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"Protective effect of dietary Conjugated Linoleic Acid against AlCl3-induced neurodegeneration: focus on the molecular mechanisms underlying the down-regulation of hallmarks of Alzheimer's disease"

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Protective effect of dietary CLA against AlCl3-induced neurodegeneration: focus on the molecular mechanisms underlying the downregulation of hallmarks of Alzheimer's disease

Abstract

The nuclear erythroid-related factor 2 (Nrf2) stands as a pivotal redox-sensitive transcription factor, serving as the master regulator of oxidoreductive and immune homeostasis within cells. In the cytoplasm, Nrf2 forms an association with the inhibitory protein Kelch-like ECH-associated Protein 1 (Keap1). Under conditions of mild oxidative stress, specific cysteine residues in Keap1 undergo alterations that allow newly synthesized Nrf2 to activate the transcription of over 200 target genes implicated in the modulation of key biochemical pathways: mitochondrial function, proteostasis, oxidative and immune homeostasis. The activation of a compensatory mechanism mediated by the Nrf2 pathway may be elicited by its direct modulation by dietary molecules like Conjugated linoleic acid (CLA) or by increasing levels of short-chain fatty acids (SCFAs) following polyphenols, microbial-accessible carbohydrates metabolism by intestinal microbiota.

Herein we demonstrate, for the first time, the modulatory ability of CLA on hallmarks (e.g. inflammation, oxidative stress, glucose dysmetabolism) in an animal model of chemically induced AD.

Thirty-five male BalbC mice were divided into 5 groups: two $AlCl_3$-intoxicated groups were treated for 5 weeks with low (L) or high (H) Al doses (8 or 100 mg/kg/day in drinking water, respectively; L or H). Two groups of animals, orally supplemented with CLA (600 mg/kg bw/day) for 7 weeks (2 preliminary weeks plus the 5-week treatment with $AlCl_3$; CLA + L, CLA + H) were used to investigate its protective effect, while untreated mice were used as control (Cntr). We provide evidence that mitochondrial dysfunction, Nrf2 alteration, inflammation and

Acetylcholinesterase (AChE) hyperactivation can occur even from low exposure. Interestingly, animal pre-treatment with an allometric CLA dose led to significant downregulation of the toxic effects elicited by low or high $AlCl_3$ dose, likely through the activation of an adaptive response.

Moreover, we provide data indicating that animal pre-treatment with CLA markedly reduced hyperactivation of compensatory mechanisms (Nrf2 and unfolded protein response) and maladaptive response (inflammation/apoptosis) In the brain cortex likely via a NOX-mediated activation of an adaptive response.

In conclusion, presented data highlight the antioxidant and anti-inflammatory effect of CLA, given its ability to modulate Nrf2 activation, representing an intriguing suitable candidate for the treatment of multifactorial disease.

1. Introduction

Ageing is an irreversible and inevitable process, associated with physical decline and alteration of molecular and cellular processes, representing by far the most impactful risk factor for many neurodegenerative diseases. Various physiological and molecular changes occur in the body during ageing, and these changes can have a significant effect on tissues composed of postmitotic cells, such as the brain.

Ageing research over time has identified more and more characteristics affecting the ageing body. The first hallmarks were identified in non-mammalian models, but during the last decade, they were confirmed in mammalian models like mouse or non-human primates, and other new ones were also discovered. These hallmarks are generally classified according to different criteria as independent processes, but they are strictly connected since they can manifest as

simultaneous or consecutive events [1]. In particular, in 2023 López-Otín reported 12 typical hallmarks of ageing [1] (**Table 1**).

Ageing Hallmark	Reference	n
Mitochondrial dysfunction	Reid Thompson et al, Chee et al,	[4] [5]
Loss of Proteostasis	Della Bella et al,	[2]
Dysbiosis	Depommier et al,	[3]
Chronic inflammation	Ridker et al,	[4]
Disabled Macro-autophagy	Yoshino et al, Brakedal et al.	[5] [6]
Altered intracellular communication	Choi et al,	[7]
Deregulated nutrient-sensing	Spadaro et al,	[8]
Cellular senescence	Justice e al, Hickson et al.	[9] [10]
Stem Cells exhaustation	Wang et al, Bhatt et al,	[11] [16]
Genomic Instability	Gordon et al.	[12]
Telomer attrition	Shim et al.	[13]
Epigenetic Alterations	Demidenko et al. Fahy et al.	[14] [15]

Table 1. Ageing hallmarks.

The misfunction of the cell processes such as redox state regulation or loss of proteostasis determines both in neurodegenerative diseases- in a stronger manner - and ageing brains, protein abnormalities and inclusion bodies. The occurrence of defective protein homeostasis is a typical element of overlap between ageing and neurodegenerative diseases or a consequence of events that contribute to brain ageing or a continuum with neurodegeneration.

Neurodegenerative diseases include a heterogeneous group of disorders characterized by the progressive deterioration of the structure and function of the central or peripheral nervous system.

Over the past 30 years, the number of patients affected by neurodegenerative diseases has significantly increased, with a percentage of almost 15% [16] representing the prominent cause of physical and cognitive impairment nowadays. The rising number of people affected by these disorders is related to the rising of the elderly population, determined by the elongation of the human lifespan. The direct consequence of this phenomenon is the growing difficulties related to the management of patients by their caregivers and the healthcare system, but particularly a significant challenge will be ensuring widespread accessibility to neurological care.

The concerted action of some physio-pathological hallmarks like mitochondrial dysfunction, loss of protein homeostasis (proteostasis), altered intracellular communication and dysbiosis, is associated with the alteration of the cellular/organism oxidoreductive (redox) homeostasis that may lead to the decline of several physiological functions and the induction of oxidative distress. In particular, increased yield of oxidant species or antioxidant depletion may harm the structure or functionality of various cellular molecules [17] leading to organ dysfunction, often representing the cause of the insurgence of a variety of chronic and age-related human diseases, like Parkinson's [18] and Alzheimer's disease [19].

Alzheimer's disease (AD), a type of dementia, is a condition characterized by a specific pattern of cognitive and functional decline related to aging, which is also associated with a distinct neuropathology [20].

Today, Alzheimer's disease has become the most prevalent type of neurodegenerative dementia in the United States, disproportionately affecting minority populations. The disease process involves alterations in the cleavage of the amyloid precursor protein (APP) and the production of the APP fragment beta-amyloid (Aβ). Additionally, the aggregation of hyperphosphorylated tau protein contributes to a decrease in synaptic strength, loss of synapses, and

neurodegeneration. The disease process also includes metabolic, vascular, and inflammatory changes, as well as coinciding pathologies. While symptomatic treatment can provide a modest, clinically observable improvement in cognition, there is a desperate need for therapies that can modify the course of the disease [20].

Reactive oxygen or nitrogen species (ROS, RNS) are generally considered responsible for the onset of oxidative stress associated with numerous human pathologies. By contrast, ROS also play a central role in modulating physiological processes [17]. For example, the oxygen peroxide produced by neutrophils during the oxidative burst is fundamental to protect cells against the attack by pathogens, otherwise, in low/moderate concentrations, it is involved in the regulation of the antioxidant response [21, 22]. It is, however, noteworthy that low levels of ROS (or RNS or natural electrophilic compounds) target Keap1 (eustress) and induce cellular response and tolerance to oxidative stress.

The brain, likely owing to its high oxygen requirements, is highly susceptible to peroxidation compared with other organs, for this reason, the preservation of redox homeostasis has been indicated as presenting a fundamental target treatment or the prevention of neurodegenerative diseases [23]. Consequentially, there is a growing research interest in the discovery of dietary modulators exhibiting the ability to prevent/mitigate oxidative damage caused by alteration of brain redox homeostasis.

Because of their antioxidant effects, the interest in natural bioactive molecules has received significant attention, being the subject of over a hundred thousand published papers. This widespread interest has led to the pervasive use of antioxidants as a solution to promote and maintain optimal health [24-26]. However, it should be underlined that most of the results attributing health benefits to antioxidants have been obtained on *in vitro* experimental models, which, not

reflecting the complex physiological mechanisms of the human body, cannot be directly translated to humans [27, 28]. Antioxidant molecules do not exhibit a unique mechanism of action and the existing differences represent additional factor that negatively influences the transferability of experimental data from *in vitro* to in vivo/clinical models.

Generally speaking, antioxidant activity can arise from 2 different biochemical mechanisms: the "direct" neutralization of pro-oxidants (scavenging capacity) or the increased efficacy of the endogenous Nrf2-activated defences (phase 2 enzymes).

1.1 The “free radical scavenger” and “antioxidant” syllogism

Extensive epidemiological data suggests that dietary antioxidants contribute to disease prevention, and in silico studies indicate that many natural antioxidants and phytochemicals may neutralize free radicals in controlled laboratory environments [29, 30]. Even though, the kinetics of these reactions suggest that radical scavenging may not be considered an efficient antioxidant mechanism defense. Despite this, a paradigm shift has occurred in recent decades, introducing another crucial aspect of nutrition's impact on health where many fruits and vegetables harboring phytochemicals should be capable of reducing the risk of diseases [31].

In particular, even today numerous food molecules, such as phytochemicals, have been demonstrated to exhibit the ability to scavenge free radicals in silico and in vitro experimental models. In these experiments, generally both the concentrations of pro-oxidants and antioxidants are much higher than those observable in physio-pathological conditions.

Moreover, it must be taken into account that the rate of an antioxidant-oxidant reaction is determined by the equation **Rate=**

k[A][B] where **k** is a second-order rate constant, **[A]** is the concentration of the antioxidant, and **[B]** is the concentration of the reactive species. Considering, for example, the hydroxyl radical, that is an extremely reactive radical reacting with nearly all molecules present in the cell, with a rate constant similar to the rate of diffusion limitation [32].

$$HO^{\bullet} + AH \rightarrow A^{\bullet} + H_2O \qquad > 10^9\ M^{-1}s^{-1}$$

The same happens with the alkoxyl radicals, produced by decomposition of lipid hydroperoxides, occurring mainly in cellular membranes [33]. Then, only thinking that any scavenger from fruits or vegetable could exert any protective activity reacting with these two radicals is meaningless. Still, the scientific literature is filled with the latest discover about powerful diet-derived scavenger molecules [34, 35]. In light of this, it is extremely clear that in vitro systems used to test “antioxidant molecules” is far from be comparable with what happens in vivo.

The non-translatability of in vitro/in silico data into in vivo/clinical models is much more difficult to hypothesize since phytochemicals, similarly to many other food molecules with antioxidant activity, are subjected to digestion and metabolic processing by the intestinal microbiota. For example, studies have shown that many flavonoids are effective antioxidants in a wide range of chemical oxidation systems, being able to scavenge peroxyl radicals, alkyl peroxyl radicals, superoxide hydroxyl radicals in aqueous and organic environments [36]. Nevertheless, if we consider tea flavonoids, often associated to a wide range of biological activities in cell models, actually have a very poor bio-availability/bio-accessibility as well as low absorption in rats [37] and humans [38].

1.2 The endogenous antioxidant activity

Although the antioxidant activity of food molecules is generally associated with the ability to neutralize pro-oxidant molecules, this mechanism, as briefly mentioned previously, does not involve the fundamental processes that underlie oxidative-reductive homeostasis. Nrf2 is the almost ubiquitously distributed transcription factor representing the master regulator of multiple organism responses, involved in crucial biochemical pathways, including oxidoreductive homeostasis, mitochondrial functioning, protein homeostasis and inflammation [39] (**Figure 1**).

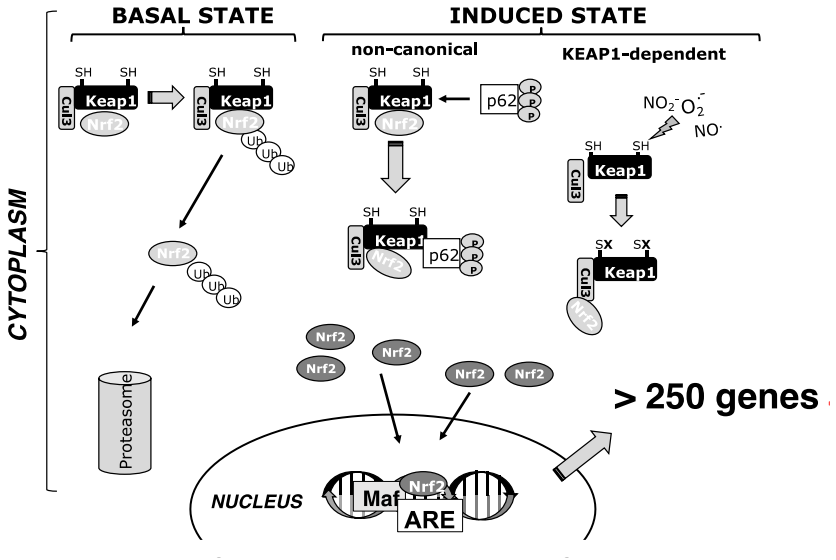


Figure.1 Schematic representation of some mechanisms involved in the regulation of the Nrf2 pathway.

Nrf2 is a redox-sensitive transcription factor, in fact it is captured by Keap1 which, owing to the exposure of specific cysteine residues, acts as a sensor of various endogenous and exogenous electrophilic molecules [40]. The oxidation of some specific cysteine thiols in the Keap1 structure, is indeed, required for triggering conformational change which prevents Keap1-Nrf2 interaction and Nrf2 ubiquitination, thus promoting nuclear accumulation of Nrf2 and transcription of phase II enzymes [41]. It is worth knowing that the

most important role of Keap1 is not just to be a Nrf2 inhibitor that regulates its turnover. Indeed, far more important is its role as “redox sensor”, function that exerts undergoing conformational changes of its structure.

Keap1 has 27/25 residues (in human and mouse respectively) and part of them are exposed to electrophiles. The oxidation of specific cysteine thiols determines changes in its structure that prevents the Keap1-Nrf2 interaction and subsequent Nrf2 ubiquitination, promoting accumulation and migration of Nrf2 in the nucleus and consequence transcription of phase II enzymes [42, 43].

Owing to their electrophilic nature Nrf2 activators are mild pro-oxidants exhibiting a dose-response activity, known as hormetic. Such a response implies that a given molecule may have either beneficial or harmful effects when present at low or high doses respectively. Notably, mild pro-oxidant molecules may initiate an adaptive response aimed at restoring the cellular oxidoreductive homeostasis (redox status) or preventing the harmful effects of pro-oxidant agents “*in the near future*” through the improvement of Nrf2-activated antioxidants/detoxifying enzymes (phase 2 enzymes) e.g. NADH quinone oxidoreductase (NQO1), γ Glutamylcysteine Ligase (GCL); Glutathione Reductase (GSR) Glutathione peroxidase (GPx), Glucose 6 phosphate dehydrogenase (G6PD), and Glutathione-S-transferase (GST) [44]. In particular, there is an increasing research activity in the discovery of the ability of dietary molecules to activate an Nrf2-mediated adaptive response.

