, 78]. Vitamin D3 deficiency is associated with reduced cognitive function, which is an important issue for stroke patients [79]. Moreover, in the rat model of stroke, vitamin D3 supplementation has been found to reduce brain damage [80] and consequent seizures [62] and oxidative stress [14]. For this reason, it should be very important to study the role of vitamin D3 in counteracting negative effects of oxidative stress *in vitro* brain. So, vitamin D3 may be considered as a potential drug for the treatment of neurodegenerative disorders.

1.6 Vitamin D specific actions on Ovarian Tissue

In recent years, scientists have increased interest in the study of vitamin D thanks to the discovery of the presence of its receptor VDR in many tissues. It has been demonstrated that in ovarian tissues a high density of VDR is present as well [81, 82]. In female reproduction, the importance of vitamin D was initially appreciated *in vivo,* as mice who were either deficient in vitamin D or lacked the vitamin D receptor (VDR), suffered from underdeveloped uterus and an inability to form normal mature oocytes, which in turn lead to infertility [83]. In humans, VDR is expressed in many female organs, including the ovaries (particularly granulosa cells),

uterus (endometrium and myometrium), and placenta [84]. These receptors are targeted by the active form of vitamin D and produce an array of effects in female reproduction. For example, vitamin D3 regulates genes involved in estrogen synthesis [84] and it also controls several genes involved in embryo implantation [85].

1.6.1 Vitamin D3 and Resveratrol

In this part of research (**Project 1**), the effects of vitamin D3 (vitD) and resveratrol (RES) were tested in ovarian cell culture and tissue. Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a natural antioxidant polyphenolic compound belonging to stilbene phytoalexins, a subgroup of non-flavonoid phenolic compounds [86, 87]. RES is contained in various vegetables such as berries, grapes, peanuts, besides red wine [88]. Particularly, red wine is the main source of RES, but a recent study discovered that peanut sprouts contain abundant RES both in cis and trans isoforms [89, 90]. Cis- and trans-isomers of RES coexist in plants and in wine. RES is rapidly metabolized *in vivo* and has a low water solubility, which reduces the rate-absorption in cells [91] reducing oral bioavailability [92]. The effectiveness of orally administered RES depends on its absorption, metabolism and tissue distribution. At intestinal level, RES is absorbed by passive diffusion or through the formation of complexes with membrane transporters, whereas in the bloodstream it can be found as glucuronide, sulfate, or free as well [93]. In some studies performed on animal models, the peak concentrations of trans-RES occur in blood and serum very rapidly, about 15 min from the beginning of the administration [94]. In different studies, RES solubility has been increased by the use of ethanol (50mg/mL) or other organic solvents [94]. Moreover, researchers have recently attempted to improve RES chemical stability, water dispersibility, bioavailability, permeability through blood–brain barrier (BBB) and therapeutic efficacy by using nanostructure-based drug delivery systems [95-97]. Epidemiologic studies have shown that RES has beneficial effects in preventing

various pathologic conditions ranging from cardiovascular diseases to cancer [98]. As reported by *in vitro* studies, RES can inhibit cell proliferation, induce apoptosis and block cell cycle progression in numerous types of human cancer cell lines, such as those of the colon, skin, breast, lung, prostate and liver, as well as pancreas [99]. In addition, in a few *in vivo* experimental models of colon and esophagus cancers the effectiveness of oral doses of RES was shown [100, 101]. RES acts as a phytoestrogen modulating estrogen receptor (ER)-mediated transcription [102]. There is a variety of RES-sensitive tissues that are ER positive and the two ER subtypes in mammals, ERα and ERβ, exhibit different tissue-specific expression profiles [103]. Specifically, effects of RES on ER include anti-inflammatory effects such as protection from trauma hemorrhage-induced injury and suppression of Interleukin-6 expression in the liver, intestine and cardiovascular system [104]. However, RES does not induce proliferation of mammary or uterine tissues, allowing it to be taken as a dietary supplement. RES binds and increases the transcriptional activity of estrogen receptors (ERα and ERβ) at 50–100 μM [103, 104-106]. RES displays a great affinity for ER behaving as either agonist or antagonist in a cell- and tissue specific manner [107]. This is important to explain the effectiveness of RES in reducing the number of vasomotor episodes and the intensity of hot flashes (HF), with the transition from moderate/severe to mild symptoms in 78.6% of patients [108]. The common incidence of hot flashes is around 75% and presently hormone replacement therapy is the gold standard in the management of moderate to severe vasomotor symptoms associated with menopause. RES has also been associated with anti-inflammatory effects, particularly in tissues that contain a large number of estrogen receptors, through this connection has been studied, but there are few studies on the mechanisms activated [109, 110].

It has been demonstrated that in ovarian tissues a high density of VDR is present as well [81, 82] and vitD acts through intracellular mechanisms similar to what observed for RES [111]. The role of vitD in cellular growth regulation is demonstrated by its ability to arrest cells in the

G1/G0 phase of the cell cycle, and by up-regulating p21, a powerful tumor suppressor gene. Thus, vitD can control cell division and proliferation [112]. VitD also has important anti-proliferative, anti-angiogenic and pro-differentiative effects in a wide range of cancers [113]. Many of the bioeffects of RES overlap with reported benefits from high circulating levels of vitD. Thus, given the ability of vitD to elicit a wide range of bio-effects via transcriptional regulation, it is interesting study RES in the context of VDR signaling to help in elucidating the molecular pathways involved by these two dietary lipophilic substances in optimizing healthspan and well aging [112]. The potential for RES to modulate vitamin D receptor signaling has recently been postulated [114, 115]. There is an overall structural symmetry and parallel configuration of RES and known VDR ligands, which could suggest that RES might serve as a low-affinity VDR ligand with the ability to activate VDR. Intriguingly, several targets emerge such as eNOS, cyclooxygenase and Akt kinase, all of which are likewise regulated by vitD [116]. Finally, vitD exerts beneficial effects on ovarian tissues preventing ROS-derived cellular injury [82]. Therefore, since these two substances have similar effects on ovarian cells, some form of interaction in exerting effects can be hypothesized. This could lead to interesting results for future clinical use in menopause-related conditions like hot flashes.

1.7 Vitamin D and Gastric Disorders

Recently, a crucial role for vitamin D in digestive system health has been described [45]. In gastric mucosa, vitamin D appears to have high preventive and therapeutic potentials [47] and is able to regulate endocrine and paracrine secretion of gastrin with secondary effects, for instance, on parietal cell HCl and pepsinogen secreting chief cells [46]. A problem related to the gastric system is the disproportionate use of nonsteroidal anti-inflammatory drugs (NSAID); indeed, around 30 million people consume NSAID to manage pain, inflammation and fever [117, 118]. The action mechanism of these drugs consists in the inhibition of the

biosynthesis of prostaglandins, the inactivation of cyclooxygenase and an increase in leukotrienes production [119], but adverse events, such as alterations in renal function, effects on blood pressure, hepatic injury and platelet inhibition, are a challenge in clinical treatment optimization [120]. However, severe gastrointestinal disorder accompanied by gastric mucosal perforation and bleeding is a major concern as well as the worst outcome of prolonged NSAID-therapy [121]: indeed, they induce gastric mucosal lesions because of their acidic properties [119]. The mechanism behind gastric damage involves a highly acidic gastric environment that favours the migration of nonionized lipophilic NSAID into the epithelial cells and at the cell surface these are dissociated into ions, trapping hydrogen ions and inducing mucosal injury. This action is further enhanced by the decrease mucosal blood flow, secretion of mucous and bicarbonates, and the defensive factors of the gastric layer [119]. Indeed, these side effects lead to reduced intestinal mucus formation, disturbed micro-circulation causing increased intestinal motility and increased mucosal permeability to many inflammatory mediators including neutrophils and cytokines [122]. The consequences can range from dyspepsia to severe peptic ulcer and bleeding; endoscopically the range from subepithelial haemorrhages and erosions to total destruction of epithelial membrane and full thickness ulcer [123]. Diclofenac, an anthranilic acid derivative with pKa of 4.0 [117], is the most widely prescribed NSAID for treating several forms of pain and inflammation [124-126]. The main clinical problem of Diclofenac is toxicity induced by oxidative tissue injury in the intra-mitochondrial environment, which appears to play a prominent role in the pathophysiology of digestive ulceration [117, 127, 128]. For these reasons, NSAID users are subjected, often empirically by doctors, to anti-acid treatments such as the proton-pump inhibitors (PPI) [129], thus, not surprisingly, NSAID and PPI are among the most frequently co-prescribed drugs worldwide [130]. Moreover, a number of clinical studies have provided the support for the ineffectiveness of PPI in preventing or reducing the NSAID-damages [131-133]. Indeed, current evidence

suggests that PPI are also associated with numerous side effects such as hypergastrinemia [134], enteric infections [135], adverse cardiovascular events [136] and increased mortality rates [137]. The need for NSAID clinical use despite their side effects encourages continuous research to create novel agents able to counteract their adverse effects with better safety profile, in particular using natural compounds. Recently, a product has been introduced that, in addition to having the antacid properties of alginates, claims to possess gastroprotective properties deriving from vitamin D3 and from plant extracts. It has been tested alone and combined with Diclofenac in order to provide an effective and safer strategy for the management of NSAID-induced gastroenteric lesions (**Project 2**).

1.7.1 Vitamin D and Alginates

There is a new group of widely used molecules that includes alginates, a polymer of alginic acid derived from the cell wall of various brown algae [138]. Alginates are polysaccharides composed of two β-d-mannuronic acid (M) and α-1-glucuronic acid (G) monomers which are held together by β 1,4 bonds [139, 140]. Recently, there is a growing interest in alginates as a therapy for Gastro-Esophageal Reflux Disease (GERD) [141]. Alginates are able to block the HCl reflux in a mechanic manner; indeed, they do not have any pharmacologic property [142]. The mechanism of action of alginates has been called “rafting”, which means that in the presence of gastric acid, they form a gel in which carbon dioxide (resulting from the splitting of bicarbonate) is trapped. Then the gel is carried to the top of the gastric contents neutralizing the acidity and preventing the ascent of acid material into the esophagus [143]. The advantage of alginate-based reflux suppressants over antacid alone is that they provide rapid and longer-lasting symptom relief [144].

It was seen that it is useful to associate alginates with natural substances. This association has been found to be effective in promoting stomach health, reducing inflammation and supporting

the immune system. Moreover, alginate/antacid system has been used as a carrier of probiotic, drugs and plant extracts [145]. For example, alginate/bicarbonate combined with two herbal gastroprotective extracts (Opuntia ficus-indica and Olea europaea) has been successfully evaluated in patients with GERD [145].

Recent studies have explored a possible role of vitD on gastroprotection [45, 146]. VitD binds its receptor VDR which is present in several tissue targets in the digestive system, in particular in the oral region and in epithelial cells of the oral cavity, tongue and gums. In addition, vitD appears to have a therapeutic role in gastric mucosa as well, stimulating cell proliferation and differentiation [147] and regulating endocrine/paracrine gastrin and pepsinogen secretions [148]. Finally, vitD acts on smooth muscle cells in the pyloric region and in different areas of the small intestine [148]. Furthermore, the low plasmatic level of vitD is found to be responsible for an insufficient emptying of the stomach, swelling, constipation and intestinal irritation [148]. After the binding between vitD and its receptor, several intracellular events involved in different mechanism start, including the protective role against oxidative stress [149], the regulation of autophagic pathways through the regulation of ATG16L1, a protein complex necessary for autophagy [35], and the ability to inhibit apoptosis by increasing the expression of endothelial nitric oxide synthase (eNOS) leading to the nitric oxide (NO) production [149]. In this context, vitD can exert some beneficial effects on gastric tissue. For this reason, its use in association with other gastroprotective agents such as alginates can increase therapeutic efficacy of the formulation in respect to the efficacy that would be obtained only with gastroprotective drugs.

1.8 Vitamin D and Cardiovascular System

VitD is also a modulator of vascular wall growth as well [150] and induces a decrease in the expression and/or secretion of proinfammatory and proatherosclerotic factors in the

endothelium [151]. The role of endothelium as a target of vitD is demonstrated by the study published by Zehnder et al. [152], in which the expression of mRNA and protein for lα-hydroxylase in human endothelium was shown for the first time. These findings demonstrated the direct effects of vitD on endothelial function, whose alteration plays an important role in the development of atherosclerosis. Indeed, vascular endothelial function is a crucial element as regards cardiovascular function and its impairment is an early manifestation of atherothrombotic disease [153, 154]. Endothelium acts as a barrier and a regulator of blood vessel activity [154] mainly through NO, which can stimulate the relaxation of the underlying smooth muscle, leading to vasodilation [154]. NO is produced by vascular endothelial cells in response to different stimuli and acts as a messenger molecule [154], activating guanylate cyclase to enhance cGMP, which in turn causes relaxation of smooth muscle and vasodilation [155]. NO is produced by nitric oxide synthases (NOS) which utilize L-arginine as their principal substrate, oxidizing it into L-citrulline and NO [153, 156]. The endothelial isoform of NOS (eNOS) is constitutively expressed in endothelial cells and is responsible for the basal release of nitric oxide from the endothelium and for the rapid change into nitric oxide flux in response to physical stimuli and molecular agonists [153, 157]. A decrease in endothelial NO production appears in the early phases of atherosclerosis development [158, 159]. Moreover, NO synthesis impairment is involved in a number of cardiovascular diseases such as peripheral vascular disease, congestive heart failure and cerebrovascular events [154, 160, 161]. The impaired NO status may cause an overproduction of reactive oxygen species in the vasculature, representing a risk factor for cardiovascular disease.

1.8.1 Vitamin D and Protective Molecules

Numerous studies have shown that acute and chronic supplementation with L-arginine improves NO production by endothelium in individuals with risk factors for atherothrombosis,

as well as in individuals with established atherothrombotic disease [153]. For this reason, extracellular L-arginine concentration is the principal determinant of intracellular L-arginine availability for eNOS [153]. Another important element to maintain ROS production at basal level is the use of "natural" substances able to modulate metabolic pathways and to treat pathological conditions. Many studies suggest that a number of natural compounds such as coenzyme Q10, vitD and other products, included in the nutraceuticals group, have the potential to target multiple pathways under these pathological conditions [162-164]. For example, with regard to cardiovascular diseases, there is strong evidence that demonstrate the link between oxidative stress and impaired mitochondrial function [165, 166]. CoQ10 is located in the mitochondria, lysosomes, Golgi apparatus and plasma membranes and provides an antioxidant action either by direct reaction with free radicals or by regeneration of tocopherol and ascorbate from their oxidised state [167, 168]. Endogenous synthesis of CoQ10 in the body decreases with age. It has been observed that up to 75% of ischemic heart disease patients exhibit low levels of CoQ10 in the plasma and in the heart in relation to the course of the disease [169, 170]. A long-term therapy with CoQ10 has been shown to decrease heart failure symptoms, to reduce major adverse cardiovascular events and mortality and to be safe and well tolerated [171]. CoQ10 has been reported to have many biological effects both *in vitro* and *in vivo* [172, 173]. CoQ10 is commonly used for prevention and treatment of many cardiovascular diseases such as myocardial infarction, congestive heart failure and other drug-induced or disease-induced cardiomyopathies [168, 174, 175]. Moreover, Q10 showed the ability to prevent oxidative stress, apoptotic cell death and monocytes cell adhesion in HUVEC [176]. On the basis of the results obtained in this research field, it is possible to speculate on the possibility of preparing a mixture of antioxidants and vitamins able to prevent cardiovascular diseases. Some studies demonstrate that a mix of micronutrients, including vitD, significantly reduces markers of lipid peroxidation and at the same time increases antioxidant potential of the

molecules contained. The importance of vitamin D in the cardiovascular system is evidenced by the observation that the deletion of VDR from the heart results in hypertrophy [177, 178] and contributes to the acceleration of atherosclerosis [4, 179]. Both experimental and clinical studies support the protective role of vitamin D in vascular system and in cardiac function through lowering blood pressure, improving endothelial function, inhibiting oxidative stress and reducing the activity of renin-angiotensin system [180]. Additional studies demonstrate the possible autocrine/intracrine mechanisms exerted by vitamin D as a modulator of endothelial functions [181]. In addition it has been demonstrated that the control on NO production exerted by vitD is mediated by VDR and related to intracellular pathways leading to eNOS activation [181]. Therefore, the VDR modulation could represent a new treatment option for all cardiovascular diseases in the future [182].

Thus, in the present study (**Project 3)** it was examined the effects of combination of vitD, Q10 and L-arginine on cell viability and NO/ROS production in endothelial and cardiac cells in order to assess a potential cooperative effect of these substances on cardiovascular function at the cellular level.

1.9 Vitamin D in Nervous System

The effects of vitamin D also affect the central and peripheral nervous system. Vitamin D receptors are in fact present throughout the brain including the primary motor cortex, the region that coordinates movement [183-185]. Specifically, VDRs have been identifIed in neuronal and glial cells in several brain areas including the cortex, deep gray matter, cerebellum, brainstem nuclei, spinal cord and the ventricular system [185.]. Furthermore, the enzyme 1α-hydroxylase, the activator of vitamin D precursors, is also present in the brain [183-187]. Vitamin D levels are associated with the conductance velocity of motor neurons and neurotransmission mediated by dopamine, serotonin, acetylcholine, GABA and the

catecholamines [186, 187]. The combination of *in vitro*, *ex vivo* and animal model data provides evidence that vitD has a crucial role in neuronal proliferation, differentiation, neurotransmission, neuroplasticity and neuroprotection and its levels correlating to the levels of several neurotrophic factors including nerve growth factors (NGF) and that of neurotrophins, which play crucial roles in the maintenance and growth of neurons [188, 189].

Some evidence implicates vitD as a candidate in influencing susceptibility to a number of psychiatric and neurological diseases, such as schizophrenia, autism, Parkinson disease (PD), amyotrophic lateral sclerosis, epilepsy, Alzheimer disease (AD) and is especially strong for multiple sclerosis (MS) [190-192].

The neuroprotective effect of vitD has been recently reported in cognitive decline of aging rats [193], and it has been extensively studied in the animal model of MS and the experimental allergic encephalomyelitis. The hormone prevents onset and reversibly blocks progression of clinical signs, but such a protective effect is absent in VDR knockout mice [191]. The effect of vitD might not be due exclusively to its neuro-immunomodulatory properties [191] since recently it has been reported that the hormone enhances neural stem cell proliferation and differentiation into neurons and oligodendrocytes, the myelinating cells of central nervous system [194, 195]. Neural stem cells constitutively express VDR, which can be upregulated by vitD [194]. VitD regulates the expression of many AD-related genes. It attenuates Aβ peptide accumulation by stimulating phagocytosis of Aβ peptide probably by modulating transcription of Toll-like receptors and cytokines together with enhancing brain-to-blood efflux transport by increasing P-glycoprotein expression [196]. Alterations in adult neurogenesis appear to be a common hallmark in different neurodegenerative diseases including PD and AD [197]. Therefore, factors that stimulate neurogenesis have been indicated as possible treatments of neurodegenerative disorders.

Moreover, the combination of anti-neurodegenerative drugs with vitD supplementation might be useful. Indeed, the supplementation of the combination nemantidine plus vitD has been shown to prevent cognitive decline more efficiently than that of the single compounds [198]. In addition, vitamin D exerts direct neuroprotective effects via the synthesis of proteins binding calcium (Ca^{2+}) ions; it is important in neuronal function and in neuronal transmission. Proper levels of neuronal calcium are critical because their excess may result in the formation of ROS with consequent neuronal damage. Indeed, vitamin D levels are inversely associated with oxidative stress which damages the brain leading to neuronal apoptosis or necrosis [199].

Vitamin D also affects neuroplasticity, a process in which neural synapses and pathways are adapted to the needs of environmental and behavioral demands adjusting the brain to noxious stimuli diseases or environmental cues [185, 200, 201]. The VDRs in glial cells play in the uptake and release of neurotransmitters, including that of GABA neurotransmission within the motor cortex [202]. GABAergic function is the principal "brake" within the brain affecting muscle relaxation via the corticospinal neurons [203].

1.9.1 Vitamin D3 and Lipoic Acid

Alpha-lipoic acid (LA) is a fatty acid containing 8 carbons and 2 sulfur molecules in a dithiolane ring [204]. Initially discovered as a bacterial growth factor in potato extract, LA was later isolated and characterized from bovine liver [205, 206]. It is naturally synthesized in the liver and other tissues and obtained from various animal and plant sources in the diet. LA is a potent antioxidant, acting as a cofactor for mitochondrial enzymes, pyruvate dehydrogenase, α-keto-glutarate dehydrogenase activity and branched chain alpha-keto acids. In addition, LA affects numerous inflammatory pathways by modulating the NF-κB-dependent gene expression [207-210]. LA's antioxidant properties were identified in 1959 when Rosenberg and Culik found that the administration of LA to vitamin C-deficient guinea pigs relieved

scurvy symptoms [211]. LA has been used for decades in Germany to treat diabetic neuropathy, cirrhosis and mushroom and heavy metal poisonings [212]. Endogenous LA occurs in the R chiral conformation (R-LA) at the C6 carbon atom. Manufacturing LA produces racemic LA, an even mix of the R-LA and S-LA chiral conformations. The bioavailability and bioactivity of racemic vs R-LA is debated [208, 213]. The functional activity of LA derives from the disulfide bond between the 2 thiol groups that cycles between the oxidized (LA) and reduced conformations (dihydrolipoic acid, DHLA). LA is highly reactive to free radicals, preventing oxidative damage by directly scavenging and neutralizing ROS [204, 210].

Several randomized clinical trials have demonstrated that LA can act as a therapeutic agent in chronic diseases such as diabetes mellitus [214], cardiovascular diseases [215] and cancers [216] by decreasing chronic inflammation and improving glucose-insulin homeostasis and endothelial function [217]. It has also been shown that LA exerts beneficial effects on the redox state of the plasma and endothelium-dependent vasodilation [218]. Previous studies have demonstrated that LA can decrease lipopolysaccharide-induced inflammatory responses and act as an anti-inflammatory agent through affecting the cyclooxygenase-2 [219, 220] and inducible nitric oxide synthase (iNOS) [221].

LA binds to Cu and Fe preventing their production of free radicals. In mitochondria, the LA/DHLA redox couple serves as a key cofactor of the pyruvate dehydrogenase complex and α-ketoglutarate dehydrogenase of the Krebs cycle, and aids synthesis of nucleic acids, thereby improving mitochondrial efficiency and reducing ROS generation [210, 212]. LA modulates also the PKB/Akt signaling pathway important for vascular endothelial integrity and the redox-sensitive transcription factor Nrf2 and NF-κB pathways, thereby indirectly promoting an antioxidant environment [222-225].

There is no doubt that it is a strong antioxidant, but due to certain reasons its use for medicinal purposes is prohibited; however, in some states it is used as a supplement and in others as a

remedy [226, 227]. These restrictions are due to some endogenous characteristics of substance by itself, such as the changeableness due to the disclosing of dithiolane ring and the emergence of disulfide bond between molecules. Other properties that limit the oral use of LA are its decreased ability to become dissolved in the gastrointestinal tract and increased rate of hepatic metabolism. In addition, besides it is widely known antioxidant potential, LA has also many other functions, as it is its involvement in mitochondria producing energy, by acting as cofactor for various enzymes involved in metabolism [226]. For its antioxidant effect, LA has been used in combination with vitD to study the capacity to prevent or repair the damage caused by oxidative stress and neurodegeneration (**Project 4**).

2. Aims of the Thesis

In recent years, vitamin D has seen growing interest among researchers, especially due to the presence of its receptor VDR in many tissues and organs. For this reason the biological effects of vitamin D3 (the active form of vitamin D, 1,25-dihydroxyvitamin D3, vitD) mediated by the interaction with VDR were studied in different tissues (ovarian, gastric, cardiac and brain).

- ➢ It has been demonstrated that in ovarian tissues a high density of VDR is present as well and vitD acts through intracellular mechanisms similar to what observed for resveratrol, a natural antioxidant polyphenol able to exert a wide range of biological effect on several tissues. Despite its important beneficial properties, it has a low water solubility, which limits its therapeutic applications in humans. The aims of this part of research (**Project 1**) were to evaluate the cooperative effects of resveratrol (RES) combined with vitD on ovarian cells studying cell viability, ROS production, activated pathways in *in vitro* study and in *in vivo* study to quantify vitD and RES, to analyze the concentration of radical oxygen species and to study activated pathways. Moreover, the modulation of specific intracellular pathways involving ER and VDR receptors has been studied to hypothesize a future clinical use in menopause related conditions like hot flashes.
- ➢ Since vitD appears to have gastroprotective properties, the effects of vitD have been studied also in stomach combined with alginates (**Project 2**); it has been tested alone and combined with Diclofenac in order to provide an effective and safer strategy for the management of NSAID-induced gastroenteric lesions. Hence, the primary objective of this research was to evaluate whether it is possible to improve the protection of the gastric mucosa during therapy with Diclofenac. Thus, the present study was designed to examine the protective effects of alginates combined with vitD assessing its effects on factors related to gastric protection such as viability, radical productions and intracellular mechanism involved.
- ➢ As regards the research part focused on vitD in cardiovascular system (**Project 3**), the effects of combination of vitD, Q10 and L-arginine on cell viability and NO/ROS production in endothelial and cardiac cells were evaluated in order to assess a potential cooperative effect of these substances on cardiovascular function at the cellular level. Moreover, the involvement of PI3K/Akt and ERK/MAPKs pathways leading to eNOS activation has been investigated. To confirm data, the same agents were also tested in an animal model to assess vasodilation, NO and ROS production.

- As regards the research part focused on effects of vitD in brain ageing (**Project 4**), the ability of vitD combined with lipoic acid (LA) has been studied to prevent or repair the damage caused by oxidative stress and iron accumulation, two different biological aspects involved in brain ageing and neurodegeneration. Cell viability, mitochondrial membrane potential and pathways activated in oxidative condition has been studied. In addition, to investigate the protection exerted by combination of vitD and LA in induced neurodegeneration using catalytic iron, cell viability, ROS production, iron concentration and activation of intracellular pathways have been studied in order to hypothesize a new human anti-ageing treatment.

3. Materials & Methods

3.1 Cell Culture

In order to investigate the effects of vitD mediated by VDR receptor in different tissues, we used several cell types which of them some directly isolated by organ of interest.

- **Project 1** (ovarian line of research)

CHO-K1 cells (Chinese Hamster Ovary cell), an epithelial cell line derived as a subclone from the parental CHO cell line initiated from a biopsy of an ovary of an adult Chinese hamster [228], have been purchased from Lonza (Basel) and cultured in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM-F12; Sigma, Milan, Italy) supplemented with 10% fetal bovine serum (FBS, Sigma, Milan, Italy), 2mM glutamine and 1% penicillin/streptomycin (Sigma, Milan, Italy) and incubated at 37°C, 5% CO_2, and 95% humidity [229]. When the cells reached 80–90% of confluence were seeded for different experiments; 1×10^4 and 2.5×10^4 cells were plated in a 96-well for MTT test and ELISA activation assay, respectively; 1×10^5 cells plated on 24-well plates to analyzed ROS production; for Western blot analysis and SOD activity the cells were seeded in 6 wells and maintained until 85% of confluence.

- **Project 2** (gastric line of research)

Primary epithelial gastric cells were obtained from the stomach of anaesthetized prepubescent pigs as previously described [45]. Briefly, the gastric cells were isolated using enzymatic solution in agitation (collagenase/dispase solution, Sigma-Aldrich, Milan, Italy) for 60 minutes at 37°C, centrifuged at 1500 rpm for 5 minutes at 4°C and then the pellet resuspended in complete medium (Ham's F12 supplemented with 10% FBS, Sigma-Aldrich, Milan, Italy) on collagen coated dishes. The cells used for the experiments were obtained from passage 3 to passage 5. The cells were used to perform different experiments; to study cell viability, ATP level and NO production $1x10^4$ cells were plated on 96 well-plates; to study ROS production $1x10^5$ cells were plated on 24 well-plates; to study the intracellular pathways by Western blot

and to analyse the activities by kit ELISA the cells were plated on 60mm dishes until confluence. To synchronize the cells, before stimulations they were maintained in DMEM without red phenol and FBS and supplemented with 1% penicillin/streptomycin, 2mM L-glutamine and 1mM sodium pyruvate in an incubator at 37°C, 5% CO_2 and 95% humidity for 18h and then to create a similar condition of human stomach, the cells were maintained for 2h on acidified medium (HCl was added at DMEM without red phenol and FBS and supplemented with 1% penicillin/streptomycin, 2mM L-glutamine and 1mM sodium pyruvate to obtain a medium with pH 4) before the stimulation. This acidified medium was maintained during the successive stimulations.

- **Project 3** (cardiovascular line of research)

Experiments were performed on both PAE and H9c2 cells.

Porcine aortic endothelial cells (PAE) were purchased from Cell Applications, Inc. (San Diego, CA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, Milan, Italy) supplemented with 10% fetal bovine serum (FBS; Sigma), 2mM L-glutamine (Sigma), 1% penicillin-streptomycin (Sigma) at 37°C with 5% CO_2, as reported in literature [230]. The cells were plated to $1x10^4$ in 96-well plates to analyze cell viability using MTT test, to evaluate NO production and ROS production using Griess assay and a rate of superoxide release, respectively. To study the intracellular pathways using Western blot, they were plated in a 6-well until confluence (about 90%).

Rat cardiac cells (H9c2) were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco modified Eagle Medium (DMEM; Sigma, Milan, Italy) supplemented with 10% fetal bovine serum (FBS; Sigma), 1% penicillin–streptomycin (Sigma) and 2mM L-glutamine (Sigma) in an incubator at 5% CO_2, and 37°C, as reported in literature [231]. The experiments were performed with cells from passages 14–17. The cells were plated to $8x10^3$ in 96-well plates to analyze cell viability using MTT test; to evaluate NO and ROS

production using Griess assay and a rate of superoxide release, respectively; finally, to study the intracellular pathways using Western blot, cells were plated in a 6-well until confluence (about 90%). Both cell lines before the experiments were maintained in DMEM 0% serum supplemented with L-glutamine, penicillin-streptomycin without red phenol (starvation medium) for 4-18h.

- **Project 4** (brain line of research)

Experiments were performed using both primary mouse astrocyte and HUVEC cells.

