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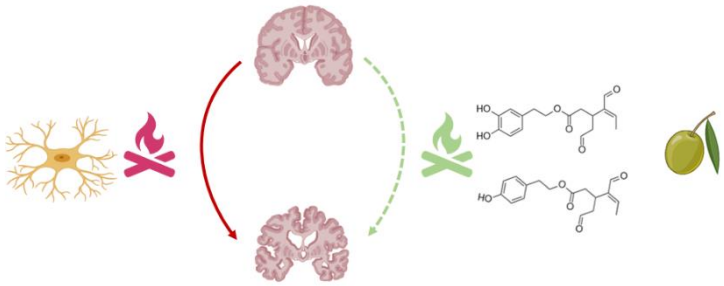
Use of olive oil polyphenols to counteract neuroinflammation and neurodegenerative diseases



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List of abbreviations

AD - Alzheimer's Disease
3NT - 3-nitrotyrosine
4HNE - 4-hydroxy-2-nonenal
4HP - 4-hydroxyphenylpyruvate
APP - Amyloid precursor protein
ARE - Antioxidant Response Element
Aβ - Beta-Amyloid
BACE-1 - Beta Secretase-1
BBB - Blood Brain Barrier
BDNF - Brain-derived Neurotrophic Factor
CAT - Catalase
CBP - CREB Binding Protein
CHPT - Choline Phosphotransferase
CNS - Central Nervous System
CVD - CardioVascular Diseases
DES - Deep Eutectic Solvents
DS - Down syndrome
ERK - Extracellular Signal-Regulated Kinase
EVOO - Extravirgin Olive Oil
GBA - Gut-Brain Axis
GPX - Glutathione Peroxidase
GSH- Glutathione
GSK3β - Glycogen synthase kinase-3 beta
GST - Glutathione S-Transferase
H_2O_2 - Hydrogen Peroxide
HO-1 - Heme-Oxygenase-1
HOMA-IR - HOmeostatic Model Assessment for Insulin Resistance
IFN-γ - Interferon Gamma
IGF-1 - Insuline-like Growth Factor-1 IGF1
IL - Interleukin
iNOS - inducible Nitric Oxide Synthase
KEAP-1 - Kelch-like ECH-associated protein 1
LPS - Lipopolysaccharide
MCI - Mild Cognitive Impairment
MDA - Malondialdehyde
MnSOD - manganese Superoxide Dismutase
MUFA - Mono-Unsaturated Fatty Acids
NaDES - Natural Deep Eutectic Solvents
NADDs - Neurodegeneration-associated dementia disorders

NF-kB - Nuclear Transcription Factor-Kappa B
NO - Nitric Oxide
NOX - NADPH oxidase
NQO1 - NAD(P)H Quinone Oxidoreductase-1
Nrf2 - nuclear erythroid 2-related factor 2
$O_2^{-\bullet}$ - Superoxide Anion
$^{\bullet}OH$ - Hydroxyl Radical
OOEP - Olive Oil Extracted Polyphenols
OOE-DES – Olive Oil Polyphenols extracted by NaDES
PD - Parkinson's Disease
PEMT - Phosphatidylethanolamine N-methyltransferase
PGC-1α - Peroxisome Proliferator-Activated Receptor-Gamma Coactivator
PKC - Protein Kinase
PLPP - Phospholipid Phosphatase
PRX - Peroxiredoxin
$ROO^{\bullet}$ - (Alkyl-)peroxyl- radical
ROOH - (Alkyl-)hydroperoxide
ROS - Reactive Oxygen Species
SOD - Super Oxide Dismutase
SPHK - Sphingosine kinase
T2DM - Type2 Diabetes Mellitus
TLR-4 - Toll Like Receptor-4
TRX - Thioredoxin
TRXR - Thioredoxin Reductase
UCP2 - Uncoupling Protein 2
αSyn - α-Synuclein
βTrCP - β-Transducin repeat-Containing Protein

Abstract

Neurodegenerative diseases represent a health emergency that we should address in the coming decades. The underlying causes of these diseases are still unclear, but they are probably closely linked to neuroinflammation and oxidative stress processes in the brain. The use of molecules that can counteract these phenomena could be an excellent tool in hampering the progression of these pathologies.

Polyphenols are molecules naturally present in plant foods that exert many biological effects, particularly against inflammation and oxidative stress. They seem to be promising molecules in the biomedical field, indeed in recent years some of them have been used in several clinical trials.

Olive oil is a good source of polyphenols, particularly of secoiridoids, a class of compounds which comprise oleocanthal and oleacein which are only found in this type of oil. The extraction of these polyphenols generally involves the use of organic solvents, such as methanol and hexane, a process which may lead to risks for the operators and environmental threats, as the oil matrix and the polyphenolic product may retain traces of organic solvents, thus being unsuitable for food use.

To solve the extraction problem, alternative extraction techniques can be used such as Ultrasound-assisted extraction (UAE), Microwave-assisted extraction (MAE), Pressurised liquid extraction (PLE) and Supercritical fluid extraction (SFE). These techniques are mainly used not only on oil but also on olive pomace and wastemill water to retrieve polyphenols lost during the process of olive oil production. Moreover they are extremely energy-demanding.

An alternative method, certainly simpler, quicker and environmentally friendly is the use of Deep Eutectic Solvents (DES). DES are binary solvents consisting of a hydrogen bond donor (nontoxic quaternary ammonium salts or amino acids) and a hydrogen bond acceptor (polyols, organic acids or carbohydrates). These two chemical families, through thermal, mechano-chemical or freeze-drying treatments, form an eutectic solvent characterised by an intense network of hydrogen bonds. When the eutectic solvent are made of natural compounds that are non toxic and even edible (such as monosaccarides, aminoacids, alcohols), they are named Natural DES (NaDES).

In this work, the bioactive capacities of polyphenols extracted from extravirgin olive oil are evaluated on different cellular and animal models of diseases, paying particular attention to inflammation and oxidative stress. In the first section, experiments are presented to evaluate whether there are biological differences between polyphenols extracted by the classical method and those extracted via NaDES, by determining the biological response (inflammation and oxidative stress) on murine BV2 microglia cells following administration

of the polyphenols before or after treatment with a pro-inflammatory stimulus. In the second section, polyphenols extracted by classical method (OOEP) were administered for 8 weeks in drinking water (10 mg/kg/day) to C57BL/6J mice under physiological or low-grade inflammation conditions (obtained by intraperitoneal injection of 0.5 mg/kg/week LPS). The mice were assessed in their cognitive capacity (long-term memory, anxiety-like behaviour) and inflammation and oxidative stress status. A broad-spectrum metabolomics study was performed in the liver and intestine. At the same time, we evaluated the impact of LPS and polyphenols on the microbiota. In the third and final section, polyphenols extracted with a NaDES made up with betaine and glycerol in a 1:2.2 molar ratio (OOE-DES) were administered for 12 weeks in drinking water (5 mg/kg/day) to euploid B6EiC3SnF1 and trisomic Ts2Cje mice. The impact of polyphenols on the mice in terms of cognition, oxidative stress and inflammation at the brain cortex and liver level was assessed, and a broad-spectrum lipidomic analysis was conducted on the brain, liver and caecum tissues.

The data obtained show that olive oil polyphenols extracted with NaDES, OOE-DES, have the same anti-inflammatory and anti-oxidant capacities as classical extracted OOEP at both the cellular and animal level. From a comprehensive point of view, olive oil polyphenols positively impact the brain directly (by modulating inflammation) and indirectly (by modifying the microbiota and metabolites produced both in the gut and liver), promoting neurodevelopment and synaptogenesis. Thus, olive oil polyphenols are an excellent tool to alleviate neuroinflammation and in this way strike at the base of neurodegeneration. Moreover, olive oil polyphenols manage oxidative stress by modulating the activation of the pathway under the control of nuclear erythroid 2-related factor 2 (Nrf2) and Peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α), regulating the expression of antioxidant enxymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX).

Introduction

Graphical introduction

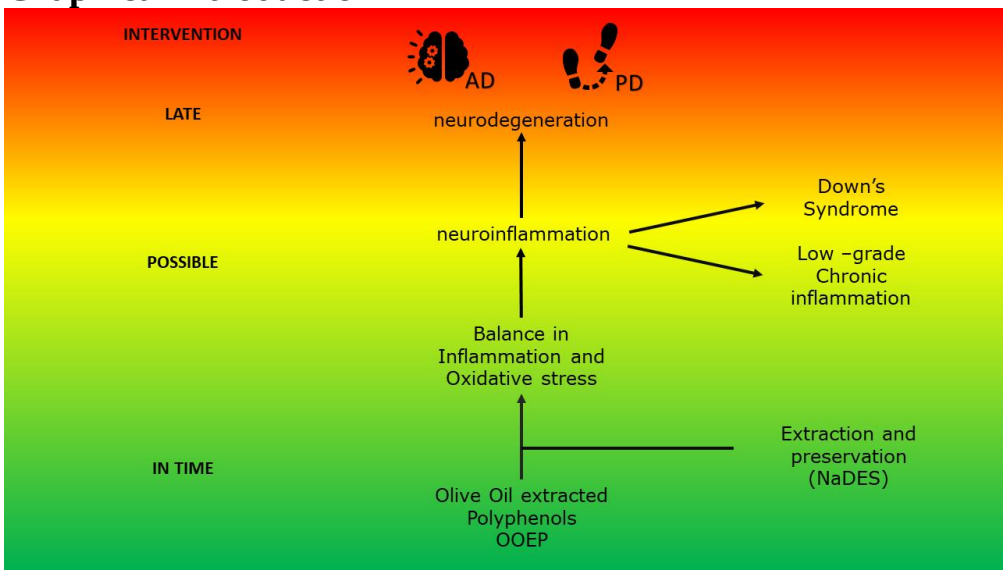


Fig. I Graphical abstract

The emergence of neurodegeneration

Neurodegeneration is a chronic and progressive loss of neurons in brain and spinal cord (Subhramanyam et al., 2019) and is a process that is common to several diseases, the main ones being Alzheimer's and Parkinson's disease (Tiwari et al., 2021.). The aetiology of these diseases is still far from being known (Marogianni et al., 2020; Zalocusky et al., 2021), which means that in-time screening and prevention are quite difficult (Ferrari & Sorbi, 2021; Porsteinsson et al., 2021). Since neurodegeneration is related to aging, the risk for each of us to suffer from a neurodegenerative disease late in life is rather high (Satyam & Bairy, 2022).

Dealing with this phenomenon is of the utmost importance because nowadays 50 million people worldwide suffer from Alzheimer's (AD) and 6 milion from Parkinson's diseases (PD) (Dorsey et al., 2018; Guzman-Martinez et al., 2019; Saez-Atienzar & Masliah, 2020) and it is estimated that these numbers will triple by 2050 (Al-kharboosh et al., 2022).

To date, the cost of care for Neurodegeneration-associated dementia disorders (NADDs, i.e., AD and PD) is around 52 billions for PD and 305 trillions for AD in the U.S. only, and it is estimated to soar to 1 trillion by 2050 (Al-kharboosh et al., 2022).

To date, there are several drugs for the treatment of the main symptoms of AD, such as acetylcholinesterase inhibitors (donepezil, rivastigmine, galantamine)

- which act by increasing the neurotransmitter acetylcholine - and the so-called anti-dementia drugs, such as memantine, which acts as a non-competitive NMDA receptor antagonist (Breijyeh & Karaman, 2020; Merighi et al., 2022). Nevertheless, these drugs only treat the symptoms and not the causes of the disease.

In recent years, new forms of treatment have entered clinical trials such as monoclonal antibodies acting against Amyloid beta (Aβ) peptide, namely Lecanemab (Swanson et al., 2021) and Aducanumab (Budd Haeberlein et al., 2022), which may represent a better treatment as they target the amyloidogenic pathway, but unfortunately, AD is not only due to an alteration of this pathway (Armstrong, 2013; Sochocka et al., 2017).

In fact, neurodegeneration is inextricably linked to neuroinflammation (Komleva et al., 2021), and much of the literature features extensive analyses of the inflammatory pathways, both systemically and at the brain level (Skaper et al., 2018).

Neurodegeneration and neuroinflammation

Neurodegeneration and neuroinflammation move hand in hand, and very often it is difficult to define which is the cause or the consequence of one another (Tiwari et al., n.d.; Uddin et al., 2021). In fact, if we consider the case of AD, the release of Aβ, due to the incorrect cut of the Amyloid precursor protein (APP) by the beta-secretase (BACE-1) and gamma-secretase, from neurons leads to their aggregation and to the activation of pro-inflammatory pathways in microglial cells, which will lead at first to the reduction of Aβ clearance, then to an increase in Aβ aggregation (Kalyan et al., 2022) and, in the long run, to neuronal death and a consecutive release of pro-inflammatory molecules (Leng & Edison, 2021).

Neuroinflammation falls into the category of chronic inflammation, which is characterized by the difficulty of eliminating the triggering agent of the inflammation itself. Underlying this phenomenon is a continuous crosstalk between neurons, microglial cells, and astrocytes, which is always present and is continuously modulated during all stages of neuron development (Araki et al., 2021) and is modified during neuroinflammation and neurodegenerative diseases (Liddelow et al., 2017; L. R. Liu et al., 2020; Vainchtein & Molofsky, 2020).

Microglia are the main immune cells of the brain and central nervous system (CNS), and play a key role in monitoring and protecting the brain, by intervening in case of damage, inflammation or infection and supporting the regeneration and repair of neural tissues. They also contribute to the modulation of synaptic functions and perform crucial tasks for the well-being

of the CNS (Czapski & Strosznajder, 2021; Heneka et al., 2015). They are extremely sensitive to their environment, such that they are activated within minutes following tissue damage (L. R. Liu et al., 2020), and can easily shift their phenotype from inactive to active (Hickman et al., 2018). Activation can lead to a pro-inflammatory M1 type, and is recorded in the presence of stimuli such as lipopolysaccharide (LPS), interferon gamma (IFN-γ), Aβ, and α-synuclein, or to an anti-inflammatory M2 type, following stimuli such as interleukin-4 (IL-4), IL-10 and IL-13 (Subhramanyam et al., 2019).
While the anti-inflammatory M2 phenotype is present during phagocytosis, synaptic remodelling, and trophic support, the pro-inflammatory M1 phenotype stimulates neuroinflammation causing excitotoxicity, synaptic pruning, and demyelinization (Leng & Edison, 2021).
Microglia indeed can become the worse enemy of neurons through:

- excessive production of superoxide anion and peroxynitrite leading to necrosis and apoptosis;
- release of proteases and metallo-proteases leading to damage that can lead to apoptosis;
- reduction of trophic factors such as Brain-derived Neurotrophic Factor (BDNF) or Insuline-like Growth Factor-1 (IGF1);
- microglial release of glutamate or overexpression of the inducible Nitric Oxide Synthase (iNOS) that leads neuronal cells to excitotoxic neuronal death(Hickman et al., 2018).

Astrocytes, for their part, work in coordination with microglia, thanks to purine and cytokine communication, and manage neuron development through synapse formation and pruning, and are also important in the regulation of neuronal excitability (Vainchtein & Molofsky, 2020). In the same way as microglial cells, astrocytes too can undergo a phenotypic switch as a result of different inflammatory stimuli. So, the astrocyte will acquire the A1 phenotype through reactive astrocytosis, in coordination with microglial M1 activation, and will acquire an A2 phenotype in the presence of M2 microglia (L. R. Liu et al., 2020).
Astrocytes also have other functions, including being a key element of the Blood Brain Barrier (BBB), taking up glucose from the bloodstream, regulating cerebral blood flow and, in addition to releasing neurotransmitters, regulating the osmolarity of the brain environment (Guillamón-Vivancos et al., 2015).

Inflammation and oxidative stress in the brain

One of the new concepts that has arisen in recent years in medicine is the one of ***inflammaging***, i.e. a state of constitutive unresolved low-grade inflammation related to aging (Skaper et al., 2018). Inflammaging is able to keep microglia attentive in a pro-inflammatory sense, in the long run leading to BBB damage (Skaper et al., 2018) and to cognitive decline, due in part to the reduction of BDNF and inhibition of hippocampal neurogenesis (Barbé-Tuana et al., 2020). A state of low-grade inflammation is sustained by other very common diseases of the elderly, such as obesity, metabolic syndrome, and diabetes (Onyango et al., 2021).

All these and other pathologies of the elderly are able to lead to alterations of the microbiota (dysbiosis) that lead to an increase in gram negative over the gram positive bacterial populations in the intestinal lumen with a concurrent increase in the passage of LPS (produced by gram negative bacteria) from the bowel into the blood (Hutchinson et al., 2020; Malesza et al., 2021; Netto Candido et al., 2018). The physiological concentration of the hematic LPS (endotoxemia) is about 10-20 pg/ml (Brown, 2019) and this concentration is granted by the hepatic clearance (Gnauck et al., 2016), but even a small increase in its concentration is enough to produce detrimental effects on immune system, on the brain and to induce a state of general discomfort and malaise in both humans and mice (Brown, 2019); moreover, LPS injection causes a weakening of epithelial integrity (X. Peng et al., 2021), especially at the intestinal (Schlegel et al., 2009) and BBB level (Veszelka et al., 2007). LPS is a very important inflammatory trigger for the body and an imbalance of its levels in the blood has been documented in AD patients (60 pg/ml) and in liver cirrhosis (Brown, 2019).

Not surprisingly, one of the most widely used methods for obtaining cellular and animal models to study neuroinflammation is the use of LPS, which can mimic both AD and PD models (Batista et al., 2019; Kalyan et al., 2022).

LPS is an endotoxin found in the outer membrane of all gram-negative bacteria and is able to be identified by immune system cells because it binds to one of the most conserved receptors in eukaryotes, the Toll Like Receptor-4 (TLR-4) (Fuke et al., 2019). Downstream of binding to this receptor, there is activation of a cellular pathway that culminates in the activation of nuclear transcription factor-kappa B (NF-κB) (MyD88-dependent pathway) or, alternatively, type I interferons (MyD88-independent pathway) (Y.-C. Lu et al., 2008).

NF-kB is able to activate the transcription of pro-inflammatory cytokines (IL-1, IL-6, IL-12, IL-17, IL-18, IL-23), chemokines (such as CCL12 and CXCL10), anti-inflammatory cytokines (IL-10, IL-4, IL-13) and proteins

involved in the inflammatory pathway (i.e. NLRP3 and iNOS) (Merighi et al., 2022).

Parallel to inflammation is the interplay of oxidative stress. Indeed, during the inflammatory process, high amounts of reactive oxygen species (ROS) can be generated as part of the body's immune response to fight infection or respond to tissue damage.

ROS are a very important family of oxidizing factors that are omnipresent in all tissues and organs. They are generated during cell metabolism and are also used as chemical messengers (Piccirillo et al., 2022), but their concentration can increase substantially under conditions of inflammation or imbalance between oxidant and antioxidant factors, triggering a condition known as oxidative stress (Forman & Zhang, 2021). They are radical molecules, hence electronically vacant, capable of interacting with lipids, proteins and nucleic acids (Piccirillo et al., 2022). Specifically, they are superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), (Alkyl-)peroxyl- radical ($ROO^{\bullet}$), (Alkyl-)hydroperoxide (ROOH) and nitric oxide ($NO^{\bullet}$). $NO^{\bullet}$ and $O_2^{-\bullet}$ are able to interact with each other and generate peroxynitrite (Forman & Zhang, 2021).

Oxidative stress is identified as a leading cause of neurodegeneration (Piccirillo et al., 2022); indeed, the brain is rich in lipids that can interact with ROS to generate lipid radicals that rapidly propagate, generating cell membrane damage and cell death (He et al., 2020) and rapidly degrade, producing malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4HNE), detectable in large quantities in the brains of those suffering from AD and mild cognitive impairment (MCI) (Chen & Zhong, 2014). Furthermore, anti-oxidant proteins and enzymes can become targets of these radicals by undergoing oxidative phenomena such as carbonylation and nitrosylation; interestingly, higher levels of 3-nitrotyrosine (3NT) were found in MCI and AD patients (Chen & Zhong, 2014; Piccirillo et al., 2022).

Two enzymes that perfectly demonstrates the connection between oxidative stress and inflammation are NADPH oxidase (NOX), which upon activation by cytokines is able to increase ROS production within cells, causing oxidative stress (M. W. Ma et al., 2017), and iNOS, which produces $NO^{\bullet}$, which is a direct proinflammatory chemical messenger and secondly can react with superoxide anion to form peroxynitrite (Radi, 2018).

Actually, tissue damage can occur due to the inflammatory process, immune cell accumulation, and cell-cell interactions that can lead to a pro-oxidant environment, increasing ROS production. At the same time, inflammation can interfere with the antioxidant systems, impairing cell's ability to neutralize ROS and maintain a redox balance (El Assar et al., 2013).

Oxidative stress is continuously controlled by antioxidant molecules (such as glutathione (GSH), vitamin E and C) and antioxidant enzyme systems (Superoxide Dismutase (SOD), catalase (CAT), glutathione-peroxidase (GPX)) (Demirci-Çekiç et al., 2022).

An important and coordinate oxidative stress response is represented by the activation of nuclear erythroid 2-related factor 2 (Nrf2) (Saha et al., 2020). Under oxidative stress, Nrf2 is released by the first regulatory protein Kelch-like ECH-associated protein 1 (KEAP1) and, if Nrf2 is not phosphorylated in its DSGIS motif by Glycogen synthase kinase-3 beta GSK3β (T. Liu et al., 2021) it does not interact with the second negative regulator β-transducin repeat-containing protein (βTrCP), and binds to the antioxidant response element (ARE) on DNA, thereby regulating the expression of a large number of genes involved in antioxidant and anti-inflammatory cellular defence as well as mitochondrial protection (Forman & Zhang, 2021). Phosphorylation on the other sites of Nrf2, which can take place by several protein kinase PKC and extracellular signal-regulated kinase ERK, is an alternative pathway for Nrf2 activation, as it weakens KEAP binding and permits the interactions with co-activator and corepressors (T. Liu et al., 2021); interestingly, Nrf2 can also be activated by polyphenolic molecules such as curcumin and resveratrol (C. Yu & Xiao, 2021).