1.3 Antioxidant effect of dietary bioactive molecules may be also mediated by gut microbiota.

Homeostatic condition of the intestinal microbiota (eubiosis) depends on the balanced diversity of these microbial population and on the

controlled growth of potentially pathogenic bacteria. In particular, the reduced diversity of the intestinal population (dysbiosis) was associated with the epithelial and mucosal functions, to an inflammatory environment in the gastrointestinal tract [45] as well as other human pathologies like Non-Alcoholic Fatty liver disease and neurodegenerative disorders.

Gut microbiota activity is essential in the host metabolism, protecting against infections from pathogens, intervening in energy homeostasis and in immune response by coordinating specific gene expression in response to different host and environmental signals [46]. In particular, the *Firmicutes* to *Bacteroidetes* ratio (F/B ratio) has been extensively examined for human and mouse gut microbiota and it has been demonstrated that the F/B ratio is associated with metabolic [47], inflammatory diseases [48], neuropsychiatric disorders and cancer [49-51].

Bacteroidetes and *Firmicutes* are the two most abundant populations of gut microbiota and are known to produce Shorth Chain Fatty Acids (SCFAs) i.e. acetate/propionate and butyrate, respectively, which were demonstrated to activate the Nrf2 pathway [52]. This evidence suggests that although biological effects of dietary antioxidants are generally associated to ROS scavenging or to the direct activation of the Nrf2 pathway, its indirect activation by SCFA may contribute to the redox homeostasis / healthy status of the entire organism.

These considerations motivated us to analyze the literature data to investigate the role played by intestinal microbiota on the antioxidant ability of bioactive molecules like carbohydrates accessible to the microbiota (MACs), polyphenols and Polyunsaturated fatty acids (PUFAs)[53].

Dietary intake of several bioactive molecules such as carbohydrates accessible to the microbiota (MACs), polyphenols, and polyunsaturated fatty acids (PUFAs) have been indeed associated with

beneficial effects on human health but the underlying mechanisms have not been fully elucidated.

In this context, the indirect modulation of oxidoreductive homeostasis by these bioactive molecules - through the alteration of gut microbiota - opened the way to the hypothesis that the antioxidant capacity of these bioactive molecules would be, at least in part, mediated by the modulation of SCFA-producing intestinal bacteria. Notably, the existence of such "indirect antioxidant effects" might shed a light on the "polyphenols paradox" which has been postulated for explaining the low bioavailability/high bioactivity exhibited by dietary polyphenols [53].

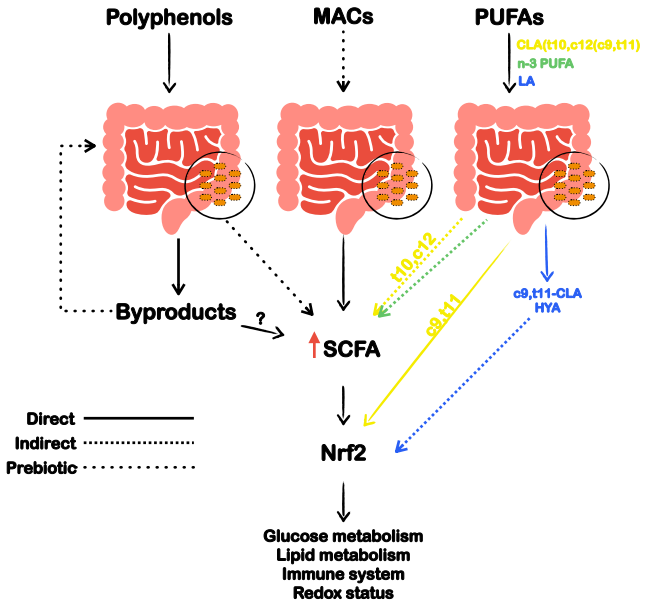


Figure 2. Antioxidant effects of polyphenols MACs and PUFAs.

2. The Nrf2 Pathway

The proper operation of essential cellular processes like differentiation, proliferation, migration and survival relies on the precise regulation of oxidation reactions, that are responsible for the

aerobic metabolism [54]. For this reason, the production of ROS and RNS (reactive nitrogen species) and their physiological flux is tightly controlled.

Nrf2 represents the master regulator of all organisms which is activated to cope with or to prevent pathological increase of ROS/RNS production. It is a ubiquitous and evolutionarily conserved transcription factor of the “cap-collar basic leucine family” [55]. Nrf2 exerts its function by acting on a regulatory region, named Antioxidant Response Element (ARE), of a number of genes involved in the antioxidant defence. These genes codify for phase II enzymes γ-glutamylcysteine ligase (GCL), peroxiredoxin, glutathione reductase (GR) and thioredoxin reductase. Other activated genes encode drug metabolizing/detoxification enzymes, including NAD(P)H quinone dehydrogenase1 (NQO1), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and haeme oxygenase 1 (HO-1) [55, 56]. All together they protect the organism against oxidative damage and detoxification of exogenous or endogenous substances [57].

2.1 Nrf2 activation mechanisms

In a basal state, the transcription factor Nrf2 is sequestered by the protein Keap1 within the 'cullin-3 (Cul3)/Ring box protein1' complex, which, in turn, associates with the enzyme E3 (also known as E3 ubiquitin-protein ligase). This complex regulates the fate of Nrf2 through a rapid turnover process involving ubiquitination. The ubiquitinated Nrf2 is then separated from Keap1, marking it for proteasomal degradation [40]. However, under conditions of mild oxidative stress or exposure to electrophilic stimuli, Keap1 can be inactivated. This inactivation allows newly synthesized Nrf2 proteins to evade Keap1-mediated ubiquitination and freely move into the nucleus. Within the nucleus, Nrf2 forms heterodimers with small

musculoaponeurotic fibrosarcoma (Maf) proteins. These heterodimers subsequently bind to antioxidant response element (ARE) sequences in the promoters of the aforementioned genes and more other [58]. This binding triggers a coordinated expression of phase II enzymes, swiftly restoring the cellular redox status.

The mechanism through which the electrophiles can mediate the conformational changes of Keap1 aimed at releasing Nrf2 is still unknown, but there are two prevailing hypothesis: the “hinge and latch” [59] and the “Keap1-Cul3 dissociation model” [60]. According to these models (non-mutually esclusive) the activation of the Nrf2 pathway is related to the escape of Nrf2 from proteasomal degradation. In the first one, the cysteine oxidation of Keap1 determine a misalignment of lysine residues that could affect the binding with Nrf2. In the second model, the same could affect the binding with Cul3.

The mechanism just described, focused on the electrophile-mediated activation of Nrf2, is considered the “canonical pathway” but, it is worth mentioning that Nrf2 function can be regulated by phosphorylation and acetylation reactions occurring at different levels [61]. These different modalities of activation of Nrf2 are Keap1-indipendent, like some phosphorylation cascades, by a number of different pathways of protein kinases (protein kinase C, mitogen-activated protein kinases, glycogen synthase kinase-3 and phosphoinositide 3-kinase) or by the limited/indirect contribution of mitogen-activated protein kinases, p38 protein, protein kinase C or phosphoinositide 3-kinase [62, 63]. Moreover, sequestosome-1 (also known as the ubiquitin-binding protein p62) can activate Nrf2 through inactivation of Keap1 via the so-called “non-canonical pathway” [64].

2.3 Nrf2 pathway activators

A number of studies have speculated the effectiveness of dietary phytochemicals in activating the Nrf2 pathway [65-69].

Phytochemicals are bioactive compounds synthesized by plants as part of their defense mechanisms. These compounds can be extracted from a variety of plant sources, including whole grains, fruits, vegetables, nuts, and herbs. The plant kingdom produces over a thousand different phytochemicals, each with its unique properties. Some noteworthy examples of phytochemicals include carotenoids, polyphenols, isoprenoids, phytosterols, saponins, dietary fibers, and certain polysaccharides [70]. Polyphenols are a large and varied group of natural compounds, which include molecules that are very different from each other. They are characterized by the presence of a polyphenolic structure which contains at least one aromatic ring and one or more hydroxyl groups [71]. A number of polyphenols like resveratrol, curcumin or quercetin have been found to activate the Keap1/Nrf2-ARE pathway significantly with different mechanisms (Table 2) [53].

	Disease/Condition	Mechanism	Model
Curcumin	Brain injury	Amelioration of white matter injury in rats following hypoxia.[72]	Rat
	Brain injury	Activation of the Nrf2-ARE axis could attenuate brain injury. [73]	Male ICR mice
	Parkinson's Disease (PD)	Activation the AKT/Nrf2 pathway could ameliorate dopaminergic neuronal oxidative damage.[74]	Male Lewis rat
Resveratrol	Spinal cord injury	Inhibition of ferroptosis via activating NRF2/GPX4 pathway. [75]	C57BL/6 mice
	Sepsis-Induced Cardiomyopathy	Ferroptosis inhibition via upregulation of Sirt1/Nrf2 signaling pathways.[76]	Rat

Quercetin	Excessive glutamate release	Decrease in depolarization-evoked glutamate release.[77]	Rat
	Epilepsy	Alleviation of seizure-like behaviors and cognitive impairment.[78]	Kainic acid-induced epileptic mice model
	Diabetic nephropathy	Inhibition of ferroptosis via activating Nrf2/HO-1 signaling pathway.[79]	Male C57BL/KsJ db/db mice
	Intervertebral disc degeneration (IDD)	Amelioration of intervertebral disc degeneration via the Nrf2/NF-κB axis.[80]	Rat

Table 2. Dietary molecules with antioxidant activity.

Another major class of compounds already mentioned that are able to activate the Nrf2 pathway are PUFA's. These molecules serve as essential components of cell membranes and are recognized for their pivotal role in addressing conditions such as non-alcoholic fatty liver, autoimmune reactions, and various chronic illnesses. Within the category of PUFAs, ω-3 and ω-6 fatty acids are particularly crucial, as the human body cannot synthesize them, and they must be obtained through dietary sources. These essential fatty acids are derived from key precursors: α-linolenic acid (ALA) for ω-3 PUFAs and linoleic acid (LA) for ω-6 PUFAs. To meet the body's ω-3 fatty acid requirements, individuals can consume seeds from plants like chia, perilla, and flax, as well as marine fish oil. Cereal products also stand out as significant sources of ω-3 fatty acids. Among the ω-3 fatty acids, α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) play crucial roles. Meanwhile, linoleic acid (LA) and arachidonic acid (ARA) are key ω-6 fatty acids.

Vegetable oils such as soybean and sunflower are primary sources of ω-6 fatty acids, with soybean and sunflower oils containing relatively lower quantities of ω-3 fatty acids. Balancing the intake of these

essential fatty acids through a diverse and well-rounded diet, incorporating sources from both plant and marine origins, is essential for maintaining optimal health and addressing various health conditions associated with PUFAs imbalances [81, 82]. The n-3 PUFA family and conjugated linoleic acid has attracted considerable attention because of their pleiotropic effects. Dietary n-3 PUFA and CLA, exhibit diverse functions through two interconnected processes known as nuclear and plasma membrane pathways. These processes are not mutually exclusive and contribute to the multifaceted impact of PUFAs on cellular functions. In the nuclear pathway n-3 PUFA/CLA demonstrate their effects by interacting with nuclear receptor proteins, exemplified by peroxisome proliferator-activated receptors (PPARs). Simultaneously, in the plasma membrane pathway, PUFAs undergo modifications within the cellular membrane. This includes the generation of eicosanoids, such as prostaglandins and leukotrienes, which are important signaling molecules involved in various physiological responses [83]. Additionally, PUFAs contribute to the alteration of membrane structure, often referred to as 'fluidity.' This property plays a role in regulating or disrupting lipid raft-associated signaling, thereby influencing cellular communication [84]. Noteworthy changes in membrane composition occur with an increased intake of PUFA through dietary sources. Specifically, an enrichment of n-3 PUFA content, often at the expense of n-6 PUFA like arachidonic acid (AA), can impact membrane fluidity and the structure of lipid rafts. This alteration in membrane properties, induced by the dietary intake of PUFAs underscores the dynamic and influential role these fatty acids play in cellular functions and signaling pathways [85].

3. Biological activity of Conjugated Linoleic Acid (CLA)

CLA is the collective name used for indicating geometrical and positional isomers derived from linoleic acid (C18.2) (28 in total). The isomers exhibiting the higher biological activity are the 'cis9,trans11' (C18:2 n-7; **c9**), and the 'trans10,cis12' isomer (C18:2 n-6; **t10**) and the best sources for CLA in the human diet are ruminant meat and dairy products in which the c9 isomer represents about the 80% of the total CLA found in these foods [86]. Of particular interest are the distinctive health effects associated with the two main CLA isomers. The t10 isomer demonstrates remarkable anti-obesity and anti-diabetic effects, effectively modulating lipid metabolism and improving glucose tolerance [87]. Vice versa, the c9 isomer stands out for its preeminent anti-cancer, antioxidant, and anti-inflammatory activities [88].

Recent research suggests that an equal mixture of the two CLA isomers could effectively activate various biological pathways due to the synergistic action of both isomers. In particular CLA, derived primarily from diet or influenced by gut microbiota, encompasses isomers with distinct health effects, and combining them in equal proportions is considered a promising approach for maximizing their positive impact on biological pathways [89]. For this reason, commercially available CLA supplements is widely used in in vivo studies and clinical trials [90-92].

Considering the biological activities of CLA on pathways like redox balance and inflammation, its possible role in ameliorating various pathological condition have been investigated over time.

The cytoprotective activity of individual CLA isomers was demonstrated in different tissues (gut, spleen and liver) of several murine models by Bergamo et al. [93-97]. Moreover, they were able to demonstrate that the combination of the two isomers has neuroprotective and immunomodulatory activity in a mouse model of neuropsychiatric lupus [98, 99].

Similarly, the healthy effects associated with dietary supplementation with CLA was confirmed in several other studies [86, 100, 101].

4. Aim of the thesis

Among the different neurodegenerative disorders, the Alzheimer's disease (AD) cannot be cured and the number of patients suffering from this pathology is destined to increase due to the average lengthening of the human life. There is consolidated evidence in the literature on the correlation between neurodegenerative pathologies and oxidative stress [102] and the potential role played by Nrf2 as a pharmacological target to attenuate the progression of these pathologies [103-105]. Nonetheless, there are only a few in vivo studies in which the neuroprotective efficacy of food-derived molecules has been evaluated in *in vivo* experimental models. Moreover, although the association of the neuroprotective ability of dietary CLA with the activation of the Nrf2 pathway was demonstrated *in vivo,* nevertheless its beneficial effect on AD has not been established yet. In particular, although the Nrf2 pathway has been recognized the key player in redox, immune and protein homeostasis only a few data are available on the neuroprotective effect of dietary Nrf2 activator (namely the CLA).