Primary mouse astrocyte cultures were prepared from both male and female C57BL/6 mouse pups, following a classical technique described elsewhere [232] according to the National Guideline for the Use and Care of Laboratory Animals. Briefly, within 24 h of birth, pups were euthanized and cortices were dissected, minced, mechanically digested, and let settle for 30 min at room temperature. Then the cell suspension was centrifuged at 800 rpm for 5min. Pelleted cells were resuspended in Neuronal Basal Medium (Sigma-Aldrich), supplemented with 5% fetal bovine serum (FBS, Gibco), 1% penicillin/streptomycin (Sigma-Aldrich), and 2mM L-glutamine (Sigma-Aldrich), plated in multiwells and maintained in culture for 6 days before treatment. Astrocytes should be separated from microglia and oligodendrocyte precursor cells by shaking, as reported in literature [233]. For the experiments, 1×10^4 cells on a 96-well plate were plated to study cell viability by the MTT test, amyloid precursor protein (APP) by the ELISA test, and ROS production by the colorimetric test; 1×10^4 cells on a black 96-well plate to analyze the oxygen consumption by a fluorescence kit; 1×10^6 on a 6-well plate to determine the iron concentration by the colorimetric assay; 1×10^6 on a 6-well plate to analyze the intracellular pathways activated by Western blot analysis; 1×10^6 on a 6-well plate to analyze p53 activity and ERK/Akt activation; and 4×10^4 on Transwell support to study the permeability, to quantify vitD and LA. Before stimulations, the cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) without red phenol and fetal

bovine serum (FBS, Sigma-Aldrich) and supplemented with 1% penicillin/streptomycin (Sigma-Aldrich) and 2mM L-glutamine (Sigma-Aldrich) in an incubator at 37°C, 5% CO_2 and 95% humidity for 3h.

Human umbilical endothelial cells (HUVEC), cells derived from the endothelium of veins from the umbilical cord, were used in order to obtain a co-culture together with astrocytes as an experimental *in vitro* model of BBB. HUVEC were purchased from ATCC®. Cells were cultured in EGM Media (Lonza) supplemented with 10% FBS (Gibco), 1% penicillin/streptomycin (Sigma-Aldrich), and 2mM glutamine (Sigma-Aldrich) in an incubator at 37°C, 5% CO_2 and 95% humidity. For the experiments, 1×10^5 HUVEC cells/cm^2 were plated in the apical compartment of 6.5mm Transwells with a 0.4μm pore size polyester membrane (Corning Costar, Sigma).

3.2 Animal Model

In order to verify the effects observed *in vitro*, some experiments were performed also in *in vivo* studies.

- **Project 1** (ovarian line of research)

Female Wistar rats weighing 300 to 350g (n = 94) purchased from Envigo^{++++} (Bresso, Italy), were housed in a room at a constant temperature of 25°C on a 12h/12h light/dark cycle with food and water available ad libitum. All experiments were conducted in accordance with local ethical standards and prospectively approved by the University OPBA (*Organismo Preposto al Benessere degli Animali*, Animal Wellness Committee). Experimental protocols were approved by national guidelines (Ministero della Salute authorization number 914/2015-PR) and in accordance with Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 86–23, 1985 revision).

- **Project 3** (cardiovascular line of research)

Male Wistar rats weighing 350 to 400g (n=125) were housed in a room at a constant temperature of 25°C on a 12-hour/12-hour light/dark cycle with food and water available ad libitum. All experiments were conducted in accordance with local ethical standards and protocols approved by national guidelines (legislative decree (DLGS), January 27, 1992, license 116) and in accordance with Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 86-23, 1985 revision). For each animal, anesthesia was performed via sevoflurane (5% for induction and 3% for maintenance) in oxygen [169]. A heat pad maintained the temperature of the animals. Under sterile conditions, rats underwent surgical procedure, thoracotomy, to expose and isolate aorta in order to perform the injection and to measure blood flow, using flowmeter probe (model 420, Transonic Systems, Inc., Ithaca, NY) positioned around the vessel. Aortic blood and tissue were sampled immediately after the end of treatments. Heart rate and respiratory rate were also continuously monitored. Aorta was the better choice to study cardiac function of drugs [234]. Additional experiments were performed to study the effects of combination of vitD, Q10 and L-arginine and after ischemia-reperfusion injury. Ischemia was induced following a classical technique [235], clamping isolated aorta downstream of the induction point. The clamp was maintained for 30min monitoring aortic blood flow. After that, the clamp was removed and reperfusion was maintained for 1h, monitoring aortic blood flow. Finally, during reperfusion, some animals were treated with QLD (Q10+L-arginine+ vitD) and monitored for 1h.

3.3 Experimental Protocol

Since the experiments explore the effects on different tissues, we subdivided the experimental protocol following this order: ovarian, gastric, cardiovascular and brain fields.

- **Project 1** (ovarian line of research)

CHO-K1 cells were treated with Resveratrol (RES) in a range of 10-100μM to determine an optimal concentration; 50μM was chosen and its efficacy verified in a time-course study (from 2 min to 48 h). The RES concentration was chosen basing on previous studies about the therapeutic range of ovarian evidence [236, 237] and on the experiments of dose-response study. RES was prepared in lipidic solvent that was also tested alone in CHO-K1 cultures. The cooperative activity of RES with vitD, was also tested, evaluating the effects of the co-stimulation with RES 50μM and vitD 100nM [238] in CHO-K1 cells during time. In addition, in order to study the bioavailability, 0.5mg RES were administered by gavage in Female Wistar rats following a standard technique [239, 240]; the quantity of RES was calculated by the conversion formula (animal-man) approved by FDA [241]. For each animal, anesthesia was performed via isoflurane (1.2–1.5 Mean Alveolar Concentration) in oxygen and gavage was carried out using probe-ended stainless-steel gastric tubes (80×1.5 mm, length×outer diameter). After treatment, rats were placed in individual cages and housed separately for the duration of the study and daily monitored. The animals were randomized in different groups: $n = 36$ treated with RES lipophilic formula; n = 36 with RES plus vitD 0.4μg lipophilic formula; $n = 18$ treated with vitD 0.4μg alone; $n = 4$ untreated (control) and sacrificed at T0. Time-point for each treatment (2, 5, 15, 30, 60, 180, 360, 720, 1440min) was conducted in triplicates. The animals were euthanized by CO_2 asphyxiation at each time point and the organs (liver, stomach, intestine, heart, kidneys and ovaries) were withdrawn to evaluate biodistribution of the different RES formulations, and to evaluate the ovarian tissue integrity by Western blot. In addition, blood samples used for RES determination by HPLC analysis, ROS and vitD quantification (by ELISA kit) were collected at each time-point using CBC tubes to obtain plasma by centrifugation at 3000 rpm for 15min at room temperature.

- **Project 2** (gastric line of research)

Primary epithelial gastric cells were used to analyze the effect of Aquilea Reflux® (Aq) and Neobianacid® (Neo) in preventing or reverting the damage caused by anti-inflammatory drugs (in particular Diclofenac) during oxidative stress or hyperacidic condition. Aq and Neo were tested in preliminary experiments before or after 200μM H_2O_2 or 1:172 (%v/v) HCl to determine the effects (prevention or repair, respectively) during oxidative stress or gastric hyperacidity. The time of stimulation was 24h, in order to observe cell viability, ATP consumption, ROS production and intracellular pathways activations. Since the greatest effects were observed during hyperacidic condition, this state was maintained during successive stimulations with anti-inflammatory drugs. In particular the protective effects on gastric epithelial cells of Aq and Neo were examined during pre and post stimulations with 250μM Diclofenac (D). The time of stimulation was 24h to study the effects on cell viability, ROS production and the intracellular pathways involved. To confirm the potential use of Aq and Neo as gastroprotective agents, some data were compared to those obtained from experiments with 10 μM Pantoprazole (P), added before Diclofenac.

- **Project 3** (cardiovascular line of research)

The study can be divided in two parts: in the former *in vitro* were performed whereas in the latter experiments were *in vivo*. In *in vitro* experiments, both PAE and H9c2 cells line were used to study the cooperative activity of different biological substances such as vitD, Q10 and L-arginine and to demonstrate the efficacy on vasodilation. Firstly, the influence of the single agents was tested on cell viability in a time-course study (from 30sec to 300sec) on H9c2 and PAE cells. The range of concentration of the single agents was reported to exert biological effects: from 2.5μM to 50μM for Q10, from 2.87mM to 11.5mM for L-arginine and from 10nM to 1μM for vitD [172, 235]. Q10 and vitD were prepared in ET-OH100%, then diluted in sterile saline 0.9% solution and then directly added to the culture medium to obtain the final concentration reported above; L-arginine was directly dissolved in sterile saline 0.9% and then

directly added to the culture medium to obtain the final concentration reported above. Secondly, the cooperative activity was demonstrated adding all agents at the same time (QLD) to analyze cell viability, NO production and ROS production on both cell lines. Finally, the intracellular pathways involved by single agents and QLD were studied by Western blot analysis. In addition, the inhibitors (100nM wortmannin, 10μM UO126 and 10mM L-NAME administered 30min before the QLD addition) [181] of intracellular pathways were tested on NO production, to verify the mechanism of action.

In *in vivo* experiments, 130 male Wistar rats (weighing 350 to 400g) were used to study blood flow, NO and ROS production, SOD activity and intracellular pathways. Rats were randomized into 4 groups: n=115 to evaluate time-course (from 30 to 300sec) of single agents for each concentration including untreated rats and solvent alone; n=10 to study QLD; n=5 to study ischemia, n=10 to study QLD after ischemia induction.

Dose selection was based on observations from *in vitro* experiments and upon a study on the translation of doses from animal to human [241]. Thus, Q10 administration resulted in 12.5mg for *in vitro* experiments and 50mg in *in vivo* experiments. Similarly, L-arginine administration was 0.5g and 2g respectively and vitD was 1.25μg and 5μg respectively. The agents administered in rats were prepared in 750μl of sterile saline 0.9% solution in a similar manner to what reported in *in vitro* experiments. Blood samples were obtained from the abdominal aorta with a sterile syringe containing 50 lL of heparin (100 United States Pharmacopeia units/mL; Sigma). Finally, abdominal aorta tract was removed immediately and placed into lysed buffer for Western blot analysis and stored at -80°C till to extractions.

- **Project 4** (brain line of research)

Primary astrocytes were used to study oxidative stress and iron-dependent damage that are two different biological aspects involved in brain ageing and neurodegeneration.

The role of vitD and LA under physiological condition was analyzed in the first set of experiments. In this step, the dose- and time-dependent studies (from 15min to 1440min) on cell viability were performed with LA (from 10μM to 100μM) to determine its optimal concentration, and then this concentration (50μM) was maintained in all successive experiments. Then the combination with 50μM LA and 100nM vitD [242, 243] was investigated in a time-course study (from 15min to 1440min) and then by the permeability assay to determine each specific concentration through BBB. In a second set of experiments, the role of oxidative stress was investigated by pretreatment for 30min with 200μM H_2O_2 on astrocytes [244]. Through MTT test the ability of vitD and LA alone and combined to prevent or restore the damage caused by oxidative stress was analyzed. Moreover, mitochondrial membrane potential measurement, amyloid precursor protein (APP) quantification, and Western blot analysis were performed at 24h. In a third set of experiments, to induce neurodegeneration, cells were pretreated with catalytic iron (Fe^{3+}) 300μM for 6 days [245] and then treated with vitD and LA alone and combined for additional 6 days to investigate the protection exerted by the combination, analyzing viability, ROS production, iron concentration, APP quantification and activated intracellular pathways.

3.4 Cell viability

The non-cytotoxic effects of vitD combined with other agents were investigated using MTT test, basing on the conversion of water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) compound to an insoluble formazan product. The measured absorbance at OD 570nm is proportional to the number of viable cells. The MTT assay can be used for determined the cytotoxicity of chemically active drugs or substances e potentially toxic. The intensity of the color of the solution obtained is directly proportional to the concentration of formazan and is corresponding to the vitality of the biological sample. MTT-

based In Vitro Toxicology Assay Kit (Sigma-Aldrich) was performed as described in literature [45] to determine cell viability after stimulations. Cells were incubated in DMEM without red phenol 0% FBS with 1% MTT dye for 2h at 37°C in incubator and then cell viability was determined measuring the absorbance through a spectrophotometer (VICTOR X4 multilabel plate reader) at 570nm with correction at 690nm. The results were obtained comparing the results to control cells (100% viable). This technique has been used in all **Projects** (**1 to 4**).

3.5 Agents preparation

In **Project 2**, alginates combined with vitD was used to prepare a commercial product (named Aquilea Reflux®, Laborest Italia srl, Milan, Italy; Aq) used as a dietary supplement indicated to counteract high acidic conditions thanks to its specific composition [45]. Aq is a dietary supplement combining the properties of calcium alginate with a tyndalized probiotic (Pylopass®) and an extract of prickly pear and olive leaves (Mucosave®) in a buffer solution. It is composed of Mucosave® (0.83mg/ml), calcium alginate (1.66mg/ml), magnesium hydroxide (2.66mg/ml), potassium citrate (4.66mg/ml), Pylopass® (0.66mg/ml) and vitamin D3 (0.000083mg/ml). It was 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, 2mM L-glutamine and 1mM sodium pyruvate (white medium).

A mixture of polysaccharides and flavonoids present as a commercial product (Neobianacid®, Aboca, Italy; Neo), is a product that improves the protection of the stomach and the esophagus thanks to the presence of Poliprotect® and a flavonoid fraction (Matricaria recutita and Glycyrrhiza glabra). This product was dissolved following the manufacturer's instructions directly in white medium and added without dilution to the cells. Diclofenac (D) and Pantoprazole (P) were prepared directly in white medium and added to the cells at final

concentrations of 250μM and 10μM respectively, as reported in literature [118, 246] before or after Aq or Neo in presence or absence of hyperacidic condition. HCl was used to prepare an acidified medium (pH 4) that was added to the cells without dilution. To create a hyperacidic condition, before stimulation 1:172 (%v/v) of HCl were also administered to the samples.

3.6 Blood-Brain Barrier Experimental Model

The blood-brain barrier (BBB) controls the passage of substances between the blood and the central nervous system (CNS) and it is a dynamic biological interface, for this reason we used this model on **Project 4**.

Astrocytes were cocultured with HUVEC cells according to methods reported in literature [247]. In brief, 4×10^4 astrocytes/cm^2 were plated on the basolateral side of the flipped 6.5mm Transwells with a polyester membrane with 0.4μm pore size (Corning Costar, Sigma-Aldrich) and left to attach for 4h. Transwells were then placed into the normal orientation and the cells left to grow for 48h. After this time, 1×10^5 HUVEC cells/cm^2 were plated in the apical compartment. The inserts were then placed in a 24-well plate. After 7 days of culture, the Transwells were treated, and permeability studies were performed [248]. To understand the ability of tested substances to cross the blood-brain barrier, the medium at the bottom side of the Transwells was quantified over time (from 15min to 1440min) by measuring the volume and the concentration of vitD and LA.

3.7 ROS Production

ROS production were analyzed on all **Projects** (**1 to 4**) to evaluate oxidative stress condition modified by the stimulation with vitD alone or combined with other agents. The rate of superoxide anion production was determined as a superoxide dismutase-inhibitable reduction of cytochrome C, following a standard technique [242]. 100μl of cytochrome C were added in

both treated and untreated cells and in another sample, 100μL of superoxide dismutase were also added for 30min in incubator (all substances from Sigma-Aldrich). After that, the absorbance was measured at 550nm using a spectrophotometer (VICTOR X4 Multilabel Plate Reader) and the O_2 was expressed as nanomoles per reduced cytochrome C per microgram of protein. In addition, only in **Project 1**, we investigated the effects also on plasma samples. The concentration of ROS in plasma was measured in a 96-well plates using the Antioxidant Assay kit (Cayman) following the manufacturer's instructions [249]. 10μl of Metmyoglobin and 150μl of Chromogen per well were added in plasma and standard samples (Trolox in Assay buffer from 0mM to 0.33mM) and the reactions started adding 40μl of Hydrogen Peroxide Working Solution to all the wells. The 96-well plate was covered, mixed for 5min at room temperature and the absorbance was measured using spectrophotometer (VICTOR X4 Multilabel Plate Reader) at 750nm or 405nm. The results were expressed as means ±SD (%).

3.8 SOD Activity Assay

Superoxide dismutase (SOD) has been measured by Cayman's Superoxide Dismutase Assay Kit basing on detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The SOD assay measures the three types of SOD (Cu/Zn, Mn, and FeSOD). The cells and tissue were lysed after treatments following manufacturer's instructions. In a 96 well, 10μl were added 200μl of the diluted Radical Detector in samples. At the same time, a standard curve was prepared (0.05–0.005 U/ml). Then, 20μl of diluted Xanthine Oxidase were added at all wells and the plate mixed for 30min at RT and then the absorbance measured through a spectrometer (VICTOR X4 multilabel plate reader) at 480nm. The results were expressed as a means (%) compared to control. This technique has been used in both **Project 1** and **3**.

3.9 Griess Assay

In **Project 3**, NO production was measured at the end of stimulations through Griess method (Promega, Milan, Italy) in a 96-well plate on both cell lines maintained in DMEM 0% FBS without red phenol, as previously described [250-252]. In addition this assay was also used to measure the level of NO production on plasma sample, as previously described [251, 252] and following the manufacturer's instructions.

Briefly, samples were mixed with Sulfanilamide Solution and incubated for 5–10 minutes at room temperature, protected from light.

After that, NED Solution was added to all wells and incubated for 5–10 minutes at room temperature, protected from light.

The absorbance were measured through a spectrophotometer (VICTOR X4 multilabel plate reader) at 550nm. NO concentration is determined by comparison of values to the Nitrite Standard reference curve.

3.10 Akt/ERK Activation Assay

The InstantOne™ ELISA is specifically engineered for accurate measurement of phosphorylated ERK 1/2 and AKT in cell lysates, following the manufacturer's instructions (Thermo-Scientific). Cells at the end of each treatments were lysed with 100µL Cell Lysis Buffer Mix, shaken for 10min at RT and 50µL/well of each sample were tested in InstantOne ELISA microplate strips including the 50µL/well Positive Control Cell Lysate and 50µL/well negative control. At each well 50µL of prepared Antibody Cocktail were added and the strips incubated for 1h at room temperature on a microplate shaker and washed 3 times with 200µL/well of Wash Buffer (1X). At the end, 100µL of the Detection Reagent were added to each well and after 20min the reaction was stopped adding to each well 100µL of Stop Solution. The strips were measured by a spectrometer (VICTOR X4 multilabel plate reader) at 450nm.

The results were expressed as means Absorbance (%) compared to control. This technique has been used in **Project 1**, **2** and **4**.

3.11 Mitochondrial Membrane Potential

The mitochondrial membrane potential was analyzed on **Project 4** by the Oxygen Consumption/Mito membrane Potential Dual Assay Kit (Cayman Chemical Company) following manufacturer's instructions [253]. The mitochondrial membrane potential was measured using JC-1 that at low concentrations (due to low mitochondrial membrane potential), it is predominantly a monomer that yields green fluorescence with emission of 530±15nm. At high concentrations (due to high mitochondrial membrane potential), the dye aggregates yielding a red to orange colored emission (590±17.5nm). Therefore a decrease in the aggregate fluorescent count is indicative of depolarization whereas an increase is indicative of hyperpolarization. The results are expressed as means ±SD (%) compared to control cells.

3.12 ATP Assay

In **Project 2**, at the end of each stimulation the medium was removed and the cells were immediately treated with the components of the ATP assay kit (nucleotide releasing buffer, ATP monitoring enzyme, enzyme reconstitution buffer, ATP), following the manufacturer's instructions. Luminescence was measured 1 min after the addition of ATP monitoring enzyme in a VICTORX4 multilabel plate reader (PerkinElmer Waltham, Massachusetts, U.S.A.), and luminescence was expressed as means ±SD% of μmol of ATP/g protein [254].

3.13 p53 Activity

In **Project 2**, p53 activity was measured by specific ELISA kit (p53 transcription factor assay kit, Cayman Chemical, Ann Arbor, Michigan, USA), examining the nuclear extracts obtained

at the end of each stimulation following the manufacturer's instructions. The nuclear extraction was obtained by classical technique using a complete buffer present in the kit. Briefly, the cells were lysed with ice-cold 1X Complete Hypotonic Buffer, supplemented with NP-40 and then centrifuged at 12.000g at 4°C for 10 minutes. The pellet was solubilized with icecold Complete Nuclear Extraction Buffer 1X supplemented with protease and phosphatase inhibitors, and then centrifuged at 12.000g for 15 minutes at 4°C; the supernatant was examined to analyse the activity of p53 related to the protein quantification through BCA assay (Thermo Fisher). This technique has been used also in **Project 4** after properly stimulation.

3.14 RES Quantification

RES quantification in CHO-K1 cells, rat plasma and tissue samples (liver, stomach, intestine, heart, kidneys and ovaries) was carried out by HPLC-MS analysis.

At the end of stimulations cells were placed in ice and supernatants were collected in 1.5ml centrifuge tubes to determine the rate of extracellular RES. 1×10^6 CHO-K1 cells at the end of stimulations were washed with cold 0.9% saline solution, lysed in ice 0.9% saline solution, mixed for 10min at 4°C and centrifuged for 20min at 13000 rpm at 4°C. Supernatants were used for quantification of intracellular RES. Samples were diluted with equal volume of acetonitrile, vortexed, centrifuged at 13000 rpm for 10min and analyzed by HPLC-UV. Tissues were homogenized in a volume of 100mg tissue/300µl of ice 0.9% saline solution (*w*/*v*) at 1600 rpm for 2min and the lysates were mixed for 20min at 4°C, and then centrifuged at 13000 rpm for 30min at 4°C. Plasma and tissue supernatants were processed as follows. An aliquot of 50µl of plasma or tissue sample was mixed with 50µl of 1M of sodium acetate buffer (pH = 5.5) and 2.5µl of β-glucuronidase/arylsulfatase from *Helix pomatia* in a 1.5ml centrifuge tube. Ethyl acetate (600µl) was added, then sample was extracted by vortexing (40s), and centrifuged at 13000 rpm for 10min. An aliquot (550µl) of the organic layer was transferred into 1.5ml

centrifuge tube and evaporated at 45°C under reduced pressure for 40min. The residue was dissolved in 100µl of acetone containing the IS (trans-4-hydroxystilbene- final concentration, 200µg/l), 25µl of 0.1N NaOH, and 100µl of 1mg/ml Dns-Cl (dansyl chloride) solution in acetone. Sample was shortly vortexed and heated at 45°C for 20min. After centrifugation (13000 rpm for 5min) the sample was analysed by HPLC-MS. Calibration curve for RES quantification was prepared by spiking blank matrixes and processed as described above, except for the addition of β-glucuronidase/arylsulfatase.

3.15 Vitamin D Quantification

In order to quantify the concentration of vitD on plasma and cells, we have been used two different assays.

- **Project 1** (ovarian line of research)

The competitive EIA assay kit has been used to detect both 25(OH)D3 and 25(OH)D2 (Cayman's Vitamin D EIA Kit) in plasma samples. At the end of each time point, plasma samples were collected using EDTA-Na2 as an anticoagulant, centrifuged for 15min at 1000×g at 4°C within 30min and then the supernatant used immediately. Before adding to wells, the SABC working solution and TMB substrate were equilibrate for at least 30min at room temperature and the strips of the plate washed twice before adding standard, sample and control. For the quantification it is necessary to plot a standard curve including control (zero well). 0.1ml of each sample and standard were added into test sample wells, the plate sealed with a cover and incubated at 37°C for 90min. After that the plate content was removed and 0.1ml of Biotin-detection antibody work solution was added into the standard and test sample for 60min at 37°C. After the plate was washed 3 times with Wash buffer 0.1ml of SABC working solution into each well was added and the plate incubated at 37°C for 30min. After the plate was washed 3 more times with Wash buffer 90µl of TMB substrate into each well was

added and the plate incubated at 37°C in dark within 15–30min. After this time 50µl of Stop solution into each well was added and the absorbance measured at 450nm in a microplate reader immediately after adding the stop solution.

- **Project 4** (brain line of research)

The competitive ELISA assay kit (FineTest) has been used to detect the metabolically active form of vitD. At the end of each stimulation, 50μl of each sample was collected and immediately used, following the manufacturer's instruction. Then, 50μl of biotin-detection and 100μl of SABC working solution were added and incubated at 37°C for 30min at each sample. At the end the supernatants were discarded, and then 90μl TMB substrate plus and 50μl of stop solution were added. Finally, the 96-well plate was analyzed by a spectrometer at 450nm (VICTOR X4, multilabel plate reader). In addition, it was necessary to plot a standard curve including the background (zero well) to perform a quantification.

3.16 Lipoic Acid Determination

The concentration of LA which crossed to BBB was measured (**Project 4**) as described in literature [255]. At the end of stimulations, the basolateral volume was analyzed through a spectrometer (VICTOR X4, multilabel plate reader) at 320nm, and the absorbance related to the standard curve was obtained from LA (200ng/ml). The results were expressed as means ±SD (%) of absorption, normalized to the control.

3.17 Amyloid Precursor Protein (APP) Quantification

Amyloid precursor protein (APP) quantification (**Project 4**) was measured by the Amyloid Beta A4 protein ELISA kit (Sigma-Aldrich) on cellular supernatants following the manufacturer's instructions. Briefly, at the end of treatments, cellular supernatants were collected and each sample was tested with the ELISA kit. The biotinylated detection antibody

specific for the target protein was added in each well and the plate was incubated for 1 hour at room temperature. Then, after 45 minutes of incubation with HRP-conjugated streptavidin, TMB substrate solution was added for 30 minutes, and subsequently, the reaction was stopped by adding stop solution. APP concentration was determined by measuring the absorbance through a spectrometer (VICTOR X4, multilabel plate reader) at 450nm and calculated by comparing results to the APP standard curve.

3.18 Iron Quantification Assay

In **Project 4**, the Iron Assay Kit (Sigma-Aldrich) which measures ferrous iron (Fe^{2+}), ferric iron (Fe^{3+}), and total iron (total iron–ferrous iron) in samples was used on astrocytes following the manufacturer's instructions [256]. The absorbance at 593nm was measured by a spectrometer (VICTOR X4, multilabel plate reader). Ferric iron concentrations are equal to the total iron (sample plus iron reducer)-Fe^{2+} (sample plus assay buffer). The iron concentration was expressed as ng/ml.

3.19 Western Blot

In order to investigate the intracellular pathways activated by vitD and other agents, Western blot and densitometric analysis were performed.

- **Project 1** (ovarian line of research)

CHO-K1 cells were lysed in ice Complete Tablet Buffer (Roche) supplemented with 2mM sodium orthovanadate, 1mM phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich), 1:50 mix Phosphatase Inhibitor Cocktail (Sigma-Aldrich) and 1:200 mix Protease Inhibitor Cocktail (Calbiochem). 35μg of proteins of each sample were resolved on 10% SDS-PAGE gel. Polyvinylidene difluoride membranes (PVDF, GE, Healthcare Europe GmbH, Milan, Italy) were incubated overnight at 4°C with specific primary antibody: anti-VDR receptor (1:400,

Santa-Cruz) and anti-ERβ (1:500, Santa-Cruz). Protein expression was normalized to the specific total protein (if possible) and verified through β-actin detection (1:5000; Sigma-Aldrich) and expressed as a mean ±SD (%).

In addition, ovarian tissues were immediately washed with ice 0.9% saline solution (*w*/*v*), weighed and homogenized in a volume of 100mg tissue/300µL of lysis buffer (0.1 M Tris, 0.01 M NaCl, 0.025 M EDTA, 1% NP40, 1% Triton X100, Sigma-Aldrich, Milan) supplemented with 2mM sodium orthovanadate, 0.1M sodium fluoride (Sigma-Aldrich, Milan), 1:100 mix of protease inhibitors (Sigma-Aldrich, Milan), 1:1000 phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, Milan), using an electric potter at 1600 rpm for 2min. Samples were mixed for 30min at 4°C, centrifuged for 30min at 13000 rpm at 4°C and 40µg of proteins for each samples resolved on SDS-PAGE gel at 15%. Proteins transferred to polyvinylidene fluoride membranes (PVDF, GE Healthcare Europe GmbH, Milan, Italy) were incubated with specific primary antibody, overnight at 4°C: anti-VDR receptor (1:400, Santa-Cruz), anti-ERβ (1:500, Santa-Cruz), anti-cyclin-D1 (1:1000, Euroclone, Milan, Italy). Protein expression was normalized and verified through β-actin detection (1:5000; Sigma-Aldrich) and expressed as a mean ± SD (%).

- **Project 2** (gastric line of research)

After each stimulation, the cells were washed with iced PBS 1X supplemented with 2mM sodium orthovanadate (Sigma-Aldrich, Milan, Italy) and lysed in iced Ripa Buffer (10mM Na2HPO4, 150mM NaCl, 2mM EDTA, 1% NP-40, 0.1% sodium dodecyl sulphate, 1% sodium deoxycholate, 50mM sodium fluoride; Sigma-Aldrich, Milan, Italy) supplemented with 2mM sodium orthovanadate (Sigma-Aldrich, Milan, Italy), 1:1000 phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich, Milan, Italy) and 1:100 protease inhibitors cocktail (Sigma-Aldrich, Milan, Italy). 35µg of proteins were resolved on 10% SDS-PAGE gels (SDS-PAGE; Bio-Rad Laboratories, Hercules, CA, USA), transferred to polyvinylidene fluoride membranes (PVDF,

GE Healthcare, Milan, Italy) anti-VDR (1:250, Santa-Cruz) which were incubated overnight at 4°C with specific antibodies: anti-p53 (1:400, Santa-Cruz), anti-Annexin V (1:1000, Sigma-Aldrich, Milan, Italy), and anti-Ki67 (1:800,Santa-Cruz), The protein expressions were normalized and verified through β-actin detection (1:5000; Sigma-Aldrich, Milan, Italy).

- **Project 3** (cardiovascular line of research)

After stimulations, H9c2 and PAE cells were washed with iced PBS 1X (Sigma) supplemented with 2mM sodium orthovanadate (Sigma) and lysed in ice with Complete Tablet buffer (Roche) supplemented with 2mmol/L sodium orthovanadate and 1:1000 phenylmethanesulfonylfluoride (PMSF; Sigma-Aldrich). 40μg from each lysate were resolved on 10% SDS-PAGE gels (SDS-page, Bio-Rad Laboratories, Hercules, CA, USA), transferred to polyvinylidene fluoride membranes (PVDF, GE Healthcare Europe GmbH, Milan, Italy) and incubated overnight at 4°C with specific primary antibody: anti-phospho-Akt^{Ser473} (1:1000; Cell Signaling Technologies, Beverly, MA, U.S.A.); anti-Akt (1:1000; Cell Signaling Technologies, Beverly, MA, U.S.A.); anti-phospho-p44/42^{thr202tyr204} (1:1000; Cell Signaling Technologies, Beverly, MA, U.S.A.); anti-p44/42 (1:1000; Cell Signaling Technologies, Beverly, MA, U.S.A.); anti-phospho-eNOS^{ser1177} (1:1000; Cell Signaling Technologies, Beverly, MA, U.S.A.); anti-eNOS (1:1000; Cell Signaling Technologies, Beverly, MA, U.S.A.); anti-p53 (1:250; Santa-Cruz Biotechnology, Dallas, Texas, U.S.A.); anti-VDR (1:250; Santa-Cruz Biotechnology, Dallas, Texas, U.S.A.). Protein expression was normalized and verified through ß-actin detection (1:5000; Sigma, Milan, Italy).

