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Ozone: a natural bioactive molecule with antioxidant property as potential new strategy in aging and in neurodegenerative disorders

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Abstract

Systems medicine is founded on a mechanism-based approach and identifies in this way specific therapeutic targets. This approach has been applied for the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Nrf2 plays a central role in different pathologies including neurodegenerative

disorders (NDs), which are characterized by common pathogenetic features. We here present wide scientific background indicating how a natural bioactive molecule with antioxidant/anti-apoptotic and pro-autophagy properties such as the ozone (O₃) can represent a potential new strategy to delay neurodegeneration. Our hypothesis is based on different evidence demonstrating the interaction between O₃ and Nrf2 system. Through a meta-analytic approach, we found a significant modulation of O₃ on endogenous antioxidant-Nrf2 ($p < 0.00001$, Odd Ratio (OR) = 1.71 95%CI:1.17-2.25) and vitagene-Nrf2 systems ($p < 0.00001$, OR = 1.80 95%CI:1.05-2.55). O₃ activates also immune, anti-inflammatory signalling, proteasome, releases growth factors, improves blood circulation, and has antimicrobial activity, with potential effects on gut microbiota. Thus, we provide a consistent rationale to implement future clinical studies to apply the oxygen-ozone (O₂-O₃) therapy in an early phase of aging decline, when it is still possible to intervene before to potentially develop a more severe neurodegenerative pathology. We suggest that O₃ along with other antioxidants (polyphenols, mushrooms) implicated in the same Nrf2-mechanisms, can show neurogenic potential, providing evidence as new preventive strategies in aging and in NDs.

Keywords: Neurodegenerative Disorders, nuclear factor (erythroid-derived 2)-like 2 (Nrf2), Ozone (O₃), Oxygen-Ozone (O₂-O₃) therapy, antioxidant system, stress oxidative biomarkers

1. Introduction

Life span has almost doubled in the last century ([WHO, 2011](#), [Wyss-Coray, 2016](#)), and consequently aging-specific diseases are becoming prevalent ([Moskalev et al., 2017](#)). However, the pathophysiologic mechanisms underlying most of them are still poorly understood and challenges regarding treatments efficacy and costs persist.

Neurodegenerative diseases (NDs, Alzheimer's disease, AD; Parkinson disease, PD; amyotrophic lateral sclerosis, ALS, Huntington Disease, HD) are the most prevalent cognitive and motor disorders of the elderly. These aging-specific diseases are characterized by the loss of homeostasis during aging, leading to low-grade stress by pathologic formation of Reactive Oxygen Species (ROS), chronic inflammation, mitochondrial dysfunction and metabolic unbalance ([Dugger, Dickson, 2017](#)). In addition, these pathophenotypes are determined by abnormal aggregation of specific proteins ([Yanar et al., 2020](#)), given the connection between excessive ROS accumulation and impairment in proteostasis network.

Despite their distinct causative factors and clinical symptoms, these diseases as well as aging have common pathogenetic features ([Aso et al., 2012](#)). This implicates potentiality in the identification of therapeutic targets on a set of disease phenotypes and physiological conditions that are mechanistically linked. Thus, contrary to a hitherto linear approach that considered one disease, one medicine, to date there is a need for a new concept of therapy condensed as "several diseases, one medicine". In this way, diseases are diagnosed not only by clinical symptoms, but mainly by the underlying molecular signatures ([Goh et al., 2007](#)). Based on this network medicine approach, [Cuadrado et al., 2018](#), [Cuadrado et al., 2019](#) reported extensive evidence about the central role playing by nuclear factor erythroid-derived 2-like 2 (Nrf2). Nrf2 is widely known and investigated as a master regulator of multiple cytoprotective responses and as a key molecular node within a cluster of a wide spectrum of diseases, including NDs. Moreover, Nrf2 activation is impaired in aging by the involvement of microRNA ([Zhang et al., 2015](#), [Schmidlin et al., 2019](#), [Silva-Palacios et al., 2018](#)). This suggests that Nrf2 could represent a common therapeutic and systems medicine target, for aging and for its related disorders. Nrf2 can transcriptionally modulate the cytoprotective genes belonging to the vitagene network. This network regulates endogenous cellular

defense mechanisms, and involves redox sensitive genes such as members of the Heat Shock Proteins (HSP) family (*Heme-Oxygenase HO-1*, *Hsp70*), but also sirtuins and the thioredoxin (Trx)/thioredoxin reductase (TrxR1) system ([Calabrese et al., 2010](#)).

Based on this rationale, in this review we present wide scientific background indicating how a natural bioactive molecule with antioxidant property such as the ozone (O₃) can be indicated as a potential new strategy to delay neurodegeneration. This hypothesis is based on the widely demonstrated evidence regarding the interaction between O₃ and Nrf2 ([Galie et al., 2018](#), [Siniscalco et al., 2018](#), [Re et al., 2014](#), [Vaillant et al., 2013](#)). We first describe the relevant, well known and documented molecular mechanisms related to antioxidant/anti-apoptotic/pro-autophagy processes targeted by the O₃ administration *via* Nrf2 biological pathway. Secondly, we report a list of the main stress oxidative biomarkers modulated by the O₃ treatment *via* Nrf2 and that, in turn are strongly involved in NDs pathophysiology as well as in aging mechanisms. Different meta-analyses have been performed to demonstrate the effect in terms of Odd Ratio (OR) of O₃ on endogenous antioxidant-Nrf2 and vitagene-Nrf2 systems.

We thus provide scientific evidence to build a consistent rationale to apply for the first time the Oxygen-Ozone (O₂-O₃) therapy in an early phase of aging decline, when it is still possible to intervene, before to develop a potential neurodegenerative pathology.

2. The Ozone (O₃) molecule and the Oxygen-Ozone (O₂-O₃) therapy

O₃ is a triatomic gaseous molecule which has been used as a powerful oxidant in medicine for more than 150 years ([Elvis, Ekta, 2011](#)). In nature, O₃ is generated during storms due to the

electrical discharges of the rays that react with atmospheric O₂ to produce O₃. In humans, a revolutionary discovery led to demonstrate that neutrophils isolated from human peripheral blood and coated with antibodies can catalyse the generation of O₃ by a water oxidation pathway, leading to efficient killing of bacteria ([Wentworth et al., 2002](#), [Babior et al., 2003](#), [Lerner, Eschenmoser, 2003](#)).

In 1785, Van Mauren was the first identifying the distinctive odor of O₃. The actual gas was later discovered by the German chemist, Christian Friedrich Schonbein at the University of Basel in Switzerland on March 13th, 1839 when working with a voltaic pile in the presence of O₂ ([Altman, 2007](#)). Friederich noticed the emergence of a gas with an electric and pungent smell, and named it ozone, which is derived from the Greek word for smell ([Bocci, 2011](#)). O₃ was used as first antiseptic for operating rooms and to disinfect surgical instruments in 1856, and in 1860 the first O₃ water treatment plant was built in Monaco to disinfect water ([Altman, 2007](#)). Nikola Tesla patented the first portable O₃ generator in 1896 in the United States. The physicist, Joachim Hansler invented the first reliable O₃ generator, and this was the breakthrough in the use of O₃ for medical applications. This invention is considered the prelude to the ozonated autohemotherapy procedure and served as the basis for O₃ therapy expansion over the last 40 years.

The O₂-O₃ therapy is a non-invasive, non-pharmacological, no-side effect and low-cost procedure applied in medicine for the treatment of more than 50 pathological processes, whose alterations in endogenous oxidative-antioxidative balance play a crucial role. Different clinical trials evidenced the effectiveness of this therapy in the treatment of degenerative disorders such as multiple sclerosis ([Smith et al., 2017](#), [Delgado-Roche et al., 2017](#), [Ameli et al., 2019](#)), but also cardiovascular, peripheral vascular, neurological, orthopaedic, gastrointestinal and genitourinary pathologies ([Bocci, 2011](#), [Elvis, Ekta, 2011](#), [Re et al., 2008](#), [Bocci,](#)

2012, [Smith et al., 2017](#), [Braidy et al., 2018](#)); fibromyalgia ([Moreno-Fernandez et al., 2019](#), [Tirelli et al., 2019](#)); skin diseases/wound healing ([Fitzpatrick et al., 2018](#), [Wang, 2018](#)); diabetes/ulcers ([Martinez-Sanchez et al., 2005](#), [Guclu et al., 2016](#), [Rosul, Patskan, 2016](#), [Izadi et al., 2019](#), [Ramirez-Acuna et al., 2019](#)); infectious diseases ([Smith et al., 2017](#), Mandhare et al., 2012, Song et al., 2018), including the recent global pandemic disease of coronavirus disease 2019 (COVID-19) ([Zheng et al., 2020](#)); dentistry ([Isler et al., 2018](#), [Khatri et al., 2015](#), [Srikanth et al., 2013](#), [Azarpazhooh et al., 2009](#)); lung diseases ([Hernandez Rosales et al., 2005](#)); osteomyelitis ([Bilge et al., 2018](#)). The potential role of O₂-O₃ as an adjuvant therapy for cancer treatment has been also suggested in *in vitro* and animal studies as well as in isolated clinical reports ([Clavo et al., 2018](#)).

At present, we have commenced a randomized double-blind clinical trial with the aim to test the efficacy of this therapy in a cognitive frailty cohort, a grant approved by the Italian Minister of Health (RF-2016-02363298). This pilot study will permit to validate the O₂-O₃ therapy in an early phase of cognitive decline, when it is still possible to intervene, before to develop a potential neurodegenerative pathology.

To date, the O₂-O₃ therapy acquires a further prestigious significance, after the medicine Nobel prize for “discovery of how cells sense oxygen” in 2019. Indeed, O₂ is the most vital element required for human life and it is the key to good health; O₃ is O₂ with an extra molecule added. The O₂ availability affects genes expression of different factors (HIFs, Hypoxia Inducible Factors), leading to the activation of trophic proteins (VEGF, Vascular Endothelial Growth Factor; PDGF, Platelet-derived growth factor) and consequently to specific biological processes, including erythropoiesis, angiogenesis and anaerobic glucose metabolism ([Zhou et al., 2019](#)). O₃ plays a role of cellular adapter to hypoxia, because it is well known its effects in increasing the levels of VEGF, PDGF, HIF ([Curro et al., 2018](#), [Zhang et al., 2014](#), [Re et al.,](#)

[2010](#)), exactly as the cell does when there is no O₂ available.

3. Focus on the biological activities of the ozone (O₃): antioxidant property

Oxidative stress is a condition where ROS and Nitrogen Species (RNS) production exceeds the cellular antioxidant defence system, leading to the imbalance between the two systems and this may contribute to the neuronal damage and the abnormal neurotransmission. It is widely known its implication in the pathogenesis and progression of NDs ([Singh et al., 2019](#)). Brain and mitochondria are the most involved systems due to their high sensitivity to oxidative damage caused by free radicals. Oxidative damage may impair the cells in their structure and function, being cause and effect of a mitochondrial reduced activity. The damage is not confined to the brain but also evident in peripheral cells and tissues.

ROS and RNS are also major factors in cellular senescence that leads to increase number of senescent cells in tissues on a large scale ([Liguori et al., 2018](#)). Cellular senescence is a physiological mechanism that stops cellular proliferation in response to damages that occur during replication. Senescent cells acquire an irreversible senescence-associated secretory phenotype (SASP), involving secretion of soluble factors (interleukins, chemokines, and growth factors), degradative enzymes like matrix metalloproteases (MMPs), and insoluble proteins/extracellular matrix (ECM) components.

Nrf2 is a member of the CNC-basic leucine zipper (CNC-bZIP) family of transcription factors. Under basal condition, Nrf2 binds to its repressor Keap1 (Kelch-like ECH-associated protein 1), an adapter between Nrf2 and Cullin 3 protein, which leads to ubiquitination followed by proteasome degradation. This Keap1-mediated degradation activity requires two reactive cysteine

residues (Cys273 and Cys288).