Genes upmodulated by Nrf2 activation that are relevant in ROS management include heme-oxygenase-1 (HO-1), SOD-1, GPX, thioredoxin reductase (TRXR), and glutathione S-transferase (GST) (Forman & Zhang, 2021).

In addition, Nrf2 is able to inhibit the activity of the inflammatory master regulator NF-kB in several ways: by blocking the translocation of its transcription factor IkB-alpha, by suppressing the NOX4/ROS/NF-kB pathway, by blocking inflammasome formation (Saha et al., 2020), by competing with the co-activator CREB Binding Protein (CBP), and by reducing the surrounding environment through HO-1 activity (Bellezza et al., 2018).

Another important player in oxidative stress management is Peroxisome proliferator-activated receptor-gamma coactivator PGC-1α, the master regulator for mitochondrial biogenesis, oxidative phosphorylation and ROS detoxification, which regulates the expression of manganese superoxide dismutase (MnSOD), CAT, peroxiredoxin (PRX), uncoupling protein 2 (UCP2), thioredoxin (TRX) and thioredoxin reductase (TRXR).

Being a GSK3β inhibitor, PGC-1α is among other things an Nrf2 activator that can translocate into the nucleus (Rius-Pérez et al., 2020). It has also been demonstrated to be a very important inhibitor of NF-kB and activator of the M2 microglial phenotype (Yang et al., 2017).

Down syndrome and the relationship with Alzheimer's disease

Down syndrome (DS) affects 1 in 1,000 new births worldwide (Martínez-Espinosa et al., 2020) and an estimated 6 million people are affected to date (Fortea et al., 2021). This syndrome is characterised by high levels of fat-related systemic inflammation (Gutierrez-Hervas et al., 2020; Huggard et al., 2020), cardiovascular problems (Dimopoulos et al., 2023), multi-organ comorbidities (Lagan et al., 2020) and cognitive deficit (Santoro et al., 2021).
According to the Alzheimer Society (www.alzheimers.org.uk), most DS patients can develop the typical neuropathology of AD by the age of 40 (Snyder et al., 2020). Underlying this relationship between the two diseases is the presence of a third chromosome 21, which leads to an imbalance of the production of some important players in the development of AD (APP and BACE-1) (Wiseman et al., 2015), of proteins involved in mitochondrial biogenesis and energy metabolism (proteins upstream of PGC-1α) (Tan et al., 2023) and in the response to oxidative stress (BACH, SOD-1) (Wiseman et al., 2015).
These and other changes have a significant impact on oxidative stress (Perluigi & Butterfield, 2012), such as increased levels of lipid peroxidation (He et al., 2016), increased oxidised and misfolded proteins that flood the protein degradation machinery (Tramutola et al., 2017), and reduction in GSH levels (Pastore et al., 2003).
Furthermore, plasma levels of inflammatory cytokines are high in DS affected individuals and there is extravasation of serum proteins in the brain (Lott & Head, 2019).

Use of lipidomics in neurodegenerative diseases: does it worth it?

Various causes have been indicated at the basis of neurodegeneration, such as genetic and environmental factors (Breijyeh & Karaman, 2020), which lead to misfolding and aggregation of proteins (Hetz & Saxena, 2017) and dysregulated immune response (Hickman et al., 2018), but a complete picture of the phenomenon is still lacking. In recent years an enormous effort has been undertaken to understand which pathways are affected in neurodegeneration, which has led to the integrated use of omics techniques (genomics, epigenomics, transcriptomics, and proteomics) (Y. Ma et al., 2020). Many of the obtained results indicate that one of the most affected pathways is the one involving ApoE receptors (T. Liu et al., 2020; Sienski et al., 2021). ApoE is a lipid carrier, which contributes to AD pathogenesis by modulating multiple

pathways including the metabolism, aggregation, and toxicity of Aβ peptide, tauopathy, synaptic plasticity, lipid transport, glucose metabolism, mitochondrial function, vascular integrity, and neuroinflammation (Kao et al., 2020; Sienski et al., 2021; Zhao et al., 2018).
Further alterations of the lipid pathway have been highlighted by the abnormal composition of brain-derived extracellular vesicles in AD patients (H. Su et al., 2021), the increased presence of 24S-hydroxycholesterol (M. Jeitner et al., 2011) and the alteration of other lipid markers (Zarrouk et al., 2018), such as cholesterol, sphingolipids, phospholipids and gangliosides (Wong et al., 2017).
In light of these new evidence, it is clear that lipids will attract more and more scientific interest in the understanding and treatment of neurodegeneration. Interestingly, the administration of lipid mediators has led to improvements in the intellectual performance of AD mice (Emre et al., 2022).

The relationship between microbiota and CNS

For decades now, the statement 'we are what we eat' has lost its philosophical connotations initially expressed by Ludwig Feuerbach, to become an extremely complex area of scientific research on the Gut-Brain Axis.
The Gut-Brain Axis (GBA) represents a complex two-way communication network between the CNS and the enteric system, involving the gastrointestinal tract and its microbiota. This communication axis is known for its role in influencing a wide range of cognitive, emotional and behavioural functions (Cryan et al., 2019).
The enteric system, redefined in the literature as the 'second brain', comprises the autonomic nervous system of the gut, which is capable of performing complex functions independently of the central brain. This enteric nervous system has millions of neurons and forms a vast interconnected network that regulates intestinal activity, nutrient movement, appetite control, and a host of other gastrointestinal functions. In addition, the gastrointestinal tract is inhabited by trillions of bacteria and other microorganisms that make up the gut microbiota (Margolis et al., 2021).
Communications between the CNS and the gut microbiota occur mainly through a variety of signals, including neurotransmitters, cytokines, hormones, metabolites, vagus and enteric nerves. These signals can act both locally in the gut and systemically, influencing communication between neurons, immune cells and cells of the gastrointestinal system. Such communication modulates brain activity, influencing emotions, cognition, sleep, appetite and various other behaviours (Cryan et al., 2019; Megur et al., 2020).
Experimental evidence supporting the importance of the GBA includes studies showing the beneficial effect of modulating the gut microbiota on certain

manifestations of neurological and psychiatric diseases (Mörkl et al., 2020). Similarly, alterations in the gut microbiota have been correlated with the development of psychiatric disorders, such as autism and schizophrenia (Góralczyk-Bińkowska et al., 2022).
The elderly often suffer from malnutrition, characterised by lack of energy and micronutrient intake leading to an increased risk for MCI and dementia (McGrattan et al., 2019). In addition, the microbial diversity of AD patients is reduced and this may lead to an increase in pro-inflammatory bacterial populations (Cryan et al., 2019; Megur et al., 2020; Ticinesi et al., n.d.) and producers of amyloid proteins (curli, tau, Aβ, α-synuclein, and prion) that stimulate human Aβ fibrillation (Kesika et al., 2021). Treatment with antibiotics and probiotics in human and mouse models of AD led to good results in terms of cognition, inflammation and Aβ aggregation (Kesika et al., 2021; Megur et al., 2020).
Polyphenols (especially flavones, flavonols and anthocyanins) also proved to be excellent modulators of the microbiota in relation to the development of senile dementia (Ticinesi et al., n.d.).

The Mediterranean diet and olive oil polyphenols

Many authors consider nutrition as one of our best allies in preventing the onset of neurodegenerative diseases (McGrattan et al., 2019; Satyam & Bairy, 2022), as many foods, especially plant foods, contain bioactive substances that could prevent/counteract the onset of neurodegeneration. One of the most comprehensive diets in this respect appears to be the Mediterranean Diet (MedDiet). The MedDiet is a traditional diet followed by people living in Mediterranean area, such as Italy, Greece, Spain and others. This diet is characterised by a balanced eating habit, rich in fruit, vegetables, whole grains, legumes, nuts, seeds, fish and olive oil (Guasch-Ferré & Willett, 2021). The benefits conferred by this diet are related to the cardiovascular system (reduced cholesterol and inflammation) (Estruch et al., 2018), metabolic diseases (diabetes type II) (Martín-Peláez et al., 2020), cancer (Mentella et al., 2019), and neurodegenerative diseases (Naureen et al., 2022).
One of the key ingredients of this kind of diet is extravirgin olive oil (EVOO). This is a complex mixture of fatty acids (especially the Mono-Unsaturated Fatty Acids (MUFA)), phytosterol, vitamins (A, E, K), tocoferols, carotenes, pigments and phenolic compounds (lignans, flavone glycosides, phenolic alcohols and secoiridoids) (Mazzocchi et al., 2019).
Polyphenols are secondary plant metabolites that exert pleiotropic effects in mammals (Guest, 2020), in a direct way, as strong antioxidants and metal chelators, and in a indirect way, by modulating cellular activity (Tsao, 2010).

Polyphenols found in olive oil exert diverse effects: they have the ability to induce apoptosis and prevent cells from initiating neoplastic transformations (Gorzynik-Debicka et al., 2018), they reduce the incidence of thrombogenic events (Ruano et al., 2007), they exert antimicrobial effects (Nazzaro et al., 2019) have antioxidant activity, provide neuroprotection and immunomodulation (Castejón et al., 2020), as well as reduce the incidence of major cardiovascular events (George et al., 2019).
Olive oil contains approximately 40 different polyphenols, which are categorised as:
- phenolic acids (caffeic, vanillic, syringic, *p*-coumaric, *o*-coumaric, protocatechuic, sinapic, *p*-hydroxybenzoic and gallic acids);
- phenolic alcohols (hydroxytyrosol (3,4-dihydroxyphenyl-ethanol (3,4-DHPEA)) and tyrosol (*p*-hydroxyphenyl-ethanol (*p*-HPEA));
- flavones (luteolin and apigenin);
- lignans ((+)-1-acetoxypinoresinol and (+)-1-Pinoresinol);
- secoiridoids (oleuropein, ligstroside, oleocanthal, oleacein)
(Servili et al., 2009).

Secoiridoids are the most representative group and most interesting from a biological point of view (Castejón et al., 2020; Costa et al., 2022). These molecules are iridoid derivatives (cyclopentane [c]pyranic monoterpenoids) formed by cleavage of the cyclopentane ring. Secoiridoids present in olive trees and derived products include oleuropein and ligstroside, their respective aglyconic forms oleuropein aglycon (3,4-DHPEA-EA) and ligstroside aglycon (HPEA-EA) and dialdehyde derivatives (oleacein, i.e. 3,4-DHPEA-EDA, and oleocanthal, i.e. HPEA-EDA) (Angeloni et al., 2017). Oleocanthal and oleacein represent the largest part of polyphenols contained in olive oil and are particularly interesting as they show remarkable anti-inflammatory and antioxidant activities. Oleuropein and ligstroside can be found in high concentrations in the leaves and in woody parts of olive trees (Ortega-García & Peragón, 2010), but in olive oil their concentration is very low, if not completely absent (Baiano et al., 2009; Bayram et al., 2012; Pedan et al., 2019).
Interestingly, recent reports indicates that oleuropein, ligstroside, oleocanthal and oleacein are also present in the roots, and decrease during ripening in olives (Ben Brahim et al., 2022; Ortega-García & Peragón, 2010).
The concentration of polyphenols in olive oil is in the range <200-600 mg/kg (Rigacci & Stefani, 2016) but may rise up to 800 mg/Kg in oils produced from specific cultivar such as the Greek Koroneiki and Kalamon or the Italian Coratina cultivar (Kouka et al., 2020). Secoiridoids present in olive trees and

drupes are naturally produced by the condensation of tyrosol or hydroxytyrosol precursors produced by the phenylpropanoid metabolism and intermediates produced by the secoiridoid biosynthesis pathways to form the glycosylated form of ligstroside and oleuropein. During olive oil production, olives crushing liberates glycosidases which remove the sugar part of ligstroside and oleuropein, producing their respective aglycones (García-Vico et al., 2021). These latter compounds undergo demethylation and spontaneous decarboxylation, giving rise to the dialdehydic forms oleocanthal and oleacein, respectively (Costa et al., 2022). Finally, oleocanthal and oleacein can be hydrolysed in tyrosol and hydroxytyrosol, both of which have biological relevance (Karković Marković et al., 2019).

Olive Oil Polyphenols as nutraceuticals

Polyphenols contained in olive oil are recognized as substances with interesting nutraceutical properties as they not only are able to be free radical scavenger, but also are able to activate cellular pathways in the fight against oxidative stress, such as Nrf2 (Bucciantini et al., 2021). This translates into beneficial effects at biological and physiological levels, protecting the body from CardioVascular Diseases (CVD) and Type2 Diabetes Mellitus (T2DM), breast and colon cancer, and cognitive decline (Rigacci & Stefani, 2016). EFSA itself has recognized the beneficial effect of hydroxytyrosol content in olive oil in protecting blood lipids from oxidative stress (European Commission, 2012.).

Plenty of scientific evidence demonstrates the beneficial effects of these polyphenols (first of all hydroxytyrosol) at the brain level, both in vitro (such as reduction of oxidative and nitrosative stress by catalase activation, reduction of cytokine production and brain cell cytoprotection) and in vivo (improvement of cognitive performance and reduction of Aβ aggregation) (Rodríguez-Morató et al., 2015). (Lauretti et al., 2017) reported increased synaptic integrity and reduced Aβ aggregation and tau protein hyperphosphorylation in a mouse model of AD after 6-month ingestion of olive oil supplemented chow.

Several clinical trials have been conducted, the meta-analyses of which concluded that olive oil intake improves markers of inflammation, such as C-reactive protein, IL-6 and TNF-α in plasma (Fernandes et al., 2020; Schwingshackl et al., 2015), and that oils with high polyphenol content, compared to those with low content, are able to improve cardiovascular disease risk factors, such as oxidized LDL, total and HDL cholesterol and MDA (George et al., 2019). In addition, a decrease in serum glucose and homeostatic model assessment for insulin resistance HOMA-IR (p-value <0.01 , <0.05))

four hours after high-polyphenols EVOO administration was reported in healthy patients; of note, the same treatment with low-polyphenol oil did not produce the same result (D'Amore et al., 2016).

Effects of olive oil polyphenols OOEP in neurodegeneration

As outlined above, up to 40 different polyphenols are present in olive oil, whose amount can vary depending on many factors, such as harvest time, soil and olive cultivar (López-Huertas et al., 2021).

In addition, the ratio among the different single compounds can change depending on the storage time of the oil, as some polyphenols can convert into others (Flori et al., 2019). This is the case for the two secoiridoids oleuropein and ligstroside, which can be converted into their respective aglycone forms (oleuropein and ligstroside aglycone) and subsequently undergo their transformation into oleacein and oleocanthal respectively. Finally, these can be hydrolysed into the phenolic alcohols hydroxytyrosol and tyrosol (Fig. 2int).

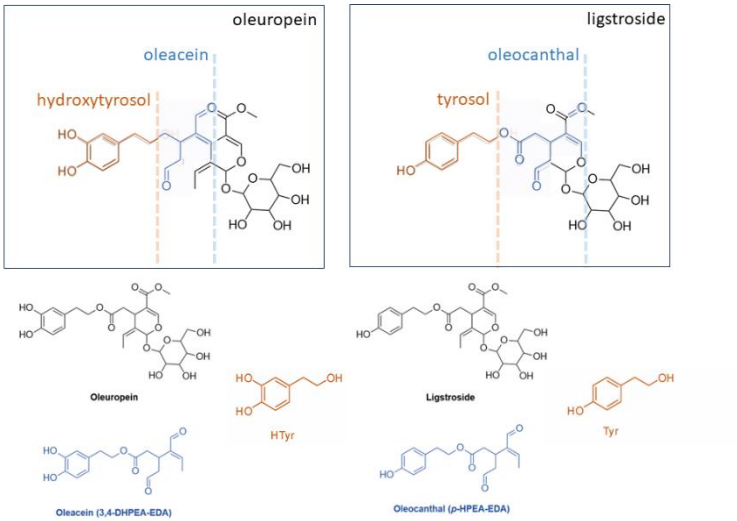


Fig. II ***Transformation of secoiridoids (in black and in blue) in phenolic acids (in red).*** *From the left, transformation of oleuropein in oleacein and hydroxytyrosol (HTYR), and, on the right, transformation of ligstroside in oleocanthal and in tyrosol (TYR) in olive oil*

It is well known that secoiridoid compounds are active as antioxidant, anti-inflammatory, and immunomodulatory compounds, particularly oleocanthal and oleacein, which have been suggested as potential therapeutic agents in inflammatory and ROS-induced diseases (Castejón et al., 2020). Apart from being radical scavengers *in vitro*, secoiridoids and phenolic alcohols can act as

antioxidants by altering the expression of antioxidant enzymes via Nrf2 activation, which modulates the expression of glutathione- and thioredoxin-related enzymes, phase 2 detoxifying enzymes, NAD(P)H quinone oxidoreductase-1 (NQO1), HO-1, etc. (Angeloni et al., 2019; Martínez-Huélamo et al., 2017). The activation of Nrf2 pathway may be at the basis of all the health promoting activities exerted by olive oil secoiridoids, some of which are described below.

This section aims to summarise the scientific evidence supporting a protective effect of individual secoiridoids or phenolic alcohols in relation to neurodegenerative diseases.

In order to exert their action, neuroprotective substances should bypass the BBB. This property was demonstrated for some of the olive polyphenols, in particular secoiridoids and phenolic alcohols.

For instance, sulfate and glucuronate derivatives of hydroxytyrosol are able to cross the BBB in rats (Galmés et al., 2021; López de las Hazas et al., 2015). Serra and coworkers (Serra et al., 2012) demonstrated that it only takes one hour after ingestion of a mixture of polyphenols for them to cross the BBB, and they were able to identify in plasma and in the brain the oleuropein secoiridoids derivatives, and tyrosol and hydroxytyrosol sulfate (originating from the degradation of ligstroside and oleuropein, respectively). On the other hand, (López-yerena et al., 2020; López-Yerena et al., 2021) demonstrated that in the rat brain, although hydroxytyrosol cannot be quantified, metabolites are present that can be traced back to first and second phase metabolism (methylated, hydrated, hydrogenated and glycuronate forms of oleocanthal or oleacein), after ingestion of an olive oil enriched in oleocanthal or oleacein.

There are plenty of reports in the literature regarding **oleocanthal**'s potential ability to counteract the pathogenesis of Alzheimer’s disease in several manners:

- it interacts with the Tau protein, forming a Schiff base with lysine 18 (W. Li et al., 2009);
- it reduces oxidative and nitrosative stress, as demonstrated *ex vivo* (De La Cruz Cortés et al., 2021), *in vitro* on chondrocytes and in neuronal SH-SY5Y cells (Barbalace et al., 2021; Iacono et al., 2010; Scotece et al., 2012) and in a rat model of brain injury (Mete et al., 2018);
- it interacts directly with Amyloid beta$_{1\text{-}42}$ (Aβ), already at concentrations of 10 nM, and reduces its aggregation, thereby preserving the dendritic spines of primary murine hippocampal cells (Pitt et al., 2009);

- when administered alone or in combination with the acetylcholine-esterase inhibitor donezepil it reduces Aβ aggregation in 5xFAD mice by increasing ApoE-dependent pathways, neprilysin and synaptogenic proteins (SNAP25 and PSD95) (Batarseh & Kaddoumi, 2018);
- when administered in powder and solid dispersion formulations by the oral route, it inhibits the C3AR1 factor upstream of STAT3 leading to improved cognitive performance in 5xFAD mice and to an attenuation of Aβ plaque formation (Tajmim et al., 2021);
- it induces the expression of proteins capable of ensuring adequate clearance of Aβ via apoE-dependent and independent pathways, also reducing astrocyte activation (as seen by (Qosa et al., 2015) in TgSwDI mice). Indeed, oleocanthal has also been shown to be an interesting modulator of these pathways in LS-180 adenocarcinoma lines (Abuznait et al., 2011), HeLa cells (Cassiano et al., 2015) and SH-SY5Y neural cells, with an increased level in the capacity of ROS scavenging, phosphorylation of AKT and upregulation of HSP90 (Giusti et al., 2018).

Other papers confirm these data. For example, (Al Rihani et al., 2019) showed that an olive oil enriched in oleocanthal was able to increase the tightness of the BBB, and to reduce Aβ plaques (-61% and -47% in the cortex, -73% and -79% in the hippocampus, respectively) in the TGSwDI AD mouse model. (Abdallah et al., 2023) also showed that oleocanthal, compared to refined olive oil with low oleocanthal content, led to a reduction in plaques, a correct cut by the α-secretase ADAM-10 resulting in the formation of sAPPalpha at the expense of the toxic sAPPbeta, and a concomitant increase in the expression of synaptic proteins, reduced astrocyte-associated and neuron-associated inflammatory markers in a 5xFAD mouse model of AD.
These studies suggest that oleocanthal may be a potential therapeutic agent for Alzheimer's disease. However, further research is needed to confirm these findings and to determine the optimal dose and delivery method for oleocanthal.
Several authors summarized the neuroprotective effects of **oleuropein**, showing that this molecule is able to inhibit aggregation and Aβ-associated damage in *C. elegans*, rat and mouse models (Klimova et al., 2019; Narayan et al., 2020; Rigacci, 2015; Romero-Márquez et al., 2023)
Oleuropein aglycone was found to interact directly with Tau protein (Daccache et al., 2011), $A\beta_{1\text{-}40}$ (Caba et al., 2021) and α-synuclein (Borah et al., 2021). (Brogi et al., 2020) conducted a very interesting *in silico* experiment where he showed that the likely point of interaction with Aβ is the 17-23 LVFFAED

motif, from which the polyphenol can then move to the same motif in another chain. This finding is in agreement with the previous work of (Bazoti et al., 2008) who demonstrated an interaction between Aβ and oleuropein. (Galanakis et al., 2011) also showed how the region (V12-N27), comprising LVFFAED, is the one that carries the greatest chemical shift in oleuropein or melatonin binding by Nuclear Magnetic Resonance Spectroscopy.