- **Project 4** (brain line of research)

Cells were washed and then lysed in the ice Complete Tablet buffer (Roche) supplemented with 2mM sodium orthovanadate, 1mM phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich), and 1:100 mix Protease Inhibitor Cocktail (Sigma-Aldrich). From each lysate, 35μg proteins was resolved into 8% and 15% SDS-PAGE gels, and polyvinylidene difluoride

(PVDF) membranes (GE Healthcare) were incubated overnight at 4°C with a specific primary antibody: anti-SOD3 (1 : 250, Santa Cruz), anti-phospho-PKA$\alpha/\beta/\gamma^{Thr198}$ (1 : 250, Santa Cruz), anti-NOS2 (1 : 250, Santa Cruz), and anti-cytochrome C (1 : 1000, Calbiochem). Protein expression was normalized and verified through β-actin detection (1 : 5000; Sigma-Aldrich) and expressed as a mean ±SD (% vs. control).

3.20 Statistical Analysis

In vitro results obtained from at least 5 independent experiments conducted in triplicates were expressed as means ±SD, using One-way ANOVA followed by Bonferroni post hoc test or the Mann-Whitney U test, to compare percentages of responses. Values of significance for $p<0,05$ were considered statistically significant (**Project 1, 2, 3** and **4**). Data collected from *in vivo* results obtained from 4 independent experiments were analyzed by two-way ANOVA and one-way ANOVA followed by Bonferroni post hoc test and the comparisons between the two groups were performed using a two-tailed Student's t-test. Multiple comparisons between groups were analyzed by two-way ANOVA followed by a two-sided Dunnett post-hoc testing. *P*-value<0.05 was considered statistically significant (**Project 1** and **3**).

4. Results

In order to explore the effects on different cell types of viD and other agents, the results has been subdivided following the **Project** order (from **1** to **4**)

- **4.1 Project 1** (ovarian line of research)

The results of this project are contained in the paper: *Biological effects of combined resveratrol and vitamin D3 on ovarian tissue. Uberti F, Morsanuto V, Aprile S, Ghirlanda S, Stoppa I, Cochis A, Grosa G, Rimondini L, Molinari C. J Ovarian Res. 2017;10(1):61*.

Dose-Response and Time-Course Study with Resveratrol in CHO-K1 Cells

To identify the dose of RES able to induce the maximal effect on cell viability during time, a dose response and a time-course study were planned. Moreover, these experiments were important to understand the cooperative effect of RES with vitD during time. As shown in Fig. 1c, RES 50μM appeared to be the dose producing the greatest effect ($p<0.05$) compared to control and to other concentrations (10, 25, 100μM) during all time periods considered (Fig. 1a–d). This concentration of RES was maintained for all successive experiments. In addition, another important finding regarded the reaction time of RES 50μM, which appeared as a biphasic curve that quickly started (at 2min), confirming its rapid metabolism. Its beneficial effect was maintained as long as 3 h.

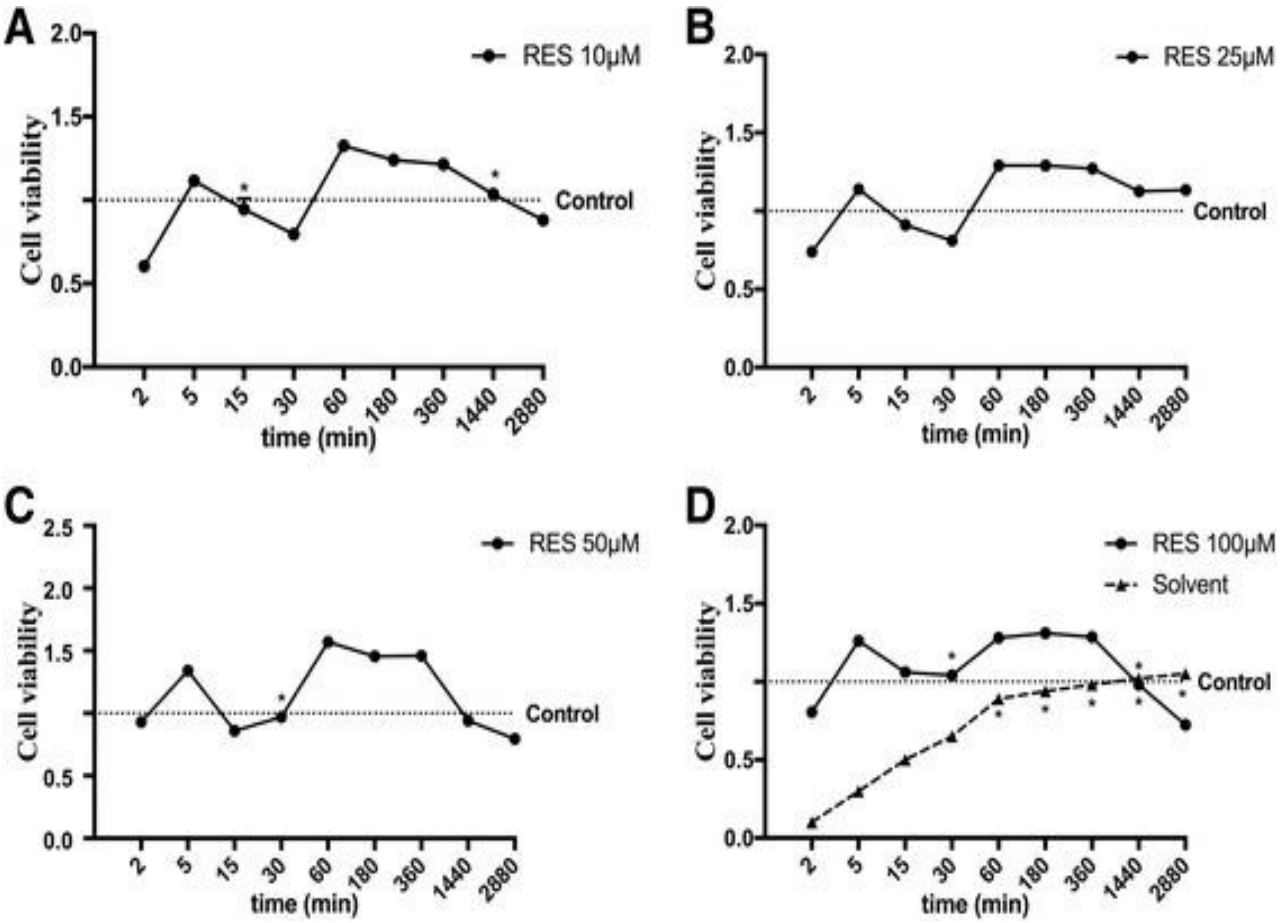


Fig. 1

Time-course and dose-response study of CHO-K1 viability measured by MTT test. **a** time-course of RES 10μM; **b** time-course of RES 25μM; **c** time-course of RES 50μM; **d** time-course of RES 100μM. Reported data are means ±SD of five independent experiments. *not significant vs control; point without symbol $p < 0.05$ vs control. RES=resveratrol. The effect of solvent alone is reported as well.

The combination of RES 50μM with vitD 100nM was able to amplify biological effects with a similar kinetic reaction to RES alone ($p<0.05$); vitD was already able to enhance the effect of RES from 2min of treatment (Fig. 2a). Then we observed a stable plateau phase around 6h and then effects began to decline for the following 48h. For this reason, we have chosen to study the kinetics ranging from 2min to 3h for all successive experiments. As shown in Fig. 2b, the ROS produced by RES alone and combined with vitD appeared to be modulated during time. In particular, in the first minutes (from 2 to 15min) a significant ROS production compared to control ($p<0.05$) was observed after treatment with RES alone and the presence of vitD was able to amplify this effect. This combination was also able to maintain a reduction of ROS up to 1h of stimulation ($p<0.05$); at 3h ROS production was comparable to control ($p>0.05$). These data on cell viability of CHO-K1 cells confirm the beneficial effects observed and the importance of the combination of RES and vitD in maintaining the beneficial effects of RES during time.

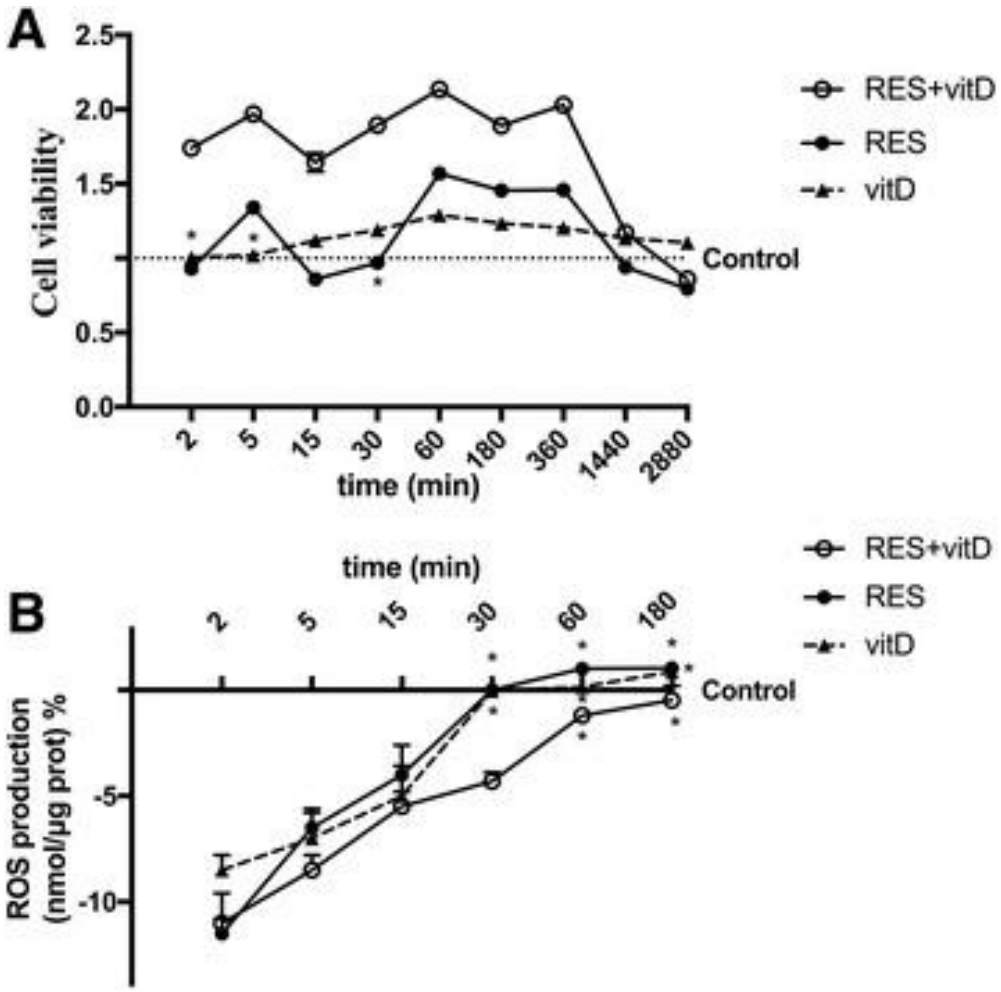


Fig. 2

The cooperative effect of RES and vitD during time on cell viability and ROS production in CHO-K1 cells. **a** cell viability and **b** ROS production measured during time-course study in presence of RES and vitD alone and combined. Reported data are means ±SD in (**a**) and they are expressed as means ±SD(%) in (**b**) of five independent experiments. *not significant vs control; points without symbol, $p<0.05$ vs control. RES=resveratrol 50µM; vitD=vitamin D3 100nM; RES+vitD=co-stimulation of RES 50µM with vitD 100nM.

Quantification of Intracellular Resveratrol with or without Vitamin D3 in CHO Cells

Since the biological effects of RES were due to its ability to be absorbed in cells and tissues, the intracellular concentration of RES in CHO-K1 during time was determined by HPLC-UV. As reported in Fig. 3, the absorption rate of RES combined with vitD was enhanced compared to RES alone (p<0.05), in particular in the first 15min of stimulation; the maximum effect was observed at 5min of stimulation (about 16.7μM). These findings confirmed previous data about the cooperative effect of RES combined with vitD; indeed, vitD was important to amplify the effects of RES influencing also the level of RES uptake in ovarian cells. Finally, the time range from 2 to 15min of stimulation was used to verify the intracellular cascade activated by RES alone and combined with vitD.

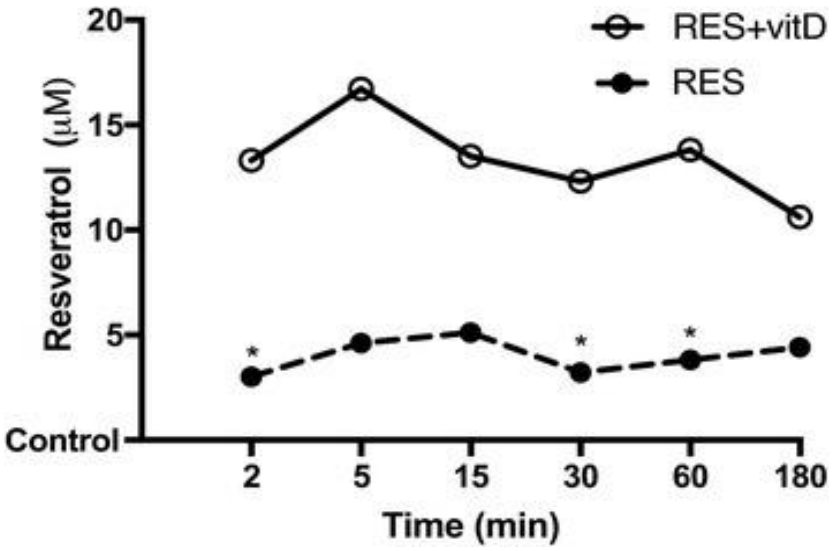


Fig. 3

Measure of intracellular concentration of RES alone and combined with vitD in CHO-K1 cells in a time-course study. Reported data are means ±SD of five independent experiments. *not significant vs control; points without symbol, p<0.05 vs control. RES=resveratrol 50μM; RES+vitD=co-stimulation of RES 50μM with vitamin D 100nM.

Analysis of the Main Intracellular Pathways Activated by Resveratrol and Vitamin D3

In order to assess which intracellular pathways were activated after intracellular uptake of RES alone and combined with vitD, ERKs, Akt, SOD, ERβ and VDR signaling were investigated in CHO-K1 cells. As reported in Fig. 4a and b, the role of ERK/MAPK and PI3K/Akt pathways was examined to explain the action mechanism of RES alone and combined with vitD; MAPK and PI3K signal transduction pathways are closely associated with the one of healthy tissue. To study changes in the activation levels of proteins associated with cellular signal transduction according to treatment time, ELISA was performed following treatment for various time periods up to 15min. The results confirmed an increase in activation of ERK and Akt due to RES alone and these effects were amplified by the use of vitD. In particular, the results showed that the activation levels of ERK and Akt started at 2min and the maximum effect was observed at 5min in both RES alone and combined with vitD compared to control ($p<0.05$) then decreased. These data confirmed the importance of vitD in amplifying the beneficial effects of RES to maintain healthy tissue. In addition, since the beneficial effects of RES included its anti-radical action, two important mechanisms involved such as SOD activity and ERβ, were also investigated by ELISA and Western blot respectively. As reported in Fig. 4c, RES combined with vitD is able to maintain ROS production low during time maintaining SOD activity similar to control. In addition, these anti-oxidant effects were obtained via ERβ activation (Fig. 4d); the involvement of ERβ was observed starting from 2min after treatment with RES alone and increasing after 5min compared to control ($p<0.05$); the presence of vitD amplified ($p<0.05$) all these levels of activations (about 20% at 2min and 65% at 5min of RES plus vitD compared to RES alone), indicating a cooperative activity exerted by RES and vitD. The importance of combined treatments with RES and vitD was also confirmed on VDR expression (Fig. 4e), in which a stable activation of VDR during time was observed ($p<0.05$), indicating its involvement in the beneficial effects previously observed.

These results indicate that RES is able to maintain tissue health through ERK, Akt, SOD, ERβ and VDR signal transduction pathways, which can help clarify that these beneficial effects exerted by vitD are a necessary condition.

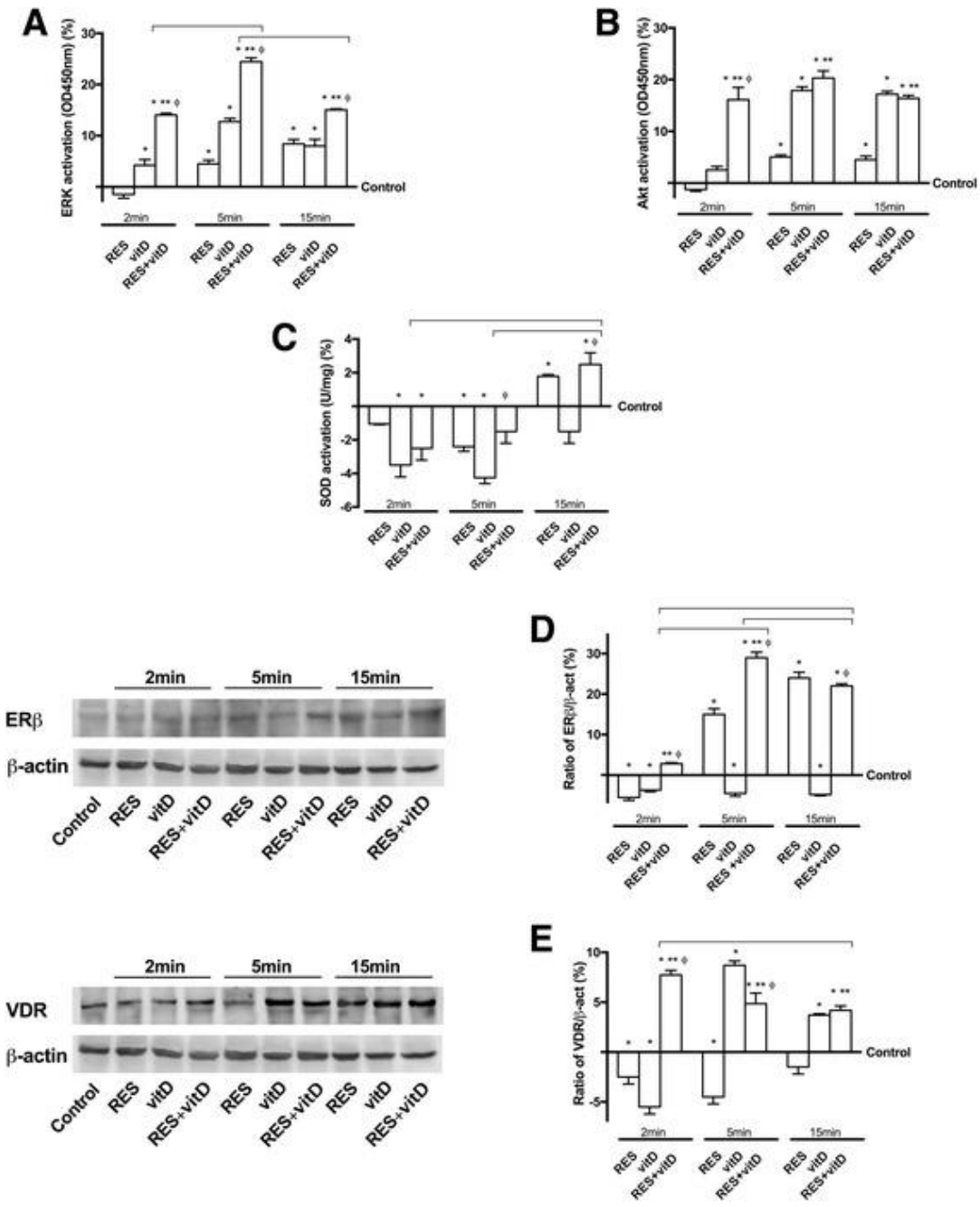


Fig. 4

Western Blot, densitometric analysis and protein activation of CHO-K1 cells stimulated with RES and vitD alone and together. In **a** (ERK/MAPK), **b** (Akt), **c** (SOD) activations by ELISA are reported as means ±SD(%) of five independent experiments. In **d** (ERβ receptor) and **e** (VDR receptor) Western blot (on the left) and densitometric analysis (on the right) are reported. In the right column the specific densitometric analysis is reported and expressed as means ±SD(%) of five independent experiments. *p<0.05 vs control; **p<0.05 vs RES alone; $^{\varphi}p$<0.05 vs vitD; the bars, p<0.05 between RES+vitD at different times. RES=resveratrol 50μM; vitD=vitamin D3 100nM; RES+vitD=co-stimulation of RES 50μM with vitD 100nM.

Bioavailability of Resveratrol with Vitamin D3

Some additional experiments were performed to demonstrate the efficacy of RES and vitD also in *in vivo* study, given their biological effects reported in an *in vitro* study. Starting from bioavailability of RES alone and combined with vitD, following a time-course experiments (2, 5, 60, 180, 360, 720min). As reported in Fig. 5a on rat plasma samples, the concentration of RES plus vitD and RES alone followed by a biphasic curve and it is time-dependent. At 2min ($p<0.05$) plasma concentration started to enhance.

The plasma of rats treated with RES alone showed a second peak of RES concentration at 1h of treatment (about 20% lower than 2min) and then the concentration decreased reaching control values. The presence of vitD significantly amplified the absorption rate of RES already in the first 2min (about 34% compared to RES alone at 2min) and obtained the maximum effects at 5min (about 220% compared to RES alone at 5min).

In addition, a second peak was extended in time (after 1h) because the absorption rate after 5min was similar to what observed with RES alone as long as 3h. At 6h the plasma concentration decreased leading to control values after a second peak. This finding about the second peak supported the hypothesis that RES can be stored in organs to explain a secondary effect in the long run.

All these data explain the role of vitD in supporting the biological activity of RES and support the importance of the cooperative activity of RES and vitD. As reported in Fig. 5b, during time the quantification of vitD in plasma samples showed an increase in quantity of vitD present in plasma when vitD is administered with RES.

After oral intake these data confirmed the mutual influence of RES and vitD on the absorption. In addition, the ROS concentration assessment in plasma of rats confirmed a positive influence of vitD in counteracting oxidative stress with RES (Fig. 5c) during time; a slow and progressive decrease starting from 1h of treatment with RES combined with vitD was observed and this

effect is aligned with the absorption test at the time the maximum absorption rate has been reached. All these data explained the ability of RES plus vitD to rapidly cross the membrane and to reach target tissues.

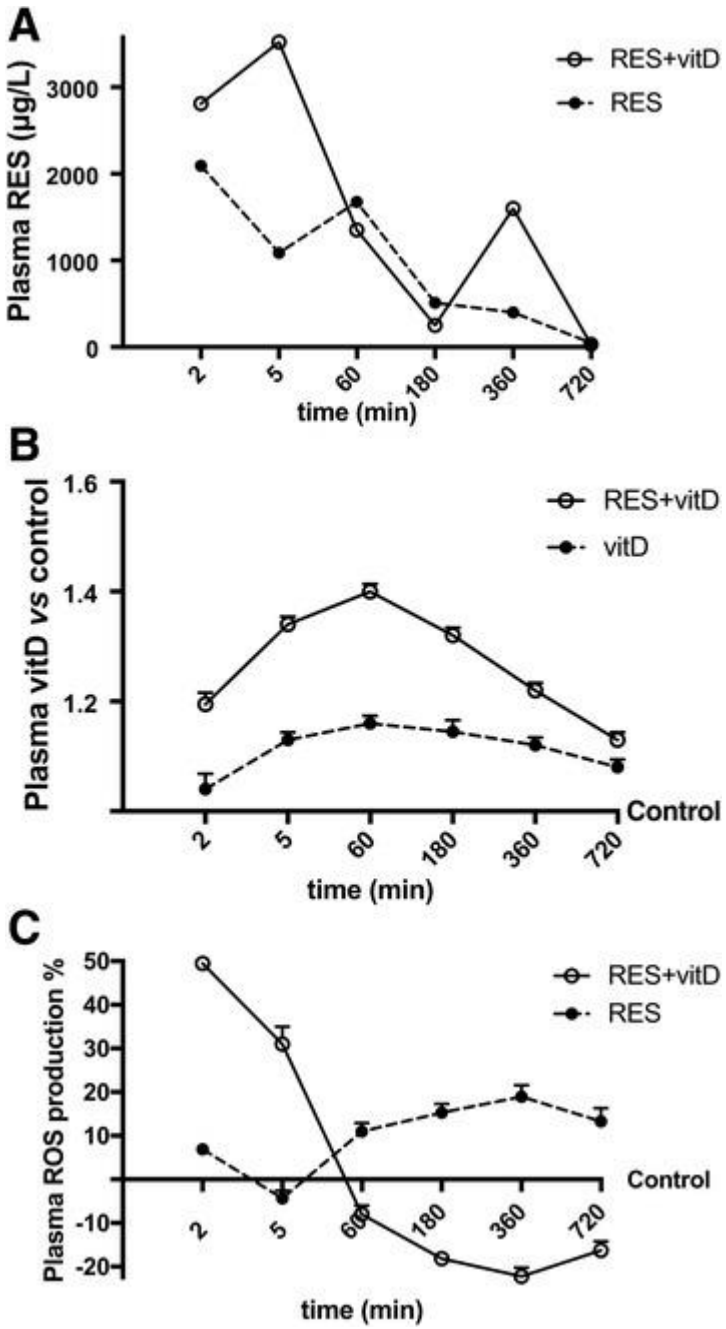


Fig. 5

Bioavailability, vitamin D quantification and ROS production in *in vivo* experiments. Female rats (n = 48) were treated with RES 0.5mg alone (n = 24), combined with vitD 0.4µg (n = 24) and with vitD 0.4µg alone (n = 18) by gavage. The animals were sacrificed at specific time-points (ranging 2–270min) and plasma samples were collected. In **a** RES plasma concentration (µg/L), in **b** vitD plasma quantification and in **c** the ROS production are reported. The results are expressed as means ±SD in panel (**a-b**) and as means ±SD(%) in panel (**c**) of 4 independent experiments. All data p<0.05 vs control. RES=resveratrol 0.5mg; RES+vitD=preparation composed of RES 0.5mg and vitD 0.4µg.

Analysis of Intracellular Pathways Activated by Resveratrol and Vitamin D3 in Ovarian Tissue

Some intracellular pathways involved in the biological effects of RES and vitD were also investigated in ovarian rat tissues in order to clarify the importance of bioavailability after oral intake of RES combined with vitD in gynecological disorders during the first minutes (2, 5, 15, 30min), following the plasmatic changes. As reported in Fig. 6, the involvement of Cyclin D1, an important regulator of G1 to S phase progression, demonstrate a better influence of RES plus vitD than RES alone ($p<0.05$) (Fig. 6a and b). The effects of RES plus vitD started at 5min compared to RES alone ($p < 0.05$) and were maintained during time, indicating an improvement in cell cycle turn-over, important to maintain the integrity of tissue. This finding was supported by a decrease in SOD activity (Fig. 6e) observed with RES combined with vitD as Cyclin D1, compared to RES alone. These improvements of the biological effects of RES were obtained due to the presence of vitD, supporting previous data on the cooperative effects of these two substances. The mechanism activated by RES plus vitD involved both ERβ (Fig. 6a and c) and VDR receptors (Fig. 6a and d), as reported. As a matter of fact, RES plus vitD showed the strongest effect on ERβ starting from 5min ($p<0.05$) compared to RES alone and on VDR starting from 2min ($p<0.05$) compared to RES alone. These effects were maintained during all time of stimulation. The *in vitro* results about the cooperative effect of RES and vitD on ovarian tissue have been supported by all these findings.

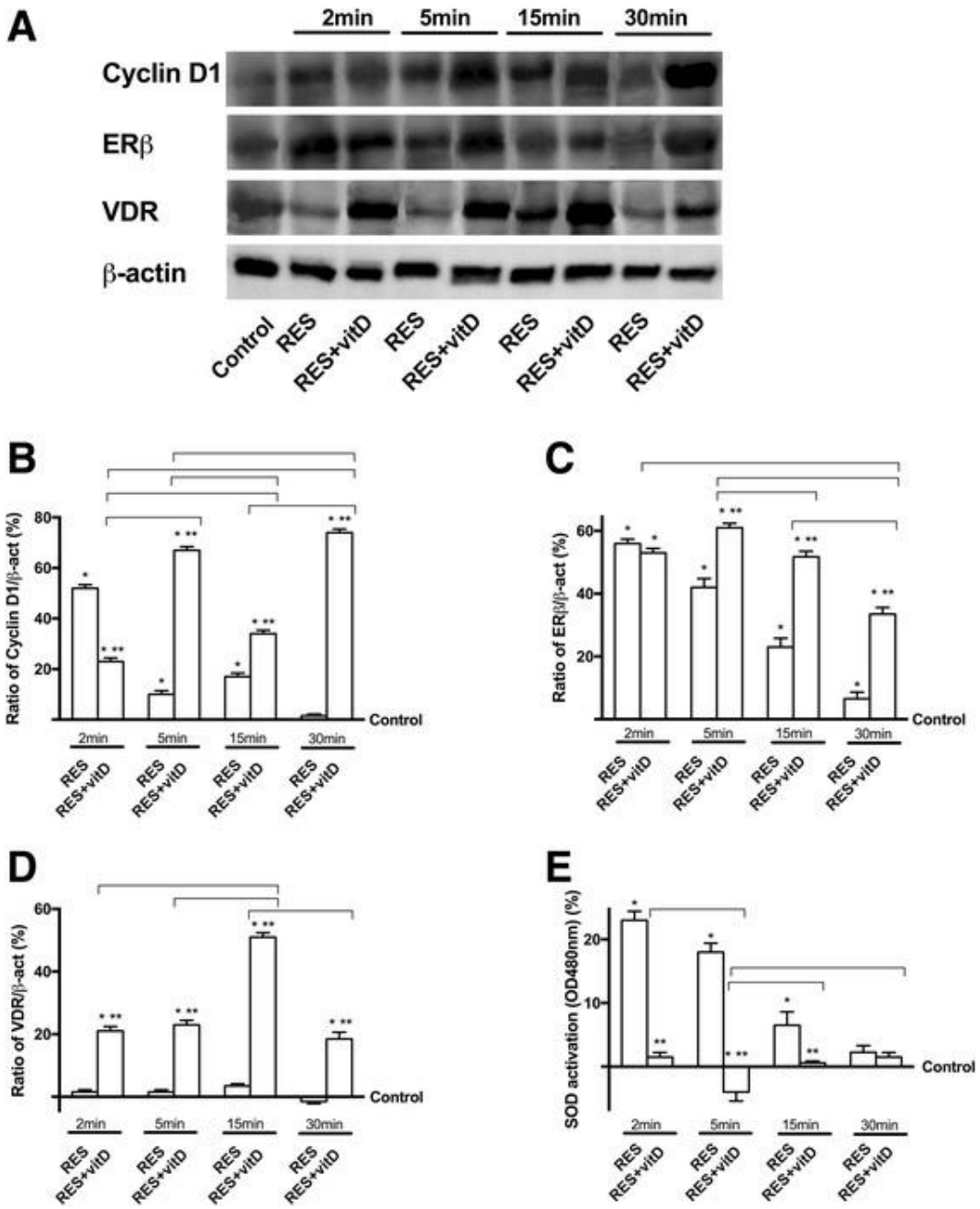


Fig. 6

Western blot, densitometric analysis and protein activity of ovarian tissue obtained from female rats (n = 32) treated with RES alone (n = 16) and combined with vitD (n = 16). In the upper (**a**) an example of Western Blot taken at different time (ranging 2–30min) of Cyclin D1, ERβ receptor and VDR receptor is reported. In the downstream the specific densitometric analysis of Cyclin D1 (**b**), ERβ receptor (**c**), and VDR receptor (**d**) is reported and expressed as means ±SD(%) of 4 independent experiments. In **e** SOD activity by ELISA was reported as means ±SD(%) of 4 independent experiments. *p<0.05 vs control; **p<0.05 vs RES alone; the bars, p<0.05 between RES+vitD at different times. RES=resveratrol 0.5mg; RES+vitD=preparation composed of RES 0.5mg and vitD 0.4μg.