The main aim of my thesis was focused on the evaluation of the potential beneficial effects produced by dietary CLA supplementation in a mouse model of chemically induced AD. In particular, as environmental exposure to essential (iron, copper, zinc and manganese) or to toxic metals (e.g. lead, cadmium and aluminium) are known to disrupt redox homeostasis [106], thus animal supplementation with aluminium chloride ($AlCl_3$, AL) is a widely used method for triggering AD in rodents.

This animal model was utilized in two different studies. The first was aimed at investigating the noxious effect of dietary $AlCl_3$ on typical signs of AD (Mitochondrial dysfunction, oxidative stress, inflammation,

glucose dysmetabolism and altered proteostasis), the neuroprotective effect of CLA and the underlying molecular mechanisms. Next, we decided to investigate the protective effects played by dietary CLA supplementation against $AlCl_3$-induced activation of compensatory mechanisms like Endoplasmic Reticulum (ER) stress, Unfolded Protein Response (UPR), Nrf2 pathway) or maladaptive response (inflammation and apoptosis) triggered by $AlCl_3$ toxicity.

The obtained results provide at first, evidence that mitochondrial dysfunction, Nrf2 alteration, inflammation and Acetylcholinesterase (AChE) hyperactivation can occur even from low doses of $AlCl_3$ (L) exposure together with CLA ability to increase the level of glucose transporters - along with its antioxidant and anti-inflammatory effect. The second series of results revealed the NADPH oxidase (NOX) involvement in the Nrf2-mediated neuroprotective effects in the brain cortex of CLA-treated mice. In particular, $AlCl_3$ neurotoxicity (as evidenced by the hyperactivation of compensatory and maladaptive mechanisms) are hampered by dietary CLA via the improvement of endogenous cyto-protections (adaptive response) triggered by NOX activation.

ABBREVIATIONS

AD Alzheimer's disease; **AL** $AlCl_3$-treated, **ATF4** activating transcription factor 4; **ATF6** activating transcription factor 6; **CLA** Conjugated linoleic acid; **ER** Endoplasmic Reticulum; **GCLC**, γ-glutamyl cysteine ligase catalytic subunit; **GRP78** glucose-related protein; **GSH**, total glutathione; **GSR** Glutathione reductase; **GST** Glutathione Transferase; **G6PD** glucose-6-phosphate dehydrogenase; **Keap1**, Kelch-like ECH-associated Protein 1, **IRE1** inositol-requiring kinase 1; **ND** neurological disorders; **NOX** NADPH oxidase; **NQO1** NAD(P)H quinone oxidoreductase; **PDI** Protein Disulfide Isomerase; ; **ROS**, Reactive

Oxygen Species; **UPR** unfolded protein response; **Xbp1s,** spliced X-box-binding protein.

5. Materials and Methods

5.1. Materials

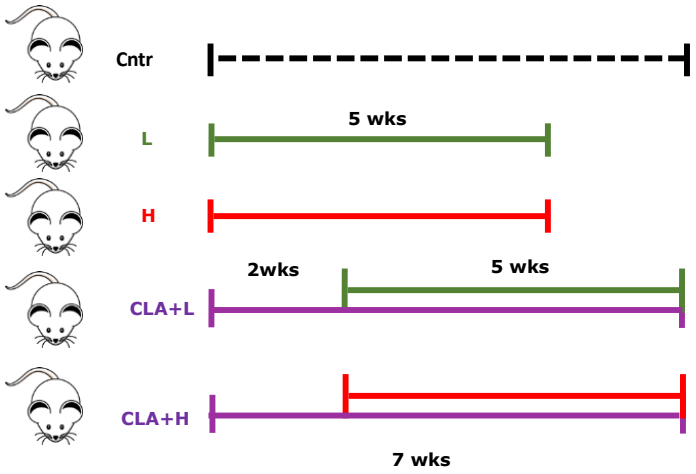
Reagents were obtained from Sigma–Aldrich (St. Louis, MO) or Serva (Serva Electrophoresis GmbH, Heidelberg, Germany). The CLA mixture (76%), containing t10, and c9 isomers (38.5% and 37.4%, respectively), was purchased from Tonalin. The list of primary antibodies used is shown in Table 3. Bovine serum albumin fraction V, acetylthiocholine iodide (ATCI), 5,5-dithiobis-2-nitrobenzoate (DTNB), salts, and buffers were purchased from Sigma-Aldrich (St. Louis, MO, USA).

5.2. Animals and experimental design

The first series of experiments was carried out on thirty-five adult (8/9 weeks) male BALB/c mice (average body weight 27.3 ±0.8 g/28.6 ± 0.3 g). The animals, originally obtained from Charles River (Charles River, Research Models, and Services, Italy) were from a colony reared in the animal facility of the Institute of Food Sciences (CNR-ISA) in a temperature-controlled room (24 °C), under a 12 h light/dark cycle in pathogen-free conditions, with free access to drinking water and standard chow (Mucedola 4RF21-GLP).

BalbC mice were divided into 5 groups (n=7 each) and individually housed. Two groups were treated for five weeks with two $AlCl_3$ doses (7.7 or 100 mg/kg; low L, or high H respectively) dissolved in drinking water. Protective effects of CLA were evaluated in 2 additional groups orally supplemented with CLA (600 mg/kg bw) before (2 weeks) and

during L or H treatment (CLA+L or CLA+H). Untreated mice were used as control (Cntr) (see Scheme 1).



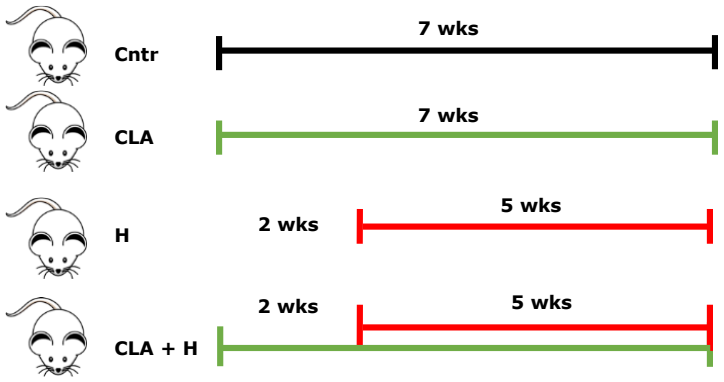
Scheme 1. Schematic representation of the experimental groups used in the first study (L= low $AlCl_3$ dose; H= $AlCl_3$ dose) .

Body weight was checked twice a week during the different treatments. $AlCl_3$ dilutions (containing 0.04 or 0.52 mg/mL) were weekly prepared and, based on the average daily water consumption (5.3 mL in 0.027 kg mouse) [107], animal intake was (approximately) 7.7 or 100 mg of $AlCl_3$ /kg bw/day. The L dose was similar to a previously published treatment (8.3 mg /kg bw/day for 28 days) which was considered compatible with a possible human exposure - [108], H dose, widely used to induce AD in rodents, was used as a positive control. These $AlCl_3$ amounts, after conversion into human equivalent doses (HED) [109], corresponded to 4.4 or 398 mg/ kg bw/ week (for an adult weighing 70 kg) and they were, approximately, 4 or 400 times higher than TWI [110].

The second series of experiments was carried out on 28 Balbc mice, which were individually housed and divided into 4 groups (n = 7 each). One group was treated for five weeks with 100 mg/kg $AlCl_3$ (H) dissolved in drinking water. The second group was orally supplemented with CLA (600 mg/kg bw, per os) for seven weeks. The

third group was orally supplemented with CLA before (2 weeks) and during $AlCl_3$ treatment (for an additional 5 weeks; CLA+H). The fourth group, composed of untreated animals, was used as a control (Cntr) (see Scheme 2). The $AlCl_3$ dose administered in this study corresponded to that commonly used to induce AD-like neurotoxic effects in mice [111] and its preparation was made essentially according to a recently published study [98].

The daily CLA dose used in both sets of experiments, upon normalization to Human Equivalent Doses, corresponded to 3 g/day, which is the average amount used in clinical [112] and in our previous in vivo studies [93-95, 98].



Scheme 2. Schematic representation of the experimental groups used in the second study (L= low $AlCl_3$ dose; H= $AlCl_3$ dose).

All experimental procedures used in these studies were carried out in accordance with national and international policies (EU Directive 2010/63), approved by the institutional committee, and authorized by the Italian Ministry of Health (n.941/2020-PR).

5.3. Sample collection and preparation

After euthanasia, the liver and brain were quickly removed, thoroughly washed in an ice-cold saline solution, blotted on filter paper, and

placed on ice. The liver was cut into pieces and stored at -80°C for subsequent analyses. Brain hemispheres were separated and dissected into two main sections, containing the cerebral cortex. The prefrontal cortex (the anterior first third) from both hemispheres was used for RT-PCR analysis. The posterior brain section from one hemisphere, containing the remaining two-thirds of the cerebral cortex (subicular cortex, hippocampus, corpus callosum, and lateral ventricle) was immediately fixed in Bouin's solution for 48 h at room temperature (r.t.), dehydrated, embedded in paraffin, and used for histological analysis. Aliquots from the cortex portion of the other hemisphere were snap-frozen using liquid nitrogen and stored at - 80 °C until biochemical (mitochondrial functioning, redox status/oxidative stress, enzyme activities) or western blotting analysis.

5.4. Evaluation of mitochondrial function

Brain mitochondria were isolated by differential centrifugation [39]. Mitochondrial respiration (100 µg of mitochondrial protein/ml) was measured in a medium consisting of 125 mM KCl, 0.1% (w/v) bovine serum albumin, 20 mM HEPES, 2 mM $MgCl_2$, and 2.5 mM KH_2PO_4, pH 7.2, by a Clark oxygen electrode at 37 °C. The real-time oxygen concentration in the reaction mixture was measured in the presence of oxidative substrates (5 mM pyruvate and 2.5 mM malate).

After 2 min, state 3 respiration was induced by the addition of 150 µM ADP. The respiratory control ratio (RCR) was calculated as the ratio of the rate of oxygen uptake in the presence of added ADP (state 3) to the rate observed in the absence of ADP (state 4). Respiratory complex activity of brain mitochondria (40 µg of protein) was carried out according to Ferramosca and collaborators. Complex I activity was determined using decylubiquinone as an electron acceptor and NADH as the donor. The oxidation of NADH was measured following the

decrease in absorbance at 340 nm. Complex II activity assay was performed following the decrease in absorbance at 600 nm resulting from the reduction of 2,6-dichlorophenolindophenol (DCPIP) as the electron acceptor and succinate as the donor. Complex III activity was determined by measuring the reduction of oxidized cytochrome c at 550 nm, whereas complex IV activity assay was performed following the decrease in absorbance at 550 nm resulting from the oxidation of reduced cytochrome c.

5.5. Redox status evaluation

Total thiols (GSHtot = GSH +GSSG) content in mouse brain cortex was quantified using the 5,5' dithio-nitrobenzoic acid (DTNB)-GSSG reductase recycling assay [37] Accumulation of oxidatively-modified proteins (carbonylated; PC) in the brain of the differently treated groups was measured accordingly to a published protocol [113] . Upon normalization to the protein content, GSHtot and PC concentrations were finally expressed as nmol/mg protein. The extent of mitochondrial protein damage was determined by quantifying PC accumulation on mitochondrial protein side chains using the OxyBlotTM Protein Oxidation Detection Kit (Chemicon) according to the manufacturer's recommendations.

5.6. Phase 2 enzyme and NOX activity assays

The activities of detoxifying phase 2 enzymes activated (GSR, G6PD, NQO1 and GST) were analyzed spectrophotometrically in brain/liver extracts using protocols used in previous studies [94]. Upon normalization to the protein content, GSR and NQO1 activity was expressed as nmoles of NADPH/mg protein/min, G6PD was expressed as UI/mg protein/min while GST activity was expressed as nmoles of chloro-dinitrobenzene (CDNB) mg^{-1} min^{-1}, respectively.

AChE activity was measured by using ATCI as a substrate in accordance to a previously described protocol [114]. Briefly, frontal cortex aliquots (about 20 mg) were homogenized in 0.3 mL of TBS (100 mM Tris–HCl, pH 8.1) and upon centrifugation (4 minutes, 2000 g, 4◦C), the rate of production of thiocholine was determined by the reaction of the thiol with DTNB to produce the yellow anion of 5-thio-2-nitro-benzoic acid. Six extract aliquots (20 µL containing 20 µg of proteins) were plated into 96 wells-plate, and well containing TBS were used as blank.

Aliquots (100 µL) of 2 mM ATCI in TBS was added to one triplicate series (assay), while 100 µL of TBS was added to the second series (control). Finally, 100 µL of TBS containing 2.4 mM DTNB was added to both series and the absorbance was measured at 405 nm after 10 min of incubation at r.t. AChE activity was corrected by subtracting the spontaneous hydrolysis of the substrate (blank) or the DTNB reaction with protein thiols (Control). Enzyme activity was finally expressed as nmol/min mg protein. Zymographic assays were carried out for the evaluation of NOX activity. In brief, protein aliquots (15 µg) from the mouse cortex were fractionated by electrophoresis on an 8% non-denaturing polyacrylamide gel. The gel was equilibrated by incubation in 10 mM Tris-HCl pH 8.0 for 5 min and for an additional 5 min in the same buffer supplemented with 0.25 mM NADPH. The reaction was developed by incubation of the gel in 10 mM Tris-HCl buffer supplemented with 0.25 mm NADPH and 5 mg/ml of nitroblue tetrazolium. Densitometric quantification of protein band intensity, as visualized by gel zymography, was carried out using the ImageJ vJ1

software (open source) and the band intensity found in differently treated groups was expressed as fold changes in comparison to untreated animals.

5.8. Western Blotting

Mitochondrial proteins (30 μg) were transferred to a nitrocellulose membrane and Western blotting analysis for the evaluation of mitochondrial complexes (CI-CV) expression (OXPHOS cocktail) or the accumulation of carbonylated proteins (OxyBlottTM Kit).

Brain cortex aliquots, obtained from different experimental groups, were minced on ice in lysis buffer (20 mM Tris–HCl pH 7.4 (TBS), supplemented with 10% NP40 0,1 %, 5 M NaCl, 50% Glycerol, 0,5 M EDTA, 1 mM Na3VO4, 10 mM EDTA, and 1% protease inhibitor mixture.