When O₃ is administered, it dissolves immediately in the plasma/serum and it reacts with PUFA (polyunsaturated fatty acids), leading to the formation of the two fundamental messengers: hydrogen peroxide (H₂O₂) as a ROS and 4-hydroxynonenal (4HNE) as a lipid oxidation product (LOP) (Bocci et al., 1998) (Fig. 1). ROS are the early and short-acting messengers, while LOPs are late and long-lasting messengers. LOPs diffuse into all cells and inform them of a minimal oxidative stress. After the oxidative/electrophilic stress challenge (4HNE, (Ishii et al., 2004), other aldehydes, (Levonen et al., 2004)), induced by O₃ (Galie et al., 2018, Siniscalco et al., 2018, Re et al., 2014, Vaillant et al., 2013), modification of the cysteine residues of Keap1 (S-HNE or S—S) inhibits ubiquitin conjugation to Nrf2 by the Keap1 complex (Brigelius-Flohe, Flohe, 2011), provoking the nuclear accumulation of Nrf2. Once in the nucleus, Nrf2 dimerizes and binds to cis-acting DNA AREs (Antioxidant Response Elements) in genes such as *HO-1*, a gene encoding enzyme that catalyses the degradation of heme in carbon monoxide (CO) and free iron, and biliverdin to bilirubin. CO acts as an inhibitor of another important pathway NF-κB (Nuclear Factor Kappa B Subunit 1) signalling, which leads to the decreased expression of pro-inflammatory cytokines, while bilirubin also acts as an important lipophilic antioxidant. Furthermore, HO-1 directly inhibits the pro-inflammatory cytokines and activates the anti-inflammatory cytokines, thus leads to balancing of the inflammatory process (Ahmed et al., 2017). Our research group confirmed that mild ozonisation, tested on *in vitro* systems, induced modulation of genes, including *HO-1* (Scassellati et al., 2017). (Fig. 1).

Fig. 1.



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Molecular mechanisms linked to antioxidant/pro-
autophagy activities of ozone (O₃) via Nrf2 signalling.

In the absence of stimuli, Nrf2 (nuclear factor erythroid 2-related factor 2) binds to its repressor Keap1 (kelch-like ECH-associated protein), an adapter between Nrf2 and Cullin 3 (Cul3) protein, which leads to ubiquitination followed by proteasome degradation. When O₃ is administered, it dissolves immediately and it reacts with PUFA (Poly-Unsaturated Fatty Acids) leading to the formation of fundamental messengers such as hydrogen peroxide (H₂O₂), 4-hydroxynonenal (4HNE) and lipid oxidation products (LOPs). These messengers can influence the modifications of cysteine residues present in Keap1 (S-HNE or —S—S) inhibiting ubiquitin conjugation to Nrf2 by the Keap1 complex and provoking the nuclear accumulation of Nrf2. Once in the nucleus, Nrf2 dimerizes and binds to cis-acting DNA AREs (Antioxidant Response Elements) in different genes: *Heme Oxygenase 1 (HO-1)*, *Superoxide dismutases (SOD)*, *Glutathione peroxidase (GSH-Px)*, *Glutathione-S-Transferase (GST)*, *Catalase (CAT)*, *GSH-reductase (GR)*, *NADPH quinone oxidoreductase 1 (NQO1)*, *Heat Shock Proteins (HSPs)*, *Cytochrome P450 monooxygenase (CYP450)*, *Thioredoxin reductase (TrxR)*, *phase II enzymes (UDP-glucuronosyltransferases, UGTs; N-acetyltransferases,*

NATs, sulfotransferases, SULTs).

A) O₃ involves casein kinase 2 (CK2), a regulator of the Nrf2 activity through its phosphorylation, and MAPK (mitogen-activated protein kinase) signalling pathway, that is inhibited with consequent inactivation of oxidative stress and apoptosis by O₃ administration.

B) O₃ modulates the degradation protein systems (autophagy), via activation of the AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) signaling pathway.

C) O₃ downregulates inducible nitric oxide synthase (iNOS), which generates nitric oxide (NO) via NF-κB (Nuclear Factor Kappa B Subunit 1) pathway. (CO = carbon monoxide).

In addition, Nrf2 regulates also the constitutive and inducible expression of antioxidants including, but not limited to, Superoxide Dismutases (SOD), Glutathione Peroxidase (GSH-Px), Glutathione-S-Transferase (GST), Catalase (CAT), NADPH quinone oxidoreductase 1 (NQO1), phase II enzymes of drug metabolism and HSPs ([Galie et al., 2018](#), [Bocci and Valacchi, 2015](#), [Pedruzzi et al., 2012](#)) ([Fig. 1](#)).

A further mechanism involves casein kinase 2 (CK2), another regulator of the Nrf2 activity through its phosphorylation. It has been demonstrated that O₃ influenced the CK2 levels together with Nrf2 phosphorylation, reducing oxidative stress and pro-inflammatory cytokines in multiple sclerosis patients ([Delgado-Roche et al., 2017](#)). Similarly, O₃ inhibits oxidative stress through inhibition of the mitogen-activated protein kinase phosphatase (MAPK) 1 signalling pathway ([Wang et al., 2018a](#)) ([Fig. 1A](#)).

Oxidative stress is one of the major drivers of protein misfolding that, accumulating and aggregating as insoluble inclusions can determine neurodegeneration ([Hohn et al., 2020](#), [Knowles et al., 2014](#)). It is known that Nrf2 promotes the clearance of oxidized or otherwise damaged proteins through the autophagy mechanism ([Tang et al., 2019](#)). Interestingly, also O₃ can modulate the degradation protein systems, not only *via* Nrf2 pathway, but also *via* activation of the AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) signaling pathway, as demonstrated in [Zhao et al. \(2018\)](#) ([Fig. 1B](#)).

O₃ can protect against overproduction of nitric oxide (NO), when NO is a toxic oxidant. NO can rapidly react with other free radicals such as O₂⁻ to generate highly reactive oxidant peroxynitrite (ONOO⁻) and other RNS, which in turn damage the biomolecules (e.g., lipids, protein, DNA/RNA), playing thus a key role in chronic inflammation and neurodegeneration ([Massaad, 2011](#), [Toda et al., 2009](#)). It has been demonstrated that O₃ downregulates inducible nitric oxide synthase (iNOS), which generates NO ([Manoto et al., 2018](#), [Smith et al., 2017](#)) *via* NF-κB signalling ([Fig. 1C](#)).

4. Focus on the biological activities of the ozone (O₃): anti-apoptotic mitochondrial property

Mitochondrial dysfunction is one of the main features of the aging process, particularly in organs requiring a high-energy source such as the heart, muscles, brain, or liver. Neurons rely almost exclusively on the mitochondria, which produce the energy required for most of the cellular processes, including synaptic plasticity and neurotransmitter synthesis. Mitochondrial dysfunctions cause increase in ROS for lowered oxidative capacity and antioxidant defence, with consequent increased oxidative damage to protein and lipids, decreased ATP production and accumulation of DNA damage ([Garcia-Escudero et al., 2013](#),

[Reutzel et al., 2020](#)). Moreover, mitochondrial bioenergetic dysfunction and release of pro-apoptotic mitochondrial proteins into the cytosol initiate a variety of cell death pathways.

Nrf2 transcribes several genes not only those implicated in antioxidant expression and energy regulation, but also those involved in mitochondria biogenesis: increases the mitophagy, mitochondrial levels of antioxidant enzymes, and resistance to redox regulated mitochondrial permeability transition pore opening ([Holmstrom et al., 2016](#)). Multiple lines of evidence showed that Nrf2 activation is part of the retrograde response aimed at restoring mitochondrial functions after stress insults, and that the impairment of Nrf2 functions is a hallmark of many mitochondrial-related disorders ([Shan et al., 2013](#)).

It has been demonstrated that O₃ administration can act on specific mechanisms to promote cell survival and proliferation, blocking the apoptotic processes. In particular, O₃ decreases the expression of *caspases 1-3-9*, *HIFa*, *Tumor Necrosis Factor-α (TNF-α)*, *Bcl-2-associated X protein (Bax)*, *poly (ADP-ribose) polymerase 1 (PARP-1)* and *p53* genes ([Fig. 2](#)) ([Yong et al., 2017](#), [Guclu et al., 2016](#), [Wang et al., 2018a](#)). Bax is located in the mitochondrial membranes and exerts pro-apoptosis effect through the mitochondrial pathway, promoting cytochrome C activation ([Mac Nair et al., 2016](#)); p53 and Caspase-3 are executive molecules of apoptosis by blocking cell cycle ([Wang et al., 2016](#)). Enzymes such as SOD, CAT, and GSH-Px, can regulate p53, Bax and Bcl-2 (BCL2 Apoptosis Regulator) ([Fig. 2](#)).

Fig. 2.



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Molecular mechanisms linked to anti-apoptotic property of ozone (O₃) via pro-apoptotic molecules inactivation.

Various apoptotic stimuli, ischemia, Reactive Oxygen Species, ROS, ipoxia can activate directly p53 that in turn can play a role as transcription factor and activate the expression of pro-apoptotic genes. Among these, Bak (Bcl-2 homologous antagonist/killer) and Bax (Bcl-2-associated X protein) can stimulate in mitochondrial membrane the activation of Cytochrome C that in turn activates Apaf1 (Apoptotic protease activating factor-1) and caspase 9 to close the circle to stimulate the activity of caspase 3. Enzymes such as SOD (Superoxide dismutase), CAT (catalase), and GSH-Px (glutathione peroxidase), can regulate p53, Bax and Bcl-2. O₃ administration decreases the expression of *caspases 1-3-9*, *Hypoxia-inducible factor (HIFa)*, *Tumor Necrosis Factor-a (TNF-a)*, *Bax* and *p53* genes. (BID (BH3-interacting domain death agonist), Nrf2 (Nuclear Factor Erytroid 2-related factor 2), CUL3 (Cullin 3), Keap 1 (Kelch-like ECH-associated Protein), H₂O₂ (Hydrogen Peroxide), 4HNE (4-hydroxynonenal), LOPs (Lipid Oxidation Products), Cyt C (Cytochrome C), PUFAs (Poly-

Unsaturated Fatty Acid), AREs (Antioxidant Response Elements)).

Moreover, O₃ stimulates the Krebs's cycle in the mitochondria by enhancing the oxidative carboxylation of pyruvate and stimulating the production of adenosine triphosphate (ATP) ([Güven et al., 2008](#)). It also causes a significant reduction of nicotinamide adenine dinucleotide (NADH), an increase of the coenzyme A levels to fuel the Krebs's cycle and oxidizes cytochrome C ([Brigelius-Flohe, Flohe, 2011](#), [Elvis, Ekta, 2011](#)).

O₃ treatment was proven to reduce mitochondrial damage in a rat heart following ischemia-reperfusion ([Meng et al., 2017](#)), as well as in a rat brain and cochlea following noise-induced hearing loss ([Nasezadeh et al., 2017](#)). Moreover, *in vitro*, O₃ increased the length of the mitochondrial cristae and the content of mitochondrial Hsp70 ([Costanzo et al., 2018](#)).

5. Pro-oxidation and antioxidant defence biomarkers influenced by ozone (O₃) and implicated in aging processes and in neurodegenerative disorders (NDs)

5.1. Stress-oxidant biomarkers modulated by the O₃ effect

A list of biomarkers (29 in total) implicated in oxidative stress, in endogenous antioxidant and vitagene systems are showed in [Table 1](#). These biomarkers have been studied and found modulated after the O₂-O₃ therapy in more of 150 studies performed in different *in vivo* (human and animal models) and *in vitro* samples and conditions. In [Table 1](#), we also reported the relative functions of these biomarkers.

Table 1.

List of the pro-oxidation and antioxidant defence biomarkers influenced by ozone (O₃) and implicated in neurodegenerative disorders (NDs) as well as in aging processes.

Ozone biomarkers	Name and Function	Involvement in NDs	Involvement in Aging processes
4-HNE	4-Hydroxynonenal: a common aldehyde byproduct of lipid peroxidation during oxidative stress. 4-HNE is highly reactive and primarily produced in the brain via lipid peroxidation of arachidonic acid, a highly abundant omega-6 polyunsaturated fatty acids (PUFA) component of neuronal membranes. HNE may modify the ATP synthase, the final step in the production of ATP from electron transport chain (ETC) inside mitochondria. 4-HNE activates Nrf2 by alkylating thiol groups of cysteine residue in Keap1.	(Moldogazieva et al., 2019 , Ayala and Munoz, 2014 , Baker et al., 2015)	(Bencal et al., 2015 , Csala et al., 2015)

8-OHdG	<p><u>8-hydroxydeoxyguanosine (8-Oxo-2'-deoxyguanosine (8-oxo-dG)</u>: oxidized derivative of deoxyguanosine. Its concentrations within a cell are a measurement of oxidative stress (DNA oxidation). Reactive oxygen species (ROS) attack guanine bases in DNA easily and form 8-hydroxydeoxyguanosine, which can bind to thymidine rather than cytosine; thus, the level of 8-OHdG is generally regarded as a biomarker of mutagenesis consequent to oxidative stress.</p>	<p>(Wang et al., 2019c, Nakabeppu et al., 2007, Poulsen et al., 2014, Polidori et al., 1999)</p>	<p>(Mec al., 20</p>
AOPP	<p><u>Advanced Oxidation Protein Products</u>: are a group of oxidatively modified protein products containing dityrosine, pentosidine, and carbonyl-containing products generated by reactive oxygen species (ROS) or formed via myeloperoxidase reaction during oxidative/chlorine stress. They are</p>	<p>(Wang et al., 2019c, Cristani et al., 2016)</p>	<p>(Mac et al., Cakar al., 20 Koc Vasse 2012 Rusai al., 20 Qing 2012 et al., Mulle 2015</p>

biomarkers of oxidant-mediated protein damage.