(Elmazoglu et al., 2021) et al outlined how the pretreatment with oleuropein of primary rat hippocampal cells subjected to hyperglycaemia and $A\beta_{1-42}$ as inflammatory stimuli, lead to antioxidant and anti-inflammatory effects by modulating the production of cytokines, the level of prooxidant markers, antioxidant enzymes and the mitochondrial membrane potential.

(Omar et al., 2017) demonstrates how oleuropein has excellent radical scavenging potential *in vitro*, as demonstrated by superoxide radical scavenging activity and ferric reducing ability of plasma.

Recent work demonstrates oleuropein's ability to inhibit Aβ aggregation, reducing cellular mortality and associated oxidative stress in SH-SY5Y cells (Leri et al., 2019), and the amyloidogenic protein S100A9, which appears to be an important player in the neuroinflammatory cascade of AD and PD, and traumatic brain injury (Leri, Chaudhary, et al., 2021). In addition, the combined treatment of oleuropein and hydroxytyrosol in a 1:1 ratio has been shown to activate autophagic flux when administered 24 hours prior to treatment with amyloid oligomers or fibrils to SH-SY5Y (Leri, Bertolini, et al., 2021). More in-depth studies on oleuropein-modulated proteins identified inhibition of BACE-1 and HDAC-2 (Luccarini et al., 2015; Omar et al., 2018) as key mechanisms. In line with this, (Kostomoiri et al., 2013) had previously brilliantly demonstrated how oleuropein increases the presence of sAPPalpha in HEK293 cells stably transfected with isoform 695 of human APP via upregulation of MMP-9 metalloprotease activity.

At the animal level, the work of (Grossi et al., 2013) demonstrates that treatment with 50 mg/kg oleuropein for 2 months is able to reduce Aβ deposition in cortex and hippocampus of TgCRND8 mice through activation of the autophagic process, as demonstrated in both murine brain and N2a murine neuroblastoma cell cultures (Grossi et al., 2013). These data are confirmed by (Pantano et al., 2017) who demonstrated that supplementation with polyphenols such as oleuropein improved behavioural performance and neuropathology in a dose dependent manner in the same mouse model.

(H. Yu et al., 2016) showed that intraperitoneal or intracerebroventricular injection of 100 mg/kg oleuropein is able to reduce ischaemic damage in the brain following reperfusion injury. Similarly, (Pourkhodadad et al., 2016) shows how a 10-day treatment of oleuropein in a rat model in which colchicine

is administered at the hippocampal CA1 level to induce cognitive dysfunction, is able to reduce oxidative stress and apoptosis. Finally, (M. Wang et al., 2020) observed how oleuropein treatment led to an increase in long-term potentiation through the facilitation of calcium permeable- amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors trafficking and synaptic transmission.
(Romero-Márquez et al., 2023) shows that oleuropein improves locomotive behavior by regulating the expression of the heterologous FOXO and Nrf2 proteins, and HSP 16.2 in a *C. elegans* model with pan-neuronal expression of the TAU protein and in *C. elegans* treated with Aβ. (Diomede et al., 2013) used the *C. elegans* CL2006 strain (which is an AD model) to show that oleuropein is able to reduce Aβ aggregation, while (Brunetti et al., 2020) demonstrates how treatment with 250 and 500 µg/ml oleuropein leads to a remarkable increase in survival of *C. elegans* following heat stress treatment. This improvement is also seen in three *C. elegans* PD models (rotenone-induced, OW13 and UA44 strain).
As a corollary to these experiments, (Marianetti et al., 2022) conducted a clinical trial by administering for 6 months a dietary supplement containing oleuropein and S-acetyl glutathione to a population at early stage of AD, describing an improvement in cognitive performance (evaluating as cognitive impairment, memory, visuospatial abilities, attention, language and speech, executive functions, behavioral disorders).
The literature has not yet fully explored the possible neuroprotective effects of oleacein and ligstroside. (Grewal et al., 2020) showed that **ligstroside** is able to increase ATP (through increased mitochondrial activity) in both SH-SY5Y-APP695 and aged female NMRI mice resulting in improved spatial working memory and life extension. However, this treatment was unable to reduce $A\beta_{1-40}$ levels in the same cell line.
Hydroxytyrosol has been shown to be a very potent NF-kB inhibitor, inhibiting lipid peroxidation and activating mitochondrial biogenesis (Killeen et al., 2014). In addition, it has great anti-oxidant potential such that it reduces copper (Cu)-induced toxicity in neuroblastoma (SH-SY5Y) cells (Omar et al., 2017).
Treatment with hydroxytyrosol in an AD mice model led to a reduction in cognitive impulsivity and an improvement in anxiety-like behaviour (Arunsundar et al., 2015). In another AD mice model (TgCRND8) there were also significant cognitive improvements, reduction in Aβ burden and reduction in inflammation after administration of a diet enriched with 50 mg/kg hydroxytyrosol for 8 weeks (Nardiello et al., 2018).
Pre-treatment with hydroxytyrosol on BV2 and C13NJ microglial lines led to an attenuation in the LPS-activated inflammatory response (Leri et al., 2023).

Hydroxytyrosol is able to interact with $A\beta_{1-42}$ and can produce fibrils that are less thick and therefore less damaging (Leri et al., 2019). Confirming this finding, (Romanucci et al., 2020) demonstrated how the catecholic function of hydroxytyrosol is able to inhibit amyloid aggregation in vitro, something that tyrosol fails to do, but rather facilitates it. In line with these data, (Romero-Márquez et al., 2022) demonstrated an improvement in paralysis due to Aβ in a *C. elegans* model following administration of polyphenols containing 20% hydroxytyrosol. On the other hand, in vivo administration of 5 mg/kg hydroxytyrosol in APP/PS1 mice produced good responses in oxidative stress and average responses in cognitive, but no effect in Aβ accumulation (Y. Peng et al., 2016).

On the other hand, **tyrosol** and **oleacein** does not appear much in the literature in relation to neurodegenerative diseases. (Taniguchi et al., 2019) reported that tyrosol is able to reduce hippocampal oxidative stress in AD (5XFAD) mice and modestly improves spatial memory, but does not reduce amyloid accumulation. Furthermore, the tyrosol structure can be used to produce derivatives such as tyrosol phosphodiester to reduce Aβ aggregation and chelate metals (Romanucci et al., 2021).

Aims of the work

Neurodegenerative diseases represent a health emergency that we should address in the coming decades. The underlying causes of these diseases are still unclear, but they are probably closely linked to neuroinflammatory and oxidative stress processes in the brain. The use of molecules that can counteract these phenomena could be an excellent tool in hampering the progression of these pathologies.

Polyphenols are molecules naturally present in plant foods that exert many biological effects, particularly against inflammation and oxidative stress. They seem to be promising molecules in the biomedical field, indeed in recent years some of them have been used in several clinical trials.

Olive oil is a good source of polyphenols, particularly of secoiridoids, a class of compounds which comprise oleocanthal and oleacein which are only found in this oil. The extraction of these polyphenols generally involves the use of organic solvents, such as methanol and hexane, a process which may leads to a risks for the operators in the sector and environmental damage threats, as the oil matrix and the polyphenolic product turn out to may have traces of organic solvents, thus being unsuitable for food use.

To solve the extraction problem, alternative extraction techniques can be used such as :

- Ultrasound-assisted extraction (UAE), which uses ultrasound waves to break down the cell walls of plant material, releasing the polyphenols,
- Microwave-assisted extraction (MAE), which uses microwaves
- Pressurised liquid extraction (PLE), which uses high pressure and temperature,
- Supercritical fluid extraction (SFE), which uses a supercritical fluid, such as carbon dioxide, to extract the polyphenols from plant material (Cai et al., 2021; Sridhar et al., 2021).

These techniques are mainly used not only on oil but also on olive pomace and wastemill water to retrieve polyphenols lost during the process of olive oil production. Moreover they are extremely energy-demanding. An alternative method, certainly simpler and quicker, is the use the use of Deep Eutectic Solvents (DES).

DES are binary solvents consisting of a hydrogen bond donor (nontoxic quaternary ammonium salts (e.g. choline chloride) or amino acids (e. g. alanine, glycine, proline, histidine, betaine)) and a hydrogen bond acceptor (organic acids (e. g. oxalic acid, lactic acid, malic acid), carbohydrates (e. g. fructose, glucose), or polyols (glycerol, propylene glicol) (Huang et al., 2021; K. Wu et al., 2022)). These two chemical families, by means of thermal,

mechano-chemical or freeze-drying treatments, form an eutectic solvent characterised by an intense network of hydrogen bonds (Wu et al., 2022).
Given the enormous potential versatility in the types of DES that can be produced, by modifying the components and molar ratios between the chemical species involved, the applications of DES move from purely chemical applications (such as metal processing and catalysis) to various biomedical applications, including anti-bacterial treatment, drug delivery and solubilisation (Swebocki et al., 2023).
When the eutectic solvent are made up of natural compounds (such as monosaccarides, aminoacids, alcohols) they acquire the name of Natural DES (NaDES) (Ruesgas-Ramón et al., 2017).
Natural Deep Eutectic Solvents (NaDES) represent a green and environmentally friendly alternative to extract polyphenols from olive products. The first article demonstrating their excellent extraction capabilities for olive oil polyphenols was published by (García et al., 2016). Over time, there has been a development in the formulation of these solvents, which has led to NaDES being more performant in extracting oleacein and oleocanthal in particular (Rodríguez-Juan et al., 2021) and polyphenols in general (Fanali et al., 2020), especially if 30% water by volume is present. In line with this, (Chanioti et al., 2021) evaluated various extraction techniques, identifying NaDES as the best solvent (compared to ethanol and methanol) for extraction to be preferably combined with the homogenization process and encapsulation to improve stability. (Rodríguez-Juan et al., 2021) showed that it is possible to remove NaDES from polyphenols extracted by chromatography without significant loss of polyphenols. (Francioso et al., 2020) also reported a chromatographic method for the extraction and purification of individual polyphenols extracted by NaDES. Among the new applications, we report the interesting work of (F. Liu et al., 2021), who exploit the presence of CO_2 inside the DES to create emulsions with the oil during the extraction phase, and (Bonacci et al., 2020), who report a method for extracting polyphenols using NaDES accompanied by microwave-assisted extraction.
In the present work, we used betaine:glycerol (1:2.2 molar ratio) NaDES to extract polyphenols and this extraction offers a number of advantages over traditional solvent extraction, including:

- it is more environmentally friendly, as the oil deprived of polyphenols can still be used as a food, as betaine is not fat-soluble, while glycerol has no limits of use according to EFSA and FDA. In addition, the polyphenol product obtained can be used directly on cells, animals and possibly on humans as NaDES consists of edible components normally used in pharmacopoeia;

- it doesn't represent an hazard for the workers, thanks to the absence of organic solvents in the extraction procedure;
- it is gentler on the plant material, which can preserve the quality of the polyphenols. Firstly, it avoids several purification and handling steps and secondly, it avoids excessive heating and oxidation of the polyphenols;
- NaDES composition can be modulated by particular needs, which gives more flexibility to researchers/manufacturers.

Given the enormous potential versatility in the types of NaDES that can be produced, by modifying the components and molar ratios between the chemical species involved, many authors report possible uses in the biomedical area in view of its economic (low production cost and free of waste), biological (biodegradability and low toxicity in both production and consumption) and chemical (simple synthesis, high solubilisation efficiency especially of low-solubility substances) characteristics (Huang et al., 2021; K. Wu et al., 2022).
Indeed, applications of NaDES already include anti-bacterial treatment, drug delivery and solubilisation (Swebocki et al., 2023).
Furthermore, it is possible to create specific NaDES that contain the bioactive molecule (active pharmaceutical ingredients, API) in their formulation (K. Wu et al., 2022).
Various biomedical applications reported are those with collagen (Grønlien et al., 2020), dexamethasone (Silva et al., 2018) and beta-lactam antibiotics (Olivares et al., 2018).
There are currently no applications of polyphenols contained in NaDES in medicine in the literature, even though NaDES seems to have an optimal potential in extracting polyphenols from biological sources (Ruesgas-Ramón et al., 2017).

In this work, the bioactive capacities of polyphenols extracted from EVOOs are evaluated on different cellular and animal models of diseases, paying particular attention to inflammation and oxidative stress.
The cumulative aim of this work is to shed light on the anti-inflammatory and antioxidant effects of polyphenols extracted from a specific polyphenol-rich Coratina cultivar oil in cellular and animal models of neuroinflammation and neurodegeneration.
The data will be presented and evaluated in the context of the specific study, also highlighting the omic changes exerted by the intake of polyphenols, beyond inflammation and oxidative stress, which could provide new inputs and theories for possbile applications of these molecules in medicine.

In the first section, experiments are presented to evaluate whether there are biological differences between polyphenols extracted by the classical method and those extracted via NaDES, by determining the biological response (inflammation and oxidative stress) on murine BV2 microglia and N2a neuronal cells. Moreover, we dedicated careful attention to a study to determine whether the response of polyphenols extracted by classical method could produce the same results before or after treatment with a pro-inflammatory stimulus.
In the second section, polyphenols extracted by classical method (OOEP) were administered for 8 weeks in drinking water (10 mg/kg/day) to C57BL/6J mice under physiological or low-grade inflammation conditions (obtained by intraperitoneal injection of 0.5 mg/kg/week LPS). The mice were assessed in their cognitive capacity (long-term memory, anxiety-like behaviour) and inflammation and oxidative stress status. A broad-spectrum metabolomics study was performed in the liver and intestine. At the same time, we evaluated the impact of LPS and polyphenols on the microbiota.
In the third and final section, polyphenols extracted with a NaDES made up with betaine and glycerol in a 1:2.2 molar ratio (OOE-DES) were administered for 12 weeks in drinking water (5 mg/kg/day) to euploid B6EiC3SnF1 and trisomic Ts2Cje mice. The impact of polyphenols on the mice in terms of cognition, oxidative stress and inflammation at the brain cortex and liver level was assessed, and a broad-spectrum lipidomic analysis was conducted on the brain, liver and caecum tissues.

Materials and methods

Extraction methods for polyphenols from olive oil

Classical method

The polyphenolic extract was obtained from an endemic monocultivar olive oil (*Olea europaea* L. var. Coratina) within 6 months of production.
Polyphenols from the olive oil were extracted using the method developed by Montedoro (Montedoro et al., 1992). Briefly, 500 µl of olive oil together with the same amount of 80:20 methanol/water are vortexed for 5 minutes and centrifuged at 14,000 *g* for 5 minutes. The aqueous fraction is collected and the remaining oil is subjected to two more extraction cycles. Finally, the three collected fractions are mixed together and dried under vacuum in a rotary evaporator. The dried extracts were finally weighed and quantified by the Folin Ciocolteau colorimetric method using hydroxytyrosol as a standard for the calibration curve.

NaDES method

The used NaDES is a betaine/glycerol 1:2.2 (molar ratio). The NaDES is prepared by mixing glycerol at a constant temperature of 70 °C into which betaine is added little by little until a clear eutectic solvent is obtained. Once cooled, it is mixed with olive oil in a 1:20 ratio, vortexed for 5 minutes and then left to decant overnight in a separating funnel. The next day, the polyphenol-enriched NaDES is collected and quantified by the Folin Ciocolteau colorimetric method using hydroxytyrosol to plot the calibration curve.

Chromatographic analysis of the polyphenolic fraction

UPLC-DAD-MS was performed on a Waters Acquity H-Class UPLC system (Waters, Milford, MA, USA), equipped with a quaternary solvent manager (QSM), a sample manager with flow through needle system (FTN), a photodiode array detector (PDA) and a single-quadruple mass detector with electrospray ionization source (ACQUITY QDa). Chromatography was performed on a Phenomenex Kinetex C18 column (100 mm × 2.1 mm i.d., 2.6 µm particle size). Solvent A was 0.1% aqueous formic acid and solvent B was 0.1% formic acid in methanol. Flow rate was 0.5 mL/min and column temperature was set at 35 °C. Elution was performed isocratically for the first 2 minutes with 2% of solvent B, then by applying a 10 minutes linear gradient from 2% to 100% B. The column was re-equilibrated for 5 minutes with 98% A and 2% B before the following injection. Samples were filtered onto 0.22 µm membranes before the analysis and 2 µL of sample were injected through

the needle. The PDA detector was set up in the range 200 to 600 nm. Mass spectrometric detection was performed in negative electrospray ionization mode using nitrogen as nebulizer gas. Analyses were performed in Total Ion Current (TIC) mode and 120–600 m/z range.

Cell culture, protein extraction and quantification for Cox inhibition activity

RAW 264.7 murine macrophages were used as a source of Cycloxygenase (COX) proteins. Cells were grown in High Glucose DMEM, supplemented with 10% foetal bovine serum (FBS), 1% Penicillin-Streptomycin and 2 mM L-glutamine.

Cells were seeded in 175 cm^2 flasks and cultured until confluence, then were harvested by scraping and centrifugation for 5 minutes at 250*g*. The pellet was kept on ice and allowed to swell in hypotonic buffer, following previously described procedures (Abmayr & Workman, 1993; Wiese & Thompson, 2001). Briefly, the homogenized pellet was lysed by 10 passages through a 26-gauge needle and centrifuged at 3,300g for 15 minutes at 4 °C. The supernatant was transferred to a new tube and centrifuged at 100,000*g* for 1 hour at 4 °C. A solution comprising 0.25 M sucrose, 10 mM Tris-acetate (pH 7.4) and 20% glycerol was prepared to resuspend the resultant microsomal pellet. Protein concentration was determined using a BCA quantification kit.

COX inhibition activity

The procedure is properly described in (Jang & Pezzuto, 1997). Briefly, 200 µg of microsome suspension were incubated for 5 minutes at 25 °C in Tris-HCl buffer 100 mM (pH 8.5), with 1 µM hemin and the test compound (i.e. oleacein, oleocanthal or ibuprofen, used as a reference), solubilized in DMSO at 50 µM final concentration. In parallel, blank samples were prepared by adding the same volume of hemin, tested molecule and microsomal resuspending buffer in Tris-HCl buffer. After incubation, N,N,N′,N′-Tetramethyl-1,4-phenylenediamine (TMPD) and arachidonic acid were added at 240 µM and 330 µM final concentration, respectively. Absorbance was read at 611 nm after 8 min of incubation with a Hitachi U-2000 spectrophotometer. Experiments were repeated four times. Blank was subtracted from each spectrophotometric measure. Data are presented as mean ± SEM in fig. 1bis A. Statistical significance was set at $p<0.05$ and analysed by one-way ANOVA.

Experiments with N2a, BV2, C8D1A cell lines

Cell maintenance

Murine neuronal N2a cells were grown in DMEM high glucose containing 10% fetal bovine serum, Penicillin–Streptomycin solution (10,000 U/ml) and 2 mM L-glutamine at 37 °C in a humidified atmosphere with 5% CO_2. Cells were plated three times a week by trypsinization (1% trypsin in PBS) in 25-cm^2 flasks. Cells were used for the experiments between the 8^{th} and 15^{th} passage.

Murine microglia BV2 cells were grown in DMEM/F-12 medium containing 10% fetal bovine serum, Penicillin–Streptomycin solution (10,000 U/ml) and 4 mM L-glutamine at 37 °C in a humidified atmosphere with 5% CO_2. Cells were plated three times a week by trypsinization (1% trypsin in PBS) in 25-cm^2 flasks. Cells were used for the experiments between the 2^{th} and 15^{th} passage.

Murine astrocytic C8D1A cells were grown in DMEM high glucose containing 10% fetal bovine serum, Penicillin–Streptomycin solution (10 000 U/ml) and 4 mM L-glutamine at 37 °C in a humidified atmosphere with 5% CO_2. Cells were plated three times a week by trypsinization (1% trypsin in PBS) in 25-cm^2 flasks. Cells were used for the experiments between the 3^{th} and 15^{th} passage.

Treatment of BV2 with LPS alone and in combination with oleacein, oleocanthal and total polyphenols

BV2 cells were plated in 6-well at the concentration of 90,000 cells/well in BV2 medium. After 24 hours all the cells were treated with LPS at the final concentration of 0.5 µg/ml and with 10 µM of oleacein or oleochantal or 30 µg/ml OOEP (classical extraction) or water (as control). 24 hours later the cells were scrapped, centrifuged and stored at -20 degrees. The following steps are properly described in "RNA extraction, retrotrascription and Real Time PCR" section. Data are presented as mean ± SD in fig. 1bis B.

NaDES toxicity curve

24 hours after the seeding, N2a, BV2 and C8D1A cells were treated with increasing volume of NaDES (0 , 0.032 , 0.16 , 0.8 , 4 %, $\%V_{NaDES}/V_{medium}$). The following steps are properly described in "Cell viability experiments (MTT assay)" section. The normalization was performed on untreated wells (0% vol/vol NaDES). Data are presented as mean ± SD in fig. 3.

Toxicity curve in the presence or absence of LPS
24 hours after the seeding, N2a, BV2 and C8D1A were treated with 0.5 µg/ml of LPS (or with the same volume of PBS, as control). Concomitantly, the cells were treated with an equal volume of water (as CTRL) or OOEP 30 µg/ml or DES (empty) or OOE-DES 30 µg/ml. The following steps are properly described in "Cell viability experiments (MTT assay)" section. The normalization was performed on control cells. Data are presented as mean ± SD in fig. 4.