After 25 min, protein extracts were obtained by centrifugation at 10.000g x 30 min at 4 ◦C and protein concentrations were measured by the Bio-Rad protein assay using Bovine Serum Albumin as the standard. Equal amounts of protein (20 μg) were fractionated by SDS-PAGE and transferred onto PVDF membranes (Biorad Laboratories, Inc., Hercules,CA, USA) using a Bio-Rad Transblot (Bio-Rad, Milan, Italy). Membranes were blocked at r.t. in milk buffer (TBS, supplemented with 3% nonfat dry milk, 0.1% Tween-20) and then incubated at 4 ◦C overnight (o.n.) with primary antibodies dilutions (Table 3). Western blot for β-Actin (CellSignaling) was performed to ensure equal sample loading. At the end of incubation with the appropriate peroxidase- conjugated secondary IgGs (1:1000, 1 h at r.t.) the membranes were washed before being incubated (5 min at r.t.) with Clarity western ECL (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The formed immunocomplexes formed were finally visualized by enhanced chemiluminescence and autoradiography according to the

manufacturer's instructions. Protein band quantification was carried out by using the (open source) ImageJ software vJ1 and normalization with the internal loading control (β-actin) and the abundance of the target proteins in differently treated animals was finally expressed as fold increase or percent (%) changes in comparison with the control group.

Antigen	Clonality	Code	Brand	Dilution	kDa
OXPHOS cocktail	mAb	ab110413	Abcam	1:500	
OxyBlott™ Kit		S7150	Chemicon/Millipore	1:1000	
GLUT 1	mAb	66290-1-Ig	Proteintech	1:1000	45-55
GLUT 3	pAb	20403-1-AP	Proteintech	1:1000	48-60
GLUT 4	mAb	666846-1-Ig	Proteintech	1:1000	48-50
GCLc	pAb	12601-1-AP	Proteintech	1:2000	73
GPx	mAb	13B2AF	Ab Frontiers	1:1000	22-23
Bax	pAb	Sc-493 (N-20)	Santacruz Biotechnology	1:1000	23
NOX1	pAb	17772-1-AP	Proteintech	1:500	65
NOX2	pAb	19013-1-AP	Proteintech	1:1000	65
ATF6	pAb	24169-1-AP	Proteintech	1:1000	75
GPR78	pAb	11587-1-AP	Proteintech	1:5000	78
PDI	pAb	11245-1-AP	Proteintech	1:1000	57
Xpb1	pAb	25997-1-AP	Proteintech	1.2000	32
SOD2	mAb	13194	CellSignaling	1:1000	22
Bcl-2	mAb	ab182858	Abcam	1:1000	26
β-Actin	mAb	ab110413	Cell Signaling	1:2000	45

Table 3. List of primary antibodies

5.9. Morphological Evaluation of Cerebral Cortex

Paraffin sections prepared from the cerebral cortex of mice were stained with hematoxylin and eosin to determine neurodegeneration. Briefly, sections were de-waxed by xylene washing for 10 minutes, followed by 10 minutes in 100% ethanol and 5 minutes in 70% ethanol and then rehydrated with 5 minutes in distilled water. The sections were then stained by placing them in hematoxylin for 15 minutes.

After washing with warm distilled water, sections were stained with eosin for 3 minutes followed by an immersion in distilled water, 70% ethanol and 100% ethanol for 2 minutes. Slides were then observed under a Zeiss Axioscope light microscope with a 40x magnification equipped with an ocular micrometer (10x=1 mm). The cell counting was carried out in an area of 1 mm^2 (10,000 μm^2). The analysis was done from five randomly selected sites in each cortex brain region by two independent observers. The average values were calculated for every region and plotted as graph.

5.7. Quantitative real-time PCR analysis

Brain cortex mRNA levels for several pro-inflammatory cytokines (IL-1β, IL-6, and TNFα) and chemokine (CXCL-1; the functional murine homolog of human IL-8) were measured to evaluate both the toxic effect of L and H doses and the protective effect of CLA. Moreover, mRNA expression of some phase-2 (GSR, and GSTμ, the catalytic subunit of GCL, GCLc) and of 2 glucose transporters (GLUT1 and GLUT3) was measured to further investigate the different mechanisms activated by Al intake or by CLA pre-treatment. while mRNA expression of selected UPR proteins (Grp78, ATF6, ATF4, Xbp1s) was evaluated to investigate the effect produced by $AlCl_3$ or by CLA pre-treatment on UPR pathway.

Total RNA was extracted from brain cortex specimens using the TRIzol reagent, according to the manufacturer's instructions (Invitrogen, Milan, Italy). cDNA was prepared from 1 mg total RNA by reverse transcription with MMLV RT Invitrogen, Milan, Italy) and the oligo-(dT)12-18 primer at 42◦C for 60 min. Real-time PCR was performed on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc, Hercules, CA) using 300nM gene-specific primers, iTaq TM Universal SYBER Green Supermix (Bio-Rad), and the following

PCR conditions: 1 cycle at 95◦C for 1.30 min and 39 cycles at 95 ◦C for 10 s and 58.7 ◦C (L-32), 62.0 ◦C (IL-1β), 59.5◦C (IL-6), 60◦C (TNFα), 55◦C (CXCL-1), 60.2◦C (GCLc and GSR), 55.7◦C (GSTμ) or 63.1◦C (GLUT1 and GLUT3) for 30 s, 1 cycle at 95°C for 1.30 min and 39 cycles at 95 °C for 10 s and 58.7 °C (L-32), 59.8°C (ATF4, ATF6, Xbp1), 60.8°C (Grp78). Gene expression levels, calculated using the ΔΔCt method [115], after normalization to the L-32 housekeeping gene were presented as fold-changes compared to controls.

Gene		**Primer sequence 5′ → 3′**
L-32	sense	AGCAGAGCTGGAGTCGCTTT
	antisense	GGAGCTGCCATCCAAAAGATACTA
IL-6	sense	GAGGATACCACTCCCAACAGACC
	antisense	AAGTGCATCATCGTTGTTCATACA
TNFα	sense	CATCTTCTCAAAATTCGAGTGACAA
	antisense	TGGGAGTAGACAAGGTACAACCC
IL-1β	sense	AGTTGACGGACCCCAAAAG
	antisense	AGCTGGATGCTCTCATCAGG
CXCL-1	sense	CTGGGATTCACCTCAAGAACATC
	antisense	CAGGGTCAAGGCAAGCCTC
GSR	sense	CTATGACAACATCCCTACTGTGGT
	antisense	CCCATACTTATGAACAGCTTCGT
GCLc	sense	TGGAGCAGCTGTATCAGTGG
	antisense	CAAAGGCAGTCAAATCTGGTG
GSTμ	sense	TGGAGCAGCTGTATCAGTGG
	antisense	TTTCTCAGGGATGGTCTTCAA
Glut 1	sense	TCAACACGGCCTTCACTG
	antisense	CACGATGCTCAGATAGGACATC
Glut 3	sense	TTCTGGTCGGAATGCTCTTC
	antisense	AATGTCCTCGAAAGTCCTGC
Xbp1s	sense	TGCTGAGTCCGCAGCAGGTG
	antisense	GACTAGCAGACT CTGGGGAAG
Grp78	sense	TTCTGCCATGGTTCTCACTAAA
	antisense	TGTTCTTCTCTCCCTCTCTCTT
ATF4	sense	CTTGCTGTCTGCCGGTTTG
	antisense	GGGAAGAGGAAAGGACACCC
ATF6	sense	GCGGATGATAAAGAACCGAGAG
	antisense	ACAGACAGCTCTTCGCTTTG

Table 4. List of used PCR Primers

5.10. Statistical analysis

Data were expressed as mean values ± standard deviation (SD). The program GraphPad Prism 6 (GraphPad Software, San Diego, CA) was

used to perform a t-test or one-way ANOVA, followed by the Tukey post-hoc test, $p < 0.05$ was considered significant.

6. Results

6.1. Animal response to $AlCl_3$ intake.

$AlCl_3$ administration is a widely used method of triggering AD in the brain of rodents. In the present study, the evaluation of some oxidative stress hallmarks of AD (i.e. mitochondrial dysfunction, AChE hyperactivation, GLUT expression, inflammation, and neuronal degeneration) was measured after 5-weeks of treatment to assess either the dose-dependent toxicity of $AlCl_3$ or the protective ability of CLA. Animal body weight of L or H groups monitored during the treatment was used to examine the general physiological effects of $AlCl_3$ on mice organisms. The obtained results indicate that the body weight of L (green circle) or H-treated mice (red circle) was comparable to that of Cntr animals (white circle) (Figure 2).

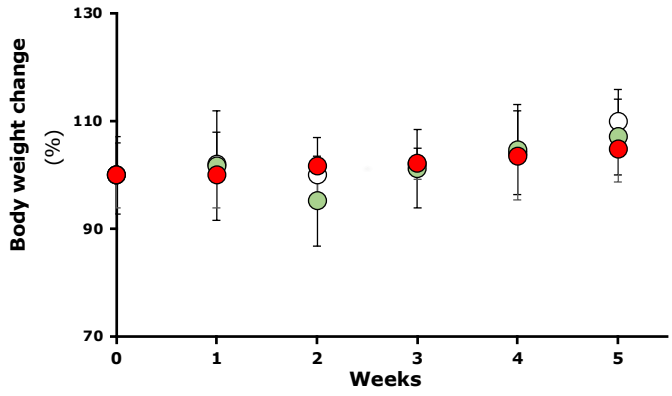


Figure 2. $AlCl_3$ effect on BalbC body weight. (Low $AlCl_3$ dose= green circles; High $AlCl_3$ dose= red circles; Control= white circles).

6.2 CLA alleviates the $AlCl_3$-induced neurodegenerative alterations in the brain cortex.

H&E-stained brain cortex sections from untreated mice revealed a normal morphological architecture with intact neurons with spherical or pyramidal perikaryon, with large nuclei (Figure 3). The cerebral cortex of L- and H-treated animals showed several neurodegenerative changes (intracytoplasmic vacuoles of neuronal cells, darkly stained shrunken nuclei, and chromatin lysis alteration) (Figure 3B, 3C, respectively). Neurodegenerative alterations associated to L or H intake are significantly alleviated by CLA supplementation ($p < 0.05$), as indicated by a re-establishment of the cerebral cortex architecture with slight degeneration, and a few nuclei with either marginated chromatin and deeply stained alteration (Figure 3D and 3E, respectively). The number of healthy neurons (blue bars) was dose-dependently reduced by L or H intake ($p < 0.001$), and their number significantly increased in CLA+L or CLA+H mice in comparison with L or H treatment, respectively ($p = 0.045$ or 0.0063). On the other hand, L or H intake resulted in a dose-dependent increase of necrotic neurons (orange bars) in comparison with untreated mice ($p = 0.002$ or 0.001) and their level was reduced following a CLA treatment, but only in CLA+H animals it reached the statistical significance ($p = 0.0015$).

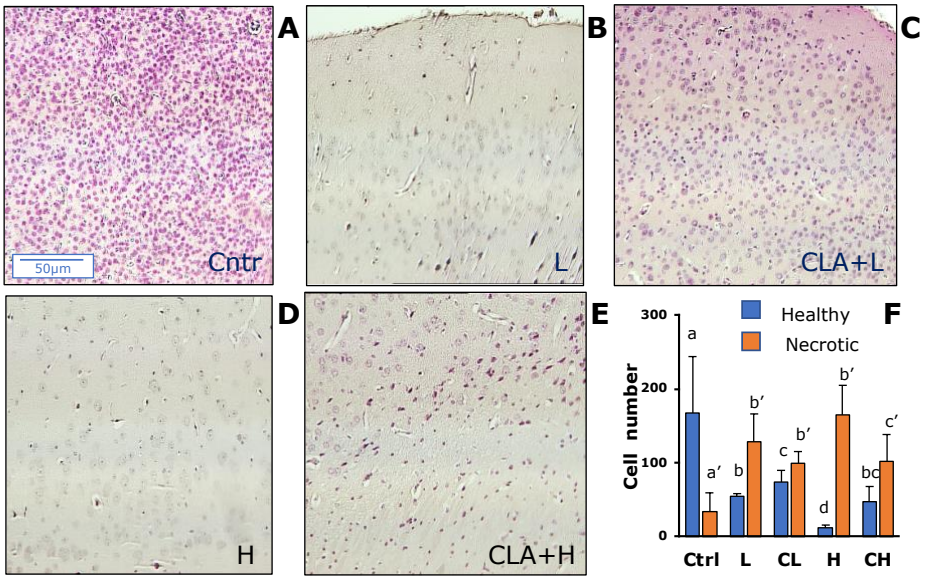


Figure 3. CLA alleviates $AlCl_3$-induced morphological degeneration of mice brain cortex Hematoxylin and eosin-stained sections of the cerebral cortex from differently treated mice (x400). **A**) Untreated mice (Cntr); **B**) Low aluminium (L); **C**) High aluminium dose (H); **D**) CLA+L; **E**) CLA+H; **F**) Mean values (± S.D.) of healthy or necrotic cells /1 mm^2 of cerebral cortex of differently treated animals. Cell count was made in at least 5-6 animals/ group. Differing superscript letters denote statistically significant differences ($p < 0.05$).

6.3. $AlCl_3$-induced decline of brain mitochondrial function is partly restored by CLA treatment.

Based on clinical data reporting the occurrence of mitochondrial dysfunction in the brain of AD patients [116] we decided to investigate the effects of L or H treatment on brain mitochondrial respiration. The toxic effect triggered by H-treatment is shown in Table 5. In particular, its noxious effect on mitochondrial respiration efficiency was indicated by the significant decline of RCR values ($p = 0.0009$), while the marked increase of oxygen consumption in the resting state of mitochondrial respiration (V4) ($p = 0.0004$) was the resultant of H uncoupling between electron transfer through respiratory complexes and ATP synthesis. The partial recovery of both RCR and V4 values in CLA +H treated mice was indicative of the protective effect of CLA.

	RCR	V_3 (nmol O_2/min/ml)	V_4 (nmol O_2/min/ml)
Cntr	3.54 ± 0.35 [a]	22.3 ± 1.5 [a]	6.3 ± 0.5 [a]
L	3.05 ± 0.34 [a]	21.2 ± 2.7 [a]	6.9 ± 0.3 [a]
H	2.13 ± 0.14 [b]	18.7 ± 2.5 [a]	8.7 ± 0.7 [b]
CLA+L	3.32 ± 0.02 [a]	20.6 ± 0.8 [a]	6.2 ± 0.2 [a]
CLA+H	2.82 ± 0.48 [a]	20.5 ± 2.0 [a]	7.3 ± 0.6 [a]

Table 5. Mitochondrial respiration efficiency in the cortex of differently treated animals. Data are means ± SD (n=3). Differing superscript letters denote statistically significant differences ($p < 0.05$) when compared with control (a) (one-way ANOVA).