CAT	<u>Catalase</u> : it catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a scavenger enzyme of reactive oxygen species (ROS), protecting the cell from oxidative damage by ROS.	(Feitosa et al., 2018)	(Veal 2018)
FRAP	<u>Ferric Reducing the Ability of Plasma</u> : total antioxidant capacity of plasma.	(Ademowo et al., 2017)	(Mull al., 2006) Rizvi 2006
Fructolysine	It is an Amadori adduct of glucose to lysine. It is a precursor of the advanced oxidation protein products, which are induced by oxidative stress, and induces oxidative stress.	-	-
GR	<u>Glutathione reductase (or glutathione-disulfide reductase, GSR)</u> : it catalyses the reduction of glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH), which is a critical molecule in resisting oxidative stress and maintaining the	(Feitosa et al., 2018, Liu et al., 2004, Rougemont et al., 2002)	(Veal 2018)

reducing environment of the cell.

GSH	<u>Glutathione</u> : it is antioxidant, capable of preventing damage to important cellular components caused by reactive oxygen species (ROS). It maintains cellular thiol status.	(Mazzetti et al., 2015 , Liu et al., 2004 , Gu et al., 2015 , Rougemont et al., 2002 , Oliveira, Laurindo, 2018)	(Mac et al., Teske 2018 , Olive Lauri 2018)
GSH-Px/GPx	<u>Glutathione peroxidase</u> : it has peroxidase activity whose main biological role is to protect the organism from oxidative damage. The biochemical function is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water.	(Mazzetti et al., 2015 , Gu et al., 2015 , Rougemont et al., 2002)	(Mac et al., Veal 2018)
GST	<u>Glutathione S-transferase</u> : it is phase II metabolic isozyme, known for the ability to catalyze the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for the purpose of detoxification.	(Mazzetti et al., 2015 , Gu et al., 2015 , Rougemont et al., 2002)	(Veal 2018)

- HIF-1 α** Hypoxia-inducible factor (HIF)-1 α : is a subunit of a heterodimeric transcription factor hypoxia-inducible factor 1 (HIF-1). It is a basic helix-loop-helix PAS domain containing protein and is considered as the master transcriptional regulator of cellular and developmental response to hypoxia. (Merelli et al., 2018) (Yeo, 2018)
- HO-1** Heme-Oxygenase-1: it catalyzes the conversion of heme into free iron, carbon monoxide and biliverdin. It possesses two well-characterized isoforms: HO-1 and HO-2. Under brain physiological conditions, the expression of HO-2 is constitutive, abundant and ubiquitous, whereas HO-1 mRNA and protein are restricted to small populations of neurons and neuroglia. HO-1 is an inducible enzyme that has been shown to participate as an essential defensive mechanism for neurons (Facchinetti, 2020) (Schiala et al., 2019)

exposed to oxidant challenges, being related to antioxidant defenses in certain neuropathological conditions.

HSP70	<p><u>Heat-Shock Protein 70</u>: it is essential for the folding and repair of damaged proteins. During stressful conditions, such as elevated temperature, it prevents protein aggregation, by facilitating the refolding or elimination of misfolded proteins. These mechanisms serve to promote cell survival conditions that would otherwise result in apoptosis.</p>	<p>(Lackie et al., 2017)</p>	<p>(Marin-Toda, Fuenfelter et al., 2015)</p>
IMA	<p><u>Ischemia-modified albumin</u>: it measures ischemia in the blood vessels</p>	<p>(Altunoglu et al., 2015, Candemir et al., 2013)</p>	-
LPO	<p><u>Lipid peroxide</u>: is the oxidative degradation of lipids.</p>	<p>(Feitosa et al., 2018, Negre-Salvayre et al., 2010)</p>	<p>(Negre-Salvayre et al., 2010)</p>
MDA	<p><u>Malondialdehyde</u>: is a marker for oxidative stress. It is a reactive aldehyde produced by</p>	<p>(Feitosa et al., 2018, Wang et al., 2019c, Ayala and</p>	<p>(Csalog et al., 2015, Maciel et al.,</p>

lipid peroxidation of polyunsaturated fatty acids. [Munoz, 2014](#))

MPO Myeloperoxidase: is a peroxidase enzyme. It requires heme as a cofactor. It is expressed in neutrophil and monocyte, and is implicated in various stages of inflammatory conditions with the production of a variety of potent oxidants. [\(Ray, Katyal, 2016, Maki et al., 2019\)](#) [\(Son 2005](#)

Nfr2/CK2 Nuclear factor erythroid 2-related factor 2: is a basic leucine zipper (bZIP) protein that regulates the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation. [\(Perez et al., 2011, Sivandzade et al., 2019\)](#) [\(Siva et al.,](#)

Casein kinase 2: a serine/threonine-selective protein kinase implicated in cell cycle control, DNA repair, regulation of the circadian rhythm, and other cellular processes. Regulator of the Nrf2 activity through its phosphorylation.

NO	<u>Nitric Oxide</u> : is an important cellular signaling molecule which is derived from L-arginine by nitric oxide synthase (NOS). It works as a retrograde neurotransmitter in synapses, allows the brain blood flow, and has important roles in intracellular signaling in neurons from the regulation of the neuronal metabolic status to the dendritic spine growth. It is able to perform post-translational modifications in proteins by the S-nitrosylation of the thiol amino acids, which is a physiological mechanism to regulate protein function.	(Hannibal, 2016, Nakamura, Lipton, 2020, Radi, 2018)	(Pico et al.,
NO-3/NO-2 (NOx)	<u>Nitrate/nitrite</u> : an index of NO production	(Hannibal, 2016, Nakamura, Lipton, 2020, Radi, 2018)	(Pico et al.,
NOS	<u>Nitric oxide synthase</u> (inducible i II, endothelial e I): it catalyzes the production of nitric oxide (NO) from L-arginine.	(Hannibal, 2016, Nakamura, Lipton, 2020)	(Jung 2012

PCC/PCO	<u>Protein carbonyl content</u> : catalyses the carboxylation reaction of propionyl CoA in the mitochondrial matrix.	(Chevion et al., 2000 , Fedorova et al., 2014)	(Cabi al., 2014 , Cakar al., 2014)
PP	<u>Protein phosphatase</u> : it is a serine/threonine phosphatase. It has been found to be important in the control of glycogen metabolism, muscle contraction, cell progression, neuronal activities, splicing of RNA, mitosis, cell division, apoptosis, protein synthesis, and regulation of membrane receptors and channels.	(Braithwaite et al., 2012 , Clark, Ohlmeyer, 2019)	(Salinas et al., 2014)
SOD	<u>superoxide dismutase</u> : are the first and most important line of scavenger antioxidant enzyme defence systems against ROS and particularly superoxide anion radicals. There are two isoforms of SOD (cytoplasmatic CuZn-SOD or SOD1 and mitochondrial Mn-SOD or SOD2).	(Feitosa et al., 2018 , Schaffert, Carter, 2020)	(Maciel et al., 2018 , Veal et al., 2018)
TAC	<u>Total antioxidant capacity</u>	(Mota et al., 2019)	(Maciel et al., 2018)

TAS	Total antioxidant status	(Mota et al., 2019)	
TBARS	Thiobarbituric acid reactive substances: byproducts of lipid peroxidation (i.e. as degradation products of fats)	(Vina et al., 2005)	(Muller et al., 2019)
TH	Total Hydroperoxides: indicator of oxidative stress.	(Tarafdar, Pula, 2018)	
TOS	Total oxidant score	(Mota et al., 2019)	

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Note: In bold the genes involved in Nrf2 signalling

From these 29 biomarkers, we focused, in this section, on those implicated in endogenous antioxidant-Nrf2 pathway (GSH; GSH-Px; glutathione reductase, GR; SOD; CAT; 4HNE; Advanced Oxidation Protein Products, AOPP in bold in [Table 1](#)). Where it was possible (available studies), we performed meta-analyses for these biomarkers on human (see supplementary material). The results showed significant increased levels of the SOD-CAT-GSH-Px-GSH-GST-GR after O₃ administration ([Fig. 3](#), Random model, $Z = 6.15$, $p < 0.00001$, OR = 1.71 95%CI:1.17-2.25; even after Bonferroni correction $0.05/6 = 0.0083$). Similar results were obtained even considering single markers, except for GR ($Z = 1.04$; $p = 0.30$) and GSH ($Z = 0.80$, $p = 0.42$). GR has been investigated only in two studies, coming from the same authors ([Hernandez Rosales et al., 2005](#)). Thus, there are not enough evidence on its single real involvement. Concerning GSH, Diaz-

Luis et al., ([Díaz-Luis et al., 2018](#)) is the only study showing a negative effect of O₃. As we followed the criteria for which the data were extracted before and after O₃ treatment (see supplementary material), this study found an increased GSH levels after O₃ administration, only when the authors performed the comparisons with control group of healthy subjects (in a sort of postconditioning). Thus, if we eliminated this study, the results of the single meta-analysis of GSH highlighted its positive increase determined by the O₃ treatment (Z = 2.30; p = 0.02, data not shown).

Fig. 3.



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Forest plot for odds ratio from meta-analysis of the endogenous Nrf2- antioxidant pathway before and after ozone (O₃) treatment.

CI, confidence interval; Chi², χ^2 test of goodness of fit; Tau², estimate of the between-study variance in a random-effects meta-analysis. Superoxide dismutase (SOD), catalase (CAT), Glutathione peroxidase (GSH-Px), Glutathione (GSH), Glutathione S-transferase (GST), Glutathione reductase (GR). RI = rectal insufflations; MATH = major

autohemotherapy

High heterogeneity in effect size across the studies ($P < 0.00001$, $I^2 = 97\%$) was observed in these meta-analyses. This is essentially explained by the presence of different factors: the type of pathology, different concentration of O_3 linked to different administration procedures and duration time treatments, age of the sample (supplementary material Table 1S).

Interestingly, different studies have been performed on aging-specific conditions. A recent work ([El-Mehi, Faried, 2020](#)) demonstrated that antioxidant properties of O_3 can ameliorate age-associated structural alterations of the rat cerebral cortex, improving age-related oxidative stress reflected in the histopathological and immunohistochemical alterations. The authors detected severe structural and cellular neurodegenerative changes in the frontal cortex of the aged rats. O_3 administration produced significant downregulation of tissue Malondialdehyde (MDA), an index of oxidative stress, and upregulation of GSH, SOD and CAT. Similarly, O_3 influenced iNOS, caspase-3, glial fibrillary acidic protein (GFAP), Ki67 and acetylcholinesterase (AChE). These findings indicate reduction not only in oxidative stress, but also in apoptosis (down-regulation caspase-3) and in gliosis (down-regulation GFAP), as well as improving in neurogenesis (upregulation of Ki-67 expression) and in cholinergic plasticity (decrease AChE activity). The authors suggest that O_3 might be useful for improving the age-related cognitive and memory deterioration, by increasing cholinergic communication.

Safwat et al. ([Safwat et al., 2014](#)) demonstrated that O_3 showed a beneficial effect on the aging reducing liver and kidney damage through its antioxidant property. O_3 was efficient in elevating the reduced hepatic and renal GSH contents as well as in normalizing

hepatic GSH-Px activity of aged rats. Moreover, O₃ succeeded in attenuating the elevated hepatic and renal MDA and protein carbonyls (PC) levels.

Another work ([El-Sawalhi et al., 2013](#)) reported that O₃ alleviated age-associated redox state imbalance, as evidenced by reduction of lipid and protein oxidation markers and lessening of lipofuscin deposition. Moreover, O₃ restored GSH levels in brain and heart tissues, and normalized GSH-Px activity in the heart tissue of the aged-rats. O₃ also mitigated age-associated energy failure in the heart and the hippocampus, improved cardiac cytosolic Ca(2+) homeostasis and restored the attenuated Na(+), K(+) -ATPase activity in the hippocampus of these rats.

Similarly, prophylactic administration of O₃ in aged-rats normalized reduced GSH content, adenosine triphosphate/adenosine diphosphate ratio, mitochondrial SOD and complex IV (cytochrome-c oxidase) activities. O₃ improved glutathione redox index (GSHRI), complex I (NADH-ubiquinone oxidoreductase) and mitochondrial mtNOS activities, and attenuated the rise MDA and mitochondrial PC levels ([Shehata et al., 2012](#)).

5.2. Stress-oxidant biomarkers implicated in aging mechanisms

Several evidence support the involvement of these biomarkers influenced by the O₃ administration in the mechanisms of aging ([Table 1](#)). We prevalently focused on those implicated in the Nrf2 signalling (in bold in [Table 1](#)).