Unstimulated and LPS-stimulated BV2 in co-administration with OOEP and OOE-DES
BV2 cells and N2a cells were plated in 6-well at the confluence of 90'000 and 300'000 cells/well respectively. 24 hours after the seeding, the cells were treated with 0.5 µg/ml of LPS (or with the same volume of PBS) in combination with OOEP or OOE-DES at the final concentration of 30 µg/ml of total poliphenols. 24 hours later the cells were scrapped, centrifuged and stored at -20 degrees. The successive RNA extraction and analysis was properly described in "RNA extraction, retrotrascription and Real Time PCR" section. Data are presented as mean ± SD in fig. 5A and 5B.

Cell viability experiments (MTT assay)
For the cell viability experiments, 96-well plates were seeded with 3'000 BV2 / 10'000 N2a / 10'000 C8D1A cells/well. 24 h after the seeding, cells were treated with the specific treatment in a final volume of 150 µl per well. Plates were incubated for 24 h in an incubator at 37°C, 5% of CO_2, and 99% RH. Thereafter, 20 µl of (3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide) (MTT) dye (stock solution 5 mg/ml in PBS) (catalog number #M2003) was added to the wells and incubated for additional 2 h at 37°C and 5% CO_2. Water-insoluble formazan crystals were dissolved in 100 µl of DMSO (catalog number #472301). For spectrophotometric measurements, Appliscan® plate reader (Thermo scientific) was used at the wavelength 570 nm, with reference set at 690 nm.

Griess assay
BV2 cells were plated in 96-well at the confluence of 150'000 cells/well. 24 hours after the seeding, all the cells were treated with 1 µg/ml of LPS in combination with water (control) / OOEP 30 µg/ml / DES (empty) or OOE-DES 30 µg/ml. 24 hours later, 50 µl of BV2 microglia culture medium was transferred into a new 96-well plate, and mixed with 50 µl of Griess reagent

(1% sulfanilamide, 0.1% naphthylethylene, 2% phosphoric acid). Then, the mixture was placed at room temperature for 10 min and the absorbance was measured at a wave length of 540 nm. The normalization was performed on control wells. Data are presented as mean ± SD in fig. 5C.

OOEP-pre/post-treatment of BV2 in the presence and absence of LPS

BV2 cells were plated in 6-well at the confluence of 90'000 cells/well. The successive day, the cells were treated with PBS (as control), with 1 µg/ml of LPS or with OOEP 30 µg/ml, according to the scheme below (T=0h). 24 hours later, the new treatment (PBS, OOEP or LPS) was added directly to the cell medium, according to the scheme below (T=24h). 24 hours later the cells were scrapped, centrifuged and stored at -20 degrees (T=48h). The successive RNA extraction and analysis was properly described in "RNA extraction, retrotrascription and Real Time PCR" section. Data are presented as mean ± SD in fig. 7.

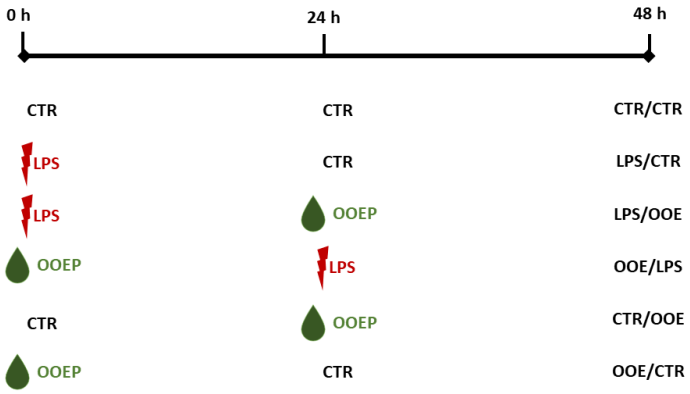


Fig. III Schematic representation of the experiment

PCA analysis on the markers studied on BV2 cells

Fold change data coming from the RT-PCR analysis were analysed through Statistical Analysis [one factor] module on Metaboanalyst 6.0 server (Y. Lu et al., 2023). Data were $\log_{10}$ transformed and auto-scaled (mean-centered and divided by the standard deviation of each variable) and the Principal Component Analysis (PCA) and the "pattern search analysis" were performed to obtain correlations between metabolites. Biplot was obtained in PCA analysis (fig. 8).

Linear regression between GPX-4 / CAT and SOD-1 after 24/48 hours of treatment was calculated and ploted using PRISM® 6.0 software (fig. 9).

Stability of polyphenols at 25 and 40 °C

3 different extra virgin olive oils were selected on the basis of polyphenol content (>600 mg/kg). 10 ml of olive oil was vortexed with 1 ml of NaDES betaine-glycerol 1:2.2 (molar ratio) (only in this experiment, the NaDES : oil ratio was 1:10) for 5 minutes and then left to spontaneously decant for 20 minutes. The OOE-DES was aliquoted (500 µl) into two 2-ml eppendorfs; parallely 500 µL of the same oil was aliquoted into two 2-ml eppendorfs (so as to obtain the same degree of atmosferic oxygen inside the eppendorf between the sample of oil and OOE-DES). In parallel, 500 µL of oil was extracted by the NaDES method and resuspended in 500 µL of water. The eppendorfs with oil, NaDES and polyphenols resuspended in water were stored in two thermostatic ovens at 25 and 40 °C. Polyphenol contents were measured at 6 months after aliquoting. Specifically, the NaDES was diluted 1:100 in water and 10 µL was injected in UPLC-MS, while the oil was extracted according to the classical method and 10 µl of the methanol/water fraction obtained was injected in UPLC-MS. 10 µl of polyphenols in water were injected directly into the instrument, taking the dilution factor into account. Data are presented as mean ± SD in fig. 2.

Animal model of subchronic inflammation

The animal model experiment of subchronic inflammation was conducted at the DMU (Disease Modelling Unit) of the University of East Anglia (UEA), in the Faculty of Medicine and Health Sciences (Norwich Medical School) located in Norwich (UK) by the research group of Professor David Vauzour.
Thirty-two male C57BL/6J mice sourced from Charles River (Margate, UK) were maintained in individually ventilated cages (n = 4 per cage, 2 cages for treatment group), within a controlled environment (21 ± 2 °C; 12 h light/dark cycle; light from 7:00 AM) and fed ad libitum on a standard chow diet (RM3-P; Special Diet Services (SDS, Horley UK) up to the age of 12 weeks, ensuring normal development and stabilisation of the microbiota. After which mice were transferred onto one of two treatment, namely water (control) and olive oil extracted polyphenols (OOE) supplement for the remaining of the experimentation. The OOEP supplement (equivalent to 10mg/kg/die olive oil polyphenol) were added to the water one other day. Diets were prepared by Research Diet Inc. (New Brunswick, USA) to comply with animal nutrition requirements. Chronic low-grade inflammation was induced through weekly intraperitoneal injections (IP) of 0.5 mg kg^{-1} LPS for 8 weeks as described

previously or a SHAM injection consisting of saline. At the end of the experiments and following the completion of behavioural testing, 4-month-old animals were sedated with a mixture of isoflurane (1.5%) in nitrous oxide (70%) and oxygen (30%) and transcardially perfused with ice-cold saline containing 10 UI heparin (Sigma-Aldrich, UK). Sera were isolated via centrifugation at 2,000g for 10 min. Brains were rapidly removed, halved, snap frozen and stored at −80 °C until biochemical analysis. Additionally, caeca were removed, weighed and contents were gently extracted. Samples were then snap-frozen in liquid nitrogen and stored at −80 °C until further analysis.

Behavioural assessment

All behavioural tests were performed at the experimental endpoint after the 8-week intervention. Prior to commencing, a visual placing test was performed on each animal to ensure animals were not visually impaired. All behavioural tests were analysed using the Ethovision software (Tracksys Ltd, Nottingham, UK).

The Open Field (OF) task used as a measure of anxiety. Animals were individually placed within the (50 cm × 50 cm × 50 cm) square arena illuminated with dim lighting (100 lux) and were allowed to move freely for a 10-minute period. Mice were tracked using Ethovision software which determined travel distance, velocity and time spent in the centre/periphery of the maze respectively.

The novel object recognition (NOR) task, a measure of recognition memory was performed as described: on day 1 (habituation), the animal was placed into an empty maze for 10 minutes. On day 2, animals were conditioned to a single object for a 10-minute period. On day 3, mice were exposed to 2 identical objects for 15 minutes. Following an inter-trial interval of one hour, mice were placed back within the testing arena now containing one familiar object and one novel object. Videos were analysed for a 5-minute period, after which if an accumulative object exportation of 8 seconds failed to be reached, the analysis continued for the full 10 min or until 8 seconds was achieved. Those not achieving 8 seconds of exploration were excluded from the analysis.40 Discrimination index was calculated as follows $DI = (TN - TF)/(TN + TF)$, where TN is the time spent exploring the novel object and TF is the time spent exploring the familiar object.

Enzyme-Linked Immunosorbent Assay (ELISA)

Cytokines were determined using dedicated ELISA kits obtained from Clinisciences. Liver samples were homogenised in RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS, 2 mM EDTA, 50 mM

NaF, 0.5% sodium deoxycholate, 1 mM PMSF and protease inhibitor SIGMAFAST™ cocktail). Lysates were incubated on ice for 30 min and centrifuged at 12,000 g for 20 min at 4 °C. Supernatants were collected and protein quantification was performed using a Bradford Assay (BIO-RAD). Equal amounts of diluted lysate (100 µl, corresponding to 80 µg protein content) were tested by ELISA kits following the manufacturer's instructions and then quantified for protein content (pg/mg tissue). Data are presented as mean ± SD in fig. 11.

Microbial 16S rRNA extraction and sequencing

Microbial DNA was isolated from approximately 50 mg of caecal content using the QIAamp PowerFecal Pro DNA Kit (Qiagen, Manchester, UK) as per the manufacturer's instructions. DNA quantity was assessed using a Nanodrop 2000 Spectrophotometer (Fisher Scientific, UK). Quality assessment was performed by agarose gel electrophoresis to detect DNA integrity, purity, fragment size and concentration. The 16S rRNA amplicon sequencing of the V3–V4 hypervariable region was performed with an Illumina NovaSeq 6000 PE250. Sequence analysis was performed by Uparse software (Uparse v7.0.1001), using all the effective tags. Sequences with ≥ 97% similarity were assigned to the same OTUs. A representative sequence for each OTU was screened for further annotation. For each representative sequence, Mothur software was performed against the SSUrRNA database of SILVA Database 13.8. OTUs abundance information was normalised using a standard of sequence number corresponding to the sample with the least sequences.

H-NMR metabolomics

Caecal metabolites were analysed and quantified by 1 H NMR analysis, according to reported methods (Tran et al., 2019; Wu et al., 2010). Briefly, frozen caecal contents were thoroughly mixed at 5,000 rpm in a Precellys® (Bertin Technologies, France) and diluted by adding deuterated phosphate buffer (1.9 mM Na_2HPO_4, 8.1 mM NaH_2PO_4, and 1 mM sodium 3-(trimethysilyl)-propionate-d4 in deuterated water (Goss Scientifics, Crewe, United Kingdom)). After mixing and centrifugation, 500 µl was transferred into a 5 mm NMR tube for spectral acquisition. High resolution 1 H NMR spectra were recorded on a 600 MHz Bruker Avance spectrometer fitted with a 5 mm TCI proton-optimized triple resonance NMR inverse cryoprobe and a 24-slot autosampler (Bruker, Rheinstetten, Germany). Sample temperature was controlled at 300 K. Each spectrum consisted of 128 scans of 65 536 complex data points with a spectral width of 20 ppm (acquisition time 2.6 s). The noesypr1d presaturation sequence was used to suppress the residual water

signal with low power selective irradiation at the water frequency during the recycle delay (D1 = 2 s) and mixing time (D8 = 0.01 s). A 90° pulse length of 11.4 µs was set for all samples. Spectra were transformed with a 0.1 Hz line broadening and zero filling, manually phased, baseline corrected, and referenced by setting the trimethylsilylpropanoic acid methyl signal to 0 ppm. Metabolites were identified using Human Metabolome Database (https://www.hmdb.ca/) and quantified using the software Chenomx® NMR Suite 8.6™.

Analysis of lipid families in low-grade inflammation mouse model

19 different lipid family were identified and quantified using H-NMR. A parametric statistical analysis (1-way ANOVA, corrected for False Discovery Rate FDR) was performed on Prism®. Only the statistically different populations were showed (Sphyngomyelin). Data are presented as mean ± SD in fig. 12.

RNA extraction, retrotrascription and Real Time PCR

Total RNA were extracted from cells or tissues using TriReagent (Merck), according to the manufacturer's instructions (phenol-chloroform extration) and extracted total RNA was quantified using NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

The reverse transcription reaction was performed using the SensiFAST™ cDNA Synthesis Kit (Bioline) according to the manufacturer's instructions. The quantitative real-time PCR (RT-PCR) was performed for each sample in technical duplicate on a CFX Real-Time detection system (BIORAD®) using the iTaq™ Universal SYBR® Green Supermix.

The used primers were designed using NCBI “pick primers” tool and are summarised in the table below.

target	*forward*	*Tmelting (°C)*	*reverse*	*Tmelting (°C)*
rps27a	AGAGGCTGATCTTTGCTGGT	58	ACCAGATGAAGGGTGGACTC	64
il1ß	TTCGTGAATGAGCAGACGC	60	CCATGGTTTCTTGTGACCCT	62
il10	CCAAGACCCAGACATCAAGG	54	GCATTCTTCACCTGCTCCAC	56
iNOS	GCAAGAGAGTGCTGTTCCAG	62	CCTGAACGTAGACCTTGGGT	64
ppar-g	GGAAGACCACTCGCATTCCT	62	TCAGCAACCATTGGGTCAGC	62
bdnf	AAAAGCAACAAGTTCCCCAGC	60	GTCGCCAGGTAAGAAACCCT	60
snap25	AGATTGACACCCAGAATCGC	62	CCACTTCCCAGCATCTTTGT	58
iba-1	GCTTTTGGACTGCTGAAGGC	62	GTTTGGACGGCAGATCCTCA	62
arg-1	ACATTGGCTTGCGAGACGTA	60	ATCACCTTGCCAATCCCCAG	62
sod-1	GGCTTCTCGTCTTGCTCTCTC	64	AACACAACTGGTTCACCGCT	60
cat	GAAGGACCGTGTTTGGTTGC	60	CCGCTGGCGCTTTTATTGTT	58
gpx-4	CCATGCACGAATTCTCAGCC	62	GGTGACGATGCACACGAAAC	60
ho-1	ATGGCGTCACTTCGTCAGAG	60	GCTGATCTGGGGTTTCCCTC	62
il-6	AAGCTGGAGTCACAGAAGGAG	64	GGTTTGCCGAGTAGATCTCAA	60
tnf-α	TGGCCTCTCTACCTTGTTGC	60	GGGAGCAGAGGTTCAGTGAT	60
trxr	AAGACGATGAACGTGTCGTG	62	TAGTCAGCCCACACTTGAGC	60
sphk	AGTCGGTGAGGATCGTGGAT	62	GTACCTCGTCAGCAACCTCC	60
plpp	CCCTCGATGTGATTTGCGTG	62	ATACGGGACGGGATGGTACT	62
pemt	CCCCCTGCAAATCATTCCCTA	62	TGAAAGGAAGGCGGCACATA	60
chpt	AAGCACCGGAACAGGTTTACA	60	GTTCCCCTAACTGCTTCTCCTC	60

Western blot analysis

Cells and tissues were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM EDTA, 5 mM NaF, 0.5% sodium deoxycholate) containing 1 mM PMSF, 1 mM Na_3VO_4 (catalog number #S6508, Merck) and SIGMAFAST™ Protease Inhibitor Cocktail (catalog number #S8820, Merck). The lysates were incubated on ice for 30 min, sonicated in the case of cell or pottered in the case of tissues, and centrifuged at 12 000 *g* for 20 min at 4 °C. Supernatants were collected and protein quantification was performed using a BCA Assay (Thermo scientific®). Equal amounts of proteins (20 µg) were separated on 4–20% SDS-PAGE and transferred to PVDF membrane probed with the primary antibody and secondary peroxidase-conjugated antibody (1:5000) (BIORAD®). The protein bands were visualized by ECL system (catalog number #1705060, BIORAD®) according to the manufacturer's instructions. Membranes of the section 2 were stripped and reprobed with housekeeping antibody (GAPDH or β-actin 1:5000) (Millipore) and secondary peroxidase-conjugated antibody anti-mouse (1:5000) (BIORAD®); whereas membrane in section 3 were normalised by stain-free™ technology (BIORAD®). Densitometric analyses were performed with ImageLab software (BioRad) (RRID:SCR_014210) and normalized to actin band or stain free ™ staining.

The antibodies used in the experiments are summarised in the table below.

target	manufacturer	code	diluition
NRF2	GeneTex	GTX 103322	1:1000
phospho-NRF2 (SER40)	Abcam	ab76026	1:5000
PGC-1α	SantaCruz	SC-13067	1:1000
ppar-γ (E8)	SantaCruz	sc-7273	1:500
4-HNE	Novus Biologicals	NB100-63093	1:2000
GAPDH (G-9)	SantaCruz	sc-365062	1:5000
β-actin	sigmaMillipore	A1978	1:5000

Bioinformatic analysis

Microbiome analysis

Microbiome data were analysed via the metaboAnalyst (Y. Lu et al., 2023), microbiomeAnalyst (Dhariwal et al., 2017) and metOrigin (G. Yu et al., 2022) servers.

Procedures with microbiomeAnalyst

The 16s rRna marker gene count data were uploaded to the dedicated section of the MicrobiomeAnalyst interface, together with the metadata file (indicating the composition of the treatment groups), the taxonomy table (according to Silva version 13.8) and the phylogenetic tree. The raw data were filtered by the programme, applying the following parameters: low-count filter (minimum count: 4; sample prevalence: 20%), low-variance filter (percentage to be removed: 10%; based on: interquartile range), data rarefaction (not), no data scaling, data transformation (Relative Log Expression RLE). At the end of this process a total of 389 low abundance features were removed based on prevalence and a total of 33 low variance features were removed based on iqr. The number of features remaining after the data filtering phase was 288.
The following analyses were conducted: stacked bar area plot, alpha diversity analysis, beta-diversity analysis, dendrogram analysis, univariate analysis.
Alpha-diversity was assessed using both Chao1 and Shannon H diversity index whilst beta diversity was assessed using Bray–Curtis index, with a PERMANOVA test (p=0.001). Univariate analysis was performed using MetagenomeSeq (0-inflated) stastical method. Adjusted p-value cutoff was 0.05. Comparisons at the phylum, family and genus level were made using classical univariate analysis using Kruskal–Wallis combined with a false discovery rate (FDR) approach used to correct for multiple testing.

Procedures with MetaboAnalyst 6.0

Metabolomics data from liver and gut cecum were studied individually using the 'statistical analysis (one factor)' module offered by metaboanalyst. No sample normalisation was performed. The data were subdued to log10 transformation associated with an auto-data scaling (mean-centred and divided by the standard deviation of each variable). The following analyses were performed: Principal Component Analysis (PCA), Significance Analysis of Microarrays (and Metabolites) (SAM), dendrogram and heatmaps. The data presented were found to be statistically significant at SAM. The histograms and the indicated significance were recalculated using the T-TEST function on PRISM®.

At the end of the procedure, the raw data were entered into the "pathway analysis" module, the metabolites were identified according to classical, HMDB and Kegg nomenclatures and underwent the same normalisation as previously specified.

Procedures with MetOrigin

Metabolomics data coming from the caecum metabolomics and microbiomics were entered into the server, conducting a Deep MetOrigin Analysis. After entering the host (*Mus musculus*) and raw data, the server was asked to conduct a Mann-Whitney U test with p value < 0.05 for statistically significant metabolites and bacteria. In order to identify which bacteria influenced the concentration of GABA, the server was asked to raise the cut-off for metabolites to 0.07. The statistical significance obtained and depicted in the heatmap for this metabolite relates to the correlation between metabolites and microbiota.

Function and correlation analysis were performed. A Spearman's correlation was used and the graphs relating to genus and family were extracted.

Animal model of Down’s Syndrome

The mouse model used are Ts2Cje mice (Rb(12.Ts171665Dn)2Cje) and euploids animals (B6EiC3SnF1). Ts2Cje are a well-established murine model of DS characterized by a triple copy of a Robertsonian fusion chromosome carrying the distal end of Chr16 and Chr12. Parental generations were purchased from Jackson Laboratories (Bar Harbour, ME, USA). Mouse colony was raised by repeated crossbreed of Ts2Cje (Ts) trisomic females with euploid (Eu) males. Since these breeding pairs produce litters containing both trisomic and euploid offspring, resultant progeny was genotyped to determine the presence of the trisomic segment using Quantitative-PCR, as previously described by Reinholdt et al. (Reinholdt et al., 2011). Mice were housed in

clear Plexiglas cages (20 x 22 x 20 cm) under standard laboratory conditions with a temperature of 22 ± 2 °C and 70% humidity, a 12-h light/dark cycle and free access to food and water.
3-month-old mice (euploid and trisomic) were divided in 4 experimental groups (Eu-Veh, Ts-Veh, Eu-DES and Ts-DES) and treated with vehicle (empty DES, VEH) or OOE-DES (n = 10 per group) for 3 months. The treatment on mice was conducted by administering the DES or Veh in their drinking water and the number of the mice was equally distributed in to the single cage (n = 4/5 per cage). The DES/Veh was replaced twice a week, and the animal's drinking was monitored. At the end of the treatment, behavioral test has been administered to all experimental groups. Ts and Eu mice were sacrificed by cervical dislocation and tissues were collected and, immediately frozen at -80°C. Trunk blood was collected in presence of EDTA and then centrifuged at 3000*g* for 15 min at 4 °C to obtain plasma. The samples were than used for Western Blot analysis and qRT-PCR.
The treatment was well tolerated and no change in body weight or in the consumption of drinking water was observed.
The experiment on the animal model was conducted in the animal enclosure of Sapienza university of Rome by the researching group of Prof. Marzia Perluigi. All the experiments were performed in strict compliance with the Italian National Laws (DL 116/92), the European Communities Council Directives (86/609/EEC).