Biodistribution of Resveratrol with Vitamin D3

The biodistribution and accumulation of RES in different organs during time (30, 60, 180, 360, 720min), such as heart (Fig. 7a), kidney (Fig. 7b) and liver (Fig. 7c) is another important parameter useful to understand the biological effects of RES combined with vitD after oral intake and blood concentration. The absorption rate in tissue of RES alone and RES plus vitD was different and time-dependent, confirming the hypothesis about the activity of RES in the second peak observed in plasma samples. In addition, a better efficacy of RES combined to vitD ($p<0.05$) in creating a tissue deposit of RES was confirmed mainly at 360min in all organs tested ($p<0.05$), indicating the importance of the combination with vitD to exert a systemic biological effect.

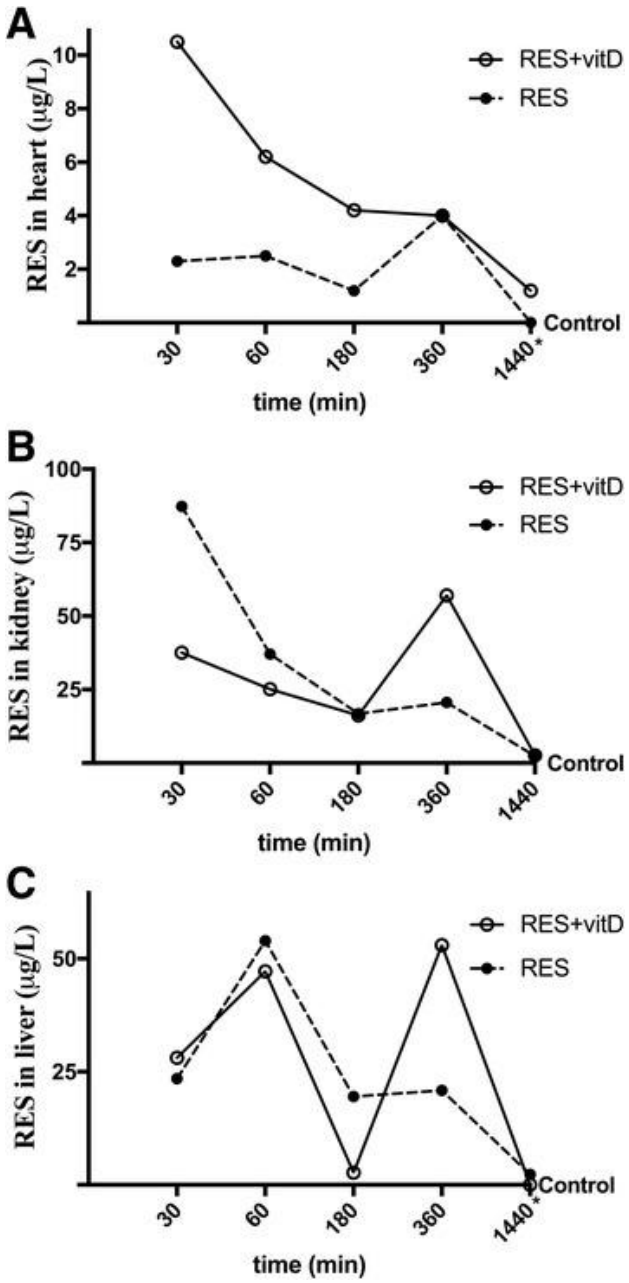


Fig. 7

Biodistribution of RES (µg/L) in *in vivo* experiments. Female rats (n = 40) were treated with RES 0.5mg alone (n = 20) and combined with vitD 0.4µg (n = 20) by gavage. The animals were sacrificed at specific time-points (ranging 30–270min) and heart (**a**), kidney (**b**) and liver (**c**) were collected. The results are expressed as means ±SD (µg/L) of 4 independent experiments. *not significant vs control; point without symbol, p<0.05 vs control. RES=resveratrol 0.5mg; RES+vitD=preparation composed of RES 0.5mg and vitD 0.4µg.

- **4.2 Project 2** (gastric line of research)

The results reported are contained in the paper: *Role of vitamin D3 and alginates in prevention of NSAID-dependent cellular injury. Molinari C, Morsanuto V, Ruga S, Stoppa I, Notte F, Farghali M, Bozzo C, Castello L, Nardone A, Magnani C, Uberti F. EC Gastroenterology and Digestive System 6.3 (2019): 211-223.*

Analysis of the Effects of Aq and Neo on Epithelial Gastric Cells Under Oxidative or Acidic Conditions

Both Aq and Neo act as a physical barrier for gastric epithelial cells and exert protection against hyperacidity, but the different compositions can determine different beneficial effects. The first set of experiments analysed the ability of act before or after the damage caused by oxidative stress and acidity of Aq and Neo after 24h of stimulation. As reported in Fig. 8, the effects on cell viability and ROS production were different between Aq and Neo alone. Aq induced a significant increase in cell viability compared to Neo (about 215%) and to control ($p<0.05$, see figure 8a); Aq also induced a significant reduction in ROS release (about 256%) compared to Neo (see fig. 8b). These preliminary data confirmed the hypothesis that the composition can influence the efficiency during gastroprotection. In order to clarify the efficacy of Aq and Neo in gastroprotection, all the following experiments were performed in presence of pre-treatment or post-treatment for 30minutes with 200μM H_2O_2 or 1:172 (%v/v) HCl. As reported in Fig. 8, the pre-treatment with H_2O_2 or HCl caused a significant reduction ($p<0.05$) of cell viability and an increase of ROS production ($p<0.05$) compared to control. Aq was able to counteract the negative effect exerted by H_2O_2 or HCl on cell viability ($p<0.05$) both before and after the damage; in particular, the most evident beneficial effects were observed in reversing the influence exerted by HCl both on cell viability and ROS production. However, regarding cell viability (see Fig. 8a) Aq seems to be more effective when it is added after pre-treatment (about 100% in oxidative and about 63% in hyperacidic conditions). On the other hand, Neo seems to have a beneficial effect only when added after the injury, reported in instructions. Between oxidative and hyperacidic pre-treatment conditions, Neo was able to have a greater effect on

cell viability when administered in presence of oxidative conditions (about 70% compared to hyperacidic conditions). As regards ROS production (see Fig. 8b), that is an important parameter to evaluate cellular damage, Aq and Neo had a different effect under oxidative and hyperacidic conditions, confirming the differences observed in cell viability. Indeed, both Aq and Neo had better results when added after pre-treatment, but Aq had a greater beneficial effect on hyperacidic conditions and Neo during oxidative stress. These data confirmed a different effect on cell viability and ROS production of Aq compared to Neo and suggest the existence of a different mechanism of protection.

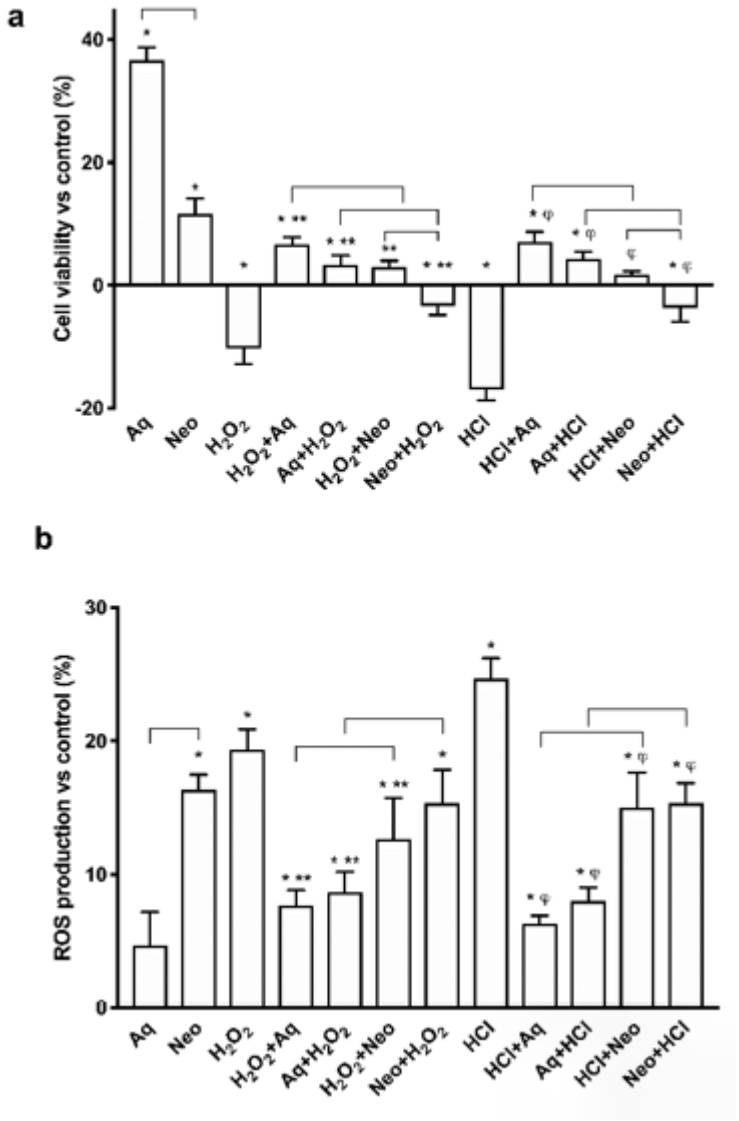


Fig. 8

Effects of Aq and Neo during acidic and oxidative stress. In panel (**a**) cell viability and in panel (**b**) ROS production measured in gastric epithelial cells treated with Aq and Neo for 24h. Aq=combination between alginates and vitamin D3, Neo=mixture of polysaccharides and flavonoids. Data are expressed as means ±SD (%) of five independent experiments normalized to control values (0% line). *$p<0.05$ vs control; **$p<0.05$ vs H_2O_2; ϕ$p<0.05$ vs HCl; arrows indicate $p<0.05$ between different groups.

Analysis of the Effects of Aq and Neo to Prevent Cell Death

Cell death is an energy-dependent process that requires ATP: typically, apoptotic cells exhibit a significant decrease in ATP level. This parameter is important in evaluating the effectiveness of Aq and Neo to prevent loss of gastric epithelial cells caused by oxidative stress or hyperacidity. As reported in Fig. 9a, ATP level was higher in presence of Aq than Neo in basal conditions ($p<0.05$), confirming data observed on cell viability. In addition, treatment with 200μM H_2O_2 or 1:172 (%v/v) HCl caused a significant reduction ($p<0.05$) of ATP indicating cell death. Aq was able to counteract the negative effects exerted by H_2O_2 or HCl on ATP production ($p<0.05$) both before and after the damage, but the best result was observed in reversing the influence exerted by pre-treatment with HCl compared to control ($p<0.05$). Neo was able to exert beneficial effects when added after the injury and confirmed a greater effect on ATP production when in presence of oxidative conditions compared to control ($p<0.05$). These data support the hypothesis of a different protection mechanism between Aq and Neo. Since p53 is a key component for cellular-induced apoptosis through mitochondrial stress markers, additional experiments were performed under the same conditions as reported before. As reported in Fig. 9b, the activity of p53 induced by Aq and Neo alone was at physiological level supported the beneficial effects observed above; moreover, in presence of H_2O_2 and HCl alone, p53 activity significantly increased compared to control, indicating a significant mitochondrial stress and a potential activation of the pathway leading to cell death. The ability to prevent and restore the damage caused by H_2O_2 and HCl of Aq ($p<0.05$) was confirmed: indeed, no significant differences were observed between pre and post treatments. Contrary to what observed for Aq, Neo was able to prevent and restore the damage caused by H_2O_2 ($p<0.05$), but a little activation was observed with HCl: this data confirms the ability of Neo to better counteract the negative effects of oxidative stress during hyperacidic conditions. All these results support the hypothesis that Aq and Neo act differently to prevent epithelial

stomach cell death. Finally, since the greater effects of Aq were observed in pre-treatment conditions with HCl, in all successive experiments only these conditions were maintained.

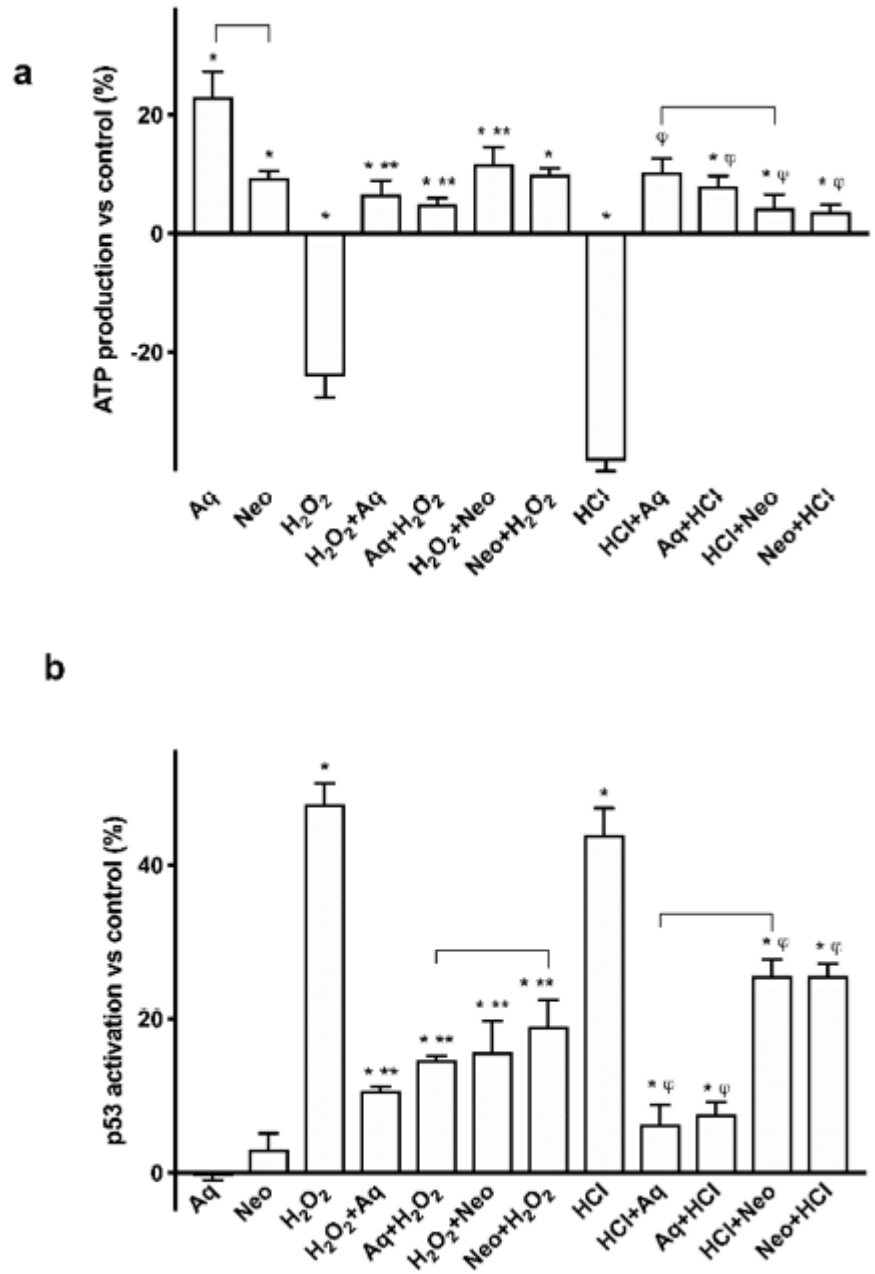


Fig. 9

Effects of Aq and Neo on apoptotic mechanism. In panel (**a**) ATP production and in panel (**b**) p53 activation in gastric epithelial cells treated with Aq and Neo for 24h during acidic condition or oxidative stress. Aq=combination between alginates and vitamin D3, Neo=mixture of polysaccharides and flavonoids. Data are expressed as means ±SD (%) of five independent experiments normalized to control values. *$p<0.05$ vs control; **$p<0.05$ vs H_2O_2; ϕ$p<0.05$ vs HCl; arrows indicate $p<0.05$ between different groups.

Aq and Neo as Gastroprotective Agents

Since the chronic use of NSAID can cause gastric ulcers, gastroprotective agents are frequently used in combination with anti-acid treatments. However, these agents often do not remove the causes of hyperacidity or oxidative stress created by NSAID in the stomach. So today scientific research is trying to find new resources of natural origin, especially derived from plants, able to reduce gastric cells damage. In this context the effects of Aq and Neo were tested in presence of Diclofenac; in particular Aq and Neo were added before and after the stimulation with Diclofenac under hyperacidic conditions. As shown in Fig. 10a, in presence of Diclofenac alone, cell viability was reduced compared to control ($p<0.05$), indicating a lower tolerability of the cells, and the pre- and post-stimulations with Aq and Neo were able to reduce this negative effect. In particular, the greatest effect was obtained using Aq compared to Neo (about 64%) when added before Diclofenac. On the contrary, during the stimulation after Diclofenac, the greatest effect was obtained with Neo compared to Aq (about 40%). These data suggest the possible ability of Aq and Neo to be used as gastroprotective agents under hyperacidic conditions in a different manner; in particular the best result was observed using Aq compared to Neo. This hypothesis was confirmed by comparing data obtained with Aq and Neo on cell viability with pantoprazole experiments (P), where similar effect of Aq and Neo preventing the loss of cell caused by Diclofenac were observed. It is noteworthy that Aq and Neo added before Diclofenac seemed to be more effective than P ($p<0.05$), indicating Aq and Neo as a new possible therapeutic approach. Finally, all these results were confirmed under acidic conditions, in which Aq and Neo counteracted the negative effects of Diclofenac better than P ($p<0.05$). In particular, pre-treatment with HCl caused a significant reduction ($p<0.05$ vs control) in cell viability induced by Diclofenac and only Aq was able to prevent (pre-stimulation) and restore (post-stimulation) the damage caused by Diclofenac under these conditions ($p<0.05$ vs control and vs Diclofenac), indicating a greater effectiveness of Aq than Neo as a gastro-protector

agent. Since a consequence of hyperacidity is the production of oxidative stress, additional experiments were carried out to investigate the ability of Aq and Neo to reverse the damage acting on ROS release. As reported in Fig. 10b, Diclofenac induced a significant increase (about 30%) in ROS production compared to control, confirming its negative effect on cell proliferation. Pre-stimulation with Aq was able to significantly reduce the ROS release caused by Diclofenac compared to the post-stimulation (about 70% vs post-treatment). Moreover, Neo seemed to have greater effects when added after Diclofenac to reduce ROS production ($p<0.05$, about 33% compared to pre-treatment). These data confirm a different ability of Aq and Neo to maintain a gastric protection. In addition, comparing the effects of Aq and Neo to P, in presence of Diclofenac, the gastro-protection mechanism was confirmed: indeed, Aq and Neo had more effectiveness than P ($p<0.05$). Similar data were observed under acidic conditions, in which Diclofenac improved its negative effects and Aq and Neo counteract it ($p<0.05$) exerting greater effects compared to P ($p<0.05$). As before, Aq had higher effects when added before Diclofenac compared to post-stimulation ($p<0.05$), supporting the idea that it can be used as a gastroprotective agent.

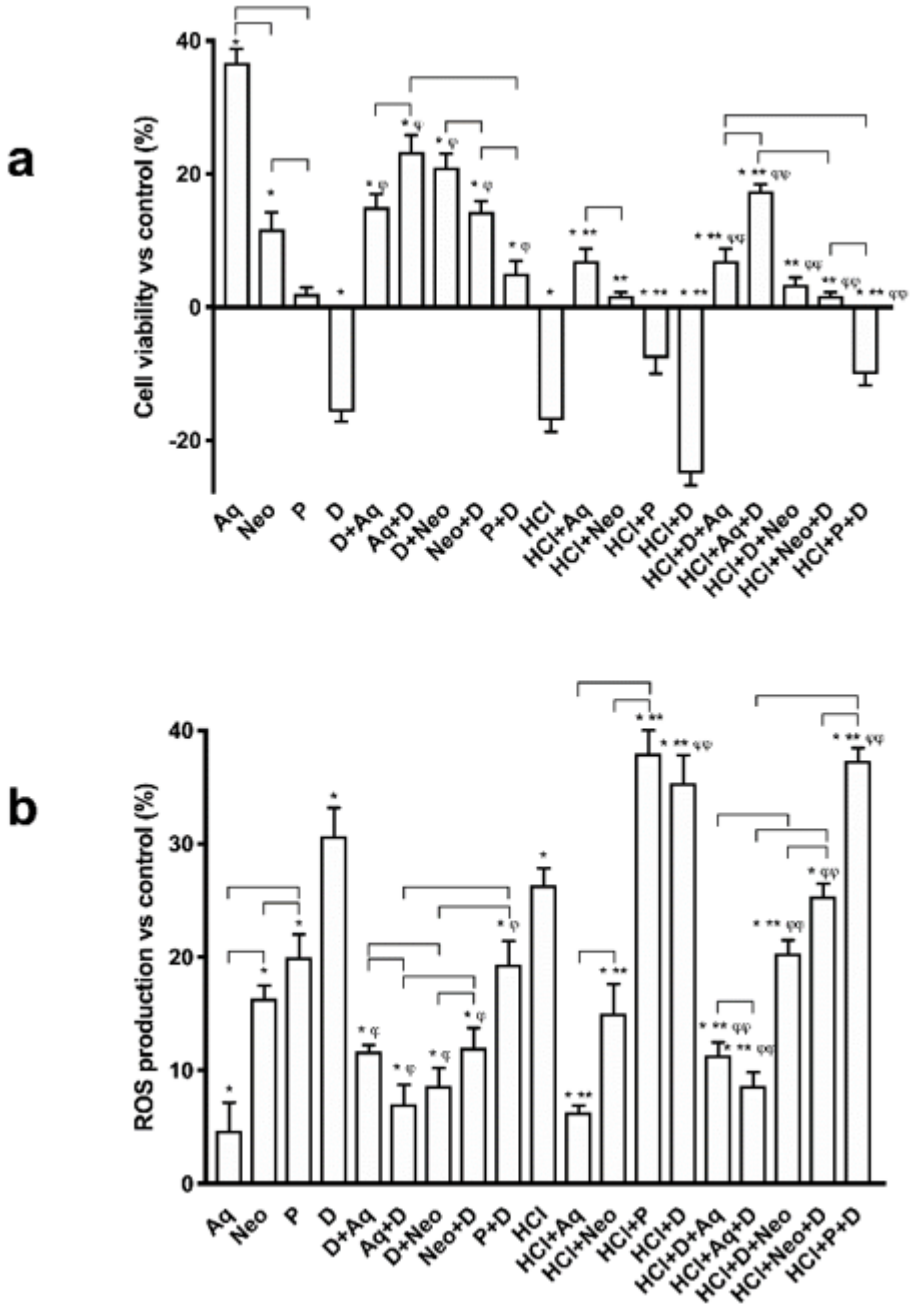


Fig. 10

Effects of Aq and Neo alone and combined with Diclofenac during acidic and hyperacidic conditions. In panel (**a**) cell viability and in panel (**b**) ROS production observed in gastric epithelial cells treated for 24h. P=pantoprazole, D=Diclofenac, Aq=combination between alginates and vitamin D3, Neo=mixture of polysaccharides and flavonoids. Data are expressed as means ±SD (%) of five independent experiments normalized to control values. *p<0.05 vs control; **p<0.05 vs HCl; ϕp<0.05 vs Diclofenac; ϕϕp<0.05 vs HCl+Diclofenac; arrows indicate p<0.05 between different groups.

Analysis of Intracellular Pathways Activated by Aq and Neo During Gastro-Protection

Since the most critical consequence for taking NSAID is hyperacidity, the latter has only been further investigated; in order to verify the gastroprotection induced by Aq and Neo, some additional experiments were performed to analyse annexin V, p53, Ki67, PI3K/Akt expressions and ERK/MAPK activity. As reported in Fig. 11a, annexin V, a marker of nuclear integrity, is important to exclude the activation of cell death pathway. Hyperacidic conditions confirmed their negative effects ($p<0.05$ vs control) and Aq and Neo alone, significantly reduced the annexin V expression compared to HCl alone ($p<0.05$). In addition, they were also able to counteract the expression of annexin V in presence of Diclofenac: in particular Aq had a better result when added before Diclofenac (about 25% vs post-stimulation) and Neo showed its best effect when added after Diclofenac (about 23% vs pre-stimulation). In order to confirm the protection exerted by Aq and Neo and to exclude a damage leading to cell death, p53 expression was evaluated under the same conditions (see Fig. 11b). Also in this case results confirmed the negative effects on p53 expression of both hyperacidity and Diclofenac, with an increase of about 36% and 32% respectively, compared to control. The positive mechanism activated by Aq on hyperacidic condition was able to reduce the activation of p53 caused by hyperacidity and Diclofenac ($p<0.05$): in particular the greater effect was observed when added before Diclofenac (about 59% vs Diclofenac). On the contrary, Neo was able to induce a beneficial effect when it was added after Diclofenac (about 25% vs Diclofenac), confirming data observed previously. Since an important factor for protection against damage, in addition to the maintenance of vitality, is the induction of cell proliferation, the Ki67 marker was also studied. The expression of Ki67 (see Fig. 11c) in presence of hyperacidic condition or Diclofenac were significantly reduced compared to control ($p<0.05$) and the pre- and post-stimulation with Aq reverted these conditions ($p<0.05$); in particular a greater effect was observed when it was added before Diclofenac (about 44% vs post-stimulation), supporting previous data about cell

viability. Neo was also able to induce an increase in cell proliferation, but the best result was observed when it was administered after Diclofenac (about 50% vs pre-stimulation), indicating a different protective mechanism compared to Aq.

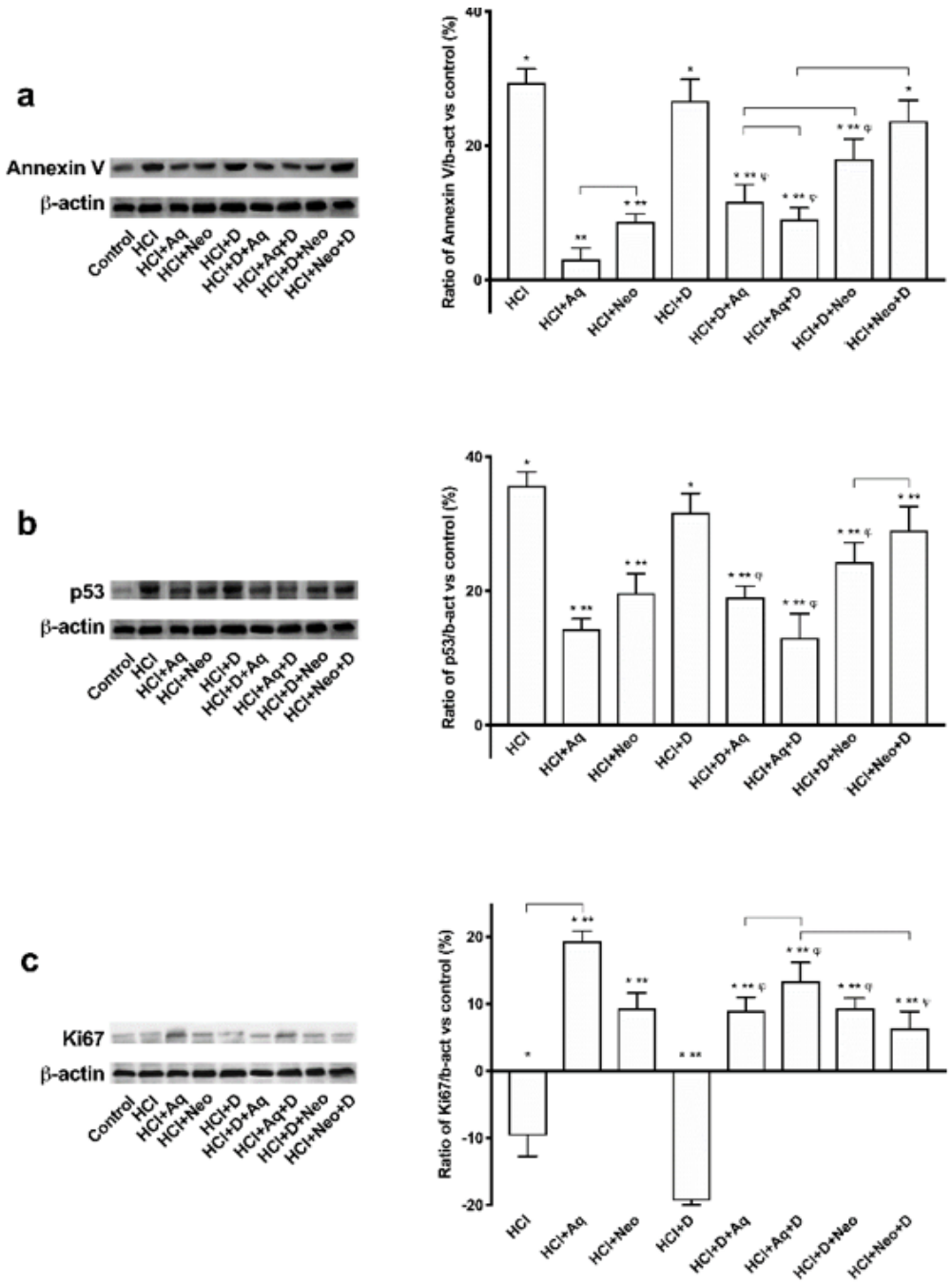


Fig. 11

Western blot and densitometric analysis of apoptotic and proliferation pathways during hyperacidic condition. In (**a**) Annexin V, in (**b**) p53 and in (**c**) Ki67 expressions analysed through Western blot (an example on the left) and densitometric analysis (on the right) of gastric epithelial cells treated for 24h. D=Diclofenac, Aq=combination between alginates and vitamin D3, Neo=mixture of polysaccharides and flavonoids. All results are expressed as means ±SD (%) normalized to control values of five independent experiments. *p<0.05 vs control; **p<0.05 vs HCl; ϕp<0.05 vs HCl+Diclofenac; arrows indicate p<0.05 between different groups.