It has been reported that the levels of lipid peroxidation products, reactive carbonyl compounds, such as 4HNE, are increased in aging tissues ([Csala et al., 2015](#)), and this increase is positively correlated with age. Impaired protein function, manifested as an

increase in PC, plays a crucial role in aging processes ([Cabiscol et al., 2014](#)). With increase of PC, the spontaneous carbonyl-amino crosslinking and accumulation were mostly irreparable changes associated with aging ([Nowotny et al., 2014](#)).

Several findings evidenced altered levels of AOPP in aging ([Komosinska-Vassev et al., 2012](#), [Rusanova et al., 2018](#), [Qing et al., 2012](#), [Silva et al., 2015](#), [Muller et al., 2015](#)). A recent work investigated the antioxidant enzymes (GSH-Px, CAT, SOD), nonenzymatic antioxidants (GR), redox status (total antioxidant capacity, TAC, total oxidant status, TOS, oxidative stress index, OSI), and oxidative damage products (AOPP, MDA) in a healthy sample divided according to age: 2-14 (children and adolescents), 25-45 (adults), and 65-85 (elderly people). They demonstrated that salivary and blood antioxidant defense is most effective in adults. Contrarily, a progressive decrease in the efficiency of central antioxidant systems (\downarrow GSH-Px, \downarrow SOD, \downarrow GSH, \downarrow TAC in erythrocytes and plasma vs. adults) was observed in the elderly. Both local and systemic antioxidant systems were less efficient in children and adolescents than in the group of middle-aged people, which indicates age-related immaturity of antioxidant mechanisms. Oxidative damage to proteins (\uparrow AOPP) and lipids (\uparrow MDA) was significantly higher in saliva and plasma of elderly people in comparison with adults and children/adolescents ([Maciejczyk et al., 2019](#)). Similarly, Cakatay et al. ([Cakatay et al., 2008](#)) found, in a young, middle-aged and elderly individuals sample, PCO and AOPP levels of the elderly and middle aged individuals higher compared with those of the young.

Although not involved in Nrf2 signaling but influenced by O₃ treatment, the increased oxidative damage to mitochondrial DNA (mitDNA) with the OH8dG (8-hydroxydeoxyguanosine) formation, represents the most common hallmark of the aging brain, marker of oxidative DNA damage. The simultaneous increased oxidation of mtDNA and deficiency of DNA repair could enhance the lesion to mitochondrial genome, potentially causing

neuronal damages ([Mecocci et al., 2018](#)).

5.3. Stress-oxidant biomarkers implicated in NDs

Several evidence support the implication of the pro-oxidation and antioxidant defence biomarkers influenced by O₃ listed in [Table 1](#) in the aetiopathogenetic mechanisms of NDs. Even for NDs, we prevalently focused on those implicated in the Nrf2 signalling (in bold in [Table 1](#)).

5.3.1. Alzheimer's Disease

AD is characterized by progressive loss of cognitive and behavioral deterioration, which leads to the impairment of daily and routine activities. It is one of the most prevalent NDs manifesting 45 million people worldwide. AD is characterized by the deposition of protein aggregates, extracellular amyloid plaques (A β), intracellular tau (τ) or neurofibrillary tangles, and loss of synaptic connections in specific regions of brain ([Schipper, 2010](#), [Mattson, 2004](#), [Selkoe, 2001](#)). The neuropathological diagnostic feature of AD is the accumulation of neurotoxic A β oligomer peptides, which, along with τ protein, mediate neurodegeneration, thus causing neuroinflammation, impairment in synaptic connection, cholinergic denervation, neurotransmitter imbalance, neuronal loss, and dendritic alterations.

Different studies indicate the relationship between A β -induced oxidative imbalance and elevated levels of by-products of lipid peroxidation (e.g., 4HNE, MDA), protein oxidation (e.g., carbonyl), and DNA/RNA oxidation (e.g., OH8dG) ([Wang et al., 2014c](#), [Zhao and Zhao, 2013](#), [Pratico, 2008](#), [Mecocci et al., 2018](#)). These alterations were observed also in peripheral lymphocytes and lymphocyte mitochondria (for review [Mecocci et al., 2018](#)). Higher levels of PC, measured in mitochondria extracted from lymphocytes, have been observed in AD (for review [Mecocci et al.,](#)

[2018](#)).

Decreased levels of antioxidant enzymes like SOD, CAT, GSH, decreased ratio of GSH/GSSG (Glutathione disulfide), and/or impaired expressions or activities of GSH-related enzymes have been observed in blood or brain of AD patients ([Singh et al., 2019](#), [Liu et al., 2004](#), [Kim et al., 2006](#), [Oliveira, Laurindo, 2018](#)).

RNS such as NO are also found to have a deleterious effect on neurons. Indeed, RNS elevation has been observed both in astrocytes as well as in neurons in an AD brain (for review [Singh et al., 2019](#)). An increase in the expression of neuronal nNOS or NOS-1, cytokine-inducible iNOS or NOS-2, and endothelial eNOS or NOS-3 isozymes has been observed in AD astrocytes. The direct association of iNOS and eNOS with A β aggregates indicating towards beta amyloid assisted in the induction of NOS to produce NO, which in turn leads to the formation of 3-nitrotyrosine (NT) ([Luth et al., 2002](#), [Luth et al., 2001](#)).

Other findings reported increased levels of CK2 in the hippocampus and temporal cortex of AD patients ([Rosenberger et al., 2016](#)) and increased levels in AOPP ([Can et al., 2013](#), [Altunoglu et al., 2015](#)), compared to non-demented controls. It has been observed that AD patients showed an increased oxidation of red blood cells GSH, which indicates oxidative stress in peripheral cells, and an increased level of plasma thiobarbituric acid reactive substances (TBARS), which indicates a higher free radical oxidation of plasma unsaturated phospholipids ([Vina et al., 2005](#)).

Moreover, HO-1 has been proposed as systemic marker in early sporadic AD ([Schipper et al., 2000](#)). Indeed, plasma HO-1 protein levels are significantly decreased in patients with probable sporadic AD ([Schipper, 2007](#)). The up-regulation of HO-1 in AD brain can be explained because of local oxidative stress. Instead,

the mechanism responsible for the downregulation of HO-1 in the blood of AD patients remains unclear, even though the existence of a HO-1 suppressor that inhibits HO-1 mRNA levels in the lymphocytes in AD plasma has been proposed ([Maes et al., 2006](#)). However, the results about HO-1 plasma levels in patients with AD are controversial. A study found no changes in the serum level of HO-1 in a big cohort of AD patients, as compared with elderly control subjects, whereas increased levels were observed in PD patients, highlighting different mechanisms involved in the peripheral response to oxidative stress in the two diseases ([Mateo et al., 2010](#)). Moreover, another study reports that in plasma of probable AD patients, both HO-1 and biliverdin reductase (BVR) levels were increased because of the enhanced oxidative stress. The authors suggested that plasma BVR status, more than HO-1, can represent a potential biochemical marker for the prediction of AD at the earliest stages of disease ([Di Domenico et al., 2012](#); for review [Nitti et al., 2018](#)).

5.3.2. Parkinson's disease (PD)

PD is the second most prevalent neurodegenerative disorder, after AD, which is characterized by the progressive degeneration of the dopaminergic neurons located in the substantia nigra (SN) pars compacta ([Spillantini et al., 1998](#)) which affects movement. The main neuropathological hallmark of PD is the presence of intracellular inclusions known as Lewy bodies (LBs) and neurites (LNs) ([Forno, 1996](#)); predominantly composed by misfolded and aggregated forms of the presynaptic protein α -synuclein (α Syn; a small protein with 140 amino acids abundant in presynaptic nerve terminals) ([Spillantini et al., 1998](#)). α Syn plays a role in synaptic transmission and dopamine levels adjustment. α Syn primarily affect tyrosine hydroxylase phosphorylation and activity and the expression level of dopamine transporter on the cell membrane.

Different evidence supported the involvement of the pro-oxidation

and antioxidant defence biomarkers influenced by O_3 listed in [Table 1](#) also with PD (focus on Nrf2). Altered levels of GSH and GSSG, decreased ratio of GSH/GSSG, and/or impaired expressions or activities of GSH-related enzymes have been detected in PD ([Liu et al., 2004](#)). TOS and OSI levels were found higher in the PD patients as compared to controls ([Mota et al., 2019](#)).

RNS also play major role in nitrosative stress in PD. NO, produced by nNOS or iNOS was found in large quantities in cells, as well as in the extracellular space around dopaminergic neurons ([Tieu et al., 2003](#)). It has been observed that in PD brains, NO obstructs various enzymes including complex I and IV of the mitochondrial electron transport chain, hinders the function of proteins by forming S-nitrosothiols, mediates lipid peroxidation, resulting in elevated levels of ROS and brain deteriorating effect. *In situ* hybridization and immunohistochemical studies also established the role of NO in PD via postmortem brain tissue analysis, which indicates an elevated level of iNOS and nNOS in basal ganglia structures ([Eve et al., 1998](#), [Hunot et al., 1996](#)). $ONOO^-$ has been shown to inhibit the presynaptic dopamine transporter, which mediates the uptake of dopamine from the synaptic cleft to stop dopamine signalling, and to refill the dopamine vesicles. Its inactivation will induce a decrease in dopamine delivery ([Picon-Pages et al., 2019](#)).

Oxidative damage in nucleic acids is likely to be a major risk factor for PD ([Bosco et al., 2006](#), [Puspita et al., 2017](#)). Oxidative DNA lesions, such as 8-oxoguanine (8-oxoG), accumulate in nuclear and mitochondrial genomes during aging, and such accumulation can increase dramatically in these patients ([Nakabeppu et al., 2007](#)).

5.3.3. Amyotrophic Lateral Sclerosis (ALS)

Among the various neurodegenerative diseases, ALS is the most common type of motor neuron disease; it is sometimes called Lou Gehrig's disease, after the famous baseball player who had this condition. ALS is characterized by the progressive degeneration of upper and lower motor neurons in the spinal cord, cortex, and brainstem ([Kikuchi et al., 2002](#)). Although for most of the last 2 decades mutation of Cu–Zn SOD1 was the only genetic aberration associated with the onset of familial ALS, recent studies have discovered additional abnormalities associated with the onset of sporadic and non-SOD1 familial ALS. These include a host of RNA/DNA-binding proteins such as the 43-kDa transactive response (TAR) DNA-binding protein (TDP-43) and the fused in sarcoma/translocated in liposarcoma (FUS/TLS). The most common genetic mutation is identified as expanded GGGGCC hexanucleotide repeat in the non-coding region of the *C9Orf72* gene located on chromosome 9p21 ([Mendez, Sattler, 2015](#)).

Wang et al., ([Wang et al., 2019c](#)) reported increased blood levels of 8-OHdG, MDA, and AOPP and decreased GSH and uric acid levels in the peripheral blood of ALS patients. These biomarkers have been found in sporadic ALS patient's urine, cerebrospinal fluid (CSF), blood, and individual tissues.

5.3.4. Huntington Disease (HD)

HD named after George Huntington in 1872, is a fatal and autosomal dominant inherited progressive neurodegenerative disorder, resulting in neuronal degeneration in the striatum followed by deterioration of the cerebral cortex and thalamus. HD is caused by a mutation in the *huntingtin (HTT)* gene. It is characterized by an abnormal extension in the cytosine–adenine–guanine (CAG) repeat in this gene, which in turn translates into an abnormally long repeat of polyglutathione in the mutant huntingtin protein. HD is mainly characterized by impaired motor and cognitive traits, personality change, and psychiatric illness

([Vonsattel, DiFiglia, 1998](#)).

Lipid peroxidation, DNA damage, and specifically protein carbonylation were found to be more pronounced in HD ([Tunéz et al., 2011](#)). Dysregulation in cysteine metabolism was observed in HD ([Paul et al., 2018](#)). Cysteine plays vital roles in redox homeostasis, being a component of the major antioxidant GSH and a potent antioxidant by itself. In HD patients, decreased GSH levels and increased lipid peroxidation were observed as compared with controls ([Oliveira, Laurindo, 2018](#)). In postmortem brain specimens of HD, a twofold increase of OH8dG in mtDNA was found in the parietal and slightly less in the frontal cortex compared to controls ([Polidori et al., 1999](#)).

6. Molecular mechanisms involving ozone (O₃), Nrf2 and vitagene network and their biological relevance in neuroprotection

At the core of adaptive responses at the cell and origin of biological organization is the concept of hormesis ([Calabrese et al., 2010](#)). Hormesis describes a process that results in ameliorating and improve cellular stress resistance, survival, and longevity in response to sub-lethal levels of stress ([Mattson, 2008](#)). Generally, a favorable biological response to low exposure to any stressor is found within the hormetic zone, whereas cell damage occurs at higher doses. The hormetic dose response results from either a direct stimulation or through an overcompensation stimulatory response following disruption in homeostasis ([Calabrese and Baldwin, 2000](#)). This theory is, to date a frontier area of neurobiological research, focal to understand and develop new/complementary therapeutic approaches to NDs. In this context, Nrf2 is considered as a hormetic-like pathway ([Calabrese et al., 2010](#)).