Behavioural assessment
The Novel Object Recognition (NOR) was conducted as previously reported in “Behavioural assessment” of low-grade inflammation mouse model.

Lipidomic analysis on total brain, liver, caecum
Lipidomic analysis were performed during my stay at the Institut des Sciences Analytiques of Lyon (France). Appoximately 10 mg of total brain, liver and caecum coming from the first sperimental subset (16 mice, divided in n=4 for group) were inserted in Precelyss evolution ® tubes with 300 µL of ammonium bicabonate buffer 150 mM and the tissues were homogenized by the system (6000 rpm for 2 cycles of 25 seconds ON and 10 seconds OFF). Tubes were centrifuged at 10,000 *g* for 15 minutes at 4°C and the upper phase was collected. The supernatants were quantified in their protein content by BCA (thermoscientific®).
The volume containing 300 µg of protein was put in a eppendorf and 300 µl of methanol were added with 5 µl of Ultimate SPLASH one ™ internal standard (Avanti® polar lipids). After 15 minutes of mixing at 900 rpm at 4 °C, 1200

µl of methyl-tert-butyl ether MTBE and 200 µl of water were added and mixed togheter for successive 60 minutes at 4 °C. Then, samples were centrifuged and upper phase was collected in new eppendorf and placed to dry under N_2 at 40 °C for 40 minutes. The dried lipid film was resuspended in 20 µl of dichloromethan / methanol 1:1 and afterwards in 180 µl of isopropanol/acetonitrile/water (2:1:1). 5 µl of the samples were injected in a HPLC -MS/MS system.
The column used for separation was a Xselect® C18 3.5 µm 2.1x100mm column (Waters®) inserted on a 1290 series HPLC device (Agilent Technologies, Waldbronn, Germany). The temperature of the auto-sampler and column were kept at 4 °C and 70 °C, respectively. Mobile phases were A (water/acetonitrile 60:40% vol/vol + 10 mM ammonium formate + 0.1% formic acid) and B (isopropanol , acetonitrile, water 90:9.5:0.5) +10 mM ammonium formate + 0.1% formic acid. The flow was 0.5 ml/min and the gradient was as follows:

minute	%A	%B
1	99	1
25	1	99
29	100	0
30	99	1

MS/MS analysis was performed on a QTRAP® 5500 LC-MS/MS System hybrid triple quadrupole/linear ion trap mass spectrometer (Sciex) equipped with a Turbo V™ ion . MS analysis was performed in positive ionization mode using an ion spray voltage of 5500 V and in negative ionization mode using an ion spray voltage of −4500 V. The nebulizer and the curtain gas flow were set at 25 and 50 psi respectively using nitrogen. The TurboV™ ion source was set at 450 °C with the auxiliary gas flow (nitrogen) set at 40 psi. The mass in Q1 and Q3 as well as the declustering potential (DP) and the collision energy (CE) values have been optimized by direct infusion into the mass spectrometer carried out from Splash® Lipidomics®. Abundance values were normalised using the deuterated family-specific internal standard.

Bioinformatic analysis on LipidMaps
Normalised data were loaded on “BioPAN: Bioinformatics Methodology For Pathway Analysis” module on LipidMaps server

(https://lipidmaps.org/biopan/) and the analysis was conducted on two groups at a time. The server calculated the active and suppressed pathways, and their statistical significance and Z-score.

Results

SECTION 1: Effect Of Olive Oil Polyphenols On Murine Microglia BV2 Cell Line

Anti-oxidant and anti-inflammatory capacity of purified olive oil polyphenols

The characterisation of Coratina oil used in this work was performed in our laboratory and described in a previous paper (Francioso et al., 2020).

The chromatographic profile of the polyphenols extracted from the Coratina olive oil showed that the oleacein content was approximately 45 per cent and oleocanthal 25 per cent of the total polyphenols (fig. 1). The oil was shown to have a total polyphenol content of 700 mg/kg and extraction in NaDES did not affect the chromatographic profile compared to polyphenols extracted by the classical method which involves the use of toxic solvents such as methanol and hexane.

Oleocanthal and oleacein were subsequently purified according to the protocol developed by our laboratory (Francioso et al., 2020) and were evaluated in vitro for their capacity to inhibit COX enzymes. Ibuprofen was used as a positive (inhibition) control.

We also evaluated the inflammatory response of purified oleacein and oleocanthal at 10 µM on BV2 cells. We compared the results obtained with results from another similar experiment with total polyphenols derived from the same oil OOEP at the highest non-toxic concentration of total polyphenols (30 µg/ml of total polyphenols, corresponding to 7.5 µg/ml (23 nM) for oleocanthal and 13.5 µg/ml (45 nM) for oleacein).

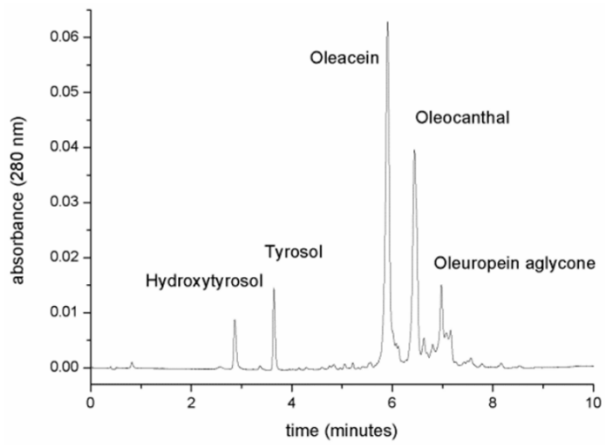
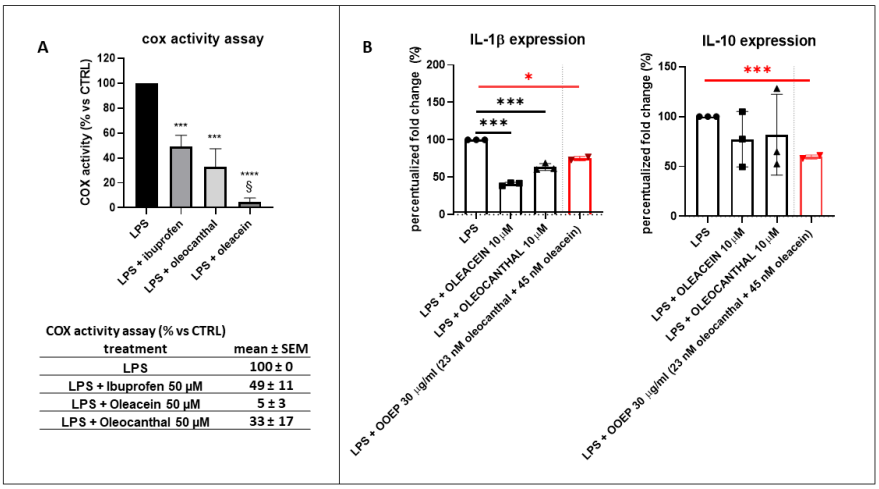


Fig. 1 ***Representative chromatogram of polyphenols extracted from Coratina olive oil using NaDES****, showing the large abundance of oleocanthal and oleacein with respect to the other polyphenols*

Being oleacein and oleocanthal the most abundant polyphenols present in the Coratina oil, we evaluated the ability of these polyphenolic compounds to inhibit cyclooxygenases in vitro (Fig. 1bis A) and found that they have notable inhibitory effect, even in comparison with a well-known COX-inhibitor, i.e. the non steroidal antiinflammatory drug ibuprofen.

In parallel, we evaluated *in vitro* on BV2 cell line the ability of these polyphenols to stimulate an anti-inflammatory response by modulating IL-1β and IL-10 expression (Fig. 1bis B). The data show a decrease in IL-1β and IL-10 expression when LPS is co-administrated with purified polyphenols or OOEP. Notably the inhibitory activity of the extract was exerted even if oleocanthal and oleacein were present in a far less concentration with respect of the purified polyphenols.



COX activity assay (% vs CTRL)

treatment	mean ± SEM
LPS	100 ± 0
LPS + Ibuprofen 50 µM	49 ± 11
LPS + Oleacein 50 µM	5 ± 3
LPS + Oleocanthal 50 µM	33 ± 17

Fig. 1bis (A) ***Cyclooxygenase activity assay: COX-inhibition by oleocanthal and oleacein****. COX activity was assayed in the presence of purified oleocanthal 50 µM, and oleacein 50 µM. Inhibitory activity was compared with that of ibuprofen 50 µM,, as a reference inhibitor, tested at the same concentration. Data are expressed as percent of control without inhibitor (CTRL) and are presented as mean ± SEM. * indicates significance vs CTRL, § indicates significance vs ibuprofen. *0.05 **0.01, ***0.001,****0.0001, according to 1-way Anova. (B)* ***RT-PCR: percentualized fold change of IL-1β and IL-10 mRNA expression in BV2 cell line*** *after treatment with LPS with/out oleacein 10 µM, oleocanthal 10 µM and total polyphenols 30 µg/ml. Data are expressed as percent of LPS-treated cells and are presented as mean ± SD. * indicates significance vs control, *0.05 **0.01, ***0.001,****0.0001 according to 1-way Anova.*

NaDES as a possible carrier of nutraceuticals

Polyphenols, especially secoiridoids, may undergo degradation and hydrolysis during storage. A pharmaceutical formulation, such as a NaDES-based capsule, could be an interesting method of delivering olive oil polyphenols and preserve polyphenol from degradation, due to the extremely limited amount of water in there contained.

To evaluate whether the stability of oleacein and oleocanthal could be altered when extracted from olive oil, we tested different preparations after being stored for 6 months at 25 and 40 °C: in olive oil, in NaDES and in ultrapure sterile water.

Gained awareness of the stability of polyphenols in NaDES and thus of their possible use in long term storage, we wondered whether NaDES could be used on cellular model. So, empty NaDES was evaluated at different concentrations on cell lines from the murine brain environment.

NaDES was evaluated in its ability to preserve polyphenols intact for six months compared to olive oil matrix and water at 25 and 40 °C (Fig. 2). Interestingly, NaDES seems to preserve the polyphenols in the same manner as olive oil, probably due to the absence of water.

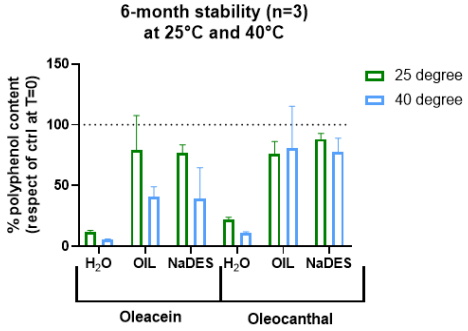


Fig. 2 ***Oleacein and oleocanthal stability*** *in water, native oil or in betaine-glycerol (1:2.2) NaDES after 6 months of storage at 25 and 40 °C. Data are expressed as percent of respective control (T=0 month) and are represented as mean ± SD (n=3).*

As a carrier of polyphenols in many experiments, empty NaDES was assayed on three different cell lines, belonging to the murine cerebral environment (i.e. murine microglia BV2, murine neurons N2a, murine astrocytes C8d1a), for its cytotoxicity (Fig. 3). It can be seen that for the three cell lines, the concentration of 0.16% vol/vol (corresponding to the concentration of 30 µg/ml in the case of NaDES loaded with polyphenols) is optimal for cell experiments, as the recorded cell viability is >80% (BV2 96±7%, N2a 90±4%, C8D1A 90±6% cell viability).

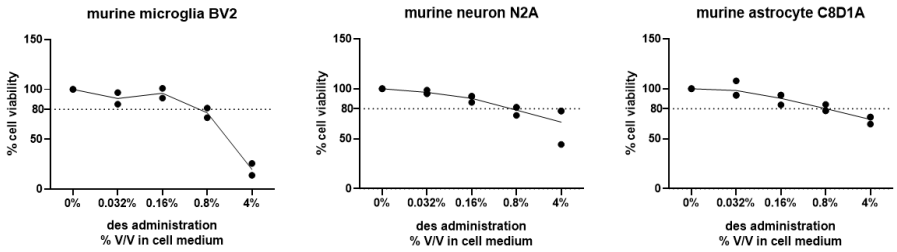


Fig. 3 ***MTT assay on BV2, N2a, C8D1a cell lines****. The experiment was performed in biological triplicate (n=3) after 24 hours of treatment with empty NaDES. Data are expressed as percentage of respective control (0% vol/vol NaDES in cell medium) and are presented as mean (solid line) and min and max (symbols).*

Evaluation of OOEP and OOE-DES toxicity on unstimulated and LPS-stimulated cells

Having identified the non-toxic NaDES concentration capable of carrying the biologically active amount of polyphenols (30 µg/ml OOEP), we assessed whether the polyphenols in NaDES could give an unpredictable toxic response. We conducted cytotoxicity experiments by using OOEP and OOE-DES at concentrations of 10 and 30 µg/ml and the same volumes of empty NaDES corresponding to these concentrations (0.05 and 0.16% V/V) as controls. In addition, we evaluated the cytotoxicity of these molecules under conditions of cellular stress due to the administration of LPS, which is known to activate cells in a pro-inflammatory manner.

Previous experiments (data not shown) allowed us to identify the concentration of 30 µg/ml OOEP as the maximum non-toxic concentration for these cell lines. This concentration corresponds to 0.16% vol/vol of empty DES as shown in the previous graphs. We opted to evaluate the cell viability with the same polyphenols on the three cell lines, even in the presence of the inflammatory stimulus LPS that will be present in the subsequent experiments.

The data show that under these specific conditions, the polyphenols contained in DES (OOE-DES) have the same effect on cell viability as compared to OOEP.

Moreover, LPS affects cell viability in the case of BV2 cell line, but the treatment with olive oil polyphenols seems to reduce the toxicity, both in OOEP and OOE-DES.

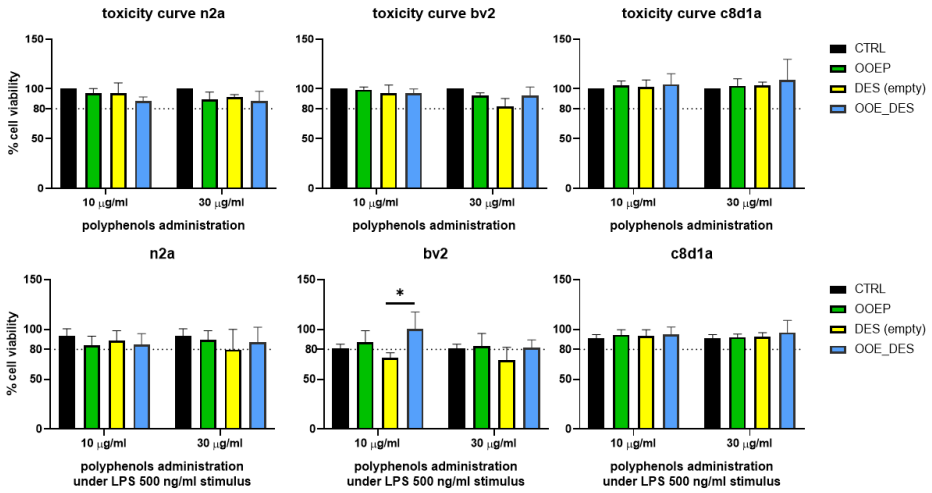


Fig. 4 ***MTT assay on proinflammatory un/stimulated BV2, N2a, C8D1a cell lines****. Upper panels show the viability of unstimulated cells under treatement with water (ctrl), polyphenols extracted by classical method 30 µg/ml (OOEP), empty NaDES (DES(empty)) and polyphenols extraxted by NaDES method 30 µg/ml (OOE_DES). Lower panels show the same experiment under inflammatory stimulus (500 ng/ml of LPS). The experiment was performed in biological triplicate (n=3) after 24 hours of treatment. Data are expressed as percent of respective untreated control and are presented as mean ± SD. * indicates significance, *0.05 **0.01, ***0.001,****0.0001 according to 1-way Anova.*

Evaluation of the OOEP and OOE-DES response on unstimulated or LPS stimulated BV2 microglial and N2a neuronal cells

Here we wondered whether the polyphenols contained in NaDES gave the same response as OOEP on BV2 microglia and N2a neuronal cell lines. We evaluated the response by mRNA expression following treatment with OOEPs and OOE-DES in normal conditions or under stimulation with LPS 0.5 µg/ml. In BV2 cells we evaluated the expression of IL-1β, iNOS and PPAR-γ, which are fundamental markers in the inflammatory response.
In N2a cells we evaluated the expression of BDNF and SNAP25, belonging to the process of neurogenesis and synaptic plasticity, while PPAR-γ is fundamental for lipid metabolism.
To validate the data obtained on iNOS on BV2, we conducted a Griess assay to quantify nitrites produced in the presence of polyphenols after inflammatory stimulus.

We evaluated the response of OOE-DES in comparison to OOEP in BV2 microglial (Fig. 5A) and N2a neuronal cells (Fig. 5B) in the presence or in the absence of pro-inflammatory stimulus (0.5 µg/ml LPS).
The data show that on these genes, the response is similar between OOE-DES and OOEP, meaning that the presence of betaine and glycerol in the DES do not impact on the effect of polyphenols on these cell lines.
In addition, we determined $NO^{\bullet}$ production on BV2 cells by the Griess assay following a pro-inflammatory stimulus, and recorded a similar response in both control (LPS and LPS+empty NaDES) and treated cells (LPS+OOEP and LPS+OOE-DES).

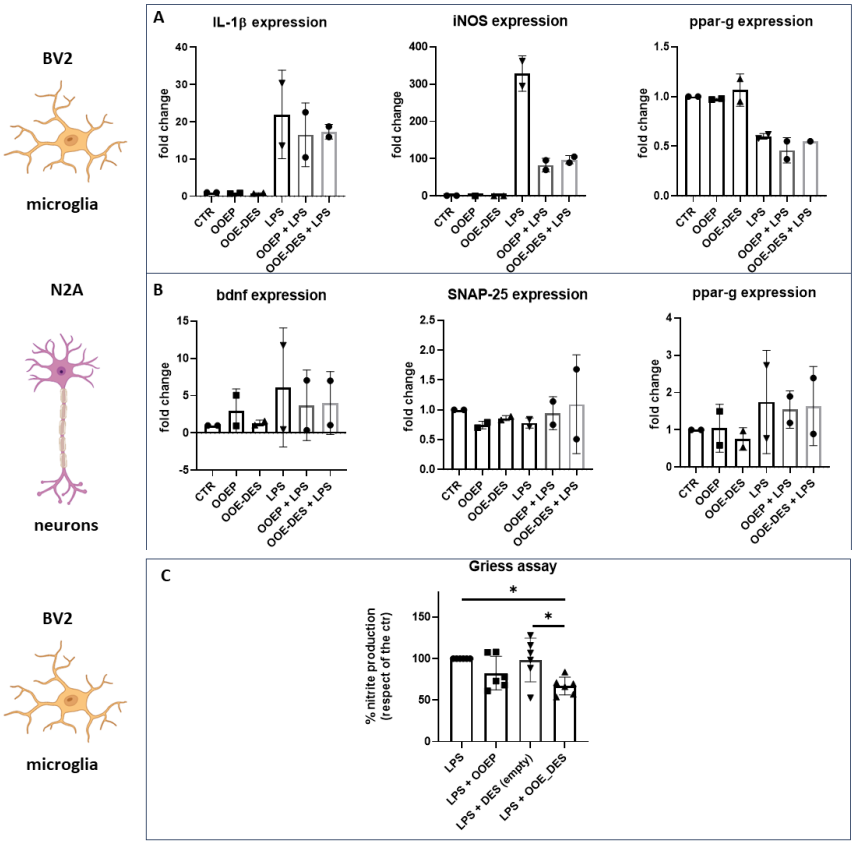


fig. 5 (A) ***RT-PCR: fold change of Il1ß, iNOS and PPAR-g mRNA expression in BV2 cell line*** *after admimnistration of water (CTR), OOEP and OOE-DES 30 µg/ml with/out proinflammatory stimulus (B)* ***RT-PCR: fold change of bdnf, snap25 and PPAR-g mRNA expression in N2a cell line*** *after admimnistration of water (CTR), OOEP and OOE-DES 30 µg/ml with/out proinflammatory stimulus. All The experiments were performed in biological duplicate (n=2) after 24 hours of treatment. Data are expressed as percentage of respective control and are presented as mean ± SD. (C)* ***Griess assay for nitrite quantification on BV2 cell line****: griess assay was performed on BV2 cell line after 24 hours of proinflammatory stimulation in combination with control (water), OOEP 30 µg/ml, empty DES or OOE-DES 30 µg/ml. The experiments were performed in biological sextuplicate (n=6) after 24 hours of treatment. Data are expressed as percentage of respective control and are presented as mean ± SEM. * indicates significance, *0.05 **0.01, ***0.001,****0.0001 according to 1-way Anova.*

Effects of pre- or post-treatment with polyphenols on LPS-induced inflammation and metabolism in a BV2 murine microglia model

The aim of this experiment was to determine whether OOEPs are able to bring about the same anti-inflammatory and anti-oxidant effect when administered before or after the pro-inflammatory stimulus LPS. BV2 microglial cells were treated according to the scheme presented in Fig. 6, such that all the

experimental conditions ended at the same time at 48 hours. The inflammatory markers used were IL-1β, iNOS, IBA-1 (indicative of microglial activation in a pro-inflammatory M1 sense) and IL-10, ARG-1(indicative of microglial activation in an anti-inflammatory M2 sense) and PPAR-γ (negative regulator of the transcription factor NF-kB). Markers aimed at studying the expression of enzymes involved in combating oxidative stress are SOD-1, CAT, GPX and HO-1.
Since inflammation and oxidative stress go hand in hand, we conducted a Principal Component Analysis PCA to identify a trend in our data.