Furthermore, cell survival is an important parameter, useful to better understand the mechanism that supports gastroprotection. In this context ERK/MAPK and PI3K/Akt were also analysed by ELISA test on hyperacidic condition. As reported in Fig. 12, Diclofenac alone induced a significant reduction on ERK and Akt activities, indicating a negative influence on cell survival ($p<0.05$ vs control). The pre- and post-stimulations with Aq were able to counteract these negative effects: indeed, ERK ($p<0.05$) and Akt activities ($p<0.05$) were significantly increased compared to Diclofenac, supporting previous data about the ability of Aq to act both before and after the damage. In addition, the major effects were observed when it was added before Diclofenac (about 156% and 55% respectively, compared to post-stimulation) supporting the hypothesized mechanism of action. On the other hand, Neo exerted a beneficial effect on ERK and Akt marker, but a greater result was observed when it was added after Diclofenac (about 18% and 100%, respectively, vs pre-stimulation). All these results are important to confirm the different potential gastroprotective activity of Aq and Neo.

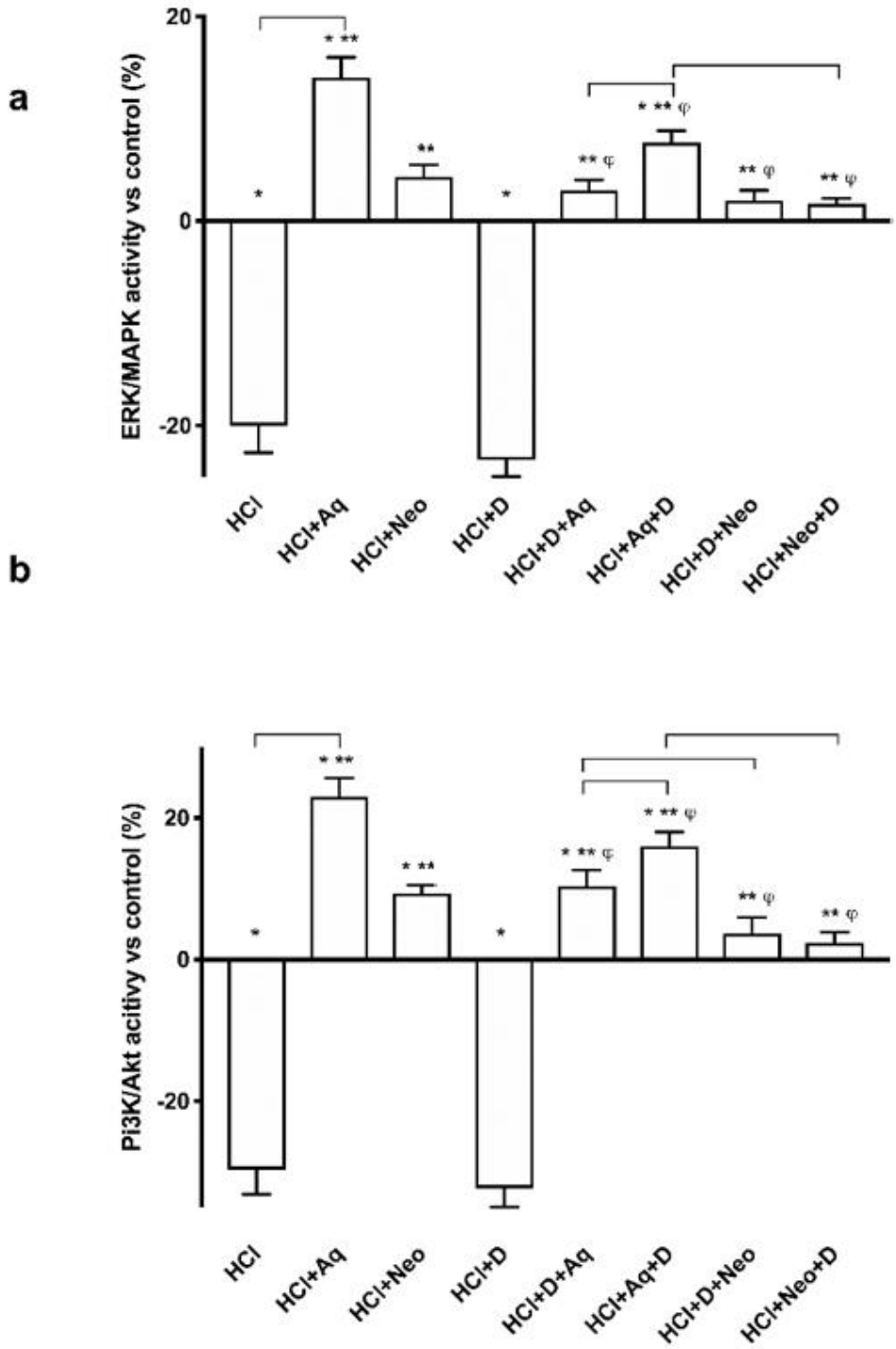


Fig. 12

Analysis of survival kinases activity on hyperacid condition. In panel (**a**) ERK/MAPK and in (**b**) PI3K/Akt activities measured by ELISA test in gastric epithelial cells treated for 24h. D=Diclofenac, Aq=combination between alginates and vitamin D3, Neo=mixture of polysaccharides and flavonoids. All the results are expressed as means ±SD (%) normalized to control values of five independent experiments. *p<0.05 vs control; **p<0.05 vs HCl; ϕp<0.05 vs HCl+Diclofenac; arrows indicate p<0.05 between different groups.

- **4.3 Project 3** (cardiovascular line of research)

The results showed in this project are contained in the paper: *Cooperative Effects of Q10, Vitamin D3, and L-Arginine on Cardiac and Endothelial Cells. Molinari C, Morsanuto V, Polli S, Uberti F. J Vasc Res. 2018;55(1):47-60.*

Effects of L -arginine, Vitamin D3 and Q10 on Cell Viability in a Time-Course Study.

As reported in Fig. 13, Q10, L-arginine and vitD were able to stimulate cell viability on both cell types during time. In particular, data showed a difference between low and high concentrations of each substances in a dose-dependent manner and the higher concentrations of Q10, L-arginine and vitD have shown to have maximum effects compared to control value ($p<0.05$). In addition, these concentrations were able to induce an increase in cell viability with a maximum effect after 90sec of stimulation ($p<0.05$). This is important to demonstrate the physiological activation of cell viability during time. The highest concentrations of Q10, L-arginine and vitD were used for all successive experiments and the time of stimulation was maintained at 90sec.

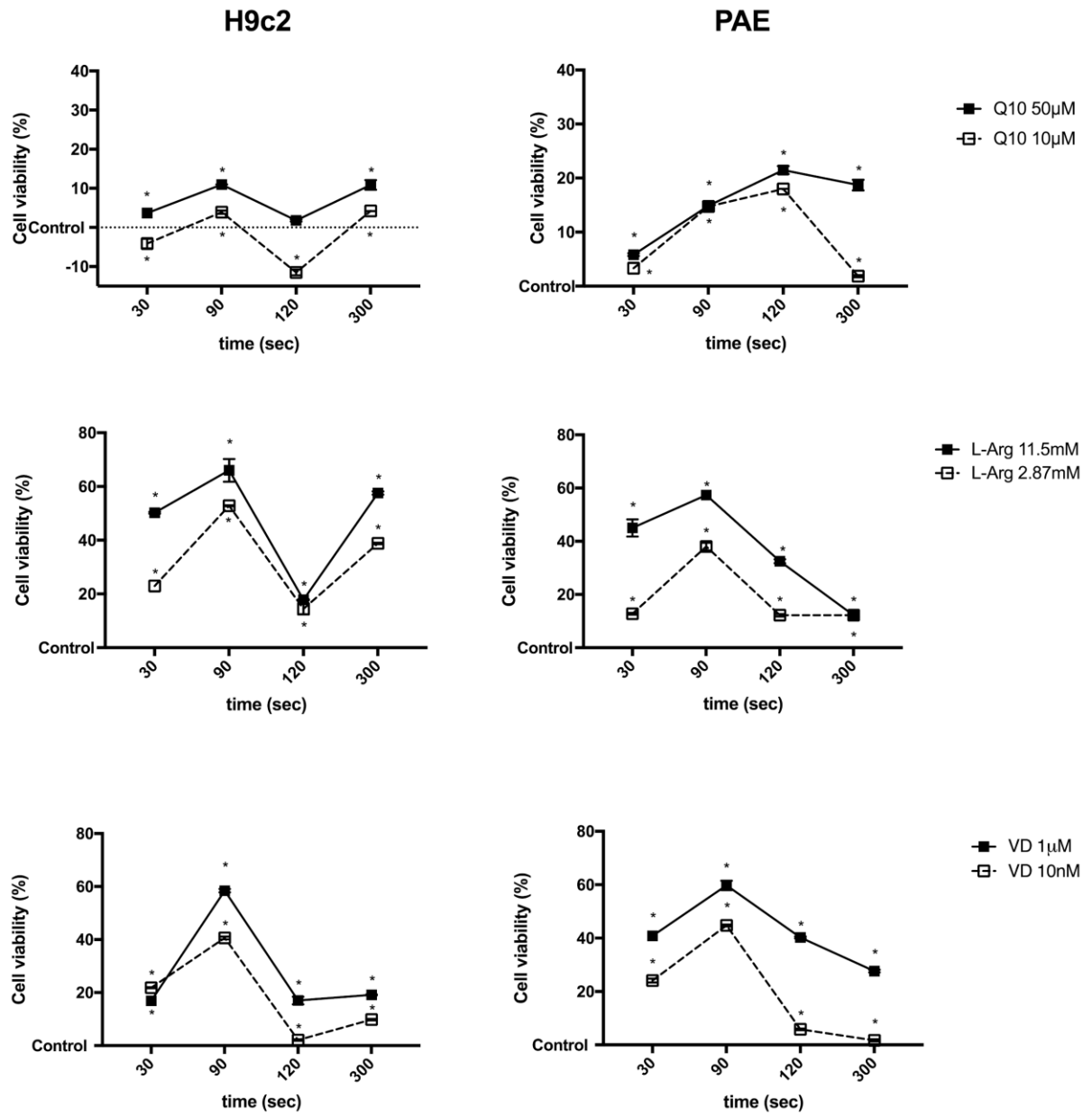


Fig. 13

Time-course and dose-response study in H9c2 and PAE cells. A time-course and dose-response of Q10, L-arginine and VD added individually in H9c2 (column on the left) and PAE cells (column on the right). The concentrations reported correspond to the higher (in black) and the lower (in white) doses used for each substance. VD=vitamin D3 (vitD). Reported data are means ±SD (%) of 5 independent experiments compared to control (line). *$p<0.05$ vs control.

Effects of Co-stimulation with L-arginine, vitamin D3 and Q10 on Nitric Oxide and Radical Oxygen Species Production.

Q10, L-arginine and vitD were applied together (referred to in this work as QLD) on H9c2 and PAE cells to demonstrate the ability of these substances to act in a cooperative manner on cell viability, NO and ROS release. As reported in Fig. 14A, the effect of QLD on cell viability in both cell types was time dependent and the maximum effect was observed at 90sec compared to control (75.5±0.71% in H9c2 and 88.75±0.35% in PAE cells), as previously observed with Q10, L-arginine and vitD individually tested. Since this study hypothesized the involvement of QLD in vasodilation, NO and ROS productions were also analyzed. As reported in Fig. 14B, NO release was time-dependent and the maximal NO release was observed at 90sec on both cell types (4.04±0.08 µMol in H9c2 and 2.63±0.18 µMol in PAE cells) compared to control. This production is comparable to the physiological release because, as shown in Fig. 14C, ROS production, measured at the same time, had an opposite trend; at 90sec ROS level was less than control on both cell types (about 42% in H9c2 and 6% in PAE cells). After this time ROS naturally augmented and NO decreased. In addition, the antioxidant activity of QLD was confirmed by the SOD activity on both cell cultures during time (Fig. 14D) in which starting from 90sec the stimulations were significant compared to control ($p<0.05$). All these findings have demonstrated for the first time a cooperative effect of QLD on promoting an increase in NO release and a possible role of these substances in vasomotility.

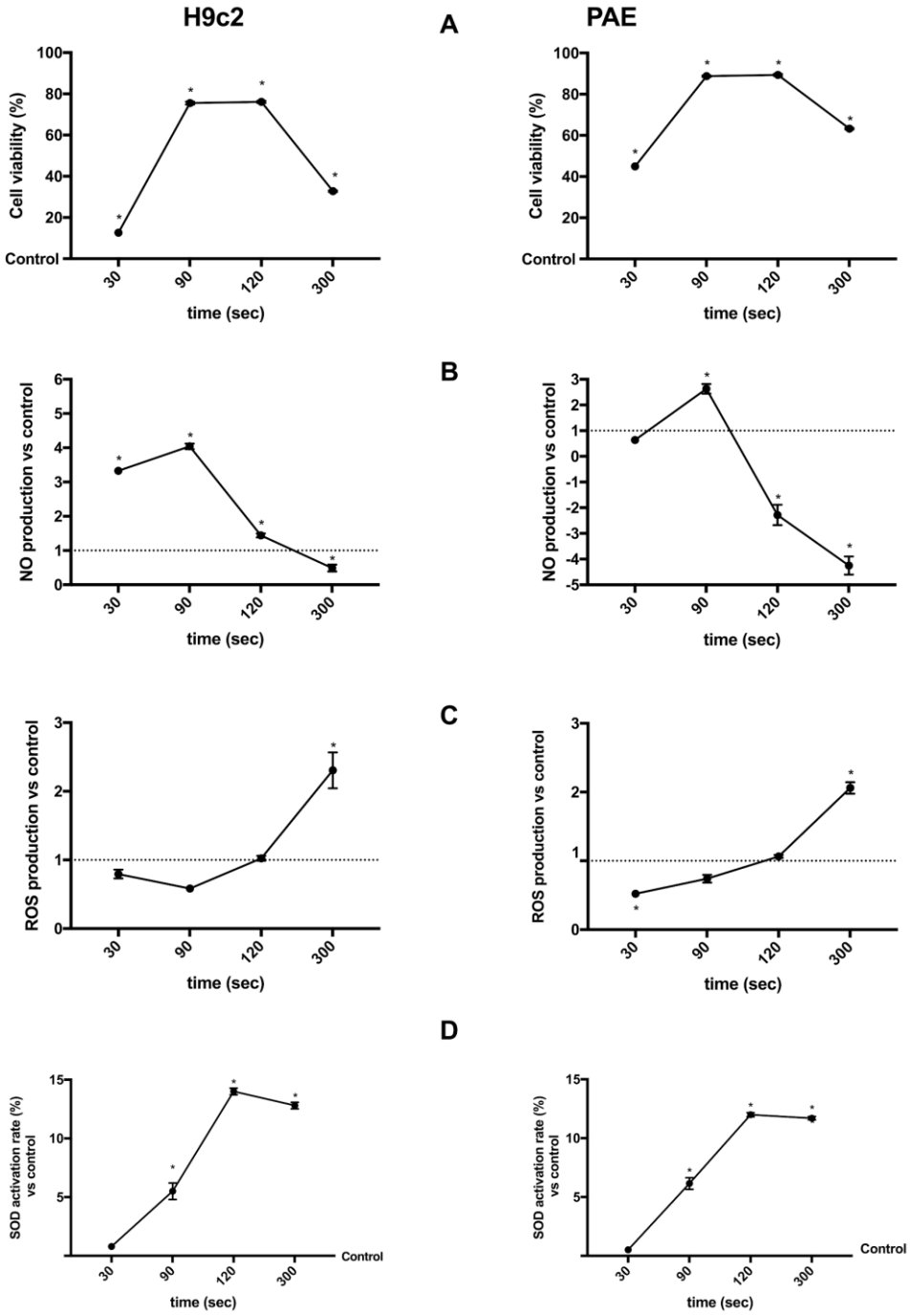


Fig. 14

Cell viability, NO and ROS productions, and SOD activity in H9c2 and PAE cells. Time course of Q10, L-arginine and VD added together, here defined QLD, in H9c2 (column on the left) and PAE cells (column on the right). In **A** cell viability was shown on both cell types. Reported data are means ±SD (%) compared to control (0%) of 5 independent experiments. *p<0.05 vs control. In **B**, nitric oxide values assessed by Griess method on cardiac and endothelial cells are shown. Data are expressed as means ±SD compared to control (line 1) of 5 independent experiments. *p<0.05 vs control. In **C** the ROS production in H9c2 and PAE cells was described. Reported data are means ±SD compared to control (line 1) of 5 independent experiments. *p<0.05 vs control. In **D** the SOD activity analysis in H9c2 and PAE cells was described. Reported data are means ±SD (%) compared to control (0%) of 5 independent experiments. *p<0.05 vs control.

Intracellular Pathways Involved in NO Release in PAE and H9c2 Cell Lines.

The main intracellular pathways involved in vasodilation leading to eNOS activation such as PI3K/Akt, ERK/MAPK and other intracellular signaling such as VDR receptor and p53, were analyzed in both cell lines by Western blot analysis, to explain the mechanisms activated by QLD. As reported in Fig. 15, Q10, L-arginine and vitD alone were able to improve ($p<0.05$) the phosphorylation of Akt, ERK and eNOS at 90sec in H9c2 and similarly ($p<0.05$) in PAE cells. Data from vitD experiments confirmed previous findings on its effects on NO production. The results about Q10 demonstrated its ability to activate the same mechanism as vitD to exert its beneficial effects ($p<0.05$). In addition, in the samples treated with QLD, the effect on Akt, ERK1/2 and eNOS expressions were amplified ($p<0.05$) in both cell types compared to control (about 20%, 31% and 38% respectively in H9c2 cells; about 38%, 27% and 23% respectively in PAE cells). These data indicate that QLD was able to activate the intracellular pathways (Akt, ERK1/2) leading to eNOS activation with consequent induction of NO release from cardiac and endothelial cells. In addition, the importance of Akt, ERK and eNOS on NO production and the involvement of these mechanisms was verified using their specific inhibitors (wortmannin, UO16 and LNAME, respectively) in presence of QLD treatment on both cell types.

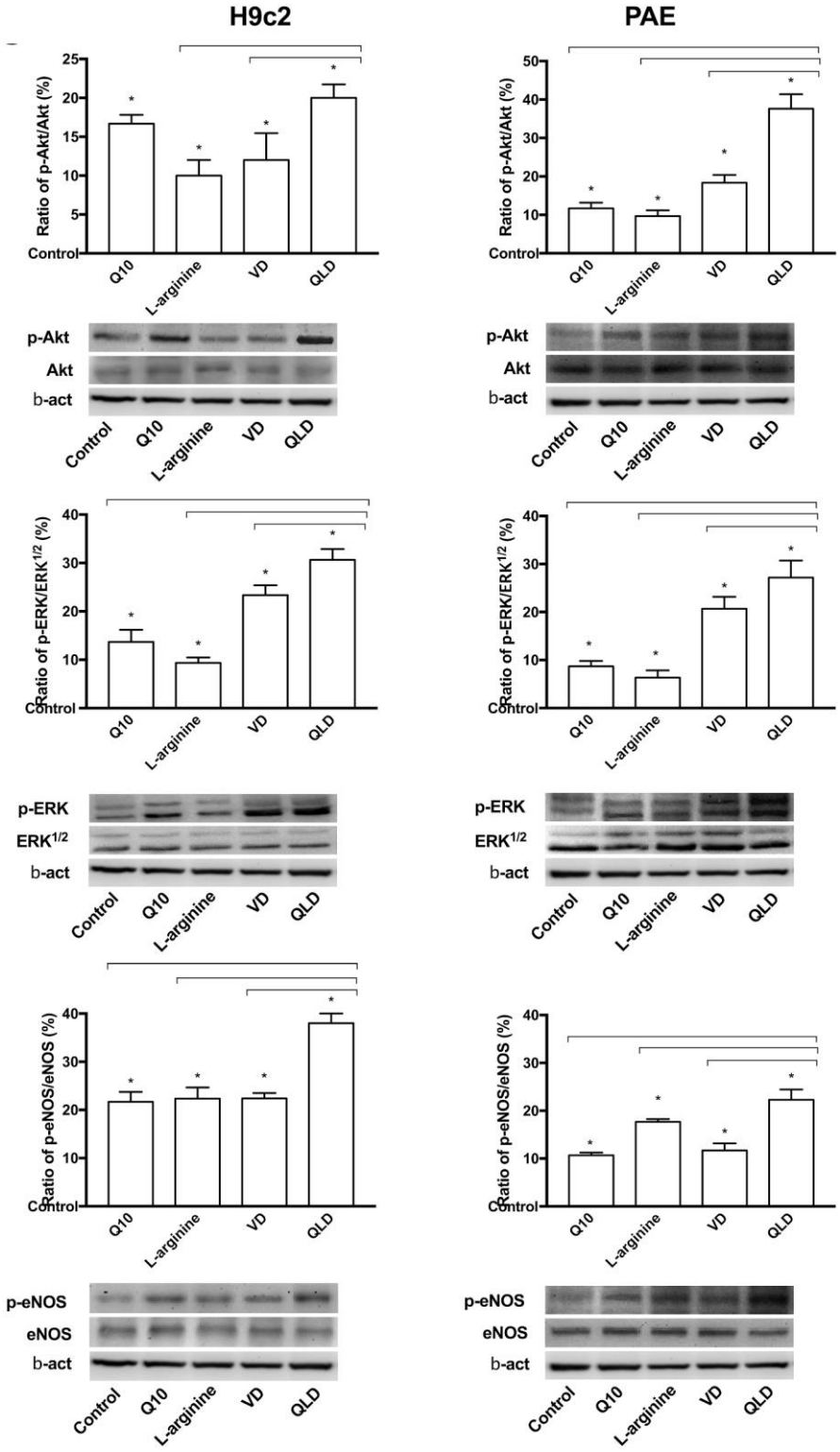


Fig. 15

Western blot and densitometric analysis of Akt, ERK1/2 and eNOS in H9c2 and PAE cells. In each panel an example of Western blot of each protein was reported. In addition, the densitometric analysis of phosphorylation relative to specific proteins are represented at 90sec of stimulation with Q10, L-arginine and VD added alone or together (QLD). Reported data are means ±SD (%) of 5 experiments compared to control (0%). VD=vitamin D3 (vitD). *$p<0.05$ vs control; bars indicate $p<0.05$ between QLD and the individual administration.

As shown in Fig. 16, the pre-treatment with the specific inhibitors caused a significant inhibition on NO production ($p<0.05$) compared to control and to QLD alone, confirming that QLD acts on Akt, ERK and eNOS to induce NO release. Since NO production can cause a damage when produced at high level, p53 was analyzed by Western blot in both cell types to exclude any cytotoxic effect.

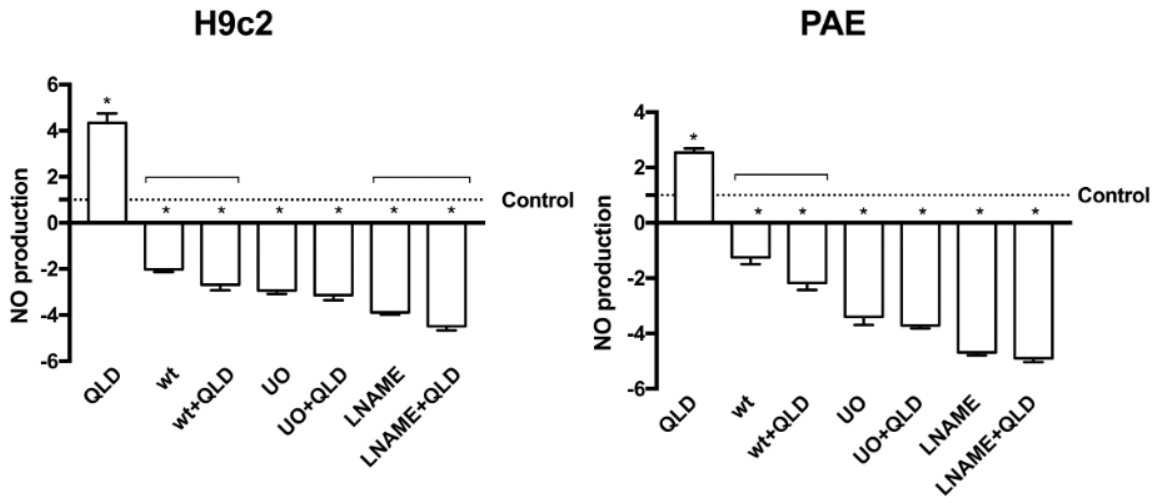


Fig.16
NO production measured by Griess method on cardiac and endothelial cells is shown. Data are expressed as means ±SD compared to control (line 1) of 5 independent experiments. *$p<0.05$ vs control. The bars indicate significant ($p<0.05$) between QLD and specific inhibitor plus QLD. QLD=Q10, L-arginine and VD. wt=100nM wortmannin; UO=10μM UO126; LNAME=10mM Nω-nitro-L-arginine methyl ester *$p<0.05$ vs control; bars indicate $p<0.05$ between QLD and the individual administration.

As reported in Fig. 17A, Q10, L-arginine and vitD individually administered did not cause any increase in p53 expression at 90sec of stimulation compared to control values on cardiac and endothelial cells; in particular, vitD induced a change of about 7% and 10% in H9c2 and PAE cells respectively compared to control, and Q10 of about 3% and 9% in H9c2 and PAE cells respectively compared to control. In addition QLD was also able to reduce the expression of p53 compared to control (about 11% and 12% in H9c2 and PAE cells, respectively) confirming its beneficial effects. Indeed, in response to low levels of oxidative stress, p53 primarily plays an antioxidant role without the involvement of nuclear activation. These findings demonstrate the importance of the cooperative activity of QLD on cardiac and endothelial cells. Since QLD includes vitD, an analysis on VDR receptor was considered important to define its role on the positive effect of QLD during vasodilation. Q10 and L-arginine alone were not able to improve the expression of VDR receptor (Fig. 17B) compared to control ($p<0.05$) in both cell types. On the contrary the stimulation with vitD alone was able to induce an enhancement ($p<0.05$) of VDR expression in cardiac (of about 16%) and endothelial cells (of about 38%) compared to control, demonstrating the effect of vitD in these cell types. In addition, the stimulation with QLD was able to improve this effect of about 23% in cardiac cells and 49% in endothelial ones compared to control and 42.5% and 30% compared to vitD alone. These findings have demonstrated for the first time an improvement of vitD effects caused by simultaneous stimulation with Q10 and L-arginine on cardiac and endothelial cells.

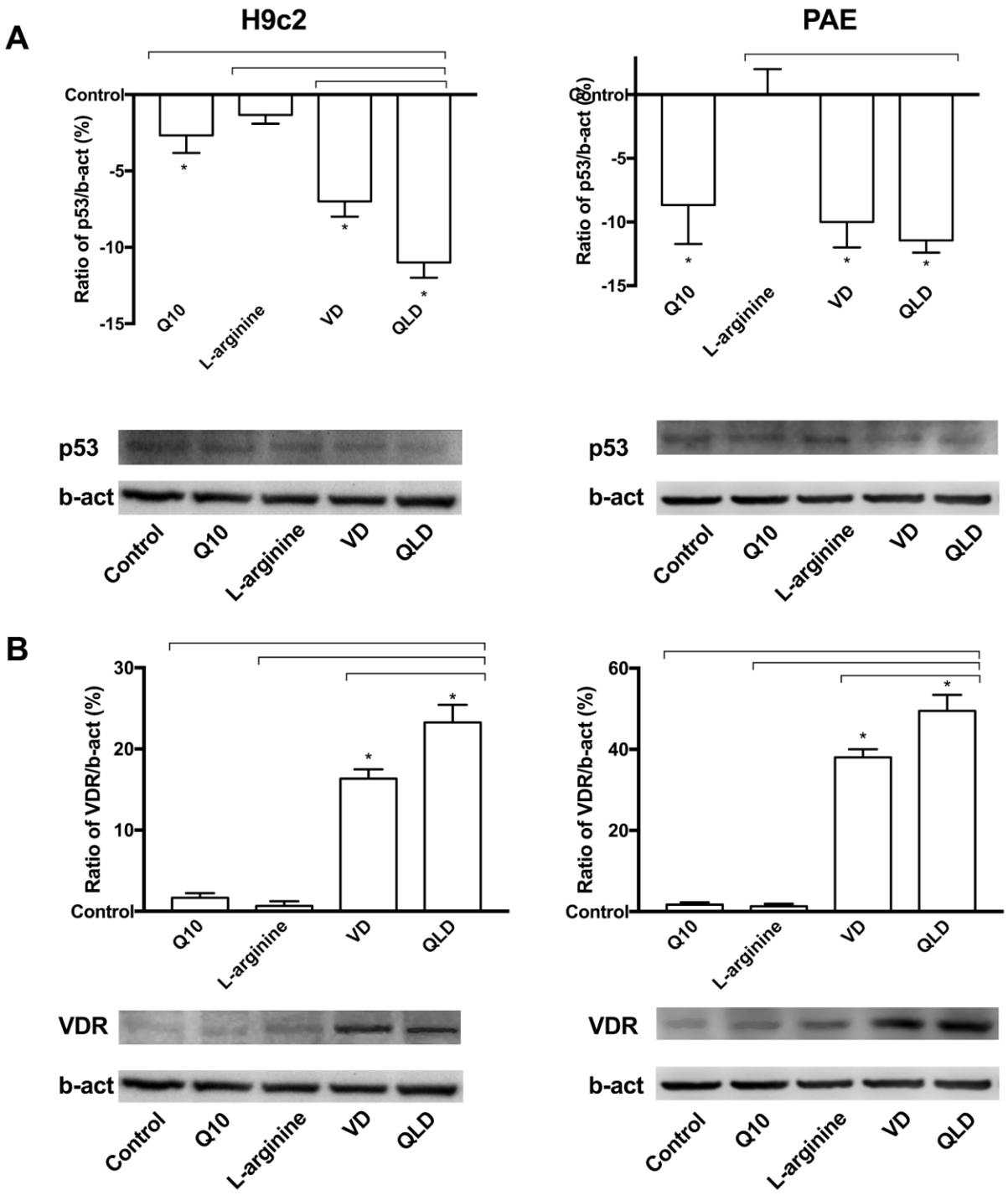


Fig. 17

Western blot and densitometric analysis of p53 and VDR in H9c2 and PAE cells. In **A** and **B** densitometric analysis and Western blot of H9c2 (left) and PAE (right) cells of p53 and VDR at 90sec of stimulation with Q10, L-arginine and VD added alone or together (QLD). The images reported are an explicative example. Reported data are the means ±SD (%) of 5 experiments compared to control (0%). VD=vitamin D3 (vitD), QLD=Q10, L-arginine and VD. *$p<0.05$ vs control; bars indicate $p<0.05$ between QLD and the individual administration.