6.4. CLA protects against H-induced decline of mitochondrial complex activity and increased protein oxidation.

Next, due to the association of AD disease with a deficit of mitochondrial complex functioning [116], the toxic effects of $AlCl_3$ on the expression/functional variations of mitochondrial complexes I-IV (CI–CIV) along with the protective ability of CLA were examined in the mice brain cortex. A significant decline in CI and III activity was found in H-treated mice compared to controls ($p <0.05$) while the decreased CIV activity in these animals did not reach statistical significance. Interestingly, CLA treatment restores CI and CIII activity at levels comparable with those found in the brain cortex of the L group (Table 5). However, changes in respiratory complex activity are not associated with an evident alteration of respiratory chain proteins (CI-CV) expression (Figure 4A). The significant accumulation of oxidatively-modified mitochondrial proteins (PC) in brain mitochondria ($p < 0.0001$) clearly indicates the pro-oxidant activity of $AlCl_3$ while the cytoprotective ability of CLA can be deduced by the inhibitory effect on $AlCl_3$-induced stress (Figure 4B). Taken together, Al-induced alteration of CI and CIII activities – which are also a site of ROS production – along with PC accumulation, are indicative of $AlCl_3$

toxicity on brain oxidoreductive homeostasis, while the protective effect of CLA is associated with their recovery in CLA+H animals.

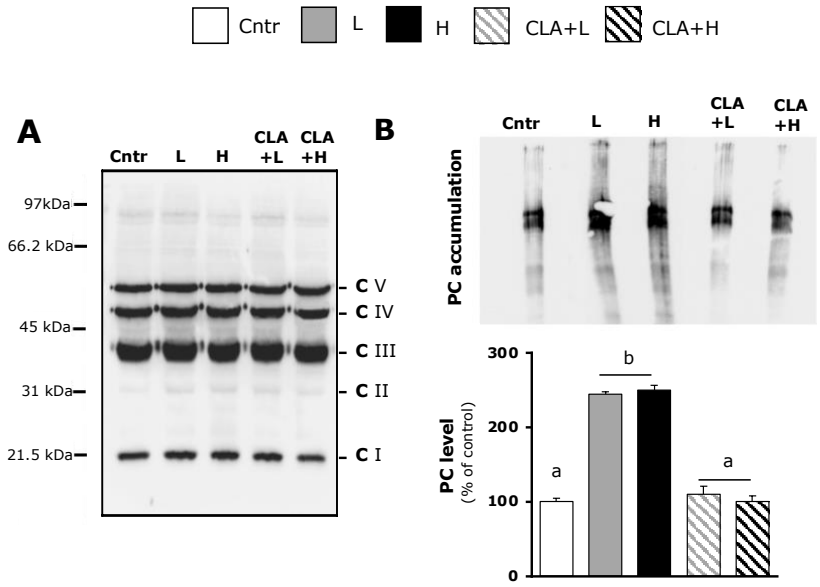


Figure 4. CLA supplementation protects against $AlCl_3$ induced accumulation of carbonylated proteins in brain mitochondria. Brain mitochondrial proteins were used to evaluate the expression of mitochondrial complexes and PC accumulation by immunochemical detection. Representative western blot analysis of mitochondrial complexes I-IV (CI–CIV) (A) and PC accumulation (B) is shown. The bands were quantified using densitometric analysis and the values were expressed as average percent increase (%) (±SD) (B, lower insert). Differing superscript letters denote statistically significant differences ($p < 0.05$) when compared with control (a).

	Complex I (nmol NADH ox/min)	**Complex II** (nmol DCPIP red/min)	**Complex III** (nmol cyt *c* red/min)	**Complex IV** nmol cyt *c* ox/min)
Cntr	16.8 ± 2.0 [a]	10.8 ± 1.1 [a]	18.7 ± 1.8 [a]	18.4 ± 2.6 [a]
L	15.1 ± 0.6 [a]	10.4 ± 0.7 [a]	15.4 ± 1.0 [b]	17.7 ± 1.9 [a]
H	13.2 ± 0.9 [b]	9.5 ± 0.9 [a]	12.5 ± 1.1 [b]	15.7 ± 0.8 [a]
CLA+L	15.9 ± 0.4 [a]	10.5 ± 0.9 [a]	18.6 ± 1.1 [a]	19.2 ± 1.0 [a]
CLA+H	15.6 ± 1.1 [a]	9.8 ± 0.6 [a]	15.5 ± 0.9 [b]	17.2 ± 0.9 [a]

Table 6. CLA improves $AlCl_3$-induced decline of mitochondrial respiratory complex activity. Data are means ±SD (n =3). Differing superscript letters denote statistically significant differences ($p < 0.05$) when compared with control (a).

6.5. $AlCl_3$-induced alteration of phase 2 enzyme is modulated by CLA intake.

Nrf2 plays a crucial role in maintaining cellular redox homeostasis and regulating inflammation response, and its activation occurs endogenously to cope with oxidative stress. On the basis of CLA ability to activate the Nrf2 pathway, we hypothesized that CLA treatment may activate an adaptive response aimed at preventing the pro-oxidant effect of $AlCl_3$. The mRNA levels of GSR and GSTμ in the brain cortex of L and H mice were not statistically different from those found in Cntr mice (Figure 5A, upper panel) while GCLc mRNA in H-treated animals was significantly higher in comparison with Cntr or L-treated mice ($p < 0.05$). On the other hand, its level in CLA+H animals was comparable to that found in untreated mice (Figure 5A, lower panel). The activity of several phase 2 enzymes (GSR, GST and G6PD) in the cortex of Al-treated mice was significantly higher than that measured in untreated mice ($p < 0.005$). Notably, their level in CLA+L and CLA+H was comparable to those of Cntr (Figure 5B).

When GCLc and GPx protein levels were evaluated by Western Blotting, we found that treatment with H was accompanied by an undetectable alteration of GCLc and by a marked decline of GPx level ($p < 0.05$). Interestingly, GCLc and GPx levels in CLA-supplemented mice (CLA+H) were significantly higher than H or Cntr mice ($p < 0.01$) (Figure 5C).

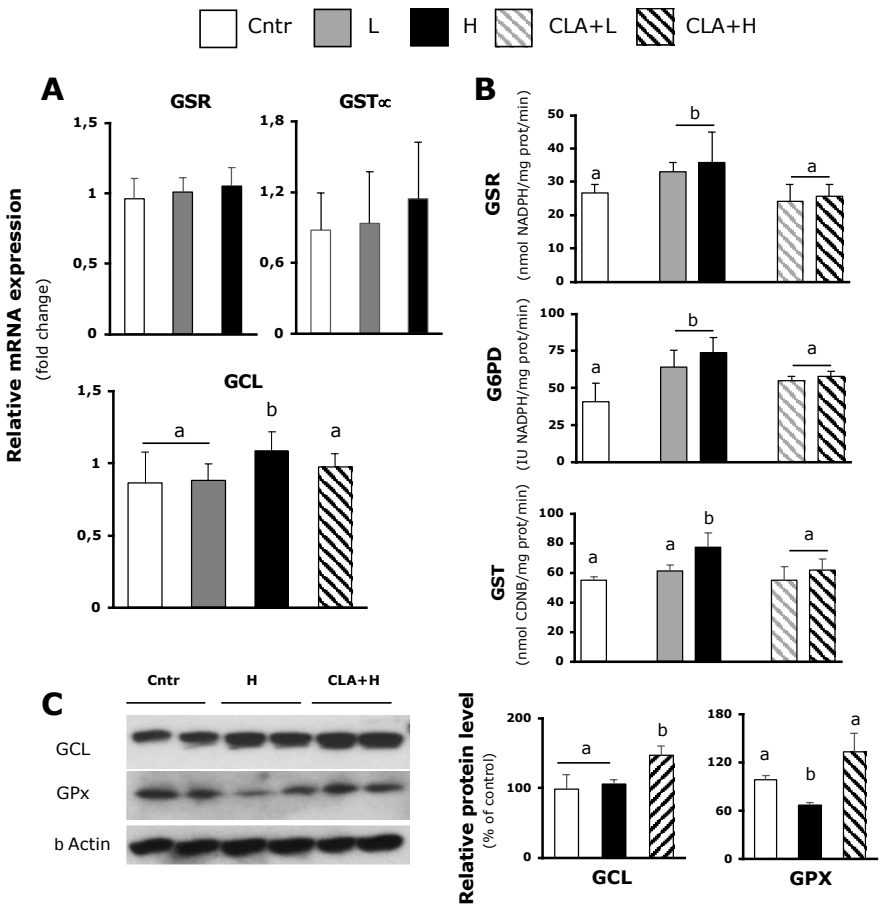


Figure 5. CLA modulates $AlCl_3$-induced alteration of phase 2 enzyme expression/activity in mouse brains.

GSR, GSTμ and GCLc mRNA levels were measured in the brain cortex of in differently treated mice (L, H, CLA+L, CLA+H) and in Cntr mice (A). GSR G6PD and GST enzyme activities were evaluated across the different groups (B). Each bar represents the mean values ±SD from a duplicate analysis of at least 6 different animals (n =6). Representative immunoblot of several Nrf2-activated protections (GCL, GPx) in brain extract from differently treated mice is shown (C, left panel). The bands were quantified using densitometric analysis from duplicate experiments and the values were expressed as average fold increase (±SD) as compared with control (C, right panel). Differing superscript letters denote statistically significant differences ($p < 0.05$).

To confirm the toxic effect produced by $AlCl_3$ treatment on cytoprotective defenses of mice, GSR, G6PD and GST activities in the liver of H-treated mice were compared with those measured in CLA+H or in Cntr groups. Data reported in Figure 6 show that the significant decline of GSR and GST activity in H-treated mice is restored/hampered by CLA intake (CLA +H) ($p <0.005$).

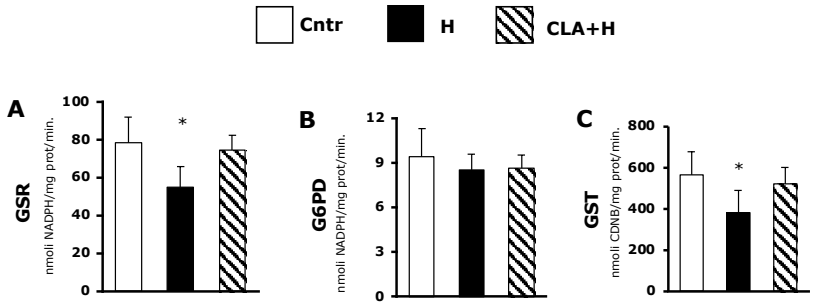


Figure 6. CLA modulates Al-induced alteration of phase 2 enzyme expression/activity in mouse liver. GSR, G6PD, and GST enzyme activities were evaluated in liver tissue of **H**, **CLA+H**, and **Cntr** mice (**A, B**, and **C**). Each bar represents the mean values ± SD from a duplicate analysis of at least 6 different animals (n = 6). Asterisk denotes statistically significant differences from **Cntr** ($p < 0.05$).

6.6. $AlCl_3$-induced oxidative stress and AChE hyperactivation is hampered by CLA supplementation.

Aluminium toxicity has been often associated with its ability to promote ROS formation [6] and oxidative stress is one of the pathognomonic signs of many neurodegenerative disorders, including AD.

Thus, GSHtot concentration and PC accumulation were measured to evaluate brain antioxidant potential and oxidative-stress extent (respectively) in $AlCl_3$-treated mice and the cytoprotective efficacy of CLA in CLA+L and CLA+H brain tissue.

The occurrence of redox status alteration is indicated by the significant decrease of GSHtot concentration ($p < 0.01$) while the marked increase of PC content in the brains of L and H mice (in comparison with untreated animals) clearly shows the occurrence of oxidative stress in these animals (Figure 7). Interestingly, the rescue of GSHtot levels (Figure 7A) and the almost complete inhibition of PC accumulation in

the brain cortex of CLA+L or CLA+H mice denotes the preventive/protective ability of CLA (Figure 7B).

A significant increase of AChE activity was dose-dependently associated with $AlCl_3$ treatment in comparison with Cntr ($p < 0.03$). Post-hoc comparisons revealed that AChE activity in the brains of L or H animals increased (of 25 or 41%, respectively) when compared to untreated animals. Enzyme activity measured in the CLA+H group was comparable to that measured in CLA+L animals ($p = 0.38$) (Figure 7C). Next, a significant negative correlation between GSHtot concentration AChE activity ($p = 0.02$; $r = 0.49$) (Figure 7D) further validates the reported link between AChE and redox homeostasis [117]. On the contrary, no significant correlation resulted between PC concentrations and AChE levels (data not shown), most likely because of the wide fluctuation of PC values in Al-treated mice.

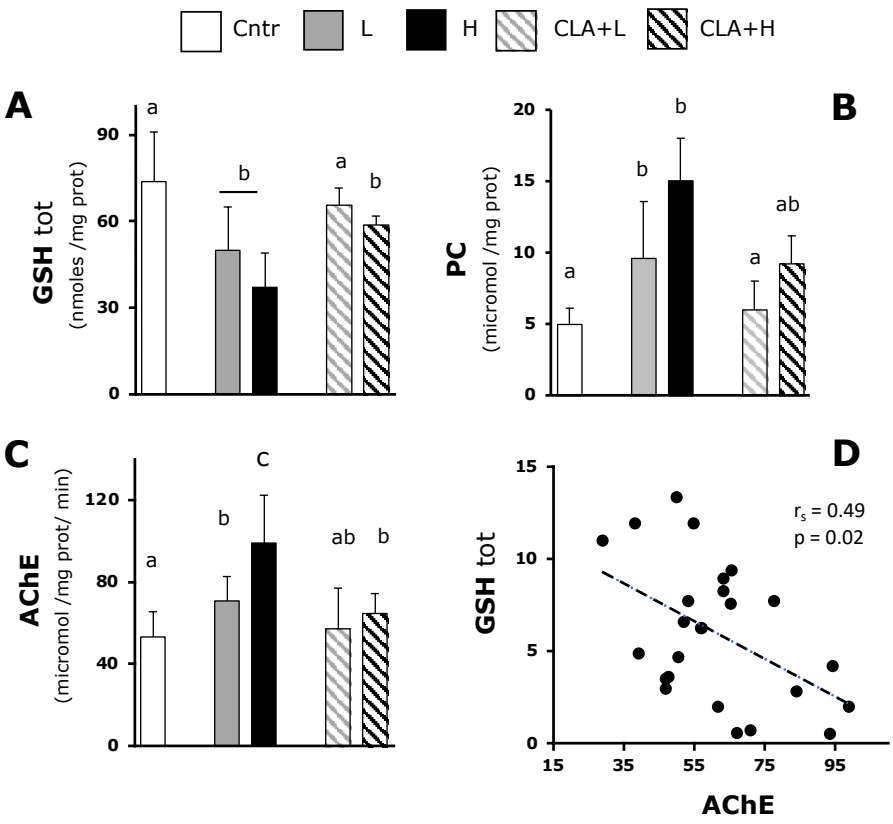


Figure 7. CLA protects against $AlCl_3$-induced oxidative stress and AChE hyperactivation. Total GSH and the accumulation of oxidatively modified proteins (PC) were used to evaluate the oxidative distress index (A, B). AChE

activity in the brain cortex of differently treated mice and its correlation with GSHtot concentration are shown in panels C and D. The results are expressed as the means ±SD from at least duplicate analyses from n= 6 animals/group. Differing superscript letters indicate statistically significant differences ($p < 0.05$).