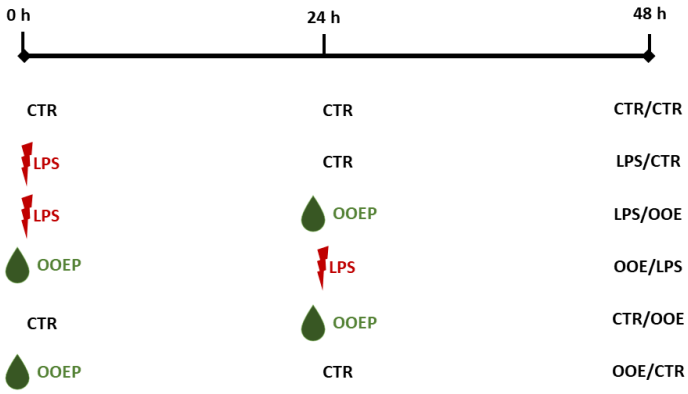


Fig. 6 Explicative treatment scheme on BV2 cell line.

As shown in Fig.7, LPS/CTR treatment triggers an inflammatory response detectable at the end of the experiment (48 hours). In fact, there is a marked increase in the expression of IL-1β and IBA-1 (pro-inflammatory markers), accompanied by iNOS (fundamental for cell-cell communication even at enormous distances) and IL-10 (fundamental for the negative feedback of the switching off of the inflammation).
Concurrent with these increases we observed a reduction in PPAR-γ (negative modulator of NF-kB), HO-1 and Arginase-1 (inhibited by iNOS activation).
Previous or subsequent administration of OOEPs leads similarly to a reduction in the pro-inflammatory markers IL1β, iNOS and IBA-1. An important difference emerges when examining IL-10. Here, the earlier administration keeps the levels low, whereas the later administration seems to increase the anti-inflammatory response.
PPAR-γ behaves in a specularly opposite manner with respect to the pro-inflammatory markers: in fact, it is a negative modulator of NF-kB. Arginase-

1 seems to be activated by OOEP administration, but, in co-administration with LPS its level remains low.
From the point of view of oxidative stress, 24 hours after OOEP treatment (CTR/OOE) HO-1 transcription is activated, whereas it is the same as the control at 48h after administration (OOE/CTR).
Treatment with LPS/CTR particularly affects CAT expression, and the same occurs when OOEP are administered alone.

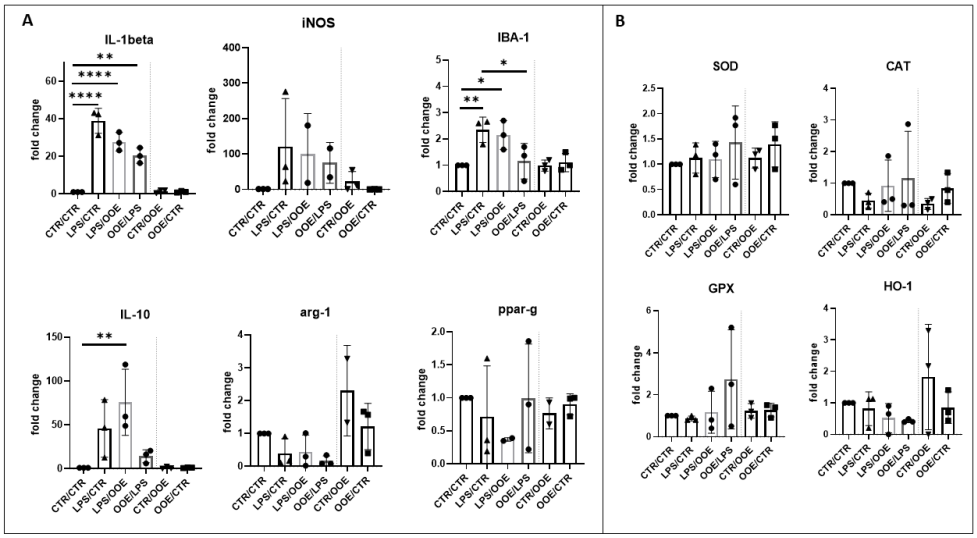


Fig. 7 ***RT-PCR: fold change of inflammatory panel (A) Il1β, IL10, IBA-1, iNOS, ARG-1, PPAR-γ and anti-oxidative panel (B) SOD, CAT, GPX, HO-1 mRNA expression in BV2 cell line*** *treated according to the previous scheme (Fig. 6). Polyphenols extracted by classical method OOEP were administered at the final concentration of 30 µg/ml, whereas LPS at the final concentration of 1 µg/ml. The experiment was performed in biological triplicate (n=3) after 48 hours of treatment. Data are expressed as percentage of respective control and are presented as mean ± SD. * indicates significance, *0.05 **0.01, ***0.001,****0.0001 according to 1-way Anova. Significance linked to CTR/OOE and OOE/CTR treatments are not showed in order to simplify the reading of the graphs.*

The PCA analysis shows that OOEP treatments (both CTR/OOE and OOE/CTR) does not change the behaviour of the markers studied compared to the control population (the red dot in Fig. 8). Conversely, treatment with LPS changes the population and drags the combined treatments with OOEP (LPS/OOE and OOE/LPS) with it.
By studying the bi-plot, it can be seen that IL-1β, IL-10, IBA-1 and iNOS form an extremely compact vector, which permits to form a stable cluster in which the LPS-treated populations reside (blue, light blue and yellow circles). The opposite vectors to this trend are PPAR-γ and Arginase-1. Subsequent correlations show how the activation of IL-1β transcription (following activation by LPS in this case) correlates with a transcriptional increase in

IBA-1, IL-10 and iNOS, while the increase in Arg-1 is negatively correlated with iNOS and IL-1β.

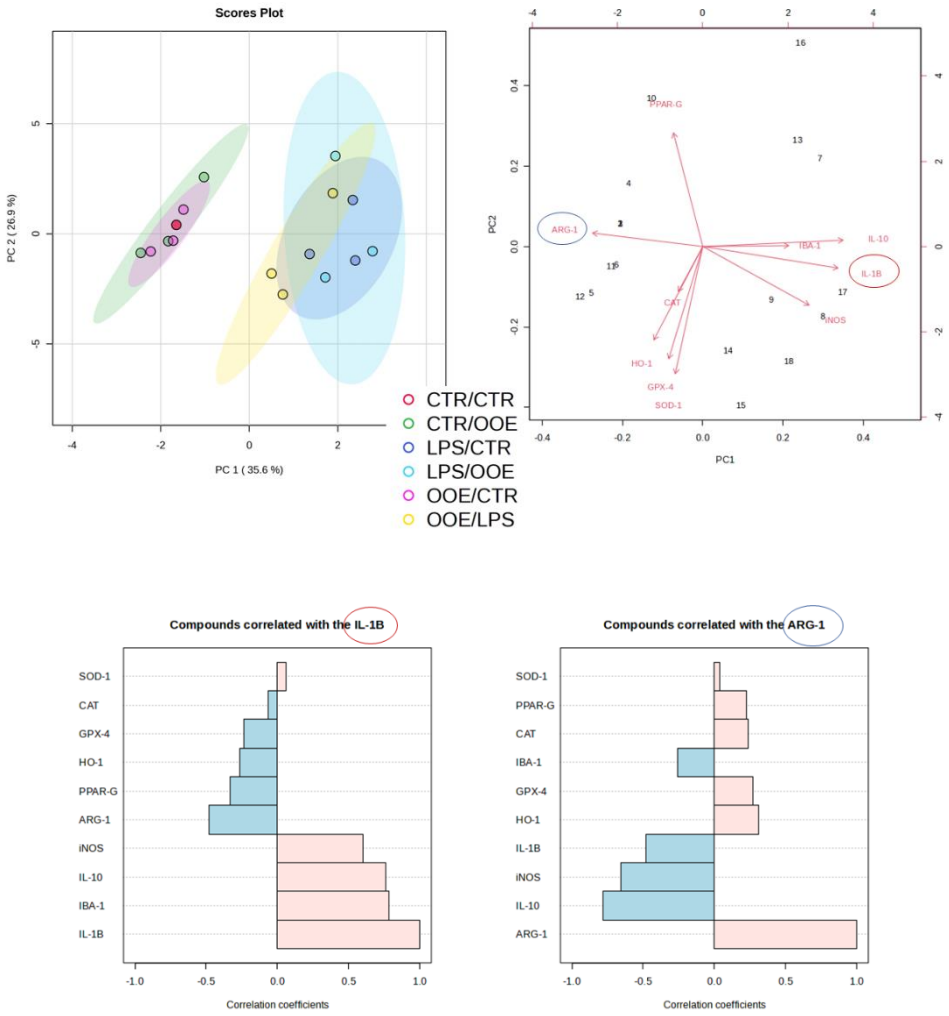


Fig. 8 ***PCA analysis on the markers studied on BV2 cells****: Upper panel, PCA analysis and Bi-Plot of the 10 studied markers in BV2 cells. Lower panel, correlation between IL-1β / Arg-1 and other studied markers. Data come from a biological triplicate (n=3).*

From the point of view of oxidative stress metabolism, it can be seen from the linear regression that OOEPs not only increase SOD-1 expression, but do so concomitantly with an increase in GPX and a reduction in catalase within 24

hours of treatment (Fig. 9). At 48 hours after treatment there are no differences between GPX-4 and CAT. The modulation at 24 hours could be explained by the mechanism proposed in Fig.9. Briefly, OOEP could increase the mitochondrial metabolism, leading to an increase of ROS. The increased level of superoxide ion can be effectively depleted by transcription and translation of SOD-1 enzyme and the subsequent increase in H_2O_2 level could be mitigated by the presence of GSH. GPX-4 will be transcribed and translated in order to convert the oxidized form in the reduced form of GSH, maintaining the redox balance.

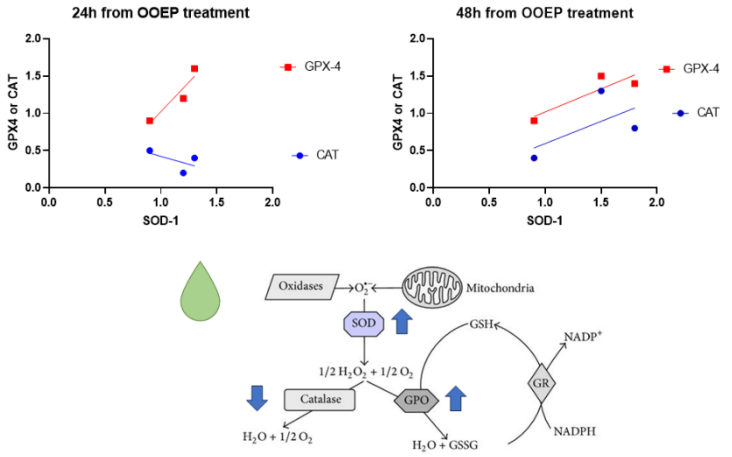


Fig. 9 ***Linear regression comparing gene expresion levels of SOD and GPX4 or CAT at 24 hours and 48 hours after OOEP treatment and a scheme presenting the proposed mechanism.*** *Data come from a biological triplicate (n=3).*

The data obtained so far have shown that olive oil polyphenols can be extracted via NaDES and that this extraction does not impact negatively the amount of recovered polyphenols or their effects on cells. In addition, we have shown how OOEPs are able to modulate inflammation on BV2 both in pre- and post-treatment in the presence of an inflammatory stimulus. Having clarified how polyphenols act at the level of inflammation and oxidative stress, we moved on to study their effects at the systemic level by administering them as OOEPs in a animal model of subchronic inflammation.

SECTION 2: Effects Of Olive Oil Polyphenols On A Mouse Model Of Subchronic Inflammation

Subchronic inflammation is a continuous pro-inflammatory stimulus that leads to different responses in the body from those we are used to in the classical inflammation. Given the antioxidant properties of olive oil polyphenols, we wondered whether their use could lead to improvements in a mouse model of subchronic inflammation.

Polyphenols extracted by the classical method (OOEP) were administered for 8 weeks in drinking water (10 mg/kg/day) to C57BL/6J mice under physiological (sham/OOE) or low-grade inflammation conditions (obtained by intraperitoneal injection of 0.5 mg/kg/week LPS) (LPS/OOE). The respective physiological (sham/water) and pathological (LPS/water) control groups were also evaluated.

The mice were assessed in their cognitive capacity (long-term memory, anxiety), inflammatory and oxidative status in liver and brain and an in-depth study on the microbiota and metabolomics of the liver and intestine was conducted

On a cognitive level

The behavioural response was assessed by means of two tests: novel object recognition and open field. Novel object recognition is expressed in terms of the discrimination index (Fig. 10A), which unfortunately is not statistically significant among the groups. Nevertheless, one must consider the variability of animal behaviour and the fact that in this case the animal's inflammatory state is not specifically exacerbated in order to study a very delicate phenomenon such as subchronic inflammation. For the purpose of understanding this phenomenon we also present spurious data, from which the discrimination index is derived. Indeed, the novel object contact time (Tn) measurements (Fig. 10A, on the left) shows a statistically significant reduction in the LPS-treated mice, which is restored to normal levels following treatment with OOEP. The difference score (Tn-To) and the discrimination index (Tn-To)/(Tn+To) show similar, but not statistically significant trends. It must be considered that these two latter indices depend to a large extent on the time the animal devoted to the Old Object and therefore a reduced interest in this may have influenced the statistical analysis. In line with the logic of the test, we consider the time taken by the animal to recognise the new object to be more important. Moreover we must note that in the discrimination index graph, 3 mice in LPS obtained negative scores, while only 1 mouse in LPS/OOE performed in this way.

With regard to the Open Field evaluation (fig. 10b), no statistical differences were found in velocity and distance, but "time in center" datum borders the significance. This parameter is important for the study of anxious-like behaviour, indeed data show that the water/LPS-treated mice move mainly along the edges of the box (p = 0.0503, borderline significant), presenting an anxious attitude, which is attenuated by the combined treatment with olive oil polyphenols OOE/LPS (p = 0.0876).

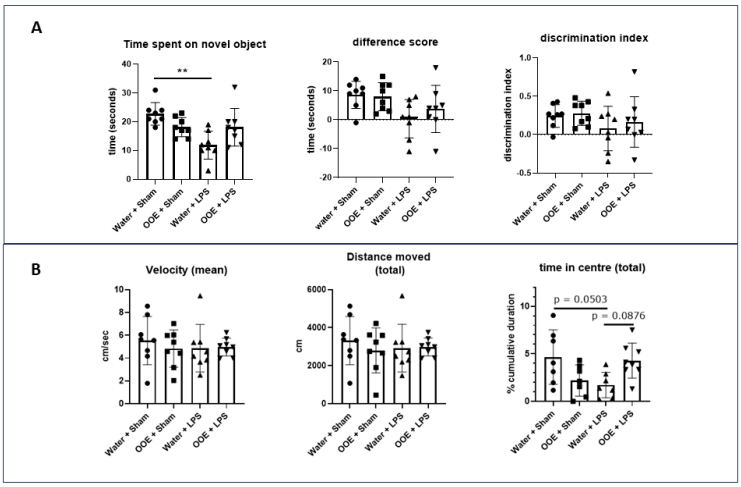


Fig. 10 ***Behavioural data****: (A) Novel Object Recognition test, expressed as time spent on novel object, difference score and discrimination index. (B) Open Field test, expressed as velocity, distance moved and time in centre. N=8 mice per group. Data are expressed as percentage of respective control and are presented as mean ± SD. Significance is expressed as * 0.05, ** 0.01, *** 0.001,**** 0.0001 (1-way ANOVA).*

On the inflammatory level in the liver

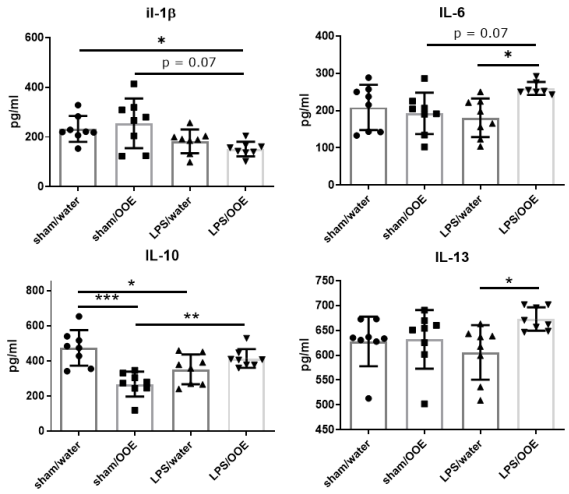


Fig. 11 ***ELISA for pro- and anti-inflammatory cytokines****: pro-inflammatory (IL1β, IL-6) and anti-inflammatory (IL-10 , IL-13) cytokines in liver are evaluated. Data are expressed in concentration (pg/ml) and are presented as mean ± SD (N=8 mice per group). Significance is expressed as * 0.05, **;0.01, ***;0.001,****;0.0001 p.value (1-way ANOVA).*

At the liver level, as expected, the LPS/water treatment did not lead to the classic acute inflammation and therefore the pro-inflammatory parameters IL-1β and IL-4 are not altered, if not decreased. The anti-inflammatory cytokines IL-10 and IL-13 are also consistently decreased compared to the sham/water control.
Moreovere there is a somewhat contradictory behaviour between the OOE-treated populations: while in the sham/OOE population the treatment leads to a decrease in IL-10 and a slight increase in IL-1β, in the OOE/LPS-treated population, there is a significant decrease of IL-1β compared to water/sham and a significant increase of IL-10 compared to the polyphenol-treated population. More interestingly, OOE/LPS treatment is able to increase IL-6 and IL-13.

On the oxidative level of the liver

The oxidative level of the liver was evaluated through 4-HNE marker, indicative of lipid peroxidation. Interestingly, 4-HNE level in the LPS/water group is increased, highlighting a significant difference against the sham/OOE group (which showed a decrease), but not against the sham/water group.

In line with this result, we evaluated the protein expression of PPAR-γ and observed a similar trend of the marker. In this case, the LPS/water group is statistically different compared to the water/sham group.
A lipidomic analysis conducted on the liver showed that of all the lipids studied, only sphingomyelins were modulated in a statistically significant manner by the OOE/LPS group, even though sphingomyelins in the LPS/water treated group did not increase markedly in comparison with sham/water group.

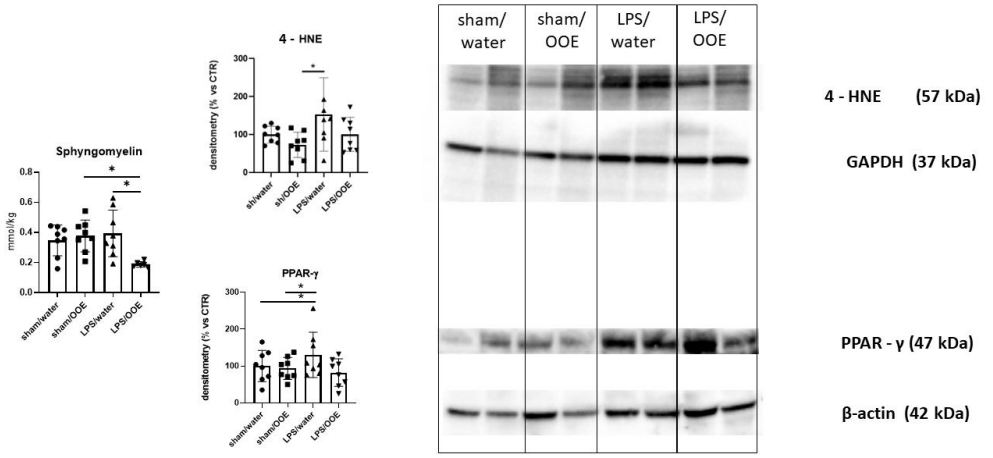


Fig. 12 ***Oxidative stress and lipidic modulation on the liver.*** *From the left, sphingomyelins content in the liver (mmol/kg), 4-HNE and PPAR-γ protein expression expressed as histograms and densitometric images. N=8 mice per group. In the case of 4-HNE and PPAR-γ data are expressed as percentage of respective control and are presented as mean ± SD. Significance is expressed as * 0.05, **;0.01, ***;0.001,****;0.0001 p.value (1-way ANOVA).*

Inflammation and oxidative stress at the brain level

Fig. 13A shows the inflammatory response in the brain. There is a pronounced increase in TNF-α in the sham/OOE and LPS/OOE population, both of which are significant compared to their respective controls. Interestingly, the expression of IL-1β also shows a similar, albeit much milder, trend, with a borderline statistical significance between Sham/OOE and LPS/water groups (p = 0.054) and LPS/OOE and LPS/water group (p= 0.058), but not between

Sham/OOE and Sham/water groups. IL-6, on the other hand, is not altered by any of the treatments.
Regarding the response to oxidative stress (Fig. 13B), no differences in the expression of TRXR were highlighted, but there is a significant increase in HO-1 in the sham/OOE population, which is not statistically different from the respective control (sham/water), but becomes significant in comparison with LPS/water.
Finally, the expression of BDNF, which is a neurotrophic factor, and of SNAP25, which is a factor for synaptogenesis were assessed (Fig. 13C), and a similar trend is evident, showing a statistically significant decrease of the two factors in the comparison between the co-treatment (OOE/LPS) and the control (water/sham). Note that the increase in TNF-α and IL-1β in the sham/OOE population does not seem to have impacted on the expression of these factors.