In vivo Study on Effects of Q10, L-arginine and vitamin D3 Added Together and Alone.

Since the cooperative effect of Q10, L-arginine and vitD acts primarily on vasodilation, these agents were tested either alone or added together in *in vivo* experiments. The single agents were tested in a time-course study and showed a maximal effect on aortic blood flow at 120sec of administration (data not shown). This treatment time was maintained for all successive experiments. An intravenous administration at 120sec showed an increase in aortic blood-flow (Fig. 18A) in a concentration-dependent manner ($p<0.05$) for all substances tested and in particular the maximum effect was obtained with the maximal concentration used (Q10 about 61%, L-arginine about 200%, vitD about 87%) compared to control and to the minimal doses used (Q10 about 40%, L-arginine about 50%, vitD about 56%). These data confirm previous observations on cell culture experiments, about the effect of Q10, L-arginine and vitD individually added. In addition, data observed during SOD activity experiments on plasma samples (Fig. 18B) confirmed the data obtained in *in vitro* experiments about the antioxidant effect. For these findings the maximal concentration of Q10, L-arginine and vitD was used in all successive experiments.

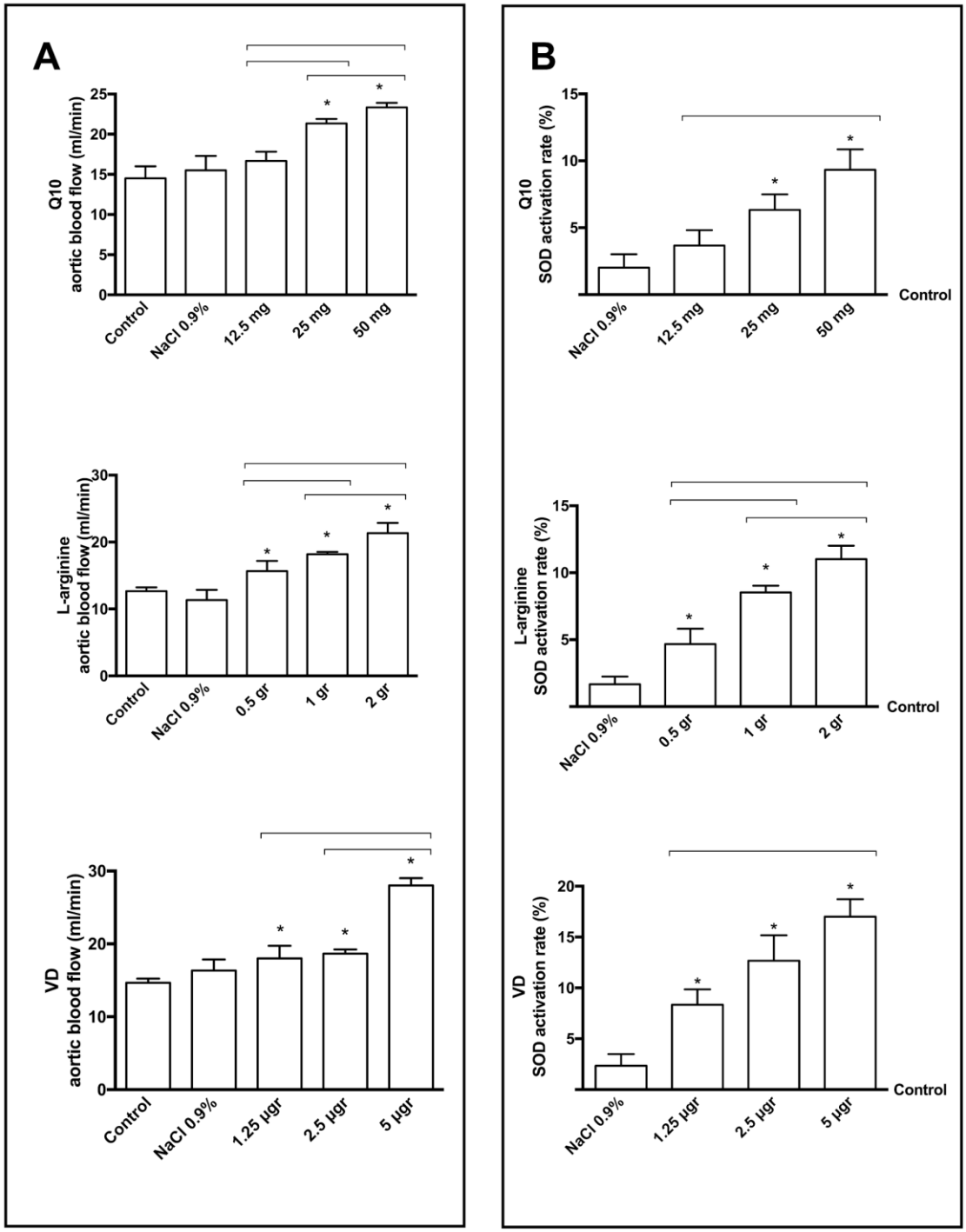


Fig. 18

Aortic blood flow measurements and SOD activity in rats. In **A** vasodilation induced at 120sec by dose-response tests with Q10, L-arginine and VD individually administered. Reported data are means ±SD (ml/min) of at least n=3 rats for each stimulation. Abbreviations are the same previously reported. The effect of the solvent (NaCl 0.9%) was showed as well. *$p<0.05$ vs control; bars indicate $p<0.05$ between different concentrations of each substances. In **B**, SOD activity on plasma sample taken at 120sec by dose-response tests with Q10, L-arginine and VD individually administered. *$p<0.05$ vs control; bars indicate $p<0.05$ between different concentrations of each substances.

QLD was added to demonstrate the cooperative effect during vasodilation; as reported in Fig. 19A, QLD at 120sec can cause an increase of aortic blood flow compared to control ($p<0.05$) of about 76% and this effect was bigger than the effect of Q10, L-arginine and vitD alone of about 30%, 230%, 7% respectively. Moreover another important element involved in vasodilation, nitric oxide, was also studied under the same conditions. NO production (Fig. 19B) induced by QLD was significant (62%, $p<0.05$) compared to control value and this production did not cause any damage because ROS production (Fig. 19C) at the same time was reduced ($p<0.05$) of about 32% compared to control. Finally, the antioxidant effect (Fig. 19D) of the combination of the three was confirmed by the analysis of SOD activity on plasma samples ($p<0.05$); the effect of the combination was greater than the single administration. The importance of cooperative effect of QLD during vasodilation was also confirmed by Western blot analysis (Fig. 20) in which the effect on Akt, ERK and eNOS was confirmed and higher than the single administrations ($p<0.05$). Finally, to verify the effects of QLD, some additional experiments were performed during ischemia-reperfusion injury (Table 2) in which QLD was seen to restore aortic blood flow ($p<0.05$ compared to ischemia). All these data have demonstrated for the first time a cooperative effect during vasodilation of Q10, L-arginine and vitD both physiological and pathological conditions.

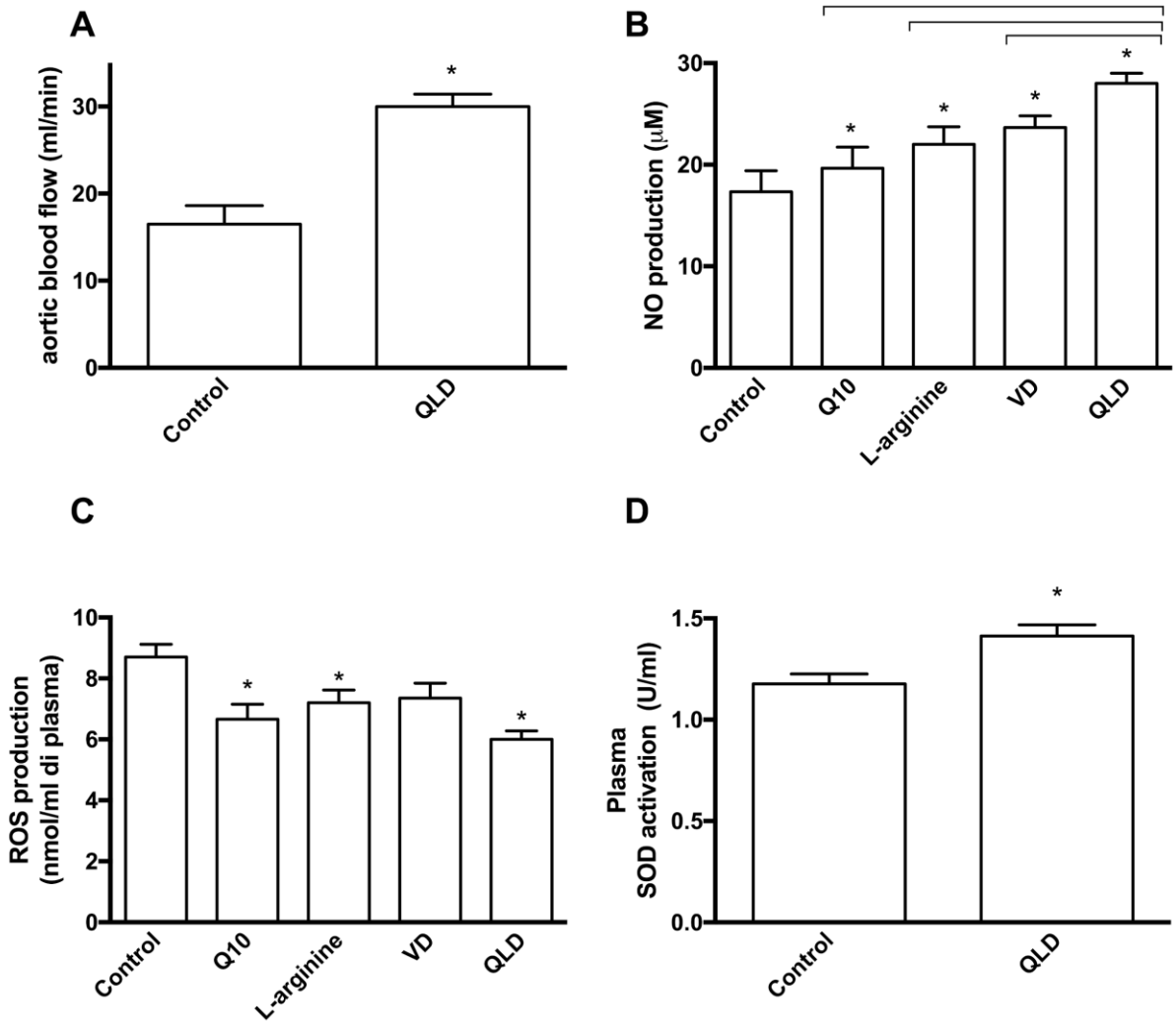


Fig. 19

QLD effects on aortic blood flow, NO and ROS production and SOD activity in plasma rats. Aortic blood flow (**A**), NO (**B**) and ROS (**C**) productions and SOD activity (**D**) are reported at 120sec after intravenous administration of QLD in rats. Reported data are means ±SD of at least n=4 rats for each stimulation. * $p<0.05$ vs control. In B bars indicate $p<0.05$ between QLD and the individual administration. VD=vitamin D3 (vitD), QLD=Q10, L-arginine and VD.

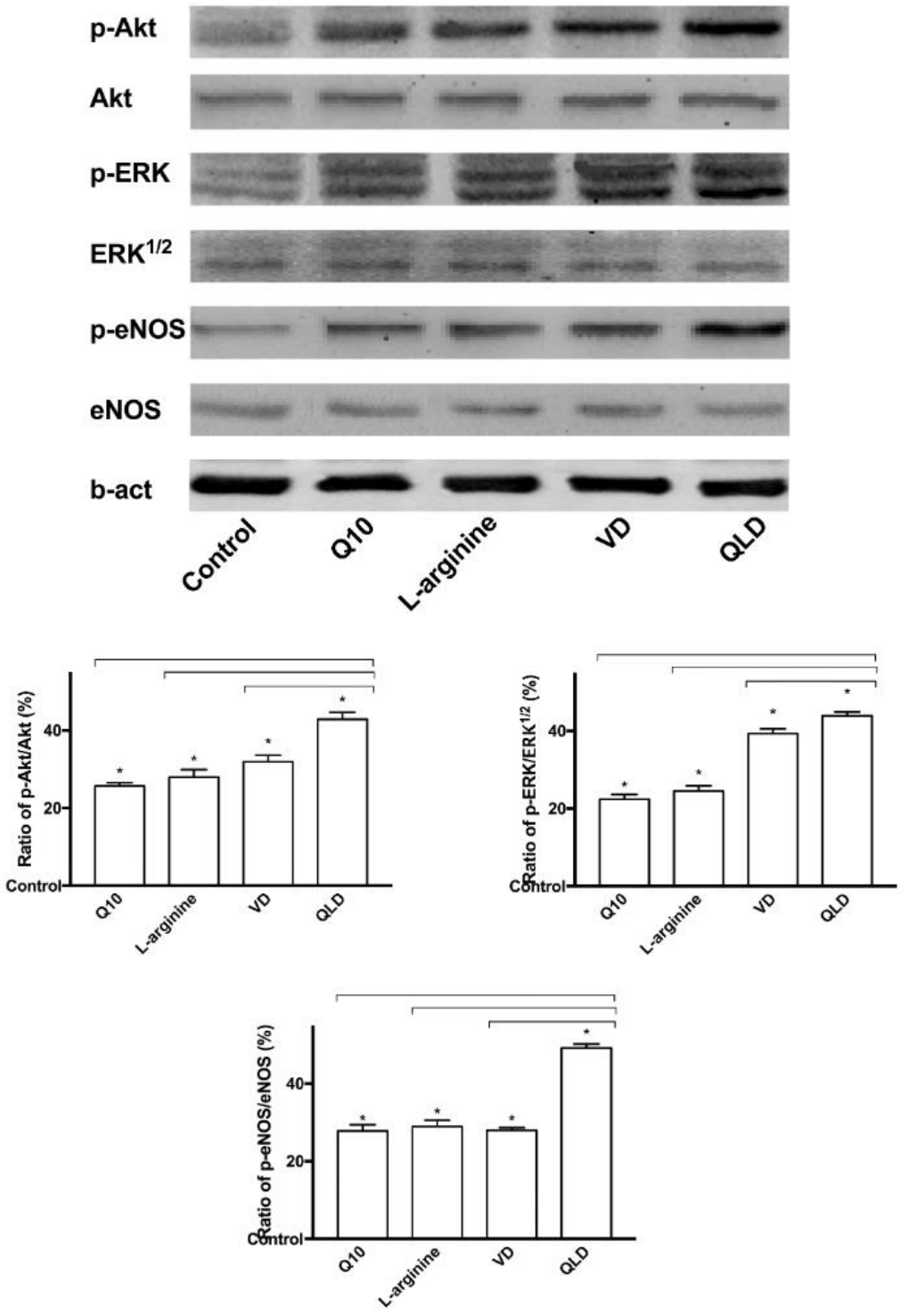


Fig. 20

Western blot and densitometric analysis of Akt, ERK1/2 and eNOS in aortic samples. In upper panel an example of Western blot and in lower panel the densitometric analysis of phosphorylation relative to specific proteins are reported. Data are means ±SD (%) of 5 experiments compared to control (0%). VD=vitamin D3 (vitD), QLD=Q10, L-arginine and VD. *$p<0.05$ vs control; bars indicate $p<0.05$ between QLD and the individual administration.

	ABF (ml/min)
Control	**24.95±5.1**
Ischemia	**13.9±4.5***
QLD	**21.2±3.7 ****

Table 2

Aortic blood flow (ABF) measured during ischemia-reperfusion experiments. Data reported are expressed as means±SD of almost 5 experiments (n=5 for each group). *$p<0.05$ vs control; **$p<0.05$ vs ischemia.

- **4.4 Project 4** (brain line of research)

The results obtained are contained in the paper: *Role of Combined Lipoic Acid and Vitamin D3 on Astrocytes as a Way to Prevent Brain Ageing by Induced Oxidative Stress and Iron Accumulation. Molinari C, Morsanuto V, Ghirlanda S, Ruga S, Notte F, Gaetano L, Uberti F. Oxidative Medicine and Cellular Longevity, 2019;2019:2843121.*

Cell Viability under Treatments with Vitamin D3 and Lipoic Acid During Time

MTT test was performed both in a dose-response and in a time-course study in order to assess the potential effect of vitD and LA alone and combined on cell viability of astrocytes. Firstly, in order to identify the best concentration to use, the concentration-dependent effect of LA alone (ranging from 10μM to 100μM) on cell viability during 24 hours (starting from 15min to 1440min) was analyzed.

As shown in Fig. 21a, 50μM LA appeared to be the dose able to induce the greatest effect (p<0.05) compared to the control and to other concentrations (10, 25, and 100μM, p<0.05) during all the time of stimulation, and the maximum effect of about 66% compared to the control was observed at 1440min. This concentration of LA was maintained for all successive experiments. Additional experiments were carried out to study the combination of 50μM LA and 100nM vitD on cell viability since the new hypothesized formulation includes LA and vitD. As reported in Fig. 21b, vitD increased cell viability of astrocytes in a time-course manner with a maximum effect, about 22% (p<0.05) at 1440min compared to the control. In addition, the combination of LA and vitD was able to significantly increase (p<0.05) cell viability during time compared to the control (p<0.05) and to 50μM LA and 100nM vitD alone starting from 60min of stimulation (p<0.05). The combination exerted a greater effect at 1440min compared to the control (p<0.05) and to 50μM LA and 100nM vitD alone (about 20.5% and 64%, respectively). For this reason, this time of stimulation was maintained for all successive experiments. In conclusion, the hypothesis of the synergistic effects exerted by vitD and LA in astrocytes indicating a possible new strategy to slow down ageing is supported by these data.

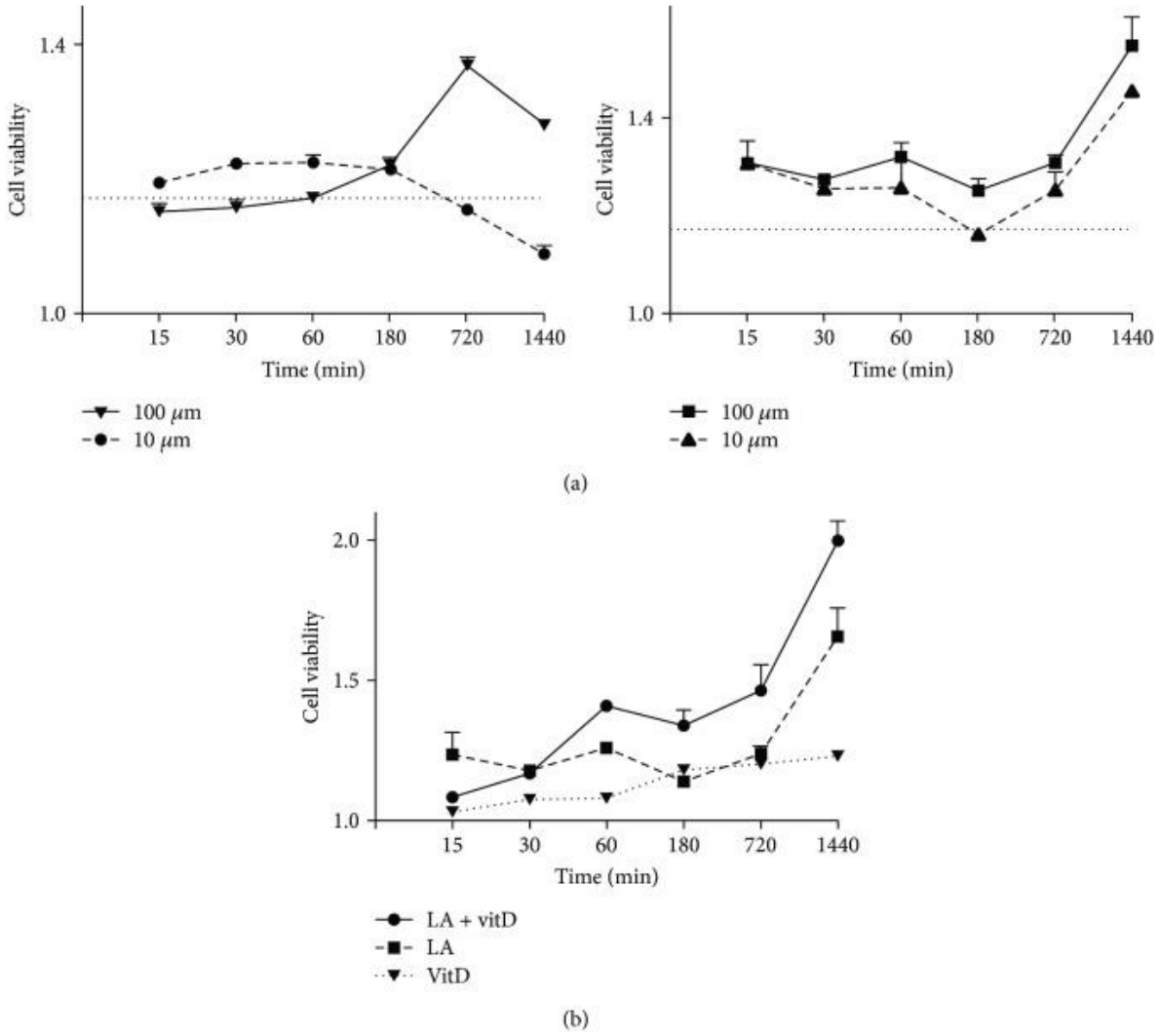


Fig. 21

Cell viability measured in time-course and dose-response studies in astrocytes. In (**a**), time-course and dose-response (ranging from 10μM to 100μM) studies of LA measured in astrocytes are illustrated; in (**b**), time-course and dose-response studies of 50μM LA combined with 100nM vitD measured in astrocytes are reported. LA=lipoic acid, vitD=vitamin D3, and LA+vitD=combination of lipoic acid and vitamin D3. Data are expressed as means ±SD (%) of five independent experiments normalized to the control (number 1 in the *y* axis corresponds to 100% control values).

Permeability of Vitamin D3 and Lipoic Acid through Blood-Brain Barrier (BBB)

A BBB permeability study was performed to better understand the ability of 100nM vitD and 50μM LA alone and combined together during time to cross the hematoencephalic barrier. As reported in Fig. 22a, the analysis of the basolateral volume showed a time-dependent increase on absorption capacity caused by 100nM vitD and 50μM LA alone compared to the control (p<0.05), and the greater effects were observed at 1440min (about 49.5% and 40.5%, respectively). The combination of vitD and LA increased the absorption capacity with respect to the control (p<0.05) during time and to their single administration starting from 60min, as previously observed on cell viability (p<0.05). These data support a cooperative effect of vitD and LA also during the permeability assay. The successive quantifications of vitD and LA were carried out to determine the specific concentration present in basolateral volume of the BBB model. In particular, the absorption of vitD and LA during time was time-dependent, and the combination of vitD and LA proved to be essential to amplify their ability to cross the barrier. Indeed, the specific quantifications of vitD (Fig. 22b) and LA (Fig. 22c) showed a greater effect of the combination compared to the separated administration (about 26% and 63%, respectively), with a maximum effect at 1440min (p<0.05 vs. control). In conclusion, the hypothesis that the combination of LA and vitD is able to exert beneficial effects directly on viability of astrocytes due to their ability to cross the BBB is supported by all these findings.

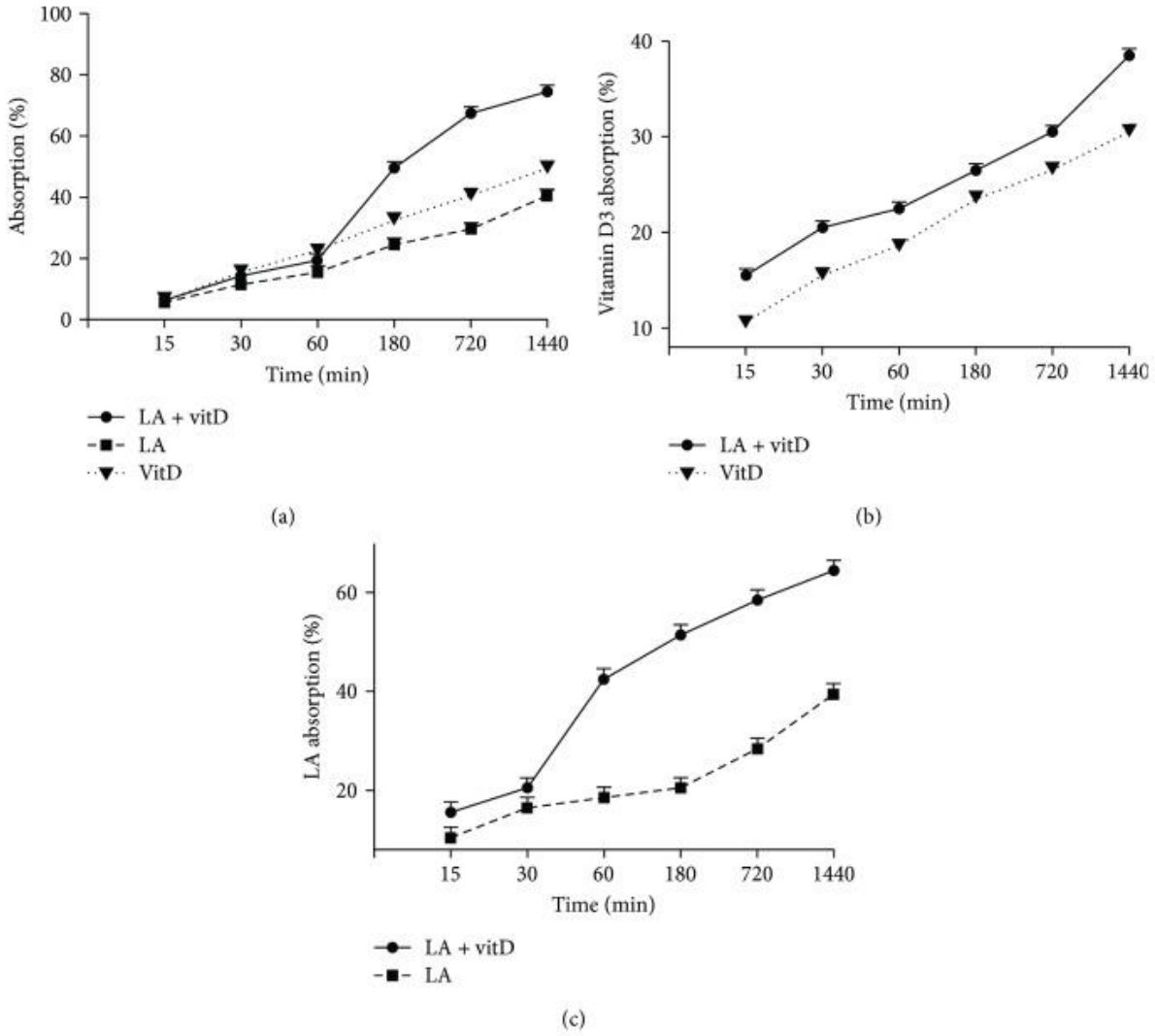


Fig. 22

BBB permeability, vitD and LA quantifications to predict their bioavailability in the brain. In (**a**), the absorption capacity through the BBB of vitD and LA alone and combined is shown; in (**b**), quantification of vitD is shown; and in (**c**), quantification of LA at basolateral environment of the barrier model are reported. LA=lipoic acid, vitD=vitamin D3, and LA+vitD=combination of lipoic acid and vitamin D3. Data are expressed as means ±SD (%) of five independent experiments normalized to control values (0% line).

Analysis of Mitochondrial Activity after Treatments with Lipoic Acid and Vitamin D3 in Oxidative Condition

Cell viability, ROS production and mitochondrial potential were evaluated in astrocytes, in order to investigate the potential action to prevent cellular ageing under oxidative condition. Exposure to 200μM H_2O_2 significantly reduced cell viability of about 46% compared to the control; conversely, following posttreatment with 50μM LA and 100nM vitD alone or combined, the cell viability was significantly increased. The greatest effect was obtained with the combination of LA and vitD which reverted the cell loss (Fig. 23a). Since the main theory at the basis of brain ageing regards the oxidative condition, additional experiments on ROS production were performed. 50μM LA, 100nM vitD, and the combination of both were able to maintain the ROS production under the physiological level (p>0.05 vs. control), supporting the hypothesis of their safety during use (Fig. 23b). Exposure of astrocytes to 200μM H_2O_2 significantly increased the intracellular ROS production as illustrated in Fig. 23b of about 30% compared to the control (p<0.05); posttreatment with 50μM LA and 100nM vitD alone significantly reduced ROS production (about 43% and 57%, respectively, vs. H_2O_2 alone), and the concomitant administration of LA and vitD improved the reduction of the ROS level compared to 200μM H_2O_2, 50μM LA, and 100nM vitD alone (p<0.05, about 78%, 62%, and 50%, respectively). Since the alteration of the formation of a proton gradient across the inner mitochondrial membrane is considered to be one of the key indicators of cellular viability, the mitochondrial potential was analyzed. Treatments with 50μM LA, 100nM vitD, and with the combination of both induced a significant increase in red fluorescence, supporting the active role of 50μM LA, 100nM vitD, and their combination on mitochondrial activity (p<0.05). In addition, the combination of LA and vitD seems to have a greater effect compared to 50μM LA and 100nM vitD alone (about 23% and 60%, respectively). H_2O_2-treated cells exhibited changes in the fluorescence signal which lead to a decreased red fluorescence signal and

increased green fluorescence signal, indicating a significant dissipation of mitochondrial potential and cell loss compared to the control (p<0.05, Fig. 23c). Post-treatment with 50μM LA, 100nM vitD alone, and with the combination of both significantly reversed dissipation of mitochondrial potential as shown in Fig. 23c compared to 200μM H_2O_2 alone (p<0.05). In particular, the combination of LA and vitD suppressed the effect of H_2O_2-induced mitochondrial dissipation, shifting the fluorescence signal from green to red (p<0.05). These results show that the combination of LA and vitD is able to attenuates the H_2O_2-induced apoptosis through the mitochondrial-mediated pathway.