It has widely been reported that the activation of Nrf2 by several

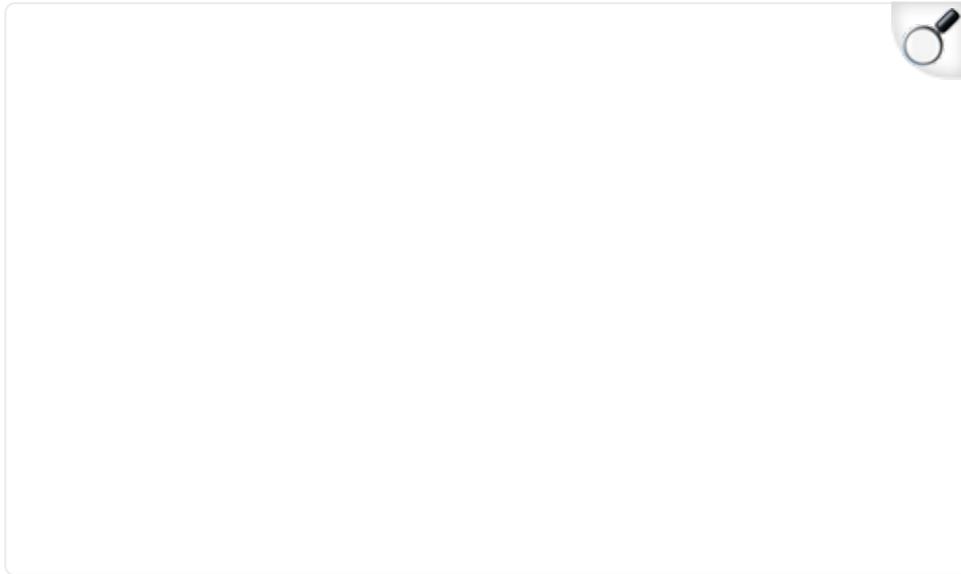
different mechanisms (calorie restriction, physical exercise, polyphenols, mushrooms) can be a way to improve life health, due to its transcriptionally modulation on the vitagene network. Calabrese et al. ([Calabrese et al., 2010](#)), performed an exhaustive review on this topic, and they described in detail each single element of the vitagene pathway. Members of the Hsp70s are, in their function as molecular chaperones, involved in folding of newly synthesized proteins and refolding of damaged or misfolded proteins, as well as in assembly and disassembly of protein complexes. Trx, is a major redox control system, consisting of a 12 kDa redox active protein Trx, and a homodimeric seleno-protein called TrxR1. TrxR1 is a flavoprotein that catalyzes the NADPH-dependent reduction of oxidized thioredoxin protein. It is usually located in the cytosol, but it translocates into the nucleus in response to various stimuli associated with oxidative stress, thereby playing a central role in protecting against oxidative stress. Sirtuins are histone deacetylases which, in the presence of NAD^+ as a cofactor, catalyze the deacetylation reaction of histone substrates and transcriptional regulators. Sirtuins regulate different biological processes, such as apoptosis, cell differentiation, energy transduction, and glucose homeostasis.

Recent reviews support wide evidence on how different nutraceuticals/antioxidants can contrast aging and combat many associated pathologies, including NDs ([Leri et al., 2020](#), [Calabrese, 2020](#)). Natural polyphenols (i.e. curcumin, resveratrol, flavonols present in *Ginkgo biloba* extracts, polyphenols present abundantly in the leaves and in the ripening fruits of the olive tree, *Olea europaea*), as well as mushrooms (*Hericium Erinaceus*, *Coriolus versicolor*) can significantly modulate Nrf2 and Nrf2-dependent vitagenes expression, showing neuroprotective action. This can potentially resolve pathologies such as AD, PD and also Meniere's Disease, another degenerative pathology ([Amara et al., 2020](#), [Trovato et al., 2016a](#), [Trovato et al., 2016b](#), [Trovato Salinaro et al., 2018](#), [Scuto et al., 2019](#)).

In line with these findings, several studies demonstrated that also O₃ can modulate the vitagene network expression.

Pharmacologically, it acts in a hormetic fashion ([Bocci et al., 2011](#), [Calabrese, 2013](#)), according an inverted V shape curve. We researched studies for meta-analyses regarding Nrf2, HO-1, Hsp70, TrxR1 and sirtuins. Whereas no studies were performed between sirtuins, TrxR1 and O₃, the results indicated that O₃ can statistically increase the expression/protein levels of Nrf2, HO-1 and Hsp70 molecules ([Fig. 4](#), Random model, $Z = 4.72$ $p < 0.00001$ OR = 1.80 95%CI:1.05-2.55, even after Bonferroni correction $0.05/3 = 0.016$). Although our work has been excluded because we performed transcriptomic analyses ([Scassellati et al., 2017](#)), we confirmed the increase of the gene encoding HO-1 (*HMOX-1*), after different concentrations of O₃. The high heterogeneity in effect size among the studies ($p < 0.0001$ $I^2 = 66\%$) is essentially determined by two factors: different sources of samples (human, cell and animal models) and different methodology (biochemical and western blot analyses, ultrastructural and immunocytochemistry evaluations) (supplementary material Table 1S). Where it was possible, we performed the analysis as homogeneously as possible: in this case, O₃ concentration (20µg/ml) and exposition time (max 24 hr) were constant in all experimental conditions.

Fig. 4.



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Forest plot for odds ratio from meta-analysis of the endogenous Nrf2- vitagene pathway before and after ozone (O₃) treatment.

CI, confidence interval; Chi², χ^2 test of goodness of fit; Tau², estimate of the between-study variance in a random-effects meta-analysis. Nuclear factor Nrf2, heme-oxygenase (HO-1), heat shock protein (HSP)

Interestingly, a study reported the benefit effect of O₃ on Menière's disease ([Pawlak-Osinska et al., 2004](#)). Moreover, as reported for polyphenols and mushrooms ([Hsiao et al., 2016](#), [Ferreiro et al., 2018](#), [Oh et al., 2014](#), [Pan et al., 2018](#), [Hasanzadeh et al., 2020](#), [Wang et al., 2019b](#)), O₃ has been found to be involved in β -catenin system ([Emon et al., 2017](#)) as well as in NLRP3 (nitrogen permease regulator-like 3) inflammasome ([Yu et al., 2017](#), [Wang et al., 2018c](#)).

All these evidence support that, as polyphenols and mushrooms, O₃ acts in the same direction. Induction of vitagenes after their supplementation/administration determines a maintained response to counteract intracellular pro-oxidant status, thus providing neuroprotection.

7. Effect of Ozone Oxidative Preconditioning on Oxidative Stress Injury

Preconditioning is a process whereby an initial low dose of a stressor agent upregulates adaptive mechanisms that enhance resilience against subsequent and acute stressor agents within a time-sensitive window of ~ 10–14 days ([Calabrese, 2016](#)). Different studies demonstrated that the supplementation with *Coriolus versicolor* ([Ferreiro et al., 2018](#), [Scuto et al., 2019](#), [Trovato Salinaro et al., 2018](#), [Trovato et al., 2016a](#)), and *Hericium Herinaceus* ([Trovato Salinaro et al., 2018](#), [Trovato et al., 2016b](#)) biomass and polyphenols ([Mao et al., 2019](#)) can maintain the response to neutralize intracellular pro-oxidant/neuroinflammatory status, preventing different neurological conditions.

Same behaviour was also widely reported for O₃. The term “ozone oxidative preconditioning” (OzoneOP) was coined when repeated administration of O₃ at nontoxic doses facilitate adaptation to oxidative stress. This occurs through mild immune system activation, enhanced release of growth factors and/or activation of metabolic pathways that help maintain redox balance (increased SOD, GSH activities, decreased peroxidation).

The first studies on OzoneOP were conducted by [Barber et al., 1999](#) ([Barber et al., 1999](#)) and Leon OS et al., 1998 ([Leon et al., 1998](#)). From 1998-1999 to date, a plethora of investigations on this topic was conducted. In [Table 2](#), we reported 65 findings, of which 55 on OzoneOP, whereas 10 are the studies conducted on

postconditioning phenomenon.

Table 2.

Preconditioning/postconditioning studies of O₃ on endogenous pro-antioxidant mechanisms *in vivo* on animal models and *in vitro* on cells.

Tissues	Dosages	Results
KIDNEY	<u>Preconditioning</u> : 0.7 mg/kg, intraperitoneally, 15 applications (once daily), before methotrexate (Mtx) (6 mg/kg).	<u>Reduction</u> : malondialdehyde (MDA) decrease; <u>Increase</u> : superoxide dismutase (SOD), glutathione peroxidase (GPx); <u>Histologically</u> : ILEUM inflammatory cell infiltration, edema, reduction in the epithelium; LIVER: significant change, decrease in cumulative prolonged in these tissues.
	<u>Postconditioning</u> : <u>Sprague Dawley rats</u> : 1, 2 mg/kg, rectal insufflations, 15 applications, once a day, ischemia/reperfusion. <u>Renal tubular epithelial cell line, NRK-52E</u> : 20, 30, 40 µg/mL in complete medium, hypoxia–reoxygenation.	<u>IN VIVO</u> : <u>Reduction manner</u> : blood urea nitrogen (BUN), creatinine (Cr), malondialdehyde (MDA), bcl-2-associated poly (ADP-ribose) polymerase 1) expression, MAPK pathway. <u>Increase manner</u> : superoxide dismutase (SOD), glutathione peroxidase (GPx); <u>Histologically</u> : ozone tubular epithelium from loss of the brush border. <u>IN VITRO</u> : <u>Reduction manner</u> : MAPK pathway, bcl-2-associated X (BAX)

	(ADP-ribose) polyme expression, apoptosis malondialdehyde (M phosphorylation of p JNK. <u>Increase dose-d manner</u> : superoxide (
<u>Postconditioning</u> :	<u>IN VIVO:Reduction</u> : i
<u>Sprague Dawley rats</u> :	nitrogen (BUN), crea
2 mg/kg, rectal	malondialdehyde (M
insufflations, 15	caspace 11, interleuk
applications, once a	Interleukin-18 (IL18)
day, after	expression/protein. I
ischemia/reperfusion.	superoxide dismutase
<u>Renal tubular</u>	<u>IN VITRO: Reductior</u>
<u>epithelial cell line</u> ,	malondialdehyde (M
<u>NRK-52E</u> : 20, 30, 40	caspace 11, interleuk
µg/mL in complete	Interleukin-18 (IL-18
medium, after hypoxia-	expression/protein. I
reoxygenation.	superoxide dismutase
	viability.
	<u>Histologic Examinati</u>
	<u>Immunofluorescence</u>
	prevented renal dam
	Jablonski grading sca
	decreased caspace 1.
<u>Postconditioning</u> : 0.5	<u>Reduction</u> : serum cre
mg/kg, rectal	blood urea nitrogen (
insufflations, after	myeloperoxidase (MI
ischemia/reperfusion.	malondialdehyde (M
A control with Oxigen	muscle actin (α-SMA
was used.	growth factor β1 (TG
	Smad 2 protein. <u>Incr</u>
	dismutase (SOD).
	<u>Histology</u> : Jablonski
	histologic appearance
	necrosis, renal areas

tubulointerstitial fibr
minimal phenomeno
Immunohistochemistry: M
SMA positive) were f
ozone-treated sample

Preconditioning: 1
mg/kg, rectal
insufflations, 15
applications, once a
day, before
ischemia/reperfusion.

Reduction: α -smooth
SMA), transforming β
(TGF- β 1) expression/
Smad7 expression/p
Morphological/immu
increase in collagen α
in α -SMA expression.

Postconditioning: 0.5
mg/kg, daily for the 10
days' reperfusion, after
ischaemia-reperfusion.
A control was
performed with
Oxygen.

Reduction: serum cre
blood urea nitrogen (C
thiobarbituric acid re
(TBARS). Increase: fi
phospholipase A2, su
dismutase (SOD).
Morphology: minima

Preconditioning: 1
mg/kg, rectal
insufflations, 15
applications, once a
day, before the kidney
transplantation.

Reduction: serum blc
(BUN), creatinine (C
malondialdehyde (M
allograft cell apoptos
superoxide dismutase
glutathione (GSH), c
nuclear factor erythro
factor 2 (Nrf-2), hem
(HO-1).

Morphological/immu
lower levels of dama
renal allograft.

Preconditioning: 0.7
mg/kg/d,
intraperitoneally, 5

Reduction: serum blc
(BUN), creatinine (C
malondialdehyde (M

days, before the induction of contrast-induced nephropathy. A control group was with Oxygen.