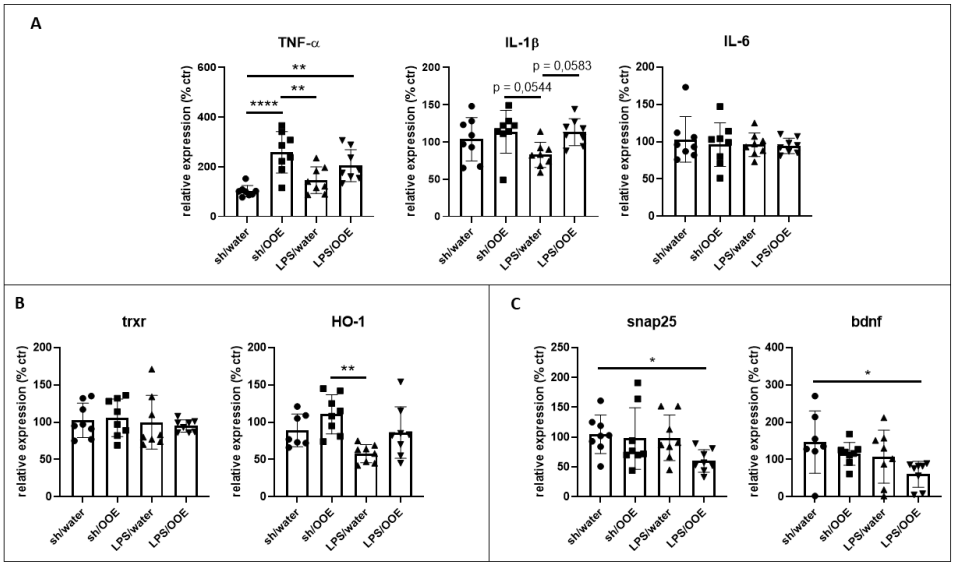


Fig. 13 ***RT-PCR of gene expression****: relative expression of (A) pro-inflammatory markers (TNF-α, IL-1β, IL-6), (B) oxidative stress response (TRXR, HO-1), (C) synaptogenic protein and neuronal growth factor (SNAP25 , BDNF) mRNA expression in cortices of treated mice. N=8 mice per group. Data are expressed as percentage of respective control and are presented as mean ± SD. Significance is expressed as * 0.05, **;0.01, ***;0.001,****;0.0001 p.value (1-way ANOVA).*

At the microbiomic level (in caecum)

The following omic analyses were conducted to check whether polyphenols were able to modify the microbiota and its metabolites. Concurrently, we also

wondered whether treatment with LPS could have influenced these same factors and was relevant to the data obtained on the brain and liver.

At the level of the microbiota, many changes were found between the treated groups. The net result of the proposed PCA analysis (Fig. 14) is that treatment with LPS caused great changes in the microbiota (blue and purple circles), despite the fact that it was administered intraperitoneally, whereas treatment in combination with OOEP (LPS/OOE) was not able to restore the water/sham situation. The subsequent dendrogram also shows how water/LPS-treatment forms a separate branch with the OOE/LPS group, far from the water/sham group.

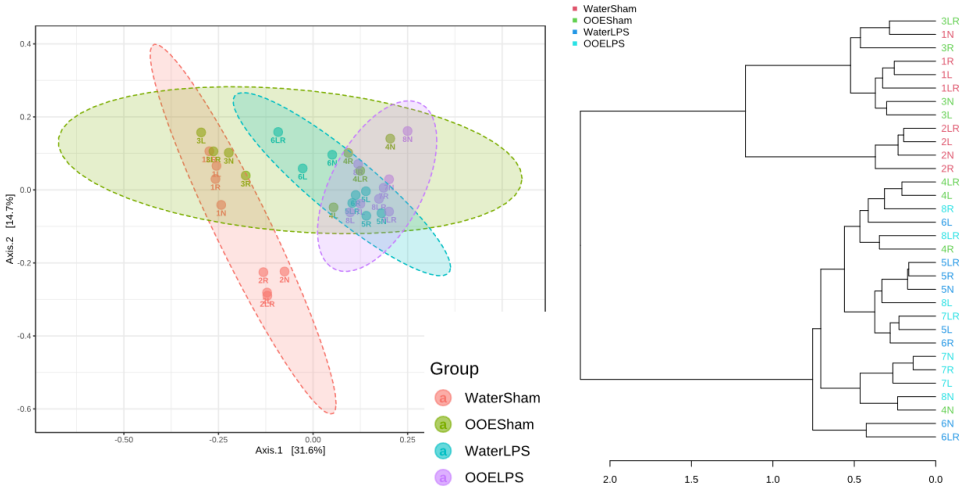


Fig. 14 ***PCA of microbiota in the gut and dendrogram analysis****. PCA is based on Bray–Curtis index and PERMANOVA test (p=0.001).*

The sham/OOE treatment brings to a change of expression of 33 Operative Taxonomy Units (OTUs) in comparison with water/sham group, whereas the

sham/LPS treatment brings to a change of expression of 36 OTUs in comparison with water/sham group (data not shown).
In the case of OOE/LPS group, the impact of OOEP on sham/LPS population was milder (modulation of 9 OTUs) (Fig. 15) most of which belong to the family of the lachnospiraceae.

	Pvalues	FDR	family	shift
OTU_109	7.00E-07	0.000188	Lachnospiraceae	↓
OTU_93	1.03E-05	0.001378	Bacteroidaceae	↓
OTU_182	0.000211	0.01883	? (class: Clostridia)	↓
OTU_222	0.000418	0.028032	Lachnospiraceae	↓
OTU_142	0.000759	0.0342	Lactobacillaceae	↓
OTU_83	0.000766	0.0342	Ruminococcaceae	↓
OTU_106	0.001272	0.04603	Lactobacillaceae	↓
OTU_10	0.001374	0.04603	Lachnospiraceae	↑
OTU_117	0.001666	0.049618	Lachnospiraceae	↑

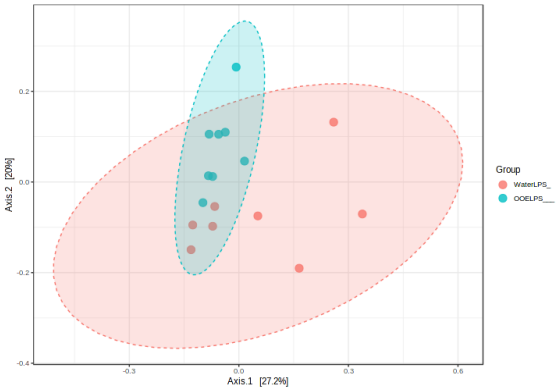
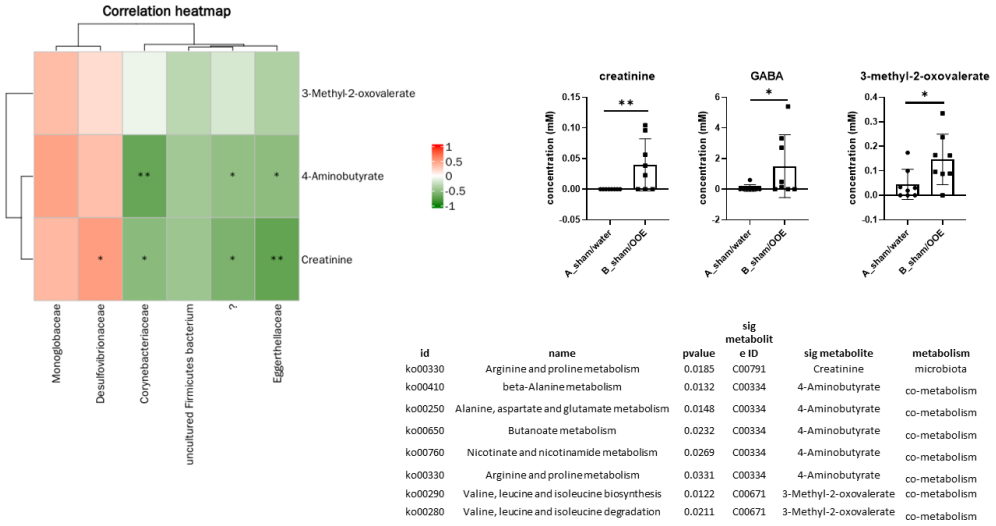


Fig. 15 ***OOEP impact on microbiota in LPS-treated population****:PCA on OOE/LPS (blue circle) in comparison with LPS/water (red circle).*

Metabolites produced in the caecum

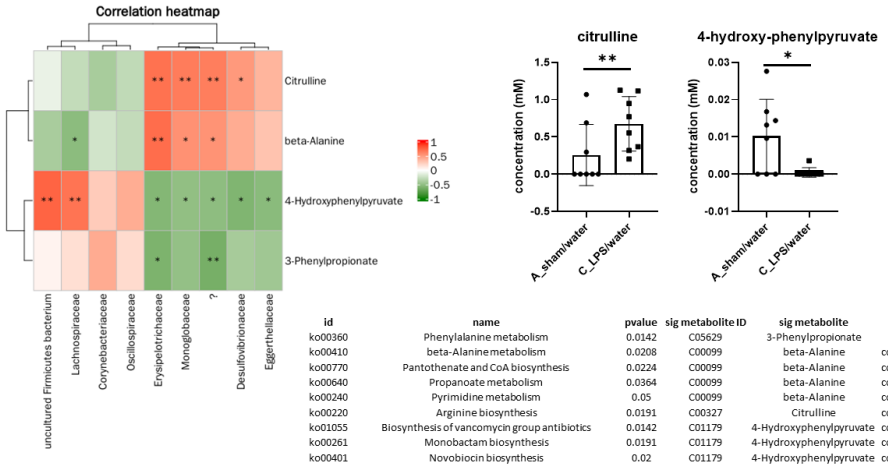
In the caecum of sham/OOE-treated mice was found a statistically significant increase in creatinine, 4-aminobutyric acid GABA and 3-methyl-2-oxovalerate.
A more detailed analysis allowed us to combine the microbiomics data with faecal metabolomics data, defining a negative correlation between GABA and creatinine and the Corynebacterium and Eggertelaceae families (Fig. 16).



id	name	pvalue	sig metabolite ID	sig metabolite	metabolism
ko00330	Arginine and proline metabolism	0.0185	C00791	Creatinine	microbiota
ko00410	beta-Alanine metabolism	0.0132	C00334	4-Aminobutyrate	co-metabolism
ko00250	Alanine, aspartate and glutamate metabolism	0.0148	C00334	4-Aminobutyrate	co-metabolism
ko00650	Butanoate metabolism	0.0232	C00334	4-Aminobutyrate	co-metabolism
ko00760	Nicotinate and nicotinamide metabolism	0.0269	C00334	4-Aminobutyrate	co-metabolism
ko00330	Arginine and proline metabolism	0.0331	C00334	4-Aminobutyrate	co-metabolism
ko00290	Valine, leucine and isoleucine biosynthesis	0.0122	C00671	3-Methyl-2-oxovalerate	co-metabolism
ko00280	Valine, leucine and isoleucine degradation	0.0211	C00671	3-Methyl-2-oxovalerate	co-metabolism

Fig. 16 ***Creatinine, GABA and 3-methyl-2-oxovalerate level in the gut of sham/water and sham/OOE groups.*** *From the left, correlation heatmap between metabolites and bacterial families. Red square indicates positive, whereas green negative correlation. Significance is expressed as * 0.05, ** 0.01, *** 0.001,**** 0.0001 (Mann Whitney U test). On the top, histograms of concentration of creatinine, GABA and 3-methyl-2-oxovalerate. Data are expressed as concentration and are presented as mean ± SD. Significance is expressed as * 0.05, ** 0.01, *** 0.001,**** 0.0001 (Student's T-test). On the bottom, Pathway analysis, p-value is expressed for each pathway (Mann Whitney U test).*

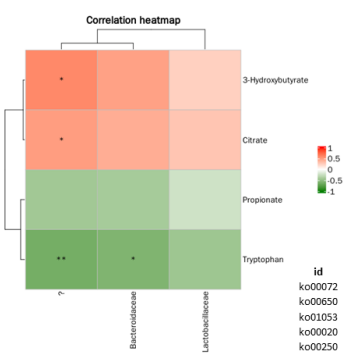
In contrast, LPS/water treatment, despite the change in bacterial populations, resulted in a statistically significant modulation in the concentration of citrulline and 4-hydroxyphenylpyruvate. This double modulation was linked to a decrease in certain bacterial families, shown in Fig. 17.

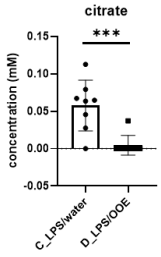


id	name	pvalue	sig metabolite ID	sig metabolite	metabolism
ko00360	Phenylalanine metabolism	0.0142	C05629	3-Phenylpropionate	microbiota
ko00410	beta-Alanine metabolism	0.0208	C00099	beta-Alanine	co-metabolism
ko00770	Pantothenate and CoA biosynthesis	0.0224	C00099	beta-Alanine	co-metabolism
ko00640	Propanoate metabolism	0.0364	C00099	beta-Alanine	co-metabolism
ko00240	Pyrimidine metabolism	0.05	C00099	beta-Alanine	co-metabolism
ko00220	Arginine biosynthesis	0.0191	C00327	Citrulline	co-metabolism
ko01055	Biosynthesis of vancomycin group antibiotics	0.0142	C01179	4-Hydroxyphenylpyruvate	co-metabolism
ko00261	Monobactam biosynthesis	0.0191	C01179	4-Hydroxyphenylpyruvate	co-metabolism
ko00401	Novobiocin biosynthesis	0.02	C01179	4-Hydroxyphenylpyruvate	co-metabolism
ko00400	Phenylalanine, tyrosine and tryptophan biosynthesis	0.0274	C01179	4-Hydroxyphenylpyruvate	co-metabolism
ko00350	Tyrosine metabolism	0.0462	C01179	4-Hydroxyphenylpyruvate	co-metabolism
ko00130	Ubiquinone and other terpenoid-quinone biosynthesis	0.0486	C01179	4-Hydroxyphenylpyruvate	co-metabolism

Fig. 17 ***Citrulline and 4-hydroxy-phenylpyruvate level in the gut of sham/water and LPS/water groups.*** *From the left, correlation heatmap between metabolites and bacterial families. Red square indicates positive, whereas green negative correlation. Significance is expressed as * 0.05, ** 0.01, *** 0.001,**** 0.0001 (Mann Whitney U test). On the top, histograms of concentration of Citrulline and 4-hydroxy-phenylpyruvate. Data are expressed as concentration and are presented as mean ± SD. Significance is expressed as * 0.05, ** 0.01, *** 0.001,**** 0.0001 (Student's T-test). On the bottom, Pathway analysis, p-value is expressed for each pathway (Mann Whitney U test).*

Finally, we evaluated the effect of OOEPs on the LPS-treated population (fig. 18) and found a statistically significant decrease in citrate, which was linked with undefined family (according to SILVA 13.8 taxonomy).



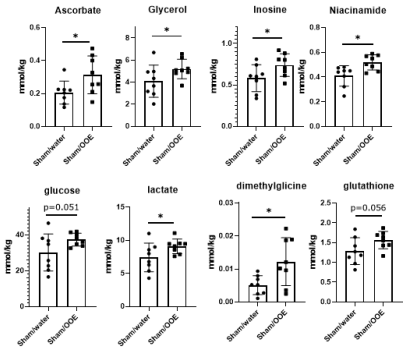


id	name	pvalue	sig metabolite ID	sig metabolite	metabolism
ko00072	Synthesis and degradation of ketone bodies	6.69E-03	C01089	3-Hydroxybutyrate	co-metabolism
ko00650	Butanoate metabolism	4.29E-02	C01089	3-Hydroxybutyrate	co-metabolism
ko01053	Biosynthesis of siderophore group nonribosomal peptides	1.89E-02	C00158	Citrate	co-metabolism
ko00020	Citrate cycle (TCA cycle)	2.22E-02	C00158	Citrate	co-metabolism
ko00250	Alanine, aspartate and glutamate metabolism	3.09E-02	C00158	Citrate	co-metabolism
ko00720	Carbon fixation pathways in prokaryotes	4.83E-02	C00158	Citrate	co-metabolism
ko00630	Glyoxylate and dicarboxylate metabolism	0.059	C00158	Citrate	co-metabolism
ko00642	Ethylbenzene degradation	1.55E-02	C00163	Propionate	co-metabolism
ko00640	Propanoate metabolism	4.83E-02	C00163	Propionate	co-metabolism
ko00760	Nicotinate and nicotinamide metabolism	0.055	C00163	Propionate	co-metabolism
ko00901	Indole alkaloid biosynthesis	1.33E-02	C00078	Tryptophan	co-metabolism
ko00400	Phenylalanine, tyrosine and tryptophan biosynthesis	3.64E-02	C00078	Tryptophan	co-metabolism
ko00404	Staurosporine biosynthesis	4.50E-02	C00078	Tryptophan	co-metabolism
ko00260	Glycine, serine and threonine metabolism	0.051	C00078	Tryptophan	co-metabolism
ko00998	Biosynthesis of various secondary metabolites - part 2	0.051	C00078	Tryptophan	co-metabolism
ko00380	Tryptophan metabolism	0.055	C00078	Tryptophan	co-metabolism
ko00970	Aminoacyl-tRNA biosynthesis	0.057	C00078	Tryptophan	co-metabolism

Fig. 18 ***Citrate level in the gut of LPS/water and LPS/OOE groups.*** *From the left, correlation heatmap between metabolites and bacterial families. Red square indicates positive, whereas green negative correlation. Significance is expressed as * 0.05, ** 0.01, *** 0.001,**** 0.0001 (Mann Whitney U test). On the top, histograms of concentration of citrate. Data are expressed as concentration and are presented as mean ± SD. Significance is expressed as * 0.05, ** 0.01, *** 0.001,**** 0.0001 (Student's T-test). On the bottom, Pathway analysis, p-value is expressed for each pathway (Mann Whitney U test).*

Metabolites produced in the liver

At the liver level, sham/OOE treatment in comparison with a control population led to an increase in certain metabolites, as shown in Fig. 19. An advanced pathway analysis showed, however, that these changes, although significant, could not be placed in a specific pathway. For the sake of accuracy, we report the only pathway (ascorbate and aldarate metabolism) in the vicinity of statistical significance ($p = 0.07$; $\log(p) = 1.15$), but which nevertheless has no significant impact on the pathway.



id	name	pvalue	sig metabolite ID	sig metabolite
ko00010	Glycolysis / Gluconeogenesis	1.77E-03	C00221; C00186	Glucose; Lactate
ko00480	Glutathione metabolism	1.89E-03	C00072; C00051	Ascorbate; Glutathione
ko00643	Styrene degradation	4.57E-02	C00186	Lactate
ko00620	Pyruvate metabolism	5.80E-02	C00186	Lactate
ko00561	Glycerolipid metabolism	6.20E-02	C00116	Glycerol
ko00030	Pentose phosphate pathway	7.40E-02	C00221	Glucose
ko00640	Propanoate metabolism	7.60E-02	C00186	Lactate
ko00052	Galactose metabolism	9.40E-02	C00116	Glycerol
ko00260	Glycine, serine and threonine metabolism	9.60E-02	C01026	Dimethylglycine
ko00053	Ascorbate and aldarate metabolism	1.03E-01	C00072	Ascorbate
ko00760	Nicotinate and nicotinamide metabolism	1.03E-01	C00153	Niacinamide
ko00051	Fructose and mannose metabolism	1.07E-01	C00186	Lactate
ko00040	Pentose and glucuronate interconversions	1.13E-01	C00116	Glycerol
ko00230	Purine metabolism	1.69E-01	C00130	Inosinemonophosphate

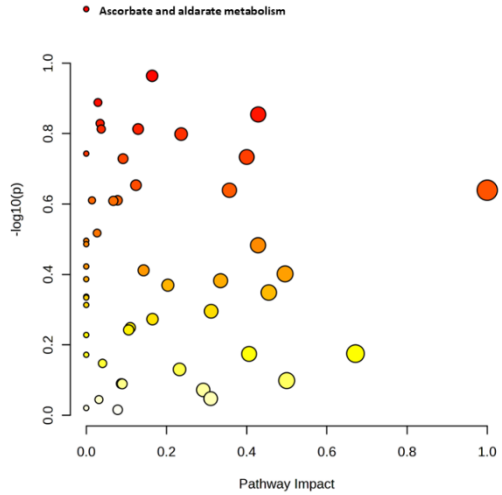
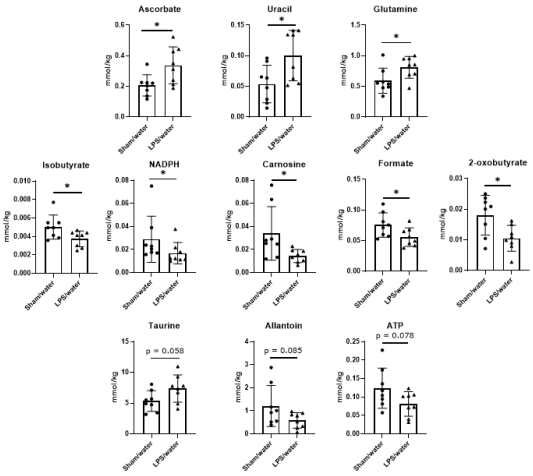