6.7. CLA intake increases glucose transporter levels in H-treated animals.

Glucose transport proteins (GLUTs) play an important role in the protection against induced oxidative stress and there are data suggesting that the alteration of GLUT1 and GLUT3 expression represents an early event in the AD pathogenesis [118]. By RT-PCR, we detected a significantly higher GLUT1 mRNA level in L and H animals in comparison with Cntr, while only H mice exhibited GLUT3 levels higher than Cntr animals. Interestingly, GLUT1 and GLUT 3 levels in CLA+H animals were markedly higher in comparison with $AlCl_3$-treated or untreated animals (Figure. 8A).

GLUT1, GLUT3 and GLUT4 protein levels were examined in Cntr, H and CLA+H mice to evaluate the protective effect of CLA, since H – among the 2 $AlCl_3$ doses – showed a more marked alteration of GLUTs mRNA. Unlike RT-PCR data, H treatment was associated with the marked decline of GLUT1 protein, and its amount was only tendentially increased by CLA (CLA+H group). GLUT3 expression was not modified by H-treatment while in these animals GLUT4 level was significantly higher when compared with untreated animals (Cntr) ($p = 0.043$). Notably, GLUT3 and GLUT4 levels in the CLA+H group were significantly higher than those found in H or Cntr animals ($p < 0.05$; Figure 8B).

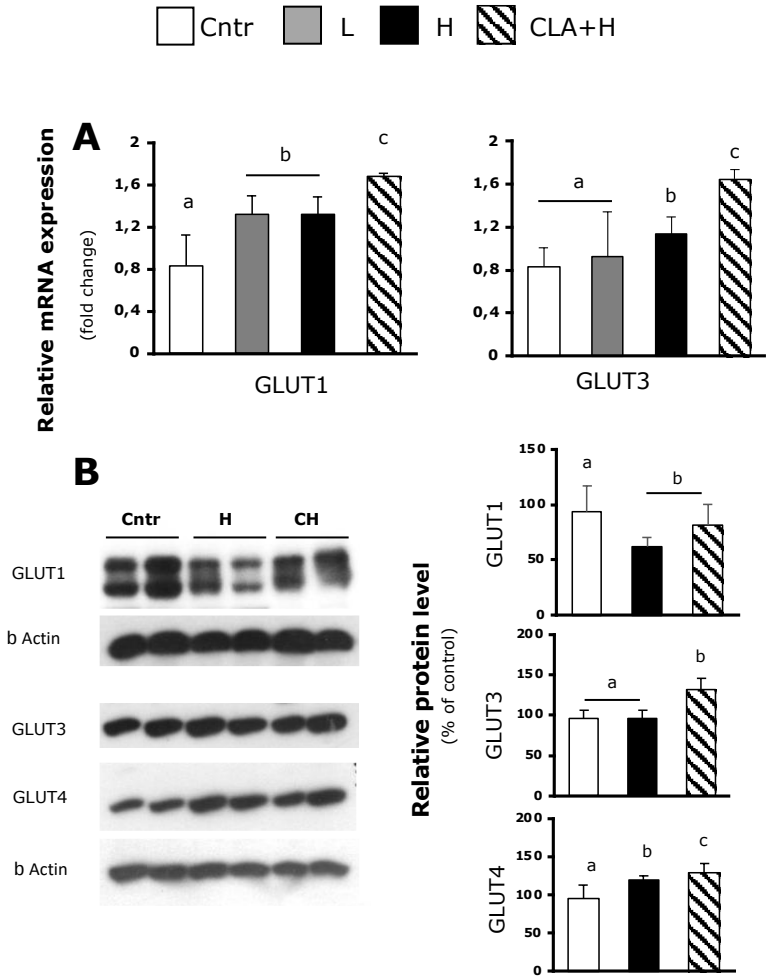


Figure 8. CLA treatment enhances GLUT levels in the brains of H-treated mice. A) mRNA levels of GLUT1, GLUT3 were measured by RT-PCR; B) Brain cortex proteins were fractionated on 8% (GLUT1) or 12% SDS-PAGE (GLUT3, and GLUT4) and glucose transporter levels were evaluated by Western blotting. Representative blots are shown (left) and band intensities calculated by quantitative densitometry and GLUT amount were finally expressed relative to β-actin level (right). Data are reported as mean ±SD of at least five animals per group (n=5). Differing superscript letters denote statistically significant differences ($p < 0.05$).

6.8. $AlCl_3$-induced pro-inflammatory activity is prevented by CLA intake.

Oxidative stress and inflammation are closely related to AD pathogenesis, where one can be easily induced by the other. In consideration of the pro-oxidant activity of $AlCl_3$ and the protective effect of CLA, pro-inflammatory chemokines/cytokines levels in $AlCl_3$ -

treated mice were finally evaluated. The significant increase of IL-1β, IL-6 and CXCL-1 mRNA levels in $AlCl_3$-treated mice is shown in Figure 10. Since a more marked increase corresponded to H intake, the protective effect of CLA was only measured in H-supplemented mice receiving CLA (CLA +H). Animal treatment with L was associated to a significant induction of IL-1β, IL-6 levels, as compared with Cntr ($p < 0.05$) (Figure. 9A, B). Levels of IL-1β, IL-6 and CXCL-1 significantly higher than those found in untreated mice (where $p < 0.05$ and $p < 0.01$) were measured in the cortex of H-treated animals. Notably, CLA intake prevented the onset of pro-inflammatory markers (IL-1β, IL-6, CXCL-1) in the brain tissue of H-treated mice (Figure. 9A, B, D). TNFα levels were increased in $AlCl_3$-treated animals (although in a not significant fashion) while CLA intake slightly reduced these levels (Figure. 9C).

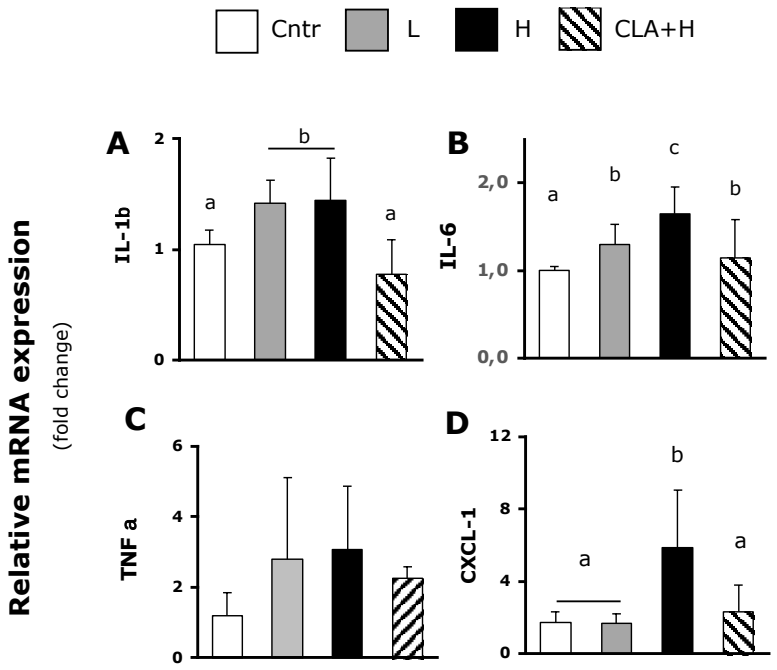


Figure 9. Pro-inflammatory activity triggered by H treatment is hindered by CLA. IL-1β, IL-6, TNFα, and CXCL-1 levels were measured by RT-PCR in the brain cortex of Cntr, L, H and CLA+H groups. Values, calculated as fold change in gene expression (Absolute Units) were expressed as mean ±SD by at least six animals per group (n=6). Differing superscript letters denote statistically significant differences ($p < 0.05$)

According to the data just shown, the short-term administration of L or H doses, albeit with different intensity, triggers the onset of oxidative-stress hallmarks of human AD (e.g. mitochondrial dysfunction, Nrf2 alteration, pro-inflammatory cytokines, AChE hyperactivation). These results indicate, for the first time, the neuroprotective effect of CLA in this animal model of AD, through the downregulation of oxidative-stress hallmarks and the decrease in the extent of neuronal degeneration.

Next, since the obtained data showed the neuroprotective ability exerted by CLA through the activation of Nrf2 pathway, we performed further experiments to deepen our understanding about the pathways underlying the protective effect against the oxidative damages inflicted by the high dose $AlCl_3$.

6.9. CLA-induced activation of NOX is associated with an adaptive response which prevents the compensatory upregulation of Nrf2 in the brain of $AlCl_3$-treated mice.

The activation of a Nrf2-mediated adaptive response was hypothesized to be responsible for the neuroprotective ability associated with the intake of CLA, but the underlying molecular mechanism has not been investigated. To this aim, an animal group - treated with CLA alone – was used to evaluate the involvement of NOX activation in the modulation of Nrf2-activated cytoprotection in the mouse brain cortex.

The modulatory effect of CLA treatment on brain NOX activity is shown in Figure 10A. Notably, CLA intake has only minor effects on GSH concentration and GST activity (Figure 10B) but it modulates GSR and G6PD activity as compared to untreated mice (Figure 10C).

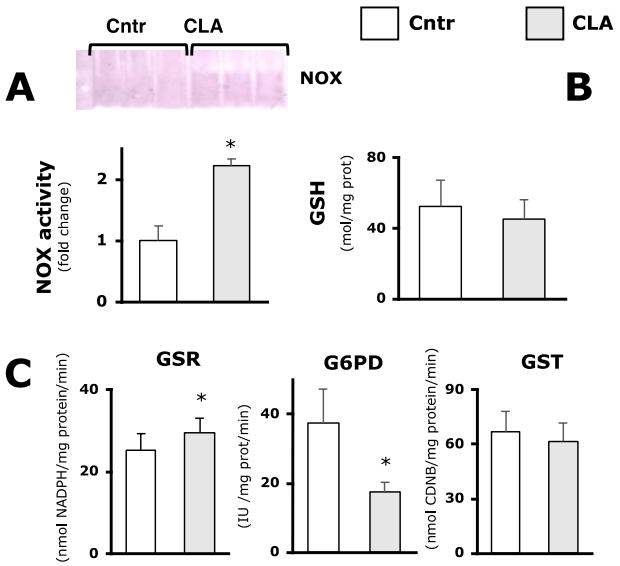


Figure 10. CLA enhances NOX activity and modulates phase 2 enzyme activity in the mouse cerebral cortex. Representative zymography of NOX activity in mouse cortex of untreated and of CLA-treated mice A (upper panel). Upon densitometric analysis, band intensity was finally expressed as an average fold increase as compared with untreated animals (Cntr) (A, lower panel).

GSH concentration (B) along with the activity of several phase 2 enzymes (GSR, G6PD, GST) (C) were measured in the brain cortex of Control and CLA-treated animals. Each bar represents the mean values ± SD from a duplicate analysis of at least 5 different animals (n=5). Asterisks indicate the presence of statistically significant differences ($p < 0.05$) when compared with Cntr.

Interestingly, the modulatory efficacy of CLA on phase 2 enzymes was further supported by the significant improvement of GSR, G6PD and NQO1 activity in liver tissue in comparison with that measured in the control group ($p<0.05$) (Figure 11).

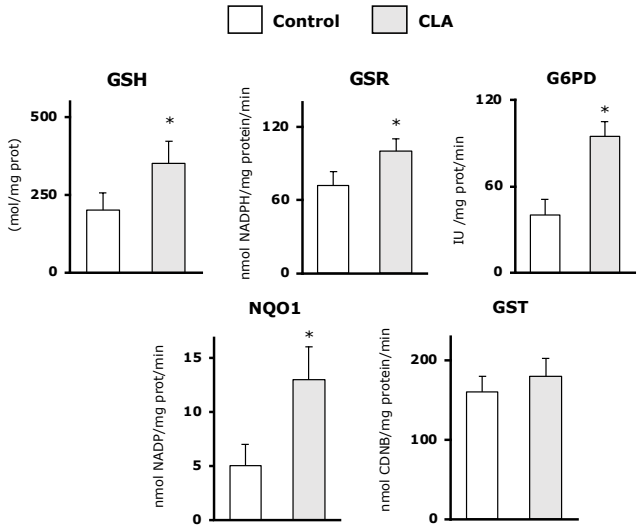


Figure 11. Dietary intake of CLA increases GSH concentration and improves phase 2 enzyme activity in the mouse liver.

These results indicate, for the first time, that a “late” mode of Nrf2 activation - likely triggered by NOX upregulation - may underlie the CLA's ability to activate an adaptive response which is likely accountable for the preventive activity against the harmful effects triggered by $AlCl_3$ intake.

6.10. CLA supplementation has only minor effects on the H-induced hyperactivation of NOXs levels/activity.

NOXs are the major cellular sources of intracellular ROS [119], thus we decided to evaluate the NOX involvement in the pro-oxidant activity of $AlCl_3$.

The results obtained from gel zymography confirmed the ability of $AlCl_3$ to significantly increase NOX activity in the murine cortex compared to control animals ($p = 0,0006$) (Figure 12A). The expression level of NOX-1 and NOX-2 protein was evaluated in the cortex of differently treated mice to investigate further NOX modulation by the treatment with $AlCl_3$ or with CLA+H. Data in Figure 12B indicate a significant upregulation of NOX-1 and NOX-2 levels in brain cortex of

AL-treated mice ($p < 0.05$) while CLA intake only produced the tendential decline of NOX activity ($p=0.07$) and the undetectable alteration of the NOX protein expression in comparison with AL-treated mice (Figure 12A, 12B).