Preconditioning: 1 mg/kg, rectal insufflations, 15 applications, once a day, before ischemia/reperfusion and/or ischemic preconditioning.

Preconditioning: IN VITRO Renal tubular epithelial cell line, NRK-52E, 20, 30, 40 µg/mL in complete medium, before hypoxia/reoxygenation.

Preconditioning: 1

status (TOS). Increase nitric acid (NO), total status (TAS). Histopathologic evaluation in degeneration of tubular dilatation of Bowman's capsule in tubular epithelium congestion.

Reduction: malondialdehyde (MDA), urea nitrogen (BUN), creatinine, and Jablonski grading scale. Increase: serum nitric oxide synthase (endothelial nitric oxide synthase, eNOS) expression, glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px).

Histological Examination/Immunofluorescence: improved renal dysfunction, reduced histological damage, decreased oxidative stress, increase presence of endothelial, eNOS and decreased iNOS.

Reduction dose-dependent: malondialdehyde (MDA), malondialdehyde (MDA) dehydrogenase (LDH), Bcl-2 associated X (BAX), Bcl-2, (polymerase 1, p16, ribose) expression. Increase in a dose-dependent manner: superoxide production. Immunocytochemistry: cleaved caspase3-positive cells.

Reduction: serum blood

mg/kg, rectal insufflations, 15 applications, once a day, before ischemia/reperfusion.

(BUN), creatinine (C malondialdehyde (M myeloperoxidase (M necrosis factor- α (TN 1β (IL- 1β), interleuki intercellular adhesio (ICAM-1), monocyte protein 1 (MCP-1), T (TLR4), nuclear fact expression/protein, c associated X (BAX), I
Morphology: decomp Jablonski scale histol

Preconditioning: 1 mg/kg, rectal insufflations, 15 applications, once a day, before ischemia/reperfusion.

Reduction: malondia serum blood urea nit creatinine (Cr), tumc α (TNF- α), interleuki interleukin-6 (IL-6), : adhesion molecule (I monocyte chemoattr: (MCP-1), Toll-Like Re and nuclear factor (N expression/protein /immunoistochemica 2-associated X (BAX)
Morphological/Immu features: relieved tub medullary haemorrh: and development of j casts, reduction in Ja

Preconditioning: 1 mg/kg, rectal insufflations, 15 treatments, once a day, before

Reduction: serum blc (BUN), creatinine (C grading scale scores,
Increase: serum nitri synthase (endothelia

ischemia/reperfusion. As control was used also Oxygen.

Postconditioning: 0.5 mg/kg, rectal insufflations, 10 applications, once a day, after ischemia/reperfusion. As control was used also Oxygen.

Preconditioning: 1.1 mg/kg, intraperitoneally, 5 days, before induction of diabetes. Other groups were diabetic rats/insulin.

Preconditioning: 25 mcg/ml, intraperitoneally, 15 days, before methotrexate (20 mg/kg).

inducible, iNOS) exp superoxide dismutase glutathione (GSH), g peroxidase (GSH-Px) Morphology: preserv histology.

Histopathological/M significant difference fraction and protein in glomerular filtrate plasma flow, creatini histological damage.

Reduction: Systolic b (SBP), Diastolic bloo Glycosylated hemogl serum blood urea nit creatinine (Cr), aldose malondialdehyde (M superoxide dismutase glutathione peroxida catalase (CAT).

Reduction: malondia Myeloperoxidase (M) necrosis factor- α (TN 1 β (IL-1 β)). Increase: (GSH).

Histopatologically: degeneration of glom glomerular congestio Bowman's space, deg proximal tubuli, deg tubuli, tubular basal wrinkling, vascular c

<p><u>Preconditioning</u>: 0.36, 0.72, 1.1, 1.8, 2.5 mg/kg, rectal insufflations, 15 applications, before cisplatin-induced nephrotoxicity (6 mg/kg).</p>	<p>interstitial edema, in cell infiltration.</p> <p><u>Reduction dose-dependent</u> creatinine (Cr) (0.72 thiobarbituric acid-reactive substances (TBARS)). <u>Increase dose-dependent manner</u>: glutathione superoxide dismutase, glutathione peroxidase (0.72, 1.1 mg/kg), creatinine.</p> <p><u>Histopathological changes</u>: 1.8 and 2.5 mg/kg, histology showed significant improved tissue</p>
<p><u>Preconditioning</u>: 1 mg/kg, intraperitoneally, 6 hours before and 6 hours after contrast-induced nephropathy agent (10 ml/kg), 5 days.</p>	<p><u>Increase</u>: total antioxidant capacity (TAC), lipocalin (NG2), increase in creatinine.</p> <p><u>Histopathological alterations</u>: improving in Renal tubular hemorrhage, cast formation</p>
<p><u>Preconditioning</u>: Major Ozonated Autohemotherapy in 5 ml blood rabbit, before ischemia/reperfusion.</p>	<p><u>Reduction</u>: interleukin-1, tumor necrosis factor-α (TNF-α), neutrophil to lymphocyte ratio (NLR), ischemia-modified albumin (IMA), total oxidant status, oxidative stress index, total antioxidant status.</p> <p><u>Histopathological changes</u>: the tubular brush border, tubular cast (TC), tubular necrosis (TN), intertubular hemorrhage, congestion (IHC), dilated Bowman space (DBS)</p>

Preconditioning: 0.5 mg/kg, rectal insufflations, 15 treatments, before ischaemia/reperfusion. Oxygen was used as further control.

Reduction: Phospholipase A2, Fructosamine. Increase: hippurate (PAH), inulin clearance, superoxide dismutase (SOD).

Morphology: increased renal blood flow (RPF), glomerular filtration rate (GFR).

Preconditioning: 0.8, 2.4, 4 mg/kg, intraperitoneally, daily for 5 days, with/without sepsis. A control was performed with Oxygen.

Reduction: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, thiobarbituric acid reactivity (TBARS), myeloperoxidase (MPO).

Increase: superoxide dismutase, glutathione peroxidase.

Preconditioning: 1 mg/kg, transrectal insufflations, once a day, 15 treatments, before the kidney transplant procedure.

Reduction: blood urea nitrogen, serum creatinine (Cr), serum albumin, Jablonski grade, serum interleukin-6 (IL-6), IL-18, cyclooxygenase-2, Malonaldehyde (MDA), nuclear factor- κ Bp65 and anti-rat antibody (HMGB1) expression/protein. Increase: Superoxide Dismutase, Glutathione peroxidase.

Morphology: alleviated morphological damage, reduced the injury of brush border, preserved renal tubular, restrained the level of NF- κ Bp65 in the kidney, suppressed the expression of HMGB1 in renal tissue.

150 mg/kg, intraperitoneally, single dose for 10 days, at the same time *Escherichia coli*.

Reduction: lactate dehydrogenase (LDH) (Liver, Kidney), creatinine.

Increase: Succinate dehydrogenase (SDH) (Lungs, Heart).

coli toxin (LPS) (20 mg/kg).

triphosphatase (ATPase), acid phosphatase (Acid Phosphatase) (Kidney, Lungs, Heart), Glucuronidase (Liver). Histochemically determined succinate dehydrogenase activity was extinguished enzymatically in the central parts of the liver, paralleled by narrow bands (Liver).

Histochemically determined lactate dehydrogenase activity showed increased activity (hepatocytes, Kupffer cells, Liver).

Histochemically determined adenosine triphosphatase activity showed decrease intensity of ATPase (Liver).

Histochemically determined acid phosphatase (Acid Phosphatase) activity showed decrease in activity (Liver).

Histochemically determined succinate dehydrogenase activity showed reaction in tubular epithelium, slightly more pronounced in the

Histochemically determined lactic dehydrogenase activity showed pronounced stimulation in principal tubules and nephrons (Kidney).

Histochemically determined adenosine triphosphatase activity showed decreased intensity of activity in renal glomeruli and in vessels, particularly in the smaller caliber (Kidney).

Histochemically detected acid phosphatase (ACP) activity of the reaction in the principal tubuli and collecting ducts (Kidney).

Histochemically detected succinate dehydrogenase (SDH) activity more pronounced after treatment.

Histochemically detected lactate dehydrogenase (LDH) activity stimulation was less pronounced (Lungs).

Histochemically detected adenosine triphosphatase (ATPase) activity changing (Lungs).

Histochemically detected acid phosphatase (ACP) activity (Lungs).

LIVER

Preconditioning: 0.2, 0.4, 1.2 mg/kg intraperitoneally, once daily, for 5 days, before lipopolysaccharide (LPS) injection (30 mg/kg).

Dexamethasone (30 mg/kg) used as a reference drug.

Preconditioning: 0.2, 0.4, 1.2 mg/kg intraperitoneally, once daily, for 5 days, before lipopolysaccharide (LPS) injection (0.1 mg/kg).

Reduction dose-dependent manner: thiobarbituric acid reagent (TBARS). Increase dose-dependent manner: glutathione

Reduction dose-dependent manner: serum Tumor Necrosis Factor- α , thiobarbituric acid reagent (TBARS). Independent manner: glutathione transferase (GST), glutathione peroxidase (GSH-Px)

Dexamethasone (30 mg/kg) used as a reference drug.

Preconditioning: 0.2, 0.4, 1.2 mg/kg, intraperitoneally, 0.2, 0.4 mg/kg, rectal application, once daily for five days, before lipopolysaccharide (LPS) injection (0.1 mg/kg).

Preconditioning: 50 µg/ml (4.4–5.0 ml), 15 treatments, one per day, before carbon tetrachloride (CCl₄). Ozone control groups were: 1. A control was with Oxygen; 2. another control was ozone without CCl₄.

Preconditioning: 1 mg/kg, rectal insufflations, 15 treatments, one per day, before ischaemia–reperfusion.

Preconditioning; 0.7

Reduction dose-depe serum Tumor Necros: alpha.

Reduction: Aspartic a transaminase (AST), hepatic lipid peroxid: thiobarbituric acid-re substances). Increase (CHEase), superoxid (SODs), Catalase (CA dependent (Ca-ATPas (GSH), glucose-6-pho dehydrogenase (G6P Morpho-metric evalu hepatic damage: redu damage area.

Reduction: Aspartic a transaminase (AST), aminotransferase (AI malondialdehyde (M hydroxyalkenals, nitr /NO₃⁻). Increase: sup dismutase (SOD), tot (TH), glutathione (G GSH/GSSG.

Reduction: serum ala

mg/kg, intraperitoneally, daily five times, before 70% partial hepatectomy. aminotransferase (ALT) and aspartate aminotransferase (AST) levels were significantly reduced. Tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6) levels were also significantly reduced. Histopathological examination: animals in the ozone group improved in liver weight, total protein, and proliferating cell nuclear antigen (PCNA) labeling index.

Preconditioning: 0.7 mg/kg, intraperitoneally, daily five times, before total body irradiation with a single dose of 6 Gy. Reduction time-dependent serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, tumor necrosis factor alpha (TNF- α), and malondialdehyde (MDA) levels were significantly reduced. time-dependent malondialdehyde (MDA) levels were significantly reduced. time-dependent superoxide dismutase (SOD).

Histopathological examination: ozone preconditioning significantly reduced the degree of hepatocellular degeneration, inflammation, sinusoidal congestion and dilatation, and central vein congestion. It also reduced the number of inflammatory cells in the lamina propria, regulated the structure of the lamina propria, abundant glycogen in the epithelium, and reduced the number of inflammatory infiltrate in the lamina propria.

Preconditioning: 0.5 mg/kg, intraperitoneally, daily five times, before lipopolysaccharide (LPS) injection (20 mg/kg). Ketamine (5 mg/kg) used as a reference drug. Reduction: Nuclear factor kappa B (NF- κ B) staining was significantly reduced. Morphology/Immunohistochemistry parameters: intact hepatic architecture, normal cell membrane integrity, and reduced inflammatory cell infiltration (p-actin and p-I κ B-positive staining).

Preconditioning: 1 mg/kg, intraperitoneally, daily five times, before LPS injection (20 mg/kg). Ketamine (5 mg/kg) used as a reference drug. Reduction: serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were significantly reduced.

mg/kg, rectal insufflations, 15 treatments, one per day, before ischemia/reperfusion. Agonist (2-chloro N6-cyclo-pentyladenosine, CCPA), Antagonist (8-cyclopentyl-1,3-dipropylxanthine, DPCPX) of A1 subtype receptor.

Preconditioning: 1 mg/kg, rectal insufflations, 15 treatments, one per day, before ischemia/reperfusion. Cycloheximide (CHX) to promote protein synthesis inhibition after OzoneOP treatment.