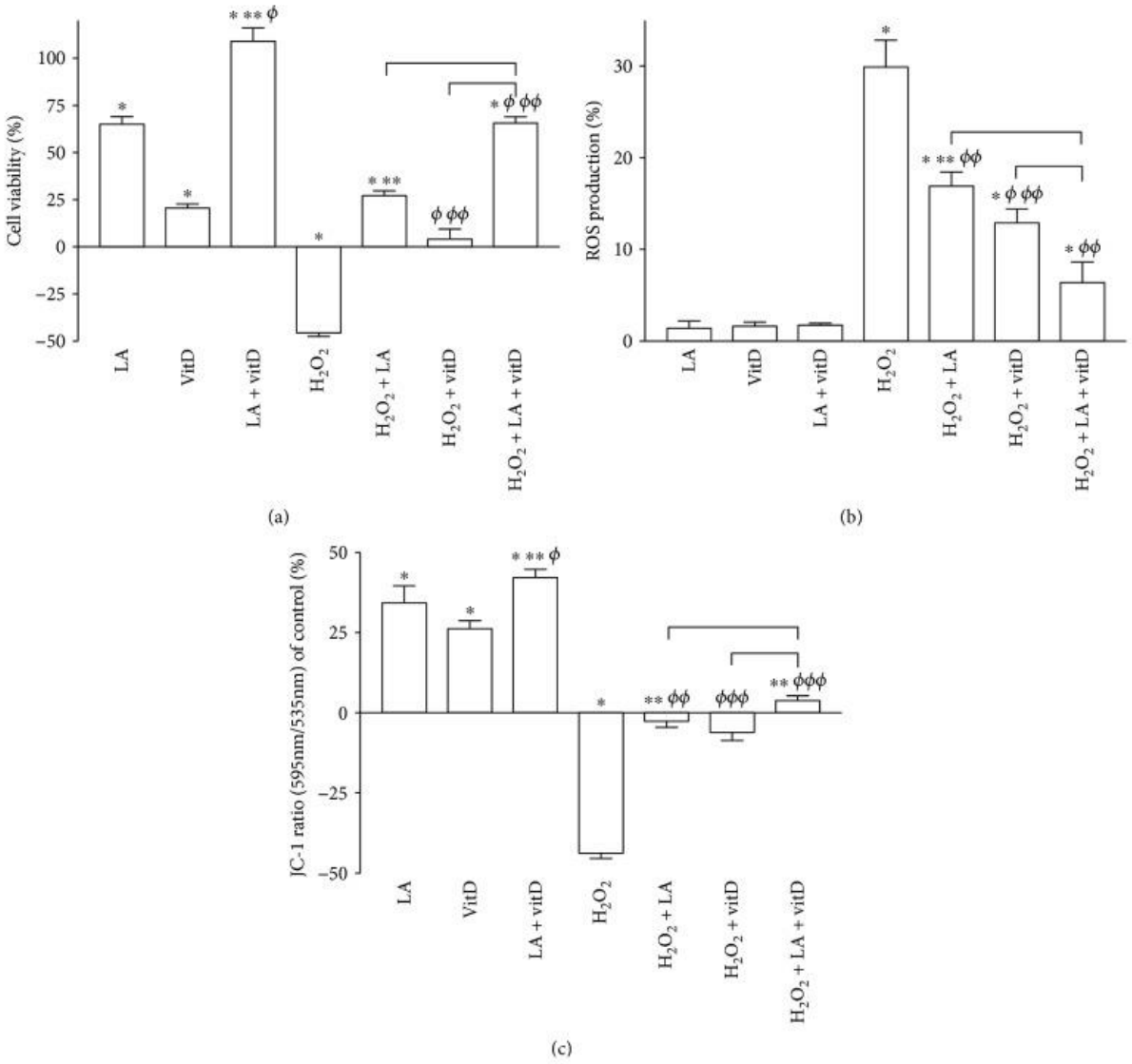


Fig. 23

Analysis of cellular viability, ROS production, and mitochondrial activity under oxidative condition. In (**a**), cell viability, in (**b**), ROS production, and in (**c**), mitochondrial membrane potential measured on astrocytes treated with vitD and LA alone and combined for 24h pre-stimulation with H_2O_2 are illustrated. LA=lipoic acid, vitD=vitamin D3, and LA+vitD=combination of lipoic acid and vitamin D3. Data are expressed as means ±SD (%) of five independent experiments normalized to control values (0% line). $^{*}p<0.05$ vs. control; $^{**}p<0.05$ vs. LA; $^{\phi}p<0.05$ vs. vitD; $^{\phi\phi}p<0.05$ vs. H_2O_2, bars: $p<0.05$ between treatments.

Study of Intracellular Pathways Activated by Lipoic Acid and Vitamin D3 in Oxidative Condition

The dissipation of mitochondrial potential under oxidative condition is known to initiate a cascade of events leading to the activation of caspases, which in turn trigger apoptosis. In this context, p53 as a key factor involved in ageing, oxidative stress, and neurodegeneration and cytochrome C as a key regulator of both cellular energetic metabolism and apoptosis were investigated in astrocytes. Data reported in Fig. 24a showed a reduction on p53 activity after the stimulation with 50μM LA and 100nM vitD alone and in combination (p<0.05 vs. control), supporting previous data about the safety of the combination. p53 activity significantly increased in H_2O_2-treated astrocytes (p<0.05 vs. control), and the subsequent stimulation with 50μM LA and 100nM vitD alone significantly reduced it (p<0.05). In addition, the combination of LA and vitD amplified the reduction compared to 200μM H_2O_2 of about 80% and the single administration of about 69% and 60%, respectively (p<0.05), resulting in survival-favored conditions. To examine the involvement of the caspase pathways, the expression of cytochrome C was analyzed under the same conditions. Fig. 24b and 25 show similar results for p53 under the stimulation with 50μM LA and 100nM vitD alone, confirming the previously observed beneficial effects. Supporting mitochondrial integrity, the combination of LA and vitD also maintained cytochrome C at the basal level. In addition, H_2O_2-treated cells showed an increase in the expression of cytochrome C which was reduced by the following stimulation with both 50μM LA and 100nM vitD alone and combined (p<0.05). In order to exclude any oxidative damage induced by the stimulations, SOD3 and iNOS expressions were investigated. As reported in Fig. 24c, 24d and 25, the SOD3 and iNOS expressions significantly increased in the presence of 200μM H_2O_2 (p<0.05 vs. control), supporting the hypothesis of the involvement of oxidative stress in astrocyte death. In addition, the post-stimulation with 50μM LA and 100nM vitD alone significantly reduced the expression of both SOD3 and iNOS compared to

200μM H_2O_2 alone, and a greater reduction was obtained by the combined stimulation with 50μM LA and 100nM vitD compared to 200μM H_2O_2 (p<0.05), indicating a beneficial effect in counteracting the ageing process. Since the neuroinflammation is a common cause of brain ageing, PKA, a key anti-inflammatory marker, was also investigated under the same conditions (Fig. 24e and 25). The expression of PKA observed in astrocytes showed a significant increase in the presence of both 50μM LA and 100nM vitD alone (p<0.05), and their combination amplified this effect supporting the anti-inflammatory activity of LA and vitD. In the presence of 200μM H_2O_2, a significant reduction compared to the control was found, and the post-stimulation with 50μM LA and 100nM vitD reverted the mechanism (p<0.05). The combination of LA and vitD added after 200μM H_2O_2 had a similar effect on PKA expression to what was observed without H_2O_2 indicating the ability of the combination to prevent the induction of inflammatory cascade under oxidative stress. A natural consequence of apoptosis is known to be cell loss, and the β-amyloid analysis demonstrated the alteration in brain tissue. As reported in Fig. 24f, the stimulation with 200μM H_2O_2 alone caused a significant increase of the APP level, supporting previous data about cell death. In addition, the post-stimulations with 50μM LA and 100nM vitD were able to reduce the damage as shown by the decrease in the APP level (p<0.05) compared to 200μM H_2O_2 alone. Finally, the greater effect was observed in the presence of the combined treatment with LA and vitD, indicating the effectiveness of the combination during brain damage.

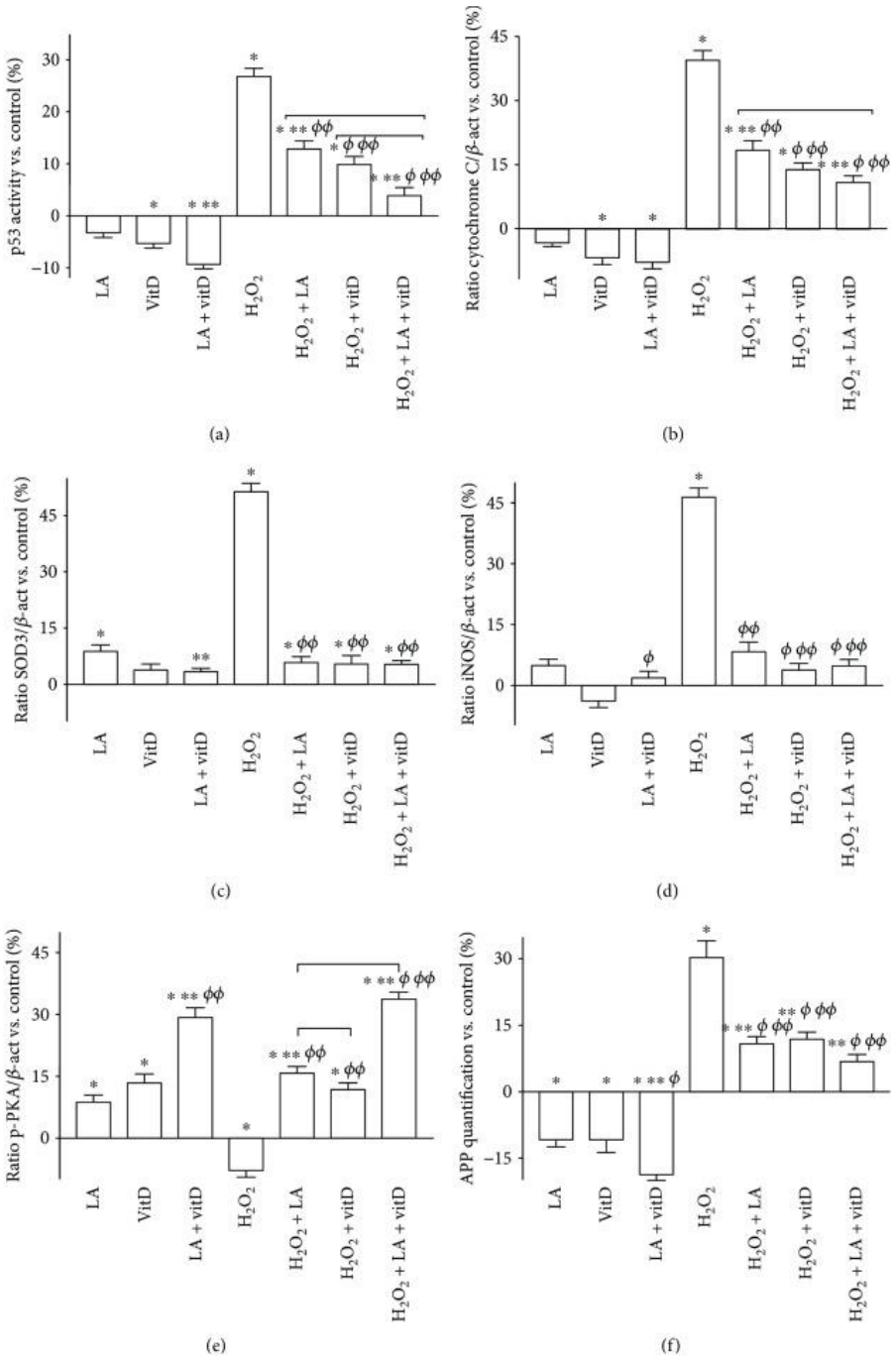


Fig. 24

Kinase activity and densitometric analysis of intracellular pathways involved under oxidative stress. (**a**) and (**f**) measurements of p53 and APP activities, respectively, measured by the ELISA test. (**b**–**e**) Densitometric analysis of cytochrome C, SOD3, iNOS, and p-PKA expressions obtained by analyzing Western blot on whole astrocyte lysates. LA=lipoic acid, vitD=vitamin D3, and LA+vitD=combination of lipoic acid and vitamin D3. Data are expressed as means ±SD (%) of five independent experiments normalized to control values (0% line) in ELISA experiments and normalized and verified on β-actin detection in densitometric analysis expressed as means ±SD (%). $^{*}p<0.05$ vs. control; $^{**}p<0.05$ vs. LA; $^{\phi}p<0.05$ vs. vitD; $^{\phi\phi}p<0.05$ vs. H_2O_2, bars: $p<0.05$ between treatments.

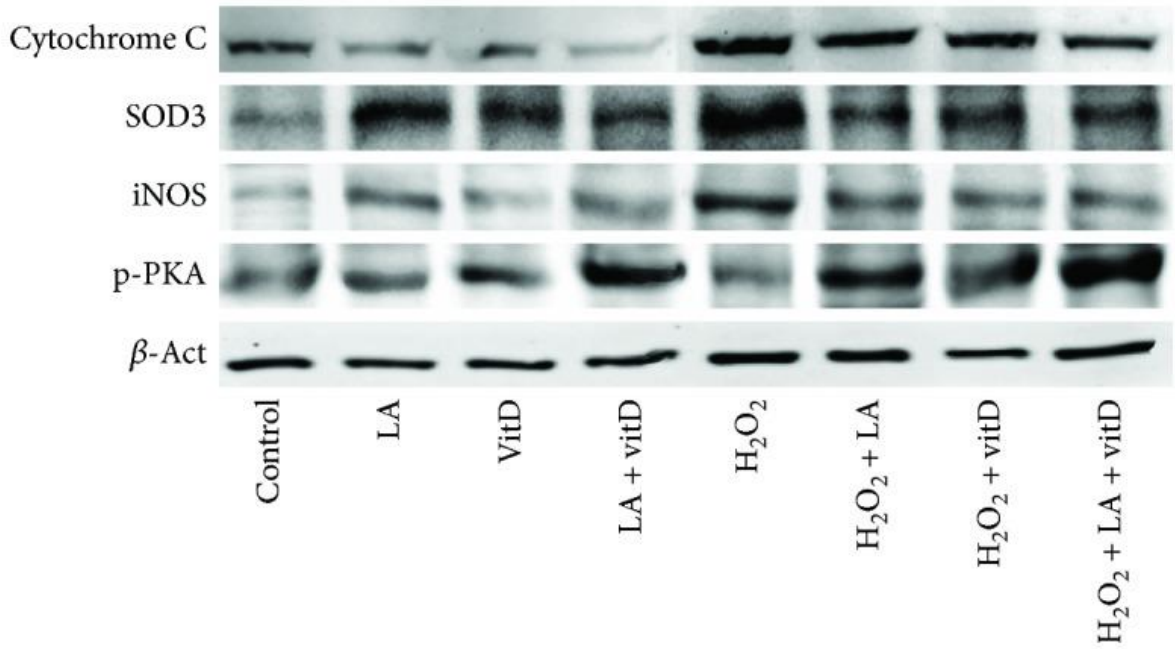


Fig. 25

Western blot of Cytochrome C, SOD3, iNOS, and p-PKA in astrocytes under oxidative stress. The images reported are an example of each protein of five independent experiments reproduced in triplicates. LA=lipoic acid, vitD=vitamin D3, and LA+vitD=combination of lipoic acid and vitamin D3.

The ERK/MAPK and PI3K-Akt pathways play a crucial role in the regulation of neuronal and brain survival. 50μM LA and 100nM vitD alone confirmed their ability to improve the viability of cells, activating ERK and Akt mediators, as reported in Fig. 26a and b. The combination of LA with vitD amplified kinase activation compared to the control and to single administration as well (< 0.05). Exposure to 200μM H_2O_2 significantly reduced ERK and Akt activities of about 6% and 7%, respectively, compared to the control; conversely, subsequent posttreatment with 50μM LA and 100nM vitD alone or combined reverted previously observed effects indicating the activation of survival pathways. In addition, the combination of LA with vitD showed a greater effect in PI3/Akt activity compared to ERK/MAPK, supporting the hypothesis that after the activation of Akt, all neuronal survival signaling was switched on.

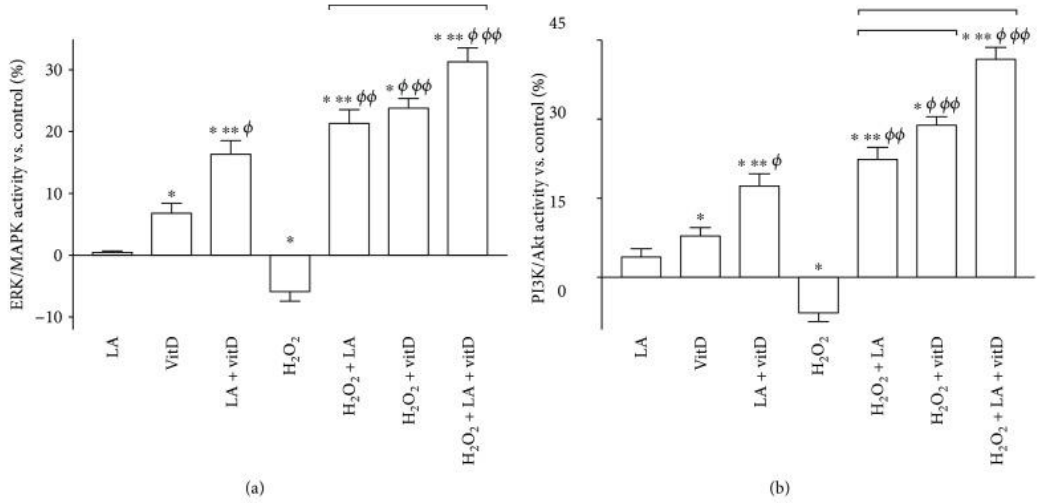


Fig. 26

Survival kinase activities measured in astrocytes under oxidative conditions. The quantification of p-ERK (**a**) and p-Akt (**b**) activities measured at 24h in astrocytes treated with vitD and LA alone and combined under pre-stimulation with H_2O_2 is reported. Data are reported as means ±SD (%) of five biological replicates normalized to control values (0% line). $^{*}p<0.05$ vs. control; $^{**}p<0.05$ vs. LA; $^{\phi}p<0.05$ vs. vitD; $^{\phi\phi}p<0.05$ vs. H_2O_2, bars: $p<0.05$ between treatments. LA=lipoic acid, vitD=vitamin D3, and LA+vitD=combination of lipoic acid and vitamin D3.

Evaluation of Lipoic Acid and Vitamin D3 Activities under Iron Accumulation

Iron can progressively be accumulated into the brain during normal ageing; in neurodegenerative disorders, it can be stored in abnormal accumulations. Since LA is a potent chelator of divalent metal ions and vitD is able to prevent the damage induced by the accumulation, additional experiments were performed on astrocytes to verify their protective ability in the brain. For this reason, cell viability and ROS production were evaluated in astrocytes after 6 days of stimulation, as reported in Fig. 27a and 27b. Exposure to 300μM Fe^{3+} significantly reduced cell viability of about 23% compared to the control ($p < 0.05$); conversely, following posttreatment for additional 6 days with 50μM LA and 100nM vitD alone, cell viability leads to control values, confirming their ability to chelate iron and repair the damage ($p>0.05$). In addition, the concomitant stimulation with LA and vitD was able to amplify the beneficial effect exerted by the single administration ($p<0.05$), supporting the idea of its possible helpful use during brain damage. To investigate the role of oxidation in the presence of iron accumulation, additional experiments on ROS production were performed. Exposure of astrocytes to 300μM Fe^{3+} for 6 days significantly increased the intracellular ROS production, as illustrated in Fig. 27b of about 60% compared to the control ($p<0.05$). Moreover, posttreatment for additional 6 days with 50μM LA and 100nM vitD alone significantly reduced ROS production (about 68% and 83%, respectively, vs. Fe^{3+}alone), and the concomitant administration of LA and vitD improved the reduction of the ROS level compared to 300μM Fe^{3+} of about 92%. These findings indicate the ability of the combination to counteract the oxidative condition caused by iron accumulation. Since the iron accumulation is an important cause of brain damage, it is essential to discover strategies to prevent it; thus, some experiments were carried out to assess the amount of iron which remains inside the cells. As reported in Fig. 27c, 300 μM Fe^{3+} increased the intracellular accumulation after 6 days of stimulation of about 69% compared to the control ($p<0.05$), indicating that 6 days with an excess of iron was a

sufficient condition to create damage in astrocytes. The post-stimulation for additional 6 days with 50μM LA and 100nM vitD alone significantly reduced iron accumulation (about 81% and 88%, respectively, vs. Fe^{3+}alone), and the concomitant administration of LA and vitD improved the prevention of accumulation compared to 300μM Fe^{3+} of about 95% and compared to the single administration (p<0.05). These findings indicate the ability of the combination to counteract the iron-dependent damage, preventing its accumulation.

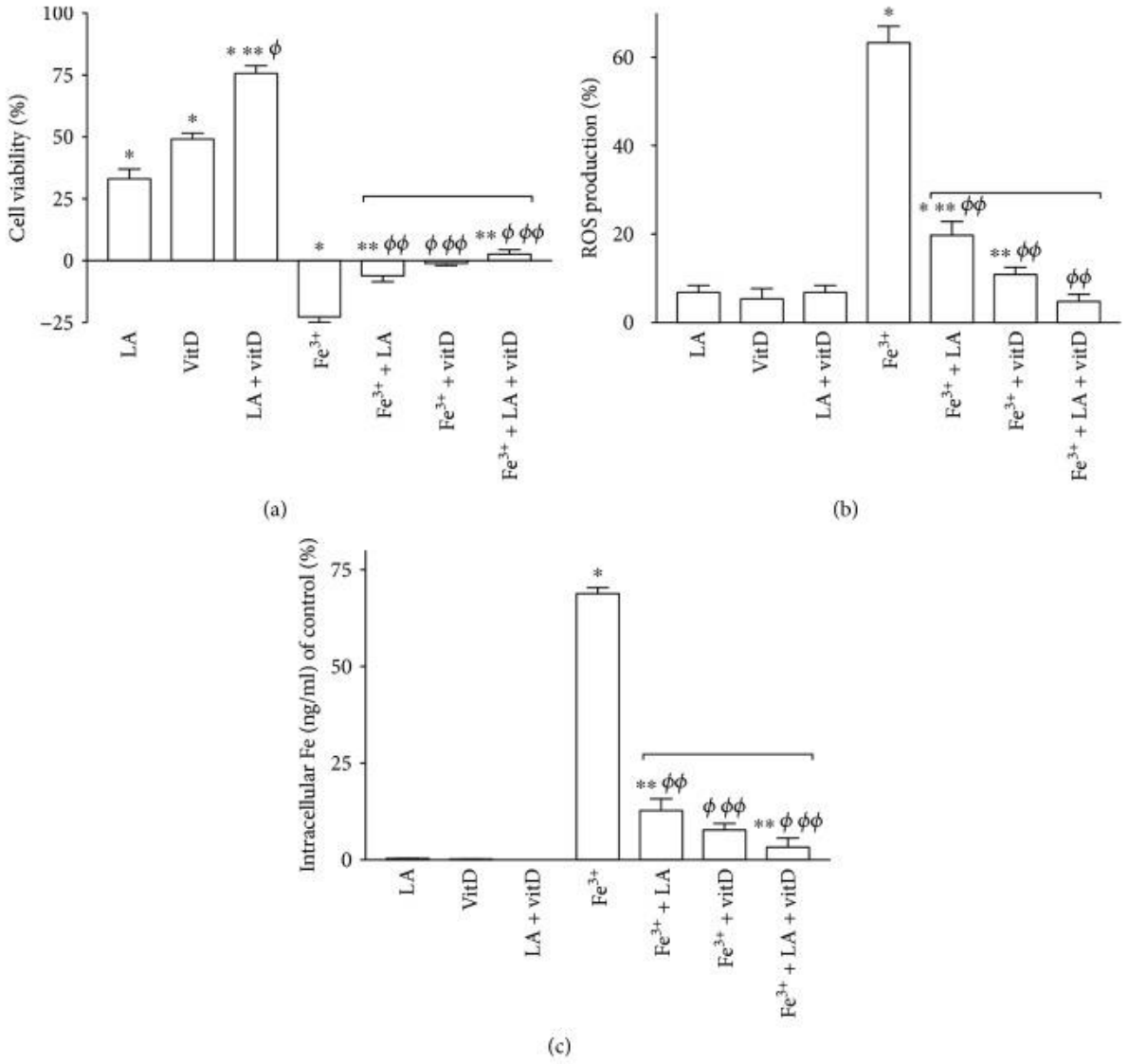


Fig. 27

Cell viability, ROS production, and iron quantification in astrocytes: (**a**) cell viability, (**b**) ROS production, and (**c**) the intracellular iron quantification measured on astrocytes pretreated with Fe^{3+} for 6 days and then with vitD and LA alone or combined for additional 6 days. LA=lipoic acid, vitD=vitamin D3, and LA+vitD=combination of lipoic acid and vitamin D3. Data are expressed as means ±SD (%) of five independent experiments normalized to control values (0% line). $^{*}p<0.05$ vs. control; $^{**}p<0.05$ vs. LA; $^{\phi}p<0.05$ vs. vitD; $^{\phi\phi}p<0.05$ vs. Fe^{3+}, bars: $p<0.05$ between treatments.

In order to confirm the protection exerted by LA and vitD, p53 activity was analyzed as well. As shown in Fig. 28a, 300μM Fe^{3+} administration for 6 days caused a significant increase in p53 activity (p<0.05) compared to the control, supporting previous observations about the viability of cells. The post-stimulation with 50μM LA and 100nM vitD alone for additional 6 days was able to reduce the p53 activation (p<0.05 vs. control) of about 84% and 88%, respectively, compared to 300μM Fe^{3+}. Finally, the combined effect of LA with vitD for additional 6 days after 300μM Fe^{3+} was able to amplify the reduction on p53 activity (p<0.05) compared to 300μM Fe^{3+} (increased about 93%) and to the single administration (p<0.05), supporting the beneficial effects previously observed. The production, accumulation, and aggregation of APP during neurodegeneration are influenced by a number of modulators, and among these is iron, so the level of APP under these conditions was also detected. As illustrated in Fig. 28b, exposing astrocytes to 300μM Fe^{3+} for 6 days significantly increased the APP level of about 43% compared to the control (p<0.05); posttreatment for additional 6 days with 50μM LA and 100 nM vitD significantly reduced the APP level (about 63% and 55%, respectively, vs. 300μM Fe^{3+} alone), and the concomitant administration of LA and vitD amplified the reduction compared to 300μM Fe^{3+}, 50μM LA, and 100nM vitD alone (p<0.05, about 82%, 53%, and 61%, respectively). These findings confirm the importance of the combination to prevent the iron-dependent damage in the brain. Cellular oxidative stress and antioxidant enzyme dysregulation are linked to age-related brain degeneration, so SOD3 expression was investigated in the same conditions previously reported. The stimulation for 6 days with 300μM Fe^{3+} caused a significant increase in SOD3 expression (p<0.05) compared to the control which was reduced by the successive stimulation for 6 days more with 50μM LA or 100nM vitD alone (p<0.05), indicating a positive effect of 50μM LA and 100nM vitD to maintain a correct balance of oxidant activity in astrocytes (Fig. 28c). In addition, the combined effect of LA with vitD added for 6 days after 300μM Fe^{3+} was able to significantly reduce the expression of

SOD3 (p<0.05) compared to both 300μM Fe^{3+}(about 78%) and 50μM LA and 100nM vitD alone (about 67% and 40%, respectively). These data allow to exclude the presence of oxidative damage and inflammatory cascade activation in the presence of iron accumulation, supporting the hypothesis that this combination could be used in human ageing. Finally, the ERK/MAPK activity was investigated in order to demonstrate that the combined stimulation with LA and vitD could prevent iron-dependent cell loss and damage, activating a rescue mechanism such as the survival pathways. Exposing astrocytes to 300μM Fe^{3+} for 6 days upregulated the ERK/MAPK activation as reported in Fig. 28d (p<0.05 vs. control), indicating a negative effect and suggesting that iron-dependent ROS generation activates this pathway; post-stimulation for additional 6 days with 50μM LA and 100nM vitD alone showed a significant decrease compared to 300μM Fe^{3+} (p<0.05), supporting the beneficial effects observed previously. In addition, the concomitant administration of LA and vitD made the observed attenuation of overexpression more pronounced compared to 300μM Fe^{3+} (p<0.05), confirming observation of a better survival of astrocytes after the damage.