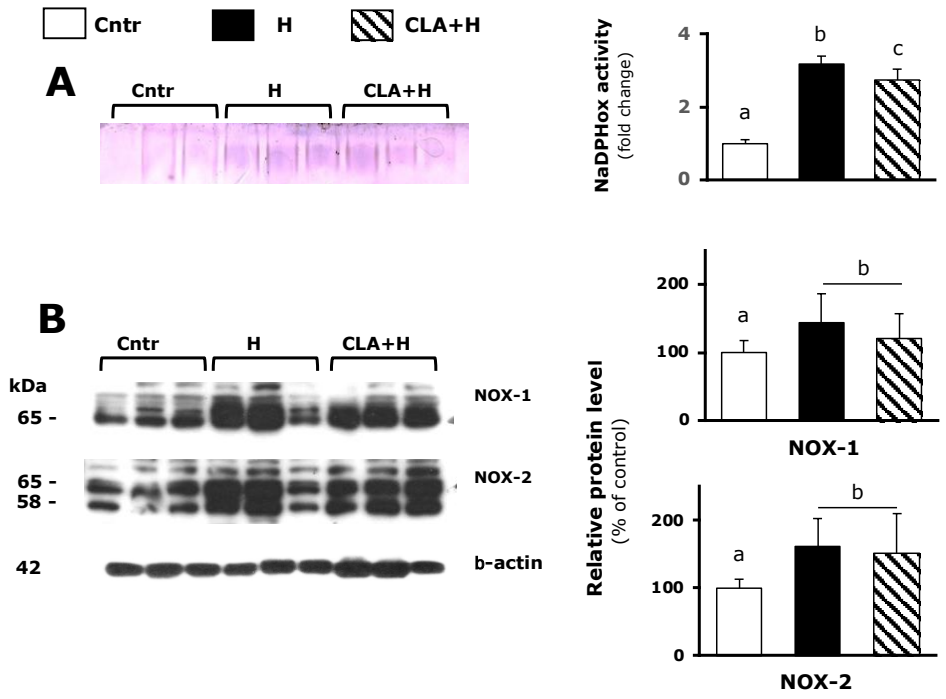


Figure 12. The antioxidant effect of CLA in the mouse cerebral cortex does not act through modulation of $AlCl_3$-induced hyperactivation of NOX activity/levels. Representative zymography showing NOX activity is shown in A (upper panel). The bands were quantified using densitometric analysis and the obtained values were finally expressed as average fold increase as compared with control samples (A, lower panel). Representative immunoblot of NOX-1 and NOX-2 expression in brain cortex of control and treated mice (AL or CLA+H) (B, upper panel). When quantitative densitometry was carried out, band intensities were calculated, and NOX1 and NOX2 levels were normalized to the β-actin level and finally expressed as a per cent change (%) in the Control samples (B, lower panels). Each bar represents the mean values ±SD from a duplicate analysis of at least 5 different animals (n=5). Data are reported as means ± SD of the mice group and asterisks indicate the presence of statistically significant differences ($p< 0.05$).

6.11. CLA downregulates the $AlCl_3$-induced hyperactivation of the UPR response in the mouse brain cortex.

Oxidative distress can disrupt the protein folding mechanism in cellular ER thus increasing the yield of misfolded proteins, leading to upregulation of compensatory pathways (Nrf2 and UPR) aimed at restoring redox homeostasis and ER function [120]. Based on data indicating the CLA efficacy in preventing the $AlCl_3$-induced alteration of cortex redox status and of Nrf2 hyperactivation [98] we hypothesized that the CLA may also modulate the $AlCl_3$-induced upregulation of UPR. To confirm this hypothesis and to investigate the noxious effects of $AlCl_3$, Western Blotting and RT-PCR experiments were carried out to evaluate the modulatory ability of CLA on $AlCl_3$-induced increase of UPR signalling (on both protein and mRNA levels).

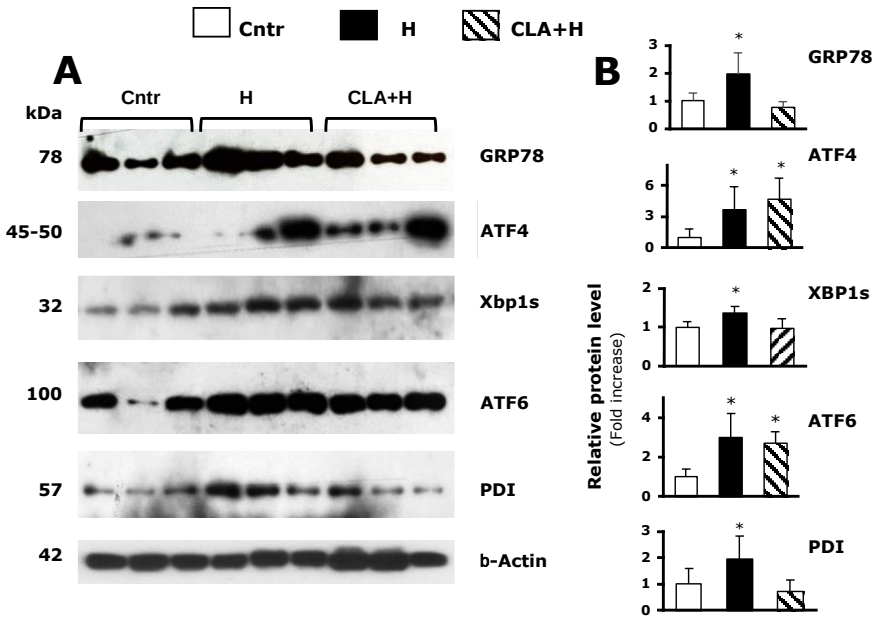


Figure 13. CLA downregulates the $AlCl_3$-induced hyperactivation of the UPR pathway in mouse brain cortex. Representative immunoblot of UPR markers (GRP78, ATF4, ATF6, Xbp1s, PDI) expression in brain cortex of untreated (Cntr), Al and CLA+H supplemented mice (A panel). Quantitative densitometry was used to calculate their relative content, upon normalized with the β-actin level, were finally expressed as fold increase in comparison with Cntr samples (B panel). Data are reported as mean ± SD of at least 5 animals per group (n=5). The asterisks denote the statistically significant differences with the Control group ($p<0.05$).

Figure 13 shows the significant hyperexpression of all the considered UPR protein levels (GRP78, Xbp1s, ATF4, ATF6, and PDI) in the cortex samples of $AlCl_3$-treated mice in comparison with the untreated group ($p < 0.01$). Notably the CLA treatment of $AlCl_3$-treated mice (CLA+H), with the only exception of ATF4, markedly downregulates GRP78, Xbp1s, ATF6 and PDI protein expression ($p < 0.05$) reaching levels comparable to that found in untreated animals (Figure 13A, B).

To further examine the modulatory role played by H and by CLA+H treatment on the UPR pathway, the mRNA levels of selected factors (namely GRP78, ATF4, Xbp1s, ATF6) were evaluated by qRT-PCR analysis. The obtained data indicates the $AlCl_3$ ability to significantly upregulate GRP78, Xbp1s, and ATF4 mRNA levels in the cortex of H-treated mice in comparison with untreated mice ($p = 0.0007$, $p = 0.008$ and $p = 0.006$, respectively) (Figure 14 A, B, C) while ATF6 expression was not modified by $AlCl_3$ treatment when compared with untreated animals (Figure. 14 D). In CLA+H animals, with the only exception of ATF4 expression, the transcript levels of GRP78 and Xbp1s was further increased in comparison with H-treated animals ($p < 0.01$).

The obtained results indicate that $AlCl_3$ hyperactivates the UPR signalling in the mouse brain cortex (at both protein and mRNA levels) and that CLA intake exhibits different modulatory effects on this compensatory mechanism.

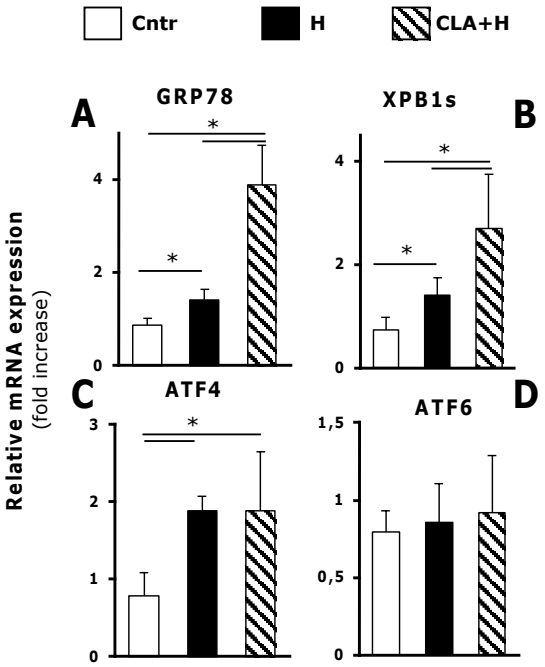


Figure 14. Treatment with CLA further increases the mRNA levels of several UPR sensors in the cerebral cortex of Al-treated mice. mRNA levels of GRP78 and three UPR sensors (ATF4, XBP1s, ATF6) in the brain cortex of differently treated animals are shown. RT-PCR analysis was carried out on at least 5 animals per group and the relative mRNA expression and after normalizing to the L-32 housekeeping gene, the data were presented as fold increase (± SD) in comparison to the control group. Each bar represents the mean values ±SD from a duplicate analysis of at least 5 different animals (n=5). The asterisks denote the statistically significant differences ($p < 0.05$).

6.12. CLA downregulates the maladaptive response triggered AL.

As insufficient UPR compensation, may be followed by the activation of a maladaptive response (driving inflammation and apoptosis events), thus the protein expression of pro- and anti-apoptotic proteins (Bax and Bcl-2, respectively) and the mRNA level of pro-inflammatory cytokines were measured in the brain cortex (Figure 15).

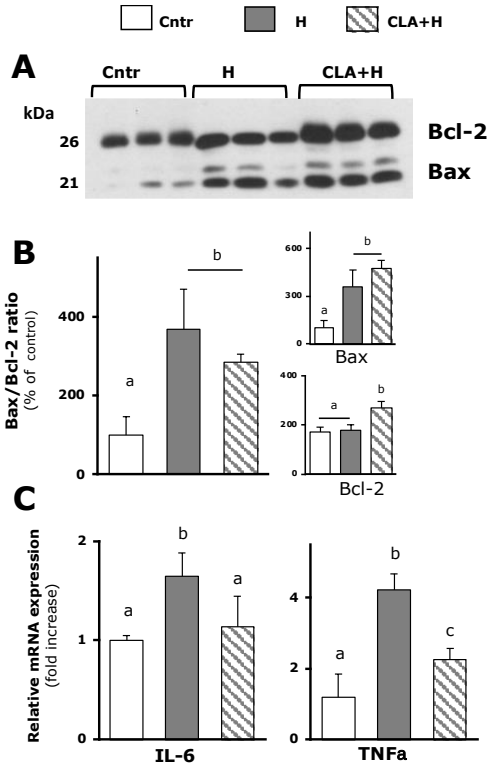


Figure 15. Animal co-treatment with CLA upregulates antiapoptotic Bcl-2 protein and reduces the level of pro-inflammatory cytokines in mouse brain cortex. Representative immunoblot showing Bcl-2 and Bax expression in brain cortex of untreated (Control), H and CLA+H supplemented mice are shown (A). Quantitative densitometry was used to calculate the fold increase in comparison with control samples and the Bax/Bcl-2 ratio) (B). mRNA levels of pro inflammatory cytokines (IL6, TNFα) in the brain cortex of differently treated animals are also shown (C). Data are reported as mean ± SD of at least 5 animals per group (n=5). Asterisks denote the statistically significant differences ($p < 0.05$).

Results in Figure 15 A show the marked increase of the proapoptotic protein Bax in the cortex of H- and CLA+H treated mice in comparison with the Control group ($p= 0.001$). The marked increase of the anti-apoptotic protein Bcl-2 level was found in the CLA+H group ($p < 0.01$) (Figure. 15 A upper panel) and, when it was used to calculate the Bax to Bcl-2 ratio, its level in the brain of CLA+H mice was significantly lower that of H animals (Figure. 15 A lower panel). The PVDF membrane used for Bax and Bcl-2 immunoblotting was finally stained with Coomassie for evidence of the equal loading of protein (Figure 15). Finally, the neuroprotective effect of CLA against the $AlCl_3$-induced maladaptive response was also indicated by the significant

decline of the mRNA levels of proinflammatory cytokines (IL-6, TNFα) in the brain cortex of CLA+H-treated mice in comparison that found in H-treated animals (Figure. 15B).

These results first showing the $AlCl_3$ efficacy in triggering a maladaptive response in the brain tissue and the marked decline produced by the CLA administration further support its neuroprotective ability.

7. Discussion

Our study indicates, for the first time, the neurotoxic effects of short-term administration of $AlCl_3$ even at an L dose. The results also demonstrate the $AlCl_3$ ability to trigger a set of pathological markers associated with AD, such as mitochondrial dysfunction, Nrf2, UPR, AChE, apoptosis and inflammation hyperactivation in the mouse brain cortex.

Notably, the presented data indicates the neuroprotective effect of dietary CLA intake against several pathological hallmarks (mitochondrial dysfunction, AChE activity, oxidative stress, inflammation, GLUT expression, of neuronal degeneration) in an animal model of AD. More interestingly, the modulatory effect of CLA on Nrf2-activated defences in the brain of healthy mice is suggestive of an adaptive response to its neuroprotective effects. In conclusion presented results provide additional support to the notion that therapies targeting Nrf2 hold promise in the treatment/prevention of neurodegenerative diseases.

The challenge in accurately assessing the impact of $AlCl_3$ in the animals is determined by the lack of reliable data on human $AlCl_3$ intake from various environmental sources. The figures provided by the Tolerable Weekly Intake (TWI) are unreliable since they could underestimate the actual exposure. In our first study we wanted to evaluate the different neurotoxicity produced by two very different doses of $AlCl_3$

respectively 4 or 400 times higher than the TWI. In this context, we demonstrated the dose-dependent neurotoxicity of $AlCl_3$ and we underline both the notable toxicity of the lowest dose and the protective effects of CLA against $AlCl_3$ toxicity.

Even if it represents the most distinctive hallmark of AD, our study does not provide the evaluation of amyloid-beta accumulation, as the CLA c9 isomer's ability to reduce it in AD animal models has been previously demonstrated by [121, 122]. Instead, our study focuses on assessing neuronal damage in brain cortex sections, which we found to be significant both with L and H exposure. Interestingly, the similar cerebral cortex architecture observed in the CLA+ L group and the control (Cntr) group suggests a preventive effect of CLA against $AlCl_3$-induced neuronal degeneration, accordingly to existing literature data [123, 124].