Preconditioning: 1 mg/kg, rectal insufflations, 15 treatments, one per day, before ischemia/reperfusion and/or ischaemic

aminotransferase (ALT), aspartate aminotransferase (AST) (NO) (nitrite/nitrate), adenosine deaminase, malondialdehyde (MDA), hydroxyalkenals, attenuated increase, NF-kB (p65) expression, tumor necrosis factor alpha (TNF- α), heat shock protein 70 (HSP70). Increase: glial cell nuclear protein-1 (GCN1) Immunohistochemistry: preservation of the liver architecture, prevent inflammatory recruit

Reduction: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), malondialdehyde (MDA), hydroxyalkenals. Increase: MnSOD, glutathione peroxidase (GSH/GSSG).

Histological lesions: morphology of the acinar cells operated. Ultrastructure: normal appearance of rough endoplasmatic reticulum, peroxisome, no alteration of structure.

Reduction: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), malondialdehyde (MDA), hydroxyalkenals. calculate total Xanthine dehydrogenase (XDH) and xanthine oxidase (XO)

preconditioning.

Oxygen was another control comparison.

Preconditioning: 1 mg/kg, rectal insufflations, 15 treatments, one per day, before carbon tetrachloride (CCl₄) (1 ml/kg). An ozone control group was ozone without CCl₄.

Preconditioning: 0.7 mg/kg, intraperitoneally, 15 applications (once daily), before methotrexate (Mtx) (6 mg/kg).

Preconditioning: 10, 30, 50 µg/ml, intraperitoneally, 5 days, before sepsis induced by intraperitoneal injection of rat fecal material (0.5 g per kg of animals weight)

sulfhydryl groups.

Improvement in histological parameters: normal in hepatic lobuli.

Reduction: uric acid, thiobarbituric acid-reactive (TBARS). Increase: hepatic liver weight (LW)/body weight ratios, superoxide dismutase (SOD), catalase (CAT).

Histopathological findings: permanence of glycogen in hepatic cells was prominent. Minimal non-parenchymal reaction co-existed at portal vein.

Reduction: malondialdehyde (MDA). Increase: superoxide dismutase (SOD), glutathione peroxidase (GPx). Histologically: ILEUM: inflammatory cell infiltration, edema, reduction in thickness of the epithelium; LIVER: no significant change, despite cumulative prolonged exposure to these tissues.

Reduction dose-dependent: LIVER/LUNG: conjugated thiobarbituric acid-reactive (TBARS), Total protein, Increase dose-dependent: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), Total antioxidant capacity (TAC)

extracted from another donor rat. A control group was performed with Oxygen.

Preconditioning: 0.8, 2.4, 4 mg/kg, intraperitoneally, daily for 5 days, with/without sepsis. A control was with Oxygen.

150 mg/kg, intraperitoneally, single dose for 10 days, at the same time *Escherichia coli* toxin (LPS) (20 mg/kg).

Reduction: serum alanine transferase (ALT), aspartate transferase (AST), creatinine, thiobarbituric acid reagent (TBARS), myeloperoxidase (MPO).

Increase: superoxide dismutase, glutathione peroxidase (GPx).

Reduction: lactate dehydrogenase (LDH) (Liver, Kidney).

Increase: Succinate dehydrogenase (SDH) (Lungs, Heart), ATPase, acid phosphatase (Acid Phosphatase) (Kidney, Lungs, Heart), Glucuronidase (Liver), Histochemically detected succinate dehydrogenase activity in the central parts of the liver was paralleled by narrow bands (Liver).

Histochemically detected lactate dehydrogenase activity increased activity (hepatocytes, Kupffer cells, Liver).

Histochemically detected adenosine triphosphatase activity decrease intensity of ATPase (Liver).

Histochemically detected acid phosphatase (Acid Phosphatase) activity decrease in activity (Liver).

Histochemically dete
succinate dehydroge
 reaction in tubular ep
 slightly more pronou
Histochemically dete
lactic dehydrogenase
 pronounced stimulat
 principal tubules and
 nephrons (Kidney).

Histochemically dete
adenosine triphosph
 decreased intensity o
 renal glomeruli and i
 vessels, particularly t
 caliper (Kidney).

Histochemically dete
acid phosphatase (Ac
 intensity of the reacti
 principal tubuli and c
 (Kidney).

Histochemically dete
succinate dehydroge
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Histochemically dete
lactate dehydrogenas
 stimulation was less
 (Lungs).

Histochemically dete
adenosine triphosph
 changing (Lungs).

Histochemically dete
acid phosphatase (Ac
 activity (Lungs),

LUNG

Preconditioning: 0.7
 mg/kg,

Reduction: malondia
 serum tumor necrosi

intraperitoneally, 5 applications (once daily), before total body irradiation (TBI) (6 Gy).

Preconditioning: 100 µg/kg, intraperitoneally, once daily for 10 days, before ischemia/reperfusion. A control was performed with Oxygen.

Preconditioning: 0.8, 2.4, 4 mg/kg, intraperitoneally, daily for 5 days, with/without sepsis. A control was performed with Oxygen.

Preconditioning: **IN VITRO A549 cell lines**, 1, 10, 20, 80 mol/L, before H₂O₂.

(TNF-α), interleukin-
Increase: superoxide
Histopathological ev
reduction in alveolar congestion, and alveolar bronchiolar hemorrhage

Reduction: malondialdehyde (MDA), myeloperoxidase (MPO), inflammasome (NLRP3) associated speck-like protein containing a caspase recruitment domain (ASC), caspase-1 cleavable cysteine-dependent aspartate-specific protease-1 (procaspase-1) (procaspase-1), apoptotic interleukin-1 beta (IL-1β), transcription factor NF-κB, superoxide dismutase (SOD).

Macroscopic and histopathological and edematous tissue, alveolar septum, rupturing alveolar hemorrhage disappeared

Reduction: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, thiobarbituric acid reactive substances (TBARS), myeloperoxidase (MPO).
Increase: superoxide, malondialdehyde, glutathione peroxidase (GSH-Px)

Reduction dose-dependent: bcl-2-associated X (BAX), bcl-2, transcription factor NF-κβ, tumor necrosis factor alpha (TNF-α), Inducible nitric oxide synthase (iNOS), nitric oxide (NO)

Increase dose-dependently catalase (CAT), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), glutathione (GSH).

Morphology: recover cells from the toxicity of hydrogen peroxide, proliferation, prevent 11.0% of cell loss.

Preconditioning: 10, 30, 50 µg/ml, intraperitoneally, 5 days, before sepsis induced by intraperitoneal injection of rat fecal material (0.5 g per kg of animals weight) extracted from another donor rat. A control group was performed with Oxygen.

150 mg/kg, intraperitoneally, single dose for 10 days, at the same time *Escherichia coli* toxin (LPS) (20 mg/kg).

Reduction dose-dependently LIVER/LUNG: conjugated thiobarbituric acid-reactive substances (TBARS), Total protein oxidation (TOS). Increase dose-dependently superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), Total antioxidant capacity (TAC).

Reduction: lactate dehydrogenase (LDH) (Liver, Kidney).

Increase: Succinate dehydrogenase (SDH) (Lungs, Heart), ATPase, acid phosphatase (Acid Phosphatase) (Kidney, Lungs, Heart), Glucuronidase (Liver).

Histochemically detected succinate dehydrogenase extinguished enzyme in the central parts of the liver, paralleled by narrow bands (Liver).

Histochemically detected

lactate dehydrogenase
increased activity (he
Kupffer cells, Liver).

Histochemically dete
adenosine triphosphatase
decrease intensity of
ATPase (Liver).

Histochemically dete
acid phosphatase (Acid
decrease in activity (C

Histochemically dete
succinate dehydrogenase
reaction in tubular ep
slightly more pronou

Histochemically dete
lactic dehydrogenase
pronounced stimulat
principal tubules and
nephrons (Kidney).

Histochemically dete
adenosine triphosphatase
decreased intensity o
renal glomeruli and i
vessels, particularly t
caliper (Kidney).

Histochemically dete
acid phosphatase (Acid
intensity of the reacti
principal tubuli and c
(Kidney).

Histochemically dete
succinate dehydrogenase
more pronounced alt

Histochemically dete
lactate dehydrogenase
stimulation was less

HEART

Preconditioning: rectal insufflations as five applications per week. In a group: 0.3 mg/kg/day in the first week, and 0.5 mg/kg/day in the second week. In another group, 0.6 mg/kg/day in the first week, and 1 mg/kg/day in the second week, before ischemia/reperfusion. A group was performed with Oxygen.

Preconditioning: 100 µg/kg/day, intraperitoneally, once daily, 5 days, before ischemia/reperfusion. A control was performed with Oxygen.

(Lungs).

Histochemically dete
adenosine triphosphæ
changing (Lungs).

Histochemically dete
acid phosphatase (Ac
activity (Lungs).

Reduction dose-depe
creatin kinase-MB (C
myeloperoxidase (M
nitrate/nitrite (NOx)
acid reactive substan

Increase dose depend
Myocardial adenine 1
ADP, AMP, TAN), glut

Histological examina
ultrastructural analys
in edema in between
and edema within m
myofibrillar arranger
slight edema around
mild mitochondrial s
decreased matrix der
disruption of mitoch
vesiculation, slight m
chromatin near nucle

Reduction: microtubu
protein 1 light chain
PTEN-induced putati
(PINK1), cytochrome
IV (COX4), Caspase 3
apoptosis. Increase: 1
(erythroid-derived 2)
glutamate-cysteine li
subunit (GCLC), glut

ligase modifier subunit
superoxide dismutase
expression.

Morphology: mild myocardial
injury.

Validation of: 1. nuclear
(TATA-binding protein
nuclear extracts), 2. cytosolic
fractions separated from
cytoplasmic fraction
oxidase subunit IV (C
detectable).

Preconditioning: 0.6
mg/kg, rectal
insufflations,
twice/week for the first
3 months, then
once/week till the age
of 15 months, in aged
rats. A control was
performed with
Oxygen.

Reduction: malondialdehyde
protein carbonyls (Pr
cytosolic Ca^{2+} (heart
Increase: glutathione
status (ATP, ADP)
(heart/hippocampus)
ATPase (hippocampus)

Preconditioning: 50, 80
mL/kg, single (1x) or
repetitive (5x)
insufflations, in rat
cardiac transplant
model.

Prolonged cardiac all
without any adjunctive
immunosuppressive treatment
alternated number of
decreased number of
increase of white blood
granulocytes.

Preconditioning; 0.3
mg/kg, rectal
insufflations, once on
alternating days for 20
sessions, before
doxorubicin (2 mg/kg).

Reduction: pro-brain natriuretic
peptide (BNP), malondialdehyde
(MDA), advanced oxidation
products (AOPP). Increase
dismutase (SOD), catalase
Morphology: slight decrease

The Oxygen group was a further control. morphology of cardiac 90% survival rate, re body weight.

150 mg/kg, intraperitoneally, single dose for 10 days, at the same time *Escherichia coli* toxin (LPS) (20 mg/kg). Reduction: lactate de (LDH) (Liver, Kidney; Increase: Succinate D (SDH) (Lungs, Heart triphosphatase (ATPase acid phosphatase (Ac Kidney, Lungs, Heart; Glucuronidase (Liver; Histochemically detected succinate dehydrogenase extinguished enzymatic central parts of the liver paralleled by narrow (Liver). Histochemically detected lactate dehydrogenase increased activity (he Kupffer cells, Liver). Histochemically detected adenosine triphosphatase decrease intensity of ATPase (Liver). Histochemically detected acid phosphatase (Ac decrease in activity (C; Histochemically detected succinate dehydrogenase reaction in tubular epithel slightly more pronounced Histochemically detected lactic dehydrogenase pronounced stimulation principal tubules and

nephrons (Kidney).

Histochemically detected adenosine triphosphatase decreased intensity of renal glomeruli and interstitial vessels, particularly the afferent arteriole (Kidney).

Histochemically detected acid phosphatase (ACP) intensity of the reaction in the principal tubuli and collecting ducts (Kidney).

Histochemically detected succinate dehydrogenase more pronounced alterations in the lungs. Histochemically detected lactate dehydrogenase stimulation was less pronounced (Lungs).

Histochemically detected adenosine triphosphatase changing (Lungs).

Histochemically detected acid phosphatase (ACP) activity (Lungs).

INTESTINE Preconditioning: 0.7 mg/kg, intraperitoneally, daily five times, before irradiation of 500 cGy.