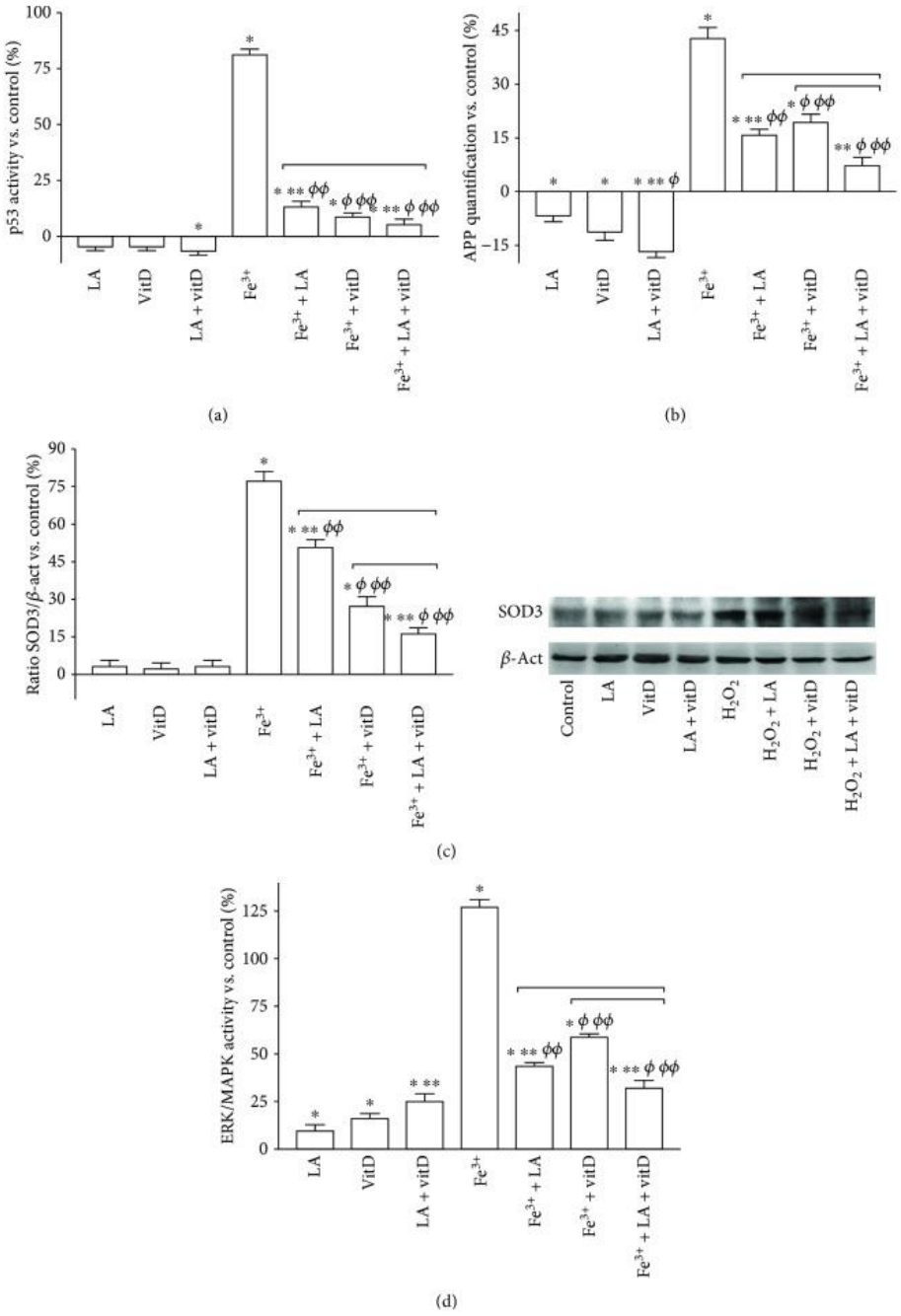


Fig. 28

Analysis of the main intracellular pathways analyzed by Western blot and the ELISA kit. In (**a**), (**b**), and (**d**), the measurements of p53, APP, and ERK/MAPK activities, respectively, are reported by the ELISA test. Data are expressed as means ±SD (%) of five independent experiments normalized to control values (0% line). (**c**) Densitometric analysis and Western blot of cytochrome SOD3 expression obtained in whole astrocyte lysates. Data are expressed as means ±SD (%) of five independent experiments normalized and verified on *β*-actin detection. LA=lipoic acid, vitD=vitamin D3, and LA+vitD=combination of lipoic acid and vitamin D3. $^{*}p<0.05$ vs. control; $^{**}p<0.05$ vs. LA; $^{\phi}p<0.05$ vs. vitD; $^{\phi\phi}p<0.05$ vs. Fe^{3+}, bars: $p<0.05$ between treatments.

5. Discussion

This research, in its different phases, demonstrated that vitD have effects in several tissues and when it has been combined with other substances it is able to influence their effectiveness. Indeed, as regards ovarian line of research, vitD is able to improve the effects of RES in ovarian cells, therefore showing a cooperative effect. In particular, in CHO-K1 RES cells combined with the vitD showed biphasic biological effects, that is, it developed the number of viable cells on the one hand and was able to reduce ROS production on the other. Moreover, the combination can modulate in a time-dependent manner, the levels of ERK/MAPK and Akt/PI3K. RES acts involving ERK and Akt pathways via attenuation of ROS generation since it is combined with vitD. These findings confirm the antioxidant effect of RES, which is mediated by SOD modulation, as shown by data collected in the *in vitro* experiments of this research. As regards the biphasic response observed, the early positive effect (found at 2 min) is followed by a decrease is present probably due to the rapid metabolism of RES and then the effect on cell viability show a significant rise that last as long as 3h. Indeed, it can be hypothesized that an activation of long-latency intracellular metabolic pathways has occurred. As a matter of fact, a biphasic RES response has also been observed in other studies [257, 258]. The observation that beneficial effects of RES on cultured ovarian cells are enhanced by the co-stimulation with vitD is novel and important and it underlines the existence of a proper regulation essential to sustain tissue homeostasis.

Moreover, it is noteworthy that cooperative effects exerted by combined RES and vitD have been made possible through the concurrent involvement of two types of receptor: ERβ and VDR. This finding assumes great relevance for the ovarian tropism, since it has been demonstrated that in the ovary, RES exhibits antiproliferative and androgen-lowering effects on theca-interstitial cells [259]. RES exerts a cytostatic, but not cytotoxic effect in granulosa cells, while inhibiting aromatization and VEGF expression [259]. In addition, RES may

increase the follicular reserve and extend the duration of ovarian life as an antiaging agent. RES studies in ovarian physiology are limited. RES was reported to exert estrogenic effects, increasing the uterine and ovarian weight [260, 261]. It is a phytoestrogen known to bind equally to estrogen receptors α and β [106, 260] and structurally similar to synthetic estrogens. The possibility of increasing the effectiveness of RES by associating vitD may be of clinical relevance in conditions linked to theca-interstitial cell hyperplasia, androgen excess and abnormal angiogenesis, such as PCOS, targeting most of the endocrine and metabolic underpinnings of PCOS. In PCOS, the typically enlarged ovaries are characterized by thecal and stromal hyperplasia [262]. This ovarian enlargement is associated with excessive ovarian androgen production and the disruption of menstrual cyclicity. Improvement of ovarian function, with restoration of ovulation and fertility, was observed with surgical reduction of ovarian size and/or partial destruction of ovarian tissues by procedures such as wedge resection and laparoscopic ovarian drilling [263, 264].

Another important information about RES is its bioavailability. The issue of bioavailability is determined by its rapid elimination and the fact that despite its highly effective absorption, the first hepatic step leaves little free RES. Indeed, only free RES can even bind to plasma proteins that could serve as a reservoir [93]. The *in vivo* phase of this research has shown that in ovarian tissue, RES exerts its effects in a cooperative manner with vitD. Specifically, in rat, RES in combination with vitD show a biphasic absorption rate not only in the ovary, but also in the heart, kidney and liver tissues, related to blood concentration. The combination is able to increase bioavailability and biodistribution and reduce ROS production confirmed by SOD activity. There is a modulation in a time-dependent manner of the levels of Cyclin D1 sustaining tissue homeostasis and a cooperative effect through the involvement of the ERβ receptor and VDR is shown. The transport into bloodstream of RES was nonlinear during time, suggesting metabolism to be rate-limiting with respect to bioavailability. After the oral dose,

the second peak of plasma level may be due to enteric recirculation of conjugated metabolites by reabsorption after intestinal hydrolysis. The execution of animal experiments is justified by the need of studying the rate of absorption of RES. After administration, RES undergoes a glucuronidation. The major form of RES transferred across the rat intestinal epithelium into the bloodstream is its glucuronide metabolite [265]. Therefore, an efficient carrier system should drive RES through the epithelial stratum to the bloodstream thus shortening its permeation time and metabolic turnover [92]. So, it was demonstrated that the effects observed in *in vivo* experiments could be related with the previously observed *in vitro* effects with RES plus vitD.

Another novelty in this study is the observation that the cooperative mechanism has also been demonstrated in the intestinal absorption phase. This is clearly stated in a new set of *in vivo* experiments where the intracellular activated cascade mechanism after absorption demonstrates the cooperative mechanism. It is important to note that until now it was assumed that the RES also acted as a VDR agonist, but primarily in anti-tumor mechanisms [112]. Due to its estrogenic action, RES appears to be an optimal candidate for use in gynecological diseases, especially in the treatment of hot flashes (HF) associated with menopause. Vasomotor symptoms, including the hot flush, are amongst the commonest symptoms of the menopause transition period. Hot flushes are a heat dissipation response characterized by flushing and sweating, probably triggered by a narrowing of the thermoneutral zone in the hypothalamus and an increased central secretion of noradrenaline. The neuroendocrine changes associated with a hot flush may have significance far beyond the immediate distress and discomfort experienced at the time [266]. Despite various therapeutic solutions for the treatment of HF have been proposed, the results obtained do not show evidence of effectiveness in the use of phytoestrogens [267]. Although there are no human studies regarding the effects of resveratrol on menopausal signs and symptoms, a recent trial demonstrated that resveratrol may enhance

mood and cognition in postmenopausal women [268]. Furthermore, vitamin D can protect against experimental serotonin depletion in rats [269] and a menopause-related decline in serotonin, a neurotransmitter with known effects on thermoregulation, may be a contributor to hot flashes [270-272]. In addition, estrogen increases the activity of the enzyme responsible for activating vitamin D [273] and so declining estrogen levels during the menopausal transition could lead to symptoms of vitamin D deficiency. Indeed, vitamin D supplementation can improve mood in non-menopausal populations [274-277]. Based on the totality of evidence from limited data, we hypothesized that higher levels of vitamin D would be associated with fewer menopause-related symptoms.

Indeed, RES is effective in reducing the number of vasomotor episodes and the intensity of HF, with the transition from moderate/severe to mild symptoms in 78.6% of patients. Resveratrol has the characteristics to be an alternative therapy in the treatment of HF in menopause [108]. The scientific community is looking for innovative strategies to implement the bioavailability of RES through drug delivery systems such as the use of nanoemulsion-based delivery systems [278] or through the ability to interact in a cooperative way with other molecules such as vitD [279].

However, the interplay between resveratrol and vitamin D must be further elucidated if the true potential of their clinical applications is to be revealed.

In conclusion, this part of study demonstrated for the first time a cooperative effect of RES and vitD on ovarian cell and tissue, mediated by main physiological intracellular mechanisms. Such results could be used as a fundamental data for the development of new therapies for gynecological conditions, such as menopause-related hot-flashes.

As regard the part of the study inherent the role of vitamin D3 in gastric disorders, vitD showed a gastroprotector effect.

Indeed, recent clinical investigations reveal that NSAID, widely used to alleviate inflammation, fever, and pain in clinical practice, have an equal risk to develop gastropathic and enteropathic damage [121] and then the relative gastric toxicity is a major consideration [119, 280]. NSAID induced gastroenteropathy has emerged as an important socioeconomic problem because there are no approved therapeutic strategies/interventions to prevent/treat NSAID-induced enteropathic damage [281].

NSAID can be divided into COX-2 selective inhibitors and non-selective COX inhibitors (COX1 and COX2 inhibitors) [282] which have a higher risk of ulcers in the gastrointestinal tract [283]. The most important non-selective COX inhibitors include aspirin, diclofenac, indomethacin, piroxicam, naproxen and ketoprofen [284]. Diclofenac is extensively used worldwide in several formulations due to its analgesic, anti-inflammatory and antipyretic activities [126]. Nevertheless, several side effects are consequences of their administration, especially with chronic use, including cardiovascular problems, renal function disorders, hepatic injuries, alterations in gastrointestinal mucosal integrity [122, 285] and electrolyte disturbances [286, 287]. A classical gastrointestinal consequence is a peptic ulcer which is a multifaceted process that includes the generation of ROS, inducing inflammatory molecules of the COX-2, cytosolic phospholipases A2, leukotriene B4, 5-lipoxygenase, PGE2, TNF-alfa, IL-1beta, and IL-6 production [288, 289], increasing LPO, xanthine oxidase, and DNA damage [290, 291] and the inhibition of antioxidant enzymes including glutathione peroxidase, glutathione reductase, glutathione S-transferase, catalase, superoxide dismutase, and heme oxygenase-1 as well as glutathione (GSH)/oxidized-GSH ratio *in vivo* [292]. The toxic effects of diclofenac on stomach mitochondria is well known [293]. Human gastric epithelial cells treated with diclofenac exhibited significant O_2 production. Ultimately these ROS collectively affected the viability of the cells by striking at the basis of cellular powerhouses or mitochondria via mitochondrial depolarization [293]. Therefore, ROS may also cause oxidative

damage to biological macromolecules and react with proteins, lipids, and mitochondrial DNA, leading to cell death by affecting various apoptotic pathways in the gastrointestinal tract [290-292]. Furthermore, NSAID also down-regulates angiogenesis, inhibits mucosal cellular restitution and promotes extracellular matrix degradation [294]. Thus, it is important to regulate the levels of ROS in the gastrointestinal tract. In addition, another important proinflammatory effect of HCl, consistent with the existing literature, is its action on synthesis [295].

Proton pump inhibitors (PPI) are currently used for the treatment and prevention of NSAID-induced gastroduodenal lesions [296]. The efficacy of PPI depends on their ability to inhibit gastric acid secretion [297]; however, in recent years, it has been proposed that acid-independent mechanisms may also contribute to the antiulcer actions of PPI [118]. Additionally, there are some reports that showing how PPI may produce gastric glands toxicity [298] and this is associated with an appreciable amount of systemic adverse effects in humans [299, 300]. Thus, the identification of more effective and safer therapies for the treatment of NSAID-induced-gastroenteropathic lesions remains an urgent priority. For this purpose, researchers are still investigating new molecules to find an ideal agent with a better safety profile. In the current study, the effects of a new compound named Aq on an *in vitro* experimental model of gastric cells have been observed. This work demonstrates that Aq can exert protective effects on gastric cells in terms of viability, ROS and NO production, thanks to vitD, which is able to improve the beneficial effects induced by alginates, supporting existing data about their mechanism of gastroprotection [45, 301]. Moreover, further *in vitro* studies demonstrate that VDR activation enhances intracellular junctions and promotes mucosal wound repair through vitD activity [302]. There is evidence that oxidative stress plays an important role in the pathogenesis of gastric injury [45]. It is noteworthy that experimental evidence shows how ROS production or superoxide are induced both by cellular exposure to an alkaline environment and/or by acidic injury [303]. It is well known that vitD may prevent

cell death inhibiting superoxide anion generation, maintaining mitochondria function and cell viability, and activating survival kinases. Our results show that Aq alone, during H_2O_2 or HCl exposure is able to improve cell viability compared to control and to other commercial products such as Neo, composed of different natural extracts. In addition, Aq significantly reduced ROS production and decreased cell viability loss when added after the injury, suggesting that cell damage and cytotoxicity can be prevented. These results suggest that Aq may exert a better gastroprotective effect through an antioxidant pathway, inhibiting apoptosis and activating survival kinases. Such effect was stronger in preventing epithelial damage than what observed using other gastroprotective agents such as Neo. Since the gastroprotection is more important during the use of Diclofenac, further experiments were carried out to validate the observed results. The results obtained from this new series of experiments have allowed us to significantly confirm beneficial effects of Aq to prevent injury caused by diclofenac under both acidic and hyperacidic conditions. Indeed, the gastroprotection exerted by Aq was similar to effect of pantoprazole indicating its effectiveness on cell viability, on reducing ROS production and on apoptotic mechanism, increasing the activation of survival kinases and cell proliferation.

As regards the research in cardiovascular field, the study has investigated the cooperative effect of Q10, L-arginine and vitD on cardiac and endothelial cells. The major results of this work indicate the importance of QLD on vasodilation through NO production and this effect is shown to be better than treatments with the individual substances. NO synthesis is involved in a number of cardiovascular diseases such as peripheral vascular disease, congestive heart failure (HF), and cerebrovascular events. An impaired NO status may cause an overproduction of ROS in the vasculature, including superoxide anion, hydrogen peroxide and derivative hydroxyl radical and hydroxide, lipid peroxides and derivatives peroxyl radicals. Q10 suppressed the generation of ROS, which subsequently attenuated the peroxidation and increased the

bioavailability of NO [168]. The results of this study have demonstrated for the first time that QLD is able to activate eNOS, one of three isoenzymes transforming L-arginine into L-citrulline and NO, in cardiac and endothelial cells. This effect is time-dependent and is accompanied by a significant increase in the level of phosphorylation of intracellular kinases. Akt kinase activates eNOS by directly phosphorylating the enzyme at Ser-1179 [304]. Akt itself is phosphorylated and activated by PI3 kinase, which in turn is activated by various agonists [305]. Also MAP kinases, important mediators of signal transduction from the cell surface to the nucleus, have been found to modulate eNOS activation [306]. Indeed, in porcine aortic endothelial cells ERK, Akt pathways have recently been implicated in the effects of various agents on NO production [307, 230]. Data from this study clearly demonstrate that the administration of QLD induced the highest production of NO and acutely increased the phosphorylation of eNOS, Akt and ERK, which are known to be involved in the intracellular signaling leading to NO production [308, 309, 252]. The relationship between NO and cellular function is complex because NO is cytotoxic at high concentration and has a protective effect at low concentration [168]. The importance of Q10, L-arginine and vitD alone on the cardiovascular system is well known. Q10 is an integral component of the mitochondrial respiratory chain for ATP production as well as an antioxidant agent, thus it could assist in improving myocardial function in HF patients [310.]. In addition, a meta-analysis on coenzyme Q10 randomized clinical trials showed that it improves the outcomes of HF patients. L-arginine improves the endothelial function modulating the nitric oxide bioactivity, increasing intracellular uptake, and acting directly with antioxidant activity [153]. In epidemiological studies, vitD deficiency has been consistently associated with an increased risk for cardiovascular disease and hypertension. Disruption of vitamin D signaling in animal models promotes hypertension, cardiac hypertrophy, and atherosclerosis. This evidence has led to prospective randomized trials on vitD supplementation in individuals at risk for cardiovascular

disease [311]. Studies carried out on VDR knockout and 1-α hydroxylase-deficient mouse models showed elevated arterial blood pressure [52, 53].

Other studies carried out on spontaneous hypertensive rats have indicated that vitamin D3 administration suppresses endothelium-dependent contraction of aorta in these models [312, 313]. Vitamin D3 has antihypertrophic, anti-inflammatory and antiproliferative properties and may reduce cardiac hypertrophy in spontaneous hypertensive rats [182]. Furthermore, vitamin D3 has a direct effect on endothelial and smooth muscle cells and may decrease coagulation and increase re-epithelialization and fibrinolysis [182].

In the animal model as well as in cell lines we have investigated the effects of antioxidant therapy with Q10, L-arginine and vitD to improve endothelial and cardiac function by means of a VDR-mediated reduction in ROS production. This finding is supported by the fact that VDR is expressed throughout the myocardium on various cell types including cardiomyocytes and endothelial cells [252, 314]. QLD was able to maintain the ability of vitD to induce its beneficial effects on cardiovascular system whereas vitD supports the effects of Q10 during vasodilation as a consequence on L-arginine effect. In addition the positive effects exerted by QLD were demonstrated thanks to a reduction of p53 activation and ROS production on both cell types. Indeed, the level of ROS observed at 300sec was not enough to cause tissue damage and always remained under physiological range. Since the translational approach is very important to confirm the mechanisms observed *in vitro*, some experiments were performed in an *in vivo* model. In rats the beneficial effects on vasodilation of Q10, L-arginine and vitD added together have been confirmed and compared to these substances alone. In addition the effects of QLD on vasodilation were demonstrated through the increase of NO production.

All these data confirm the importance of Q10, L-arginine and vitD during vasodilation and demonstrate for the first time a possible cooperative use in amplifying the positive effect of each of them. Using QLD to counteract increased free radical production is a potential method

to reduce myocardial injury in patients and to reduce aging effects on the heart. This is relevant in the light of the possible use of QLD supplementation as a potential tool to treat or prevent cardiovascular diseases.

In addition, as regards the research part focused on vitamin D3 in brain ageing, several studies were conducted which shows the association between vitamin D and brain health and the impact of vitamin D deficiency on the brain. In 2016, Miller BJ et al. conducted a human study which shows that vitamin D increases plasma Aβ in older adults, indicative of decreased brain Aβ [315]. Annweiler C et al. conducted a study which demonstrates the neurosteroid actions of vitamin D in the regulation of calcium homeostasis, β-amyloid deposition, antioxidant and anti-inflammatory properties. It also discusses neuroprotection action of vitamin D against neurodegenerative process associated with Alzheimer's disease and cognition [316]. Furthermore, a study conducted by Annweiler et al. shows that hypovitaminosis D is commonly seen in adults and 65-year-old patients. They show sign of dementia and cognition impairment [317]. Also in early life vitamin D plays a vital role in neuronal development. Some studies conducted recently shows the effect of vitamin D on early life brain development. A study conducted by Yates et al. shows that deficiency of vitamin D in maternal and offspring causes some disabilities in early life including learning and memory problems and grooming behaviours. There was also some evidence of increased lateral ventricle volume and altered neural expression of genes involved in dopamine and glucocorticoid-related pathways suggesting autism and schizophrenic-like disorders [318].

Di Somma et al., shows optimal levels of vitamin D in the bloodstream are necessary to preserve the neurological development and protect the adult brain [319]. Balanced dietary intake is a well-established lifestyle factor in maintaining cognition during ageing. A recent study shows that vitamin D helps in keeping cognitive function in older adults [320].

Furthermore, recent studies hypothesize also the use of antioxidant molecules combined with symptomatic drugs to reduce oxidative stress, thus improving cognitive functions during ageing and in age-related diseases [321]. It is well known that the brain is highly susceptible to oxidative damage due to high concentrations of polyunsaturated fatty acids and transition of metals that are involved in the generation of the hydroxyl radical [322, 323]. In an adult brain, astrocytes are responsible in maintaining neuronal and synaptic functions [324] and the oxidative stress plays a key role in astrocyte loss, mainly due to the highly active mitochondria metabolism [323].

According to literature, the brain has a poor catalytic activity and has low levels of protective antioxidant enzymes; for this reason, in this part of research, the efficacy of LA and vitD in oxidative mechanisms involved in the ageing process was tested. Indeed, it was demonstrated that the combination of LA and vitD exerts a synergistic and cooperative effect on astrocyte activity indicating a possible new strategy to slow down ageing. The combination of LA and vitD is able to perform beneficial effects directly on viability of astrocytes, since these substances are able to cross the blood-brain barrier. Moreover, the combination of LA and vitD increases the absorption rate of the two substances compared to the control during time and to their single administration starting from 60min, supporting the cooperative effects of LA and vitD also during permeability. A recent work by Farr et al. [325] demonstrated that lipoic acid improves memory and reverses indices of oxidative stress in old mice but decreases the lifespan. This feature has not been studied in this research. This could be a limitation of this research, which could be addressed in the future. However, vitD, with its antioxidant action, allows to use lower doses of lipoic acid, and this could improve the safety of the molecule. Another limitation of this study could be the use of HUVEC rather than a brain-derived endothelial cell. However, some studies indicate that this experimental model can present structural and functional equivalency with *in vivo* BBB while still using HUVEC [326].

Oxidative stress has a key role in ageing and for this reason additional experiments on ROS production were performed. Treatment with LA and vitD improves the reduction of the ROS level in oxidative conditions. The alteration of the formation of a proton gradient across the inner mitochondrial membrane is considered to be one of the key indicators of cellular viability; the combination of LA and vitD suppressed the effect of H_2O_2-induced mitochondrial dissipation. Thus, this study demonstrates that combined LA and vitD can attenuate the H_2O_2-induced apoptosis through the mitochondrial-mediated pathways. Furthermore, the specifics to be used in combination have been determined evaluating the intracellular pathways activated by LA and vitD during oxidative condition. Our findings suggest that there is a reduction on p53 activity after the stimulation with LA and vitD that it is correlated to survival-favored conditions. Moreover, the involvement of the caspase pathway has been examined analyzing the expression of cytochrome C. The combination of LA and vitD is able to reduce cytochrome C expression during oxidative damage, supporting mitochondrial integrity. Besides, it has been demonstrated that stimulation with LA and vitD under oxidative condition can reduce cell death and increase cell survival, by the activation of ERK and Akt mediators. Even the major pathways (SOD3 and iNOS) involved in oxidative stress were inhibited by administration of LA and vitD, indicating better cell survival.

Since neuroinflammation is a common cause of brain ageing, PKA, a key anti-inflammatory marker, was also investigated under the same conditions. The cAMP-dependent PKA signaling is one of the best-characterized signal transduction mechanisms in the central nervous system [327] and plays a diverse role in neuronal functions, such as regulating synaptic plasticity, learning, and memory [328]. PKA is a key phosphorylating enzyme, which triggers a wide variety of physiological involvement in cell survival, synaptic plasticity, and activation or repression of gene expression [327]. The PKA role in inflammation consists in its involvement in the cross talk between different signaling mechanisms such as the inactivation of

phospholipase Cβ; the phosphorylation of inositol 1,4,5-tris phosphate receptors, thereby modulating Ca^{2+} influx; and the phosphorylation of calcium-calmodulin kinase. In addition, cAMP stimulates mitogen-activated protein kinase activity in cultured neurons [327]. In addition, some studies by Salinthone et al. demonstrated that cAMP/PKA inhibits NF-κB function by slowing down its translocation into the nucleus and inhibits NF-κB activity [329]. The expression of PKA observed in astrocytes showed a significant increase in the presence of LA and vitD, and their combination amplified this effect, supporting the hypothesis of an anti-inflammatory activity of combined LA and vitD. The combination of LA and vitD added after H_2O_2 had a similar effect on PKA expression. This finding allows to exclude the involvement of inflammation cascade during treatment with LA and vitD combined.

Neuroinflammation and iron accumulation are hallmarks of a variety of adult neurodegenerative diseases [330], and particularly, iron is recognized to influence the biochemistry of proteins involved in neurodegeneration (for instance, APP), as well as those playing a crucial role in neuronal development and efficiency [331]. Moreover, it was demonstrated that the exposure to Fe^{3+} significantly reduced cell viability and increased ROS production and the combination of LA and vitD can prevent Fe^{3+}-dependent oxidative injury in astrocytes. It is well known that LA is a potent chelator of divalent metal ions and vitD is able to prevent the damage induced by the iron accumulation [242, 332]. However, processes involved in age-related and disease-related accumulations of iron and iron-induced inflammation in specific brain regions and cells are poorly understood. Post-treatment with LA and vitD was able to restore the damage caused by iron accumulation. Furthermore, it has been demonstrated that LA and vitD alone or together were able to prevent intracellular iron accumulation damage by their ability to prevent iron deposition promoting its elimination (in fact, the treatment was added after 6 days of iron administration). Besides, even if the presence of iron caused a harmful condition for the cells, the response to treatment significantly activated

the cellular survival mechanisms (ERK) by switching off apoptosis mechanisms (p53). Indeed, these effects were found to be mediated by the inhibiting effect of LA on oxidative (SOD3) and inflammatory (APP) systems.

In conclusion, this study demonstrates for the first time that the combination of LA and vitD is an effective treatment for astrocytes under oxidative stress conditions, indicating the possibility of developing new strategies to treat brain ageing in all stages. Besides, the combined treatment with LA and vitD improved the negative effects of pre-neurodegenerative conditions, so there are the preludes to develop a new formulation to slow down brain ageing and neurodegenerative diseases, like Alzheimer and Parkinson diseases, such as a new food supplement Cebral®.

Vitamin D, has long been known to help build strong bones by increasing the body's absorption of calcium and phosphorous. But beginning in 2000, research into vitamin D's role in other health conditions began to expand rapidly especially due to the presence of its receptor (VDR) in many tissues and organs. The role of vitamin D in calcium metabolism has been extensively studied and there is strong support for vitamin D's role in bone health. However, it is showing surprising effects distributed throughout the body and the study of the extraskeletal role of vitamin D has become a very promising line of research in recent years. This thesis fits perfectly into the category of research aimed at developing new therapeutic applications that can be foreseen for this precious molecule.

6. Future Perspective

Since vitamin D deficiency is usually associated with several human disease, further studies should be performed in order to verify if the correct dosage and use of vitamin D supplementation can improve the quality of life of subjects. In this context, many researchers proposed to use vitamin D3 and/or its derivatives at low dosage combined with other substances, such as natural compounds to potentiate their beneficial effects and not the toxicity one. For instance, plant polyphenols, e.g., carnosic acid (CA) from rosemary and silibinin from milk thistle, markedly potentiate the differentiation-inducing effects of low concentrations of Vitamin D and its derivatives on AML cell lines and patient-derived leukemic blasts [333]. Indeed, in recent years there is a growing interest in the study of natural substances to support the health of the body; the use of natural substances it is mainly used for the treatment of phlogistic states, but also in other pathologies such as senile dementia, peptic ulcer and gastrointestinal disorders, prostatic hypertrophy, memory loss, beneficial effects can be observed. In addition, these substances are able to strengthen the immune system and reduce plasma cholesterol levels. [334]. For example, studies have shown that diet-ingested polyphenols are associated with reduce risk in some chronic diseases including cardiovascular disease, neurodegenerative disorders, diabetes, cancer and osteoarthritis [335]. Another interesting example regards curcumin which is the active substance contained in turmeric, well known for its antioxidant action, anti-inflammatory and modulatory effect on the main enzymes involved in the regulation of the cellular cycle [336]. The protective effects are expressed on different tissues and include cardiovascular protection, hepatoprotection and protection from gastric ulcers [337]. Thanks to experimental studies, the food industry has also recently focused its attention on plants and fruits with a high polyphenol content and is introducing many functional foods and dietary supplements onto the market. [338]. Polyphenols have antioxidant and anti-inflammatory capacities, that are able to protect cells from oxidative stress and to keep

cholesterol levels within physiological limits. Moreover, they also have antibacterial, antipruritic, antiparasitic and cytotoxic properties.

For this reason, the study of an alternative strategy for the development of a new vitamin D-based therapy that passes through the enhancement of its activity by associating other compounds seems to be particularly promising. The combination of these agents of different molecular classes has led in various experimental systems and clinical protocols to greater efficacy than the compounds administered individually. Some compounds of plant origin and antioxidants and also some compounds that do not belong to these groups, but which have potential as co-inductors of vitamin D3, will be studied in the near future in order to improve the knowledge of this vitamin and its combined effects in different conditions for develop some possible new therapeutic strategies for humans.

7. References

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