Previous studies associated AD with brain oxidative damage related to mitochondrial dysfunction. [125] We discovered that L and H have a notable impact on oxygen consumption during the resting phase of mitochondrial respiration. This observation suggests the occurrence of a disruption in the coordination between the transfer of electrons through respiratory complexes and the synthesis of ATP. Since respiratory complexes I and III are pivotal in maintaining a balance between mitochondrial bioenergetics and the homeostasis of ROS, a decrease in mitochondrial respiration efficiency is often linked to an elevated production of reactive species [126]. Notably, our results highlight the specific involvement of complex III in the pathological processes induced by Al. The correlation between the decline in complex III activity and the onset of oxidative stress markers (such as a decrease in total glutathione and the accumulation of protein carbonyls) in both L and H dose-treated mice underscores the pathological role played by this mitochondrial complex. This study is the first to investigate the effect of CLA on brain mitochondria. We found that CLA can restore complex I and improve complex III and IV

activities, as well as mitochondrial function, even in H treated mice. However, considering the role of the Nrf2 pathway in regulating mitochondrial function in liver tissue [127], it is possible that the involvement of Nrf2 cannot be completely excluded in the ability of CLA to rescue $AlCl_3$-induced dysfunction.

The neuronal death observed is coherent with the dose-dependent increase of AChE activity, being this enzyme able to induce apoptosis [128]. AChE activity is a recognized marker of AD progression, and it has been extensively demonstrated its link with redox homeostasis, as its inhibition in vitro determines high levels of Haeme-oxygenase, a phase II enzyme [129]. The increased GSHtot content we found in the mice brains further confirms this negative correlation and supports the hypothesis that a CLA-mediated modulation of the redox state/Nrf2 pathway is involved in the downregulation of AChE activity, which otherwise is significantly increased in H- mice brain.

Being Nrf2 a key role player in the modulation of cellular redox homeostasis, we proceeded with the evaluation of the phase II enzymes activity in this chemically induced AD model. Our data show a marked inhibition of several phase II enzymes activity (GSR, G6PD, and GST) and an increase of GPx and GCLc protein levels in CLA+H mice in contrast with H treated mice, remarking the activation of an Nrf2 adaptive response exerted by CLA. However, the mRNA levels of the mice brain do not allow us to fully assess the alteration produced by the treatment with Al or the protective effect of CLA.

Al-induced hyperactivation of G6PD is consistent with the onset of oxidative stress, but its hyperactivity may also be functional in modulating glucose metabolism. Glucose is typically metabolized by G6PD through the pentose phosphate pathway to prevent NADPH loss and maintain reduced glutathione levels. Considering that the diffusion of glucose by GLUT transporters relies on a concentration gradient between the blood and brain [130], it can be speculated that increased G6PD activity in the brain of Al-treated mice may aim to

enhance glucose metabolism and facilitate glucose uptake from the bloodstream. However, further studies are required to confirm this hypothesis. These findings are consistent with the reported interplay between Nrf2/G6PD activation and glucose metabolism [131]. Additionally, the ability of CLA to increase GLUT1, GLUT3, and GLUT4 expression at both the RNA and protein levels is in good accordance with the efficacy of certain Nrf2 activators such as metformin in improving GLUT1 expression [132], as well as the positive correlation between Nrf2 efficiency and GLUT1 and GLUT4 levels [133]. The significant increase in GLUT levels in CLA +H mice, compared to the H or Cntr groups, strongly supports the positive association between redox homeostasis/Nrf2 function and glucose metabolism. Moreover, considering the therapeutic role of increased GLUT levels [134], these findings suggest CLA as a promising agent for a multi-target therapy useful for the treatment and prevention of AD.

The unfolded protein response (UPR) is a cellular process that plays a key role in restoring cellular homeostasis when perturbed by the accumulation of potentially toxic misfolded proteins [135]. There is growing evidence indicating the relevance of ER stress/UPR pathway in the pathogenesis of AD [136]. In the brain tissue of rodent models of AD, the accumulation of GRP78, a major contributor to protein quality control in the ER, has been used as a theranostic biomarker of ER-stress/UPR upregulation [137]. The high affinity of GRP78 for misfolded proteins triggers the activation of downstream UPR signaling pathways, including PERK, ATF4, and IRE-1, which aim to maintain protein homeostasis and cell viability [138]. PERK and IRE-1 function as translational and post-translational modifiers, while XBP1s, ATF6, and ATF4 translocate to the nucleus and act as transcription factors [139]. Increased expression of ATF4, ATF6, and XBP1s has shown protective effects against the accumulation of amyloid precursor protein (APP) or amyloid proteins, indicating their potential roles in AD pathogenesis [140, 141].

PDI, whose levels are modulated via the IRE-1 – Xbp1s axis [142], promotes disulphyde bridge formation in the ER, thereby facilitating protein folding. As ROS are generated during oxidative protein folding, the rearrangement and formation of disulphyde bonds using PDI activity may help re-establish redox homeostasis. Overexpression of PDI has been associated with apoptosis, while its inhibition has shown neuroprotective effects in models of Huntington's disease [143]. The accumulation of GRP78, ATF4, ATF6, XBP1s, and PDI proteins, as well as the upregulated mRNA levels of GRP78, ATF4, and XBP1s, in the brain cortex of H-treated mice suggest the hyperactivation of the UPR pathway by Al and provide further insight into this animal model of AD. Similarly to the UPR, the hyperactivation of phase 2 enzyme activity in the cortex of H-treated mice may be considered an adaptive response. Overall, the hyperactivation of both Nrf2 and UPR in the brains of AL-treated mice is not unexpected, considering the compensatory role and bidirectional functioning of the Nrf2-UPR axis [144].

Our study demonstrates the efficacy of CLA against UPR hyperactivation for the first time. The protein levels of UPR markers/chaperones, including GRP78, Xpb1s, ATF6, and PDI, significantly declined in the brains of CLA-AL treated mice. However, despite the downregulation of protein levels, the mRNA levels of these UPR markers in CLA+H-treated mice remained elevated. This could be due to the longer duration of ER-stress quality control mRNA compared to the recovery of unfolded protein load, suggesting a shift in the activation/decline times of mRNA encoded UPR proteins in response to CLA treatment. On the other hand, CLA does not contribute to the stimulation of UPR genes as it does not trigger stressing effects. Moreover, the modulatory role of CLA on UPR and Nrf2-activated phase 2 enzyme activity/level supports the functional link between Nrf2 and UPR pathways, indicating Nrf2's involvement in preserving ER integrity and protein homeostasis.

Apoptosis and inflammation are cellular processes interconnected with the alteration of cellular mechanisms like ER/UPR occurring, albeit to a different extent, in neurodegenerative diseases but also in normal ageing [145]. In particular, insufficient UPR compensation or prolonged stress is known to be responsible for maladaptive signalling [146] which includes inflammation and programmed cell death. Inflammation plays a significant role in the brain pathology of AD and is known that pro-inflammatory cytokines such as IL-1β, IL-6, TNFα, and IFN-γ can contribute to the formation of amyloid plaques [147]. IL-1β, in particular, is an early upregulated pro-inflammatory cytokine in AD patients, its level remains elevated as the disease progresses and specific polymorphisms associated with higher IL-1β production have also been linked to an increased risk of AD [148]. IL-6 is a multifunctional cytokine that can exhibit either pro-inflammatory or anti-inflammatory effects, depending on the amount and conditions under which it is released. It plays a crucial role in the normal homeostasis of neuronal tissue. Insufficient IL-6 signalling leads to reduced microglial activation, while overproduction of IL-6 leads to chronic neuroinflammation and neurodegeneration [149].

The increased levels of IL-1β and IL-6 transcripts observed in L and H animals are consistent with clinical data reporting their elevated expression in the cortex of AD patients [150]. This study is the first to demonstrate the anti-inflammatory efficacy of CLA in brain tissue, aligning with recent clinical data [151] and the recognized immune-modulatory effects of CLA.

Apoptosis is associated with AD pathogenesis and can activate various proteins such as Bax, Bad, Bid, Bcl-XS, Bcl-XL, and caspases. This cascade of events ultimately leads to apoptosis. The simultaneous increase in mRNA levels of pro-inflammatory cytokines and the upregulation of the pro-apoptotic protein Bax in the brains of H-treated mice led to the hypothesis that $AlCl_3$ has the potential to induce a maladaptive response in the UPR pathway. Specifically, the

significant decrease in cytokine levels and the notable increase in the anti-apoptotic protein Bcl-2 align with the downregulating effects that CLA exhibits on the molecular mechanisms preceding the onset of these neurotoxic effects, such as oxidative distress and upregulation of compensatory mechanisms.

NADPH oxidases (NOXs) and mitochondria are the major sources of intracellular ROS production. Next, part of this work was aimed at evaluating the NOX involvement in the pro-oxidant activity of $AlCl_3$ and in the adaptive response elicited by CLA intake.

Being $AlCl_3$ able to induce oxidative damage, we investigated its ability to stimulate pro-oxidant (NOX) [106] or to upregulate ER stress/UPR responses. The results obtained indicate that NOX is, at least in part, involved in the modulatory activity of CLA on Nrf2-mediated redox homeostasis (eustress) in the mouse brain cortex likely via the activation of an adaptive response. On the contrary, NOX hyperactivation in $AlCl_3$-treated mice, was associated with the upregulation of oxidative distress markers (GSH depletion accumulation of oxidatively-modified proteins).

Our data suggest that AL intake is associated with the upregulation of NOX activity/NOX1-NOX2 expression and the downstream hyperactivation of compensatory mechanisms (UPR, Nrf2), as well as the overexpression of pro-apoptotic/pro-inflammatory markers. It is worth noting that CLA did not effectively downregulate NOX levels, which might contrast with its beneficial effects. This observation is consistent with literature data highlighting the prominent role of mitochondrial dysfunction in the neurotoxic effect of $AlCl_3$ [111].

These results indicate that the pro-oxidant mechanism triggered by H exposure is accompanied by the upregulated NOX activity/level. Data reporting the lack of modulatory efficacy of CLA on this cellular mechanism suggested that NOX modulation is not involved in the neuroprotective effect of CLA. Additionally, the modulatory role of food-derived Nrf2 activators on mitochondrial function [152] strongly

suggests the major role played by mitochondria in the neuroprotective effect of CLA.

CLA's potential to induce an adaptive response through Nrf2 activation has been previously suggested to contribute to its neuroprotective effects [153]. However, our study is the first to demonstrate the association between NOX activation and the modulatory effects on phase II enzyme activity in the brains of CLA-treated mice. Interestingly, the different effectiveness of CLA in inducing phase 2 enzyme activity in the brain and liver tissues is not entirely unexpected, as different organs may have different vulnerability and responsiveness to alterations in redox homeostasis [154].

Conclusions

In conclusion, drugs capable of modulating multiple therapeutic targets are considered the best pharmacological strategy for the treatment of multifactorial diseases such as AD. Our data confirm the ability of CLA to downregulate multiple pathological mechanisms (mitochondrial dysfunction/damage, oxidation protein, decreased AChE activity and inflammation) or show a modulatory ability on glucose metabolism, suggesting its suitability as a drug candidate for the treatment of AD. It can be assumed that the choice of Nrf2 as a therapeutic target for the treatment of multifactorial diseases, such as neurodegenerative ones, is consequent to the high number of genes modulated through this nuclear factor. These genes belong to interconnected pathogenic processes (oxidative distress, UPR hyperactivation, apoptosis inflammation) activated by AL intake in the mouse brain cortex. More interestingly, the modulatory ability of CLA on AL-induced derangement of the UPR pathway in this animal model of chemically induced AD was herein demonstrated for the first time. In particular, since oxidative distress plays an important role in the

onset/progression of the pathogenesis of AD, it is conceivable that a prompt reactivity of the brain defences (probably triggered via an adaptive response) could attenuate the intensity of the pathological phenomena occurring downstream this stressful/triggering event. In this context, since it has been hypothesized that the lack of clinical efficacy of dietary antioxidants against AD is caused by the use of molecules that target a single mechanism [144], Nrf2 activators through the activation of a pleiotropic mechanism or via the improvement of the endogenous mechanisms responsible for maintaining the oxidoreductive homeostasis of the brain may have greater therapeutic efficacy on AD. It is possible to hypothesize that chemical instability, the low bio-availability/solubility of many Nrf2 activators used so far in many in vivo studies, along with the non-allometric conversion of the dose, could explain the discouraging results obtained so far in clinical studies [155]. Finally, it can be hypothesized that the use of an allometric dose (HED) of a stable, bioavailable and safe Nrf2 activator such as CLA could ensure greater translation success. More clinical studies are however needed to support this hypothesis.

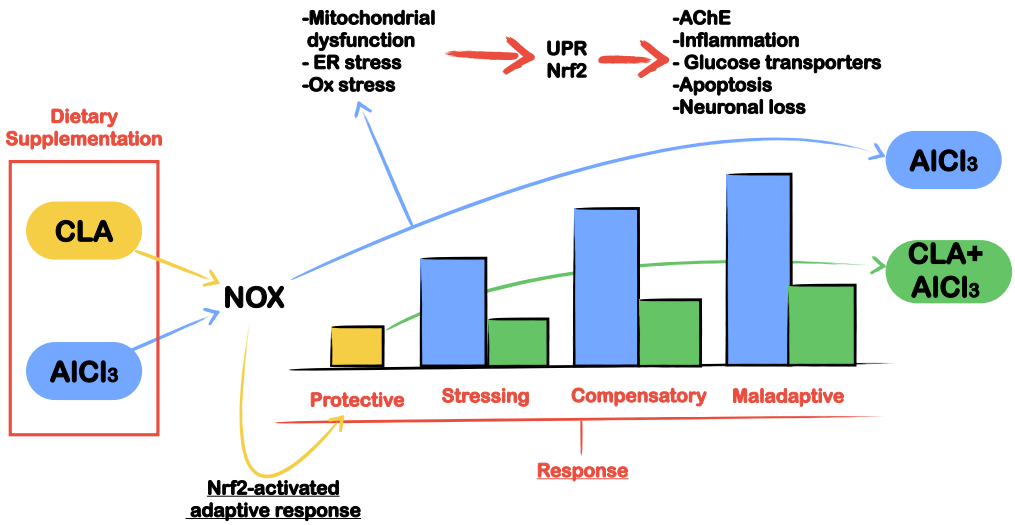


Figure 17. Graphical abstract

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