Reduction: malondialdehyde, myeloperoxidase (MPO), bursting pressure value, anastomosis, Hydroxyl superoxide dismutase. Histopathological evaluation: improving in anastomosis healing, granulation tissue development and histology corresponding to the

<p><u>Preconditioning:</u> 0.7 mg/kg, intraperitoneally, daily five times, before total body irradiation with a single dose of 6 Gy.</p>	<p>inflammatory response</p> <p><u>Reduction time-dependent:</u> serum alanine aminotransferase, aspartate aminotransferase, tumor necrosis factor-α, malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT).</p> <p><u>Histopathological examination:</u> reduction in hepatocellular degeneration, inflammation, congestion and dilatation of sinusoids and central inflammatory cell infiltration in lamina propria, regular structure, abundant goblet epithelium, reduced inflammatory infiltrate in the lamina propria.</p>
<p><u>Preconditioning:</u> 0.7 mg/kg, intraperitoneally, 15 applications (once daily), before methotrexate (Mtx) (6 mg/kg).</p>	<p><u>Reduction:</u> malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT).</p> <p><u>Histologically:</u> ILEUM: reduction in inflammatory cell infiltration, edema, reduction in the epithelium; LIVER: no significant change, despite cumulative prolonged ischemia in these tissues.</p>
<p><u>Postconditioning:</u> 0.7 mg/kg/day, intraperitoneally and intraluminally, laparotomy and/or ischemia/reperfusion.</p>	<p><u>Macroscopic Appearance:</u> mucosal weight in jejunum, mucosal weight in jejunum, DNA and protein in jejunum, villus height in jejunum and ileum, cell proliferation in jejunum.</p>

COCHLEAR

Preconditioning: 1 mg/kg, intraperitoneally, 7 days, before ischemia/reperfusion.

Postconditioning: 60 µg/mL, rectal and/or intratympanic, 7 days, after cisplatin-induced ototoxicity (5-mg/kg/day). The rats were tested with distortion product otoacoustic emissions (DPOAE).

Postconditioning: 30 µg/ml, intravenous, daily administration for 14 days, at the same time with noise exposure.

ERK protein. Reduction: Score in jejunum and enterocyte apoptosis ileum, caspase 3.

Reduction: apoptotic malondialdehyde (MDA), oxidant score (TOS), superoxide dismutase (SOD), glutathione peroxidase (GPx), antioxidant capacity (CAT).

Histological evaluation: numbers of glial cells, ganglion, reduced level of vascularization.

Statistically significant DPOAE results.

Histopathological section: stria vascularis damage, inner-outer hair cell loss.

Reduction: malondialdehyde (MDA), % mitochondrial swelling, mitochondrial membrane potential (MMP), Glutathione S-transferase (GST), cytochrome c (Brain, cochlear), ATP.

Histopathological findings

		mitochondrial memb (MMP) collapse, mito swelling, cytochrome
SKELETAL	<u>Preconditioning</u> : 0.7 mg/kg, intraperitoneally; 4 doses, before ischemia.	<u>Reduction</u> : malondia Serum nitrite-nitrate nitric oxide synthase immunostaining. <u>Incr</u> peroxidase (GSH-Px) dismutase (SOD).
	<u>Preconditioning</u> : 0.7 mg/kg, 6 days, before ischemic period and/or hypothermia.	<u>Reduction</u> : malondia interleukin-1 β (IL-1 β kinase (CK), aspartat aminotransferase (AS oxide (NO). <u>Increase</u> peroxidase (GSH-Px) dismutase (SOD). <u>iNOS immunohistoch</u> mild intensity.
PANCREAS	<u>Preconditioning</u> : 50 μ g/kg, intraperitoneally, once a day for seven days. Streptozotocin (STZ) (2 ml). A control was performed with Oxygen.	<u>Reduction</u> : 4-hydroxy HNE), Poly (ADP-ribo (PARP-1), glucagon, ; <u>Increase</u> : nuclear fac glutathione-s-transfe insulin, leptin. <u>Immunohistochemist</u> tissue degeneration e partial restoration of population size of isl and absence of islet c <u>Immunofluorescence</u> death, decreased DN.
	<u>Postconditioning</u> : 0.7- mg/kg, intraperitoneally, daily	<u>Reduction</u> : serum am lipase, aspartate ami (AST), alanine aminc

for 3 days, induction of acute necrotizing pancreatitis. A control was performed with Oxygen.

(ALT), γ -Glutamyl transaminase, malondialdehyde (MDA), Alkaline phosphatase, glutathione peroxidase, superoxide dismutase. Increase in weight. Liver in infected rats.

Histopathologic analysis: degrees of necrosis and infiltration. Improvement in histological injury score.

ARTHRITIS Postconditioning: 80 mg/kg, articular space 3 times/week (3.5 weeks) after PG/PS-induced arthritis. A control was performed with Oxygen.

Reduction: TNF α and expression/protein, and Fructolysine. Increase in Superoxide Dismutase (SOD), calcium. Ameliorate the joint score of arthritis index.

Histological results: improved morphology.

TESTICULAR Preconditioning: 1 mg/kg, intraperitoneally, before detorsion for 2 hours.

Reduction: Ischemia (IMA), Total Oxidant, Oxidative Stress Index. Histopathological score.

OTHER Preconditioning: 1 mg/kg, rectal insufflations, 15 sessions in 5 weeks, in alternated days, 2 mL/kg of lipofundin. A control group was performed with Oxygen.

Reduction: malondialdehyde peroxidation potential, oxidation protein carbonyl, nitric oxide (NO). Increase in glutathione (GSH).

Histopathology: smaller aortas, smaller intima.

[Open in a new tab](#)

We observed that OzoneOP exerts a protective effect on ischemia-reperfusion injury (IRI) in rat models of cochlear, hepatic, intestinal, renal, cardiac, lung and skeletal ischemia through an oxidative preconditioning mechanism that prevents the increase of the endogenous pro-oxidant and stimulates antioxidant mechanisms ([Table 2](#)). Some authors also developed an *in vitro* Hypoxia/Reoxygenation (H/R) model to simulate OzoneOP, using normal rat kidney epithelial (NRK-52E) cells. This to eliminate confounding variables linked to animal models ([Wang et al., 2014a](#), [Wang et al., 2018a](#)). Interestingly, the results confirmed those obtained in *in vivo* animal model ([Table 2](#)).

OzoneOP prevents also other different kind of injury: lipopolysaccharide (LPS) injection, carbon tetrachloride, partial hepatectomy, total body irradiation, methotrexate, intraperitoneal injection of rat fecal material, sepsis, kidney and cardiac transplantation, contrast-induced nephropathy, induction of diabetes, cisplatin-induced nephrotoxicity, contrast-induced nephropathy agent, H₂O₂, doxorubicin, ototoxicity, noise exposure, hypothermia, lipofundin ([Table 2](#)).

Different methodological systems have been implemented in these studies. The different authors analysed differences in mRNA gene expression levels as well as protein levels in Western Blot and biochemical analyses. All authors performed morphological, histopathological, immunofluorescence, and immunohistochemistry evaluations, in parallel and in concordance with molecular investigations. Interestingly, in some cases, the effects observed were strongly dose and time-dependent ([Table 2](#)).

In some cases (10 in total), the studies have been performed in

postconditioning, obtaining the same outcomes. León Fernández et al. ([Leon Fernandez et al., 2012](#)) investigated the systemic redox status of patients with low back pain and neck pain, and if O₃ oxidative postconditioning modified the pathological oxidative stress and protected against oxidative protein damage. In 33 patients with diagnosis of disc hernia (DH), 100% showed a severe oxidative stress. Major changes in SOD, total hydroperoxides, AOPP, fructolysine, and MAD were observed. After O₃ postconditioning, there was a re-establishment of patients' cellular redox balance as well as a decrease in pain in both DH. This demonstrated that O₂-O₃ therapy protected against oxidation of proteins and reduced the pain.

8. Conclusions

According to [Cuadrado et al. \(2018\)](#), [Cuadrado et al. \(2019\)](#), systems medicine identifies a cluster of chronic disease pathophenotypes including NDs in which Nrf2 plays a fundamental role. Similarly, Nrf2 is strongly implicated in aging processes ([Zhang et al., 2015](#), [Schmidlin et al., 2019](#), [Silva-Palacios et al., 2018](#)). These condition/diseases share common mechanisms and the results represent a first attempt to structure Nrf2 as a common therapeutic and systems medicine approach.

We here have presented extensively research and strength on the antioxidant activities of O₃ correlated with the interaction with Nrf2 ([Galie et al., 2018](#), [Siniscalco et al., 2018](#), [Re et al., 2014](#), [Vaillant et al., 2013](#)), along with anti-apoptotic functions by acting on mitochondrial Bax, caspases, p53 and HIF α molecules ([Yong et al., 2017](#), [Guclu et al., 2016](#)), pro-autophagy and bioenergetic activities on Krebs' cycle. This paper provides a road map for mechanism-based systems medicine where O₃-Nrf2-vitogene network plays a crucial role in the modulation of the cellular redox balance, in the reduction of the formation of ROS/RNS, in the change of apoptotic and autophagy mechanisms ([Vikram et](#)

[al., 2017](#)). This underlines the evidence to become potential new therapeutic targets for NDs, and at the same time to reduce the aging physiological mechanisms and cognitive decline, potential risk factors to develop more severe neurodegeneration damage.

Challenges regarding treatments efficacy and costs still persist for NDs. Thus, we suggest that O₂-O₃ therapy could represent a useful, safe, no-invasive, no-pharmacological, economical, effective treatment for these neurodegenerative conditions. In the medical setting, this therapy employs a gas mixture of O₂/O₃, obtained from the modification of medical-grade O₂ using certificated O₃ generator device (Bocci, V, 2011). Based on the basic mechanisms of action of O₃ in blood, the therapeutic range of O₃ has been precisely calculated and found to be 10–80 µg/ml of O₃ in blood ([Schwartz-Tapia et al., 2015](#)). O₃ medical preparations are classified into three types: ozonized water, ozonized oil and ozonized gas, whereas different and main routes of application with relative concentrations of O₃ are widely described in Schwartz-Tapia et al., 2015 ([Schwartz-Tapia et al., 2015](#)).

The side effects are minimal; the World Federation of Ozone therapy (WFOT) estimates the incidence of complications at 0.0007%. Moreover, the treatment is not only perfectly tolerated but most of patients have reported a feeling of wellness and euphoria throughout the cycle. This fact explains why the compliance of the patients remains excellent throughout the years.

The mechanisms of the positive effects of O₃ are attributed not only to up-regulation of cellular antioxidant enzyme activity, but also to the activation of the immune and anti-inflammatory systems, modulation of NLR3 inflammasome, action on proteasome, enhancement in the release of growth factors from platelets, improvement in blood circulation and O₂ delivery to damaged tissues, and enhancement of general metabolism, along

with being a potent bactericide, fungicide and virucidal with potential effect on gut microbiota (for review [Scassellati et al., 2020](#)). Consequently, these combinatorial effects could impact on cognitive and neurodegenerative domains, directly or indirectly through the mediation of gut microbiota ([Cattaneo et al., 2017](#)). Nrf2-ARE and vitagene network, but also NF- κ B, NFAT (nuclear factor activated T-cells), AP-1 (Activated Protein-1), HIF α are the principal signalling pathways on which O₃ exercises its effects (for review [Scassellati et al., 2020](#)). These effects could be sharable with those involved in NDs, where high inflammation and oxidant state, mitochondria dysfunctions, metabolic alterations, and slowdown in regenerative processes and immune system characterize these disorders.

As reported in [Smith et al., 2017](#), to date systems are available and proposed to have a more precise measurement of the redox state of a patient. One system proposes simultaneously measuring different biological markers in the blood such as GSH, GSH-Px, GST, SOD, CAT, conjugated dienes, total hydroperoxides, and TBARS. Using an algorithm, information can be gathered about the total antioxidant activity, total pro-oxidant activity, redox index, and grade of oxidative stress. Thus, systems like this can provide insights to the correct dosage and response to O₃ therapy based on oxidative stress levels seen in the patient.

With the awareness that further studies are needed, this review reports substantial scientific evidence for building a rationale of using the O₂-O₃ therapy to delay aging processes and neurodegeneration, exploiting well documented omni various functions of O₃. This therapy could represent a convenient, inexpensive monodomain intervention, working in absence of side effects that will permit to modulate the oxidant, but also immune, inflammatory, metabolic, microbiota and regenerative processes impaired in NDs.

There is a recent consistent upsurge of interest in complementary

medicine, especially dietary supplements and foods functional in delaying the onset of age-associated NDs. O₃ along with other antioxidants (polyphenols, mushrooms) can open new neuroprotective strategies, and could represent therapeutic targets to minimize the deleterious consequences associated to oxidative stress, such as in brain aging and NDs.

Authors' Contributions

Catia Scassellati and Antonio Carlo Galoforo contributed equally to this work.

Declaration of Competing Interest

The authors have declared no conflict of interest.

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Footnotes

Appendix A Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.arr.2020.101138> .

Appendix A. Supplementary data

The following is Supplementary data to this article:

[mmc1.docx](#) (36.7KB, docx)

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Associated Data

This section collects any data citations, data availability statements, or supplementary materials included in this article.

Supplementary Materials

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