Fig. 19 ***OOEP impact on hepatic metabolites, analysis between sham/OOE and sham/water groups.*** *From the left, histograms of concentration of metabolites. Data are expressed as concentration and are presented as mean ± SD. Significance is expressed as * 0.05, ** 0.01, *** 0.001,**** 0.0001 (Student's T-test) in the histograms and as raw p value in the table (SAM analysis). On the bottom, pathway analysis.*

The case is different for LPS treatment, which caused a very strong change in several metabolites in the treated group.
The increase in uracil and reduction in carnosine led to a significant impact on beta-alanine metabolism, as shown in the graph.

metabolyte	statistical test	P value	P value summary	shift
2-oxobutyrate	Unpaired t test	0,0154	*	down
Ascorbate	Unpaired t test	0,0182	*	up
Uracil	Unpaired t test	0,0225	*	up
carnosine	Unpaired t test	0,0366	*	down
Isobutyrate	Unpaired t test	0,037	*	down
glutamine	Unpaired t test	0,0394	*	up
formate	Unpaired t test	0,0425	*	down
NADPH	Mann Whitney test	0,0468	*	down
taurine	Unpaired t test	0,0579	ns	up
ATP	Unpaired t test	0,0781	ns	down
Allantoin	Unpaired t test	0,0850	ns	down



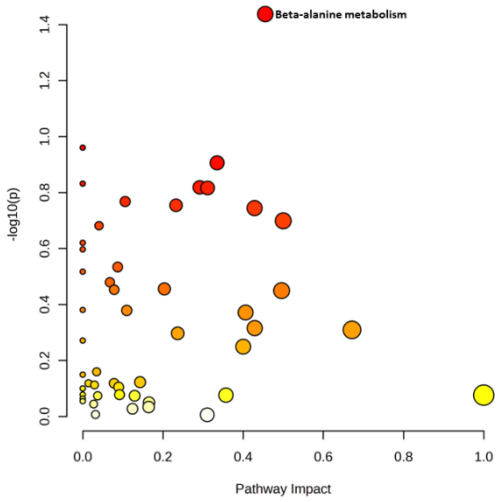
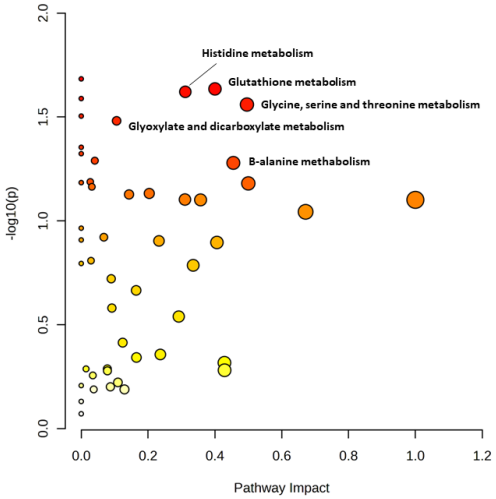


Fig. 20 ***LPS impact on hepatic metabolites, analysis between LPS/water and sham/water groups.*** *From the left, summary table and histograms of concentration of metabolites. Data are expressed as concentration and are presented as mean ± SD. Significance is expressed as * 0.05, ** 0.01, *** 0.001,**** 0.0001 (Student's T-test) in the histograms and as raw p value in the table (SAM analysis). On the bottom, pathway analysis.*

Finally, several changes were observed in the co-treated mice compared to LPS treatment. The graph below attempts to summarise the five metabolic pathways affected by the treatment.



Pathway Name	p	-log(p)	Holm p	FDR	Impact
Glutathione metabolism	0.023172	1.635	1	0.18869	0.40022
Histidine metabolism	0.02393	1.6211	1	0.18869	0.31147
Glycine, serine and threonine metabolism	0.027551	1.5599	1	0.18869	0.4959
Glyoxylate and dicarboxylate metabolism	0.033007	1.4814	1	0.18869	0.10582
beta-Alanine metabolism	0.052613	1.2789	1	0.18869	0.45522

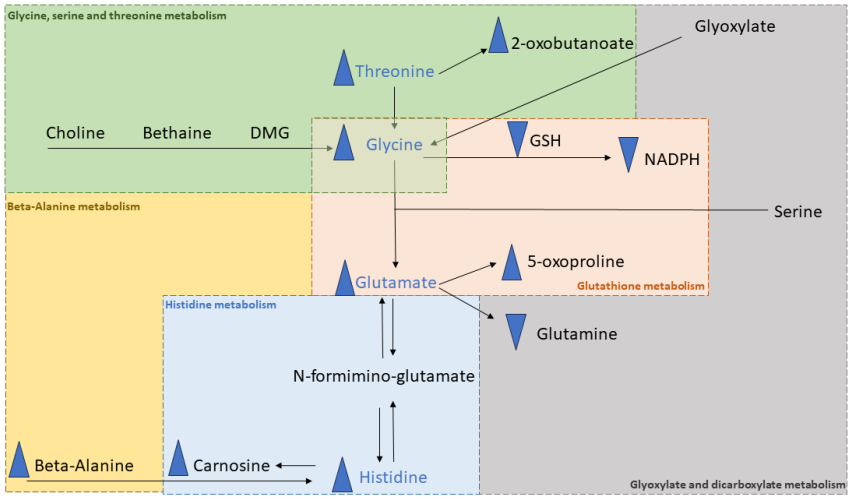


Fig. 21 ***OOEP impact on hepatic metabolites, analysis between OOE/LPS and water/LPS groups.*** *Upper panel, Pathway analysis and table with raw p value (SAM analysis). On the bottom, proposed mechanism of the pathways that are divergent between LPS/OOE and LPS/water groups. In the graph, blue triangles (up and down) indicate the impact of OOEP on water/LPS population.*

SECTION 3: Effects Of Olive Oil Polyphenols On A Normal And Down Syndrome Mouse Model

Down Syndrome is a condition characterised by unresolved state of inflammation. This state is due both to a gene imbalance of certain proteins essential to the maintenance of oxidative balance and to feedback loops due to disease-related problems such as insulin resistance. In this third and final section of the thesis, we study the effects of olive oil polyphenols extracted and administered as NaDES (5mg/kg/day) in drinking water to euploid and trisomic mice. At the end of the experiment, cognitive, inflammatory and oxidative responses of the animal were evaluated. Finally, the alteration of certain lipogenesis-related parameters such as PGC-1alpha and PPAR-γ stimulated our research to perform a broad-spectrum lipidomic analysis on an experimental subset of 4 mice per group.

On a cognitive level

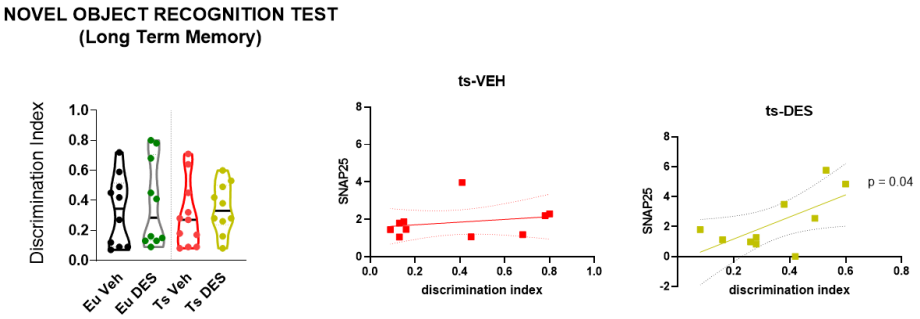


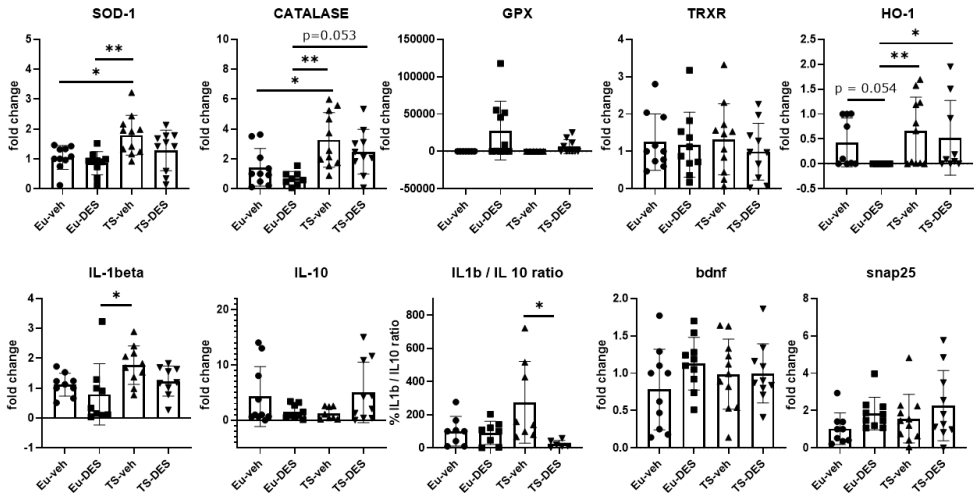
Fig. 22 ***Behavioural data****: on the left, Novel Object Recognition test, expressed as discrimination index in violin plot. Solid line represent median of the data. N=10 mice per group. On the right, Linear regression of SNAP25 vs discrimination index in TS-VEH and TS-DES groups.*

The effect of the treatment on behaviour was evaluated by means of the NOR. At 6 months of age the TS mice begin to show the first symptoms of cognitive retardation and a slight decrease in the cognitive capacity of the mice is evident from the violin plot, whereas the polyphenol-treated groups (EU-DES and TS-DES) show a slight increase. A correlation was made between the results of the discrimination index and the expression of Snap25 mRNA, and while no relationship is apparent in the Ts-Veh mice, a good positive correlation between the two parameters is evident in the Ts-DES group (p=0.04).

At the brain and liver level

Trisomic mice show higher baseline levels of SOD and CAT in the brain (Fig. 23). Treatment with polyphenols lowered the expression of these these genes, although not in a statistically significant manner. GPX and TRXR, two other proteins involved in the response to oxidative stress, are not impacted by the pathology; of note, treatment with olive oil polyphenols significantly impacted GPX expression in the euploid population.

On the inflammatory side, there was an increase in IL-1β expression in the trisomic population and a concomitant decrease in IL-10 expression compared to healthy animals. Conversely, treatment with polyphenols reduced the expression of IL-1β and increased IL-10. With regard to the expression of proteins related to neuronal plasticity, an increase could be seen in animals treated with olive polyphenols (Eu-DES and Ts-DES), but this was not statistically significant. Most interestingly, the correlation between SNAP-25 and the NOR shows that in the treated Ts-DES population it regained a linear relationship following treatment (Fig. 22).



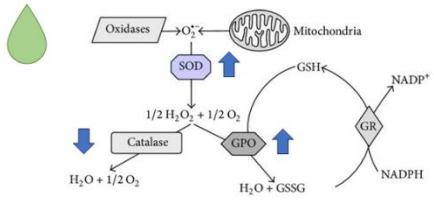


Fig. 23 ***RT-PCR on cortices****: fold change of oxidative stress response (SOD-1, CAT, GPX, TRXR, HO-1), inflammatory markers (IL1β, IL10, IL1B/IL10 ratio), synaptogenic protein and neuroal growth factor (SNAP25, BDNF) mRNA expression in cortices of treated mice. N=10 mice per group. Data are expressed as percentage of respective control and are presented as mean ± SD. Significance is expressed as * 0.05, **;0.01, ***;0.001,****;0.0001 p.value (1-way ANOVA). On the bottom, the proposed mechanism illustrated in BV2 cellular model.*

Treatment with OOE-DES appears to have slightly impacted BDNF and SNAP25, but not in a statistically significant manner.
Surprisingly, polyphenol treatment led to a reduction in HO-1 in euploid mice but not in trisomic mice. We validated this finding by conducting a Western blot (Fig. 24) on the phosphorylated protein Nrf2 and it appears that the untreated trisomic mice show higher values of phosphorylated Nrf-2, thus explaining the normal HO-1 levels in the cortex.
Also, the lipid transcription factor PPAR-γ and PGC-1α were evaluated. At the same time, the same analyses were conducted on the liver. Treatment with polyphenols appears to increase the percentage of phosphorylated Nrf2 protein in the trisomic population.

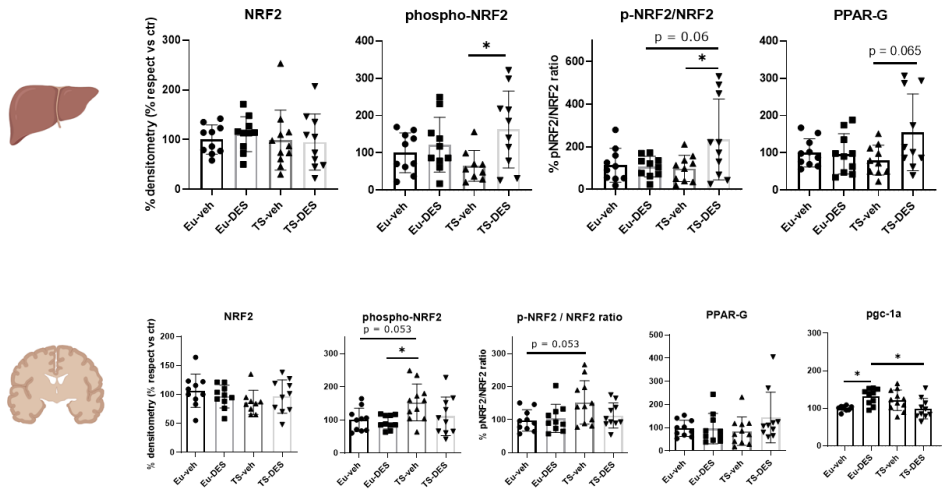
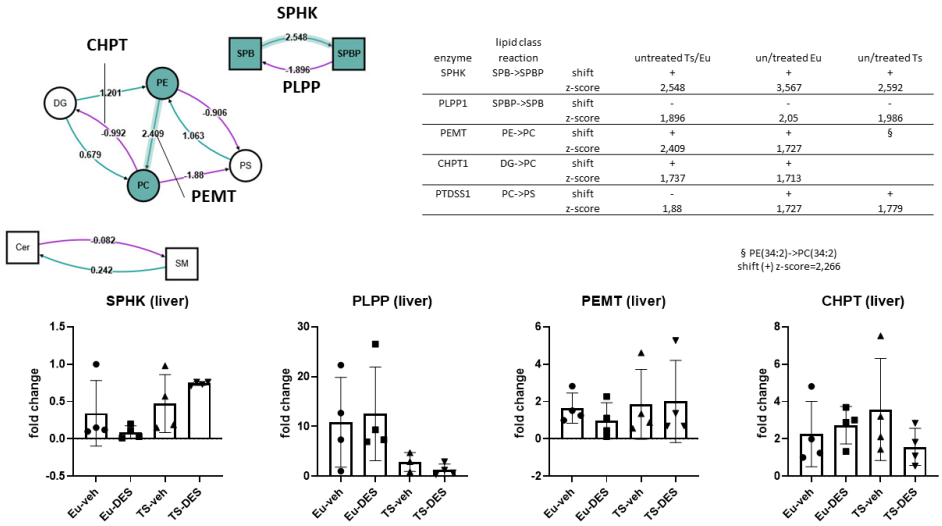


Fig. 24 ***Western blot analysis****: protein expression in livers and cortices of treated mice. Nrf2, phosphorilated Nfr2, p-Nrf2/Nrf2 ratio, PGC1α and PPAR-γ protein expression are showed as histograms. N=10 mice per group. Data are expressed as percentage of respective control and are presented as mean ± SD. Significance is expressed as * 0.05, **;0.01, ***;0.001,****;0.0001 p.value (1-way ANOVA).*

At the lipidomic level

From a lipidomic point of view, the euploid and trisomic populations are divergent for certain lipid parameters, and treatment with polyphenols is also capable of modifying the profile of certain lipids in the brain, liver and caecum. The data presented are preliminary (n=4 mice per group) and were therefore used in an exploratory manner in order to identify the molecular pathways modified by polyphenol treatment and the trisomy 16.



enzyme	lipid class reaction		untreated Ts/Eu	un/treated Eu	un/treated Ts
SPHK	SPB->SPBP	shift	+	+	+
		z-score	2,548	3,567	2,592
PLPP1	SPBP->SPB	shift	-	-	-
		z-score	1,896	2,05	1,986
PEMT	PE->PC	shift	+	+	§
		z-score	2,409	1,727	
CHPT1	DG->PC	shift	+	+	
		z-score	1,737	1,713	
PTDSS1	PC->PS	shift	-	+	+
		z-score	1,88	1,727	1,779

Fig. 25 ***Lipidomic results on liver****: on the left, pathway analysis with highlighted lipid families impacted by the trisomy (TS-Veh vs EU-Veh). On the right, predicted enzymes involved in lipid changes between different populations (TS-Veh vs EU-Veh, EU-Des vs EU-Veh, TS-Des vs TS-Veh) in the table. On the bottom, mRNA expression fold change for SPHK, PLPP, PEMT, CHPT enzymes in the liver (n=4 mice per group).*

The BioPan module of the Lipidmaps server hypothesised that the detected lipid changes could be traced back to a modulation of specific key enzymes in lipogenesis (Fig. 25). We attempted to test most of the hypotheses on the liver of mice.

The conducted lipidomic analyses showed that the trisomic population is different from the euploid population in terms of lipids. Specifically, we found an increase in the production of sphingosine-1-phosphate in the trisomic population. This was found to be due to reduced expression of the phospholipid phosphatase PLPP enzyme, which remains low even after treatment with OOE-DES. Very interestingly, lipdmaps had proposed an increase in Sphingosine kinase SPHK enzyme activity with a consecutive reduction in PLPP activity; however, RT-PCR data show that there is no real modulation of transcription following treatment. Phosphatidylethanolamine N-methyltransferase PEMT and Choline Phosphotransferase CHPT are also more expressed in the trisomic population than in the euploid population, although the experimental number taken into consideration must be taken into account. We believe that increasing the experimental number will give new meaning to the preliminary data.

From the point of view of lipidic family changes, at the level of the brain the polyphenols reduced the expression of triglycerides only in the euploid population; in the trisomic population they were already low (Fig. 26).
Moving to the liver level, polyphenols impacted on both populations by upmodulating lysophosphatidylcholines and phosphatidylcholines.
At the level of the intestinal mucosa, there was an increase in lactosyl-ceramides in the trisomic mice, which were promptly lowered by treatment with polyphenols.

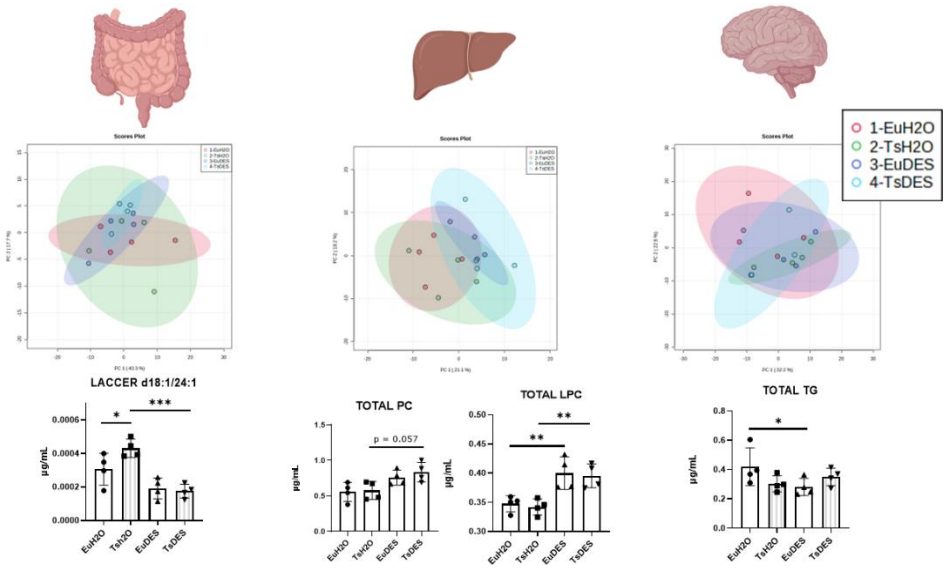


Fig. 26 ***Representative lipidomic results****: Main impacted lipid families in caecum, liver and brain (PCA and hystogram representation). Data are expressed as concentration (µg/ml) and are presented as mean ± SD. Significance is expressed as * 0.05, **;0.01, ***;0.001,****;0.0001 p.value (1-way ANOVA).*

Discussion

Section 1: In vitro experiments in cell cultures

Activity of olive oil polyphenols

An uncontrolled inflammatory process can lead to neuroinflammation that triggers neurodegeneration (Barbé-Tuana et al., 2020). This process is finely regulated by microglia (Skaper et al., 2018). In our experiments we demonstrated how treatment with OOEPs leads to a significant reduction of the acute inflammatory response (reduction of IL-1β, iNOS and IBA1) in a inflammatory-stimulated microglial population. In addition, pre-treatment with OOEP led to the maintenance of IBA-1 expression at control levels. This means: 1) that OOEP is not seen by the highly receptive microglia as potentially toxic molecules and 2) that OOEP-pre-treated microglia do not manifest an overt M-1 phenotype, characterised by a markerd increase of IL-1β, iNOS e IBA-1.

The downmodulation of IBA-1 by olive oil polyphenols has been previously reported by (D'Andrea et al., 2020; Taticchi et al., 2019).

Another fact to be discussed is the different behaviour between pre- and post-treatment with polyphenols. Indeed, the experiments in the cell model were aimed at assessing whether or not the inflammatory and oxidative response could be modulated by pre-treatment or post-treatment with OOEPs and whether there was a difference between these two treatments.

It is clear that treatment with LPS has a dramatic impact on cell metabolism as it leads to a very strong pro-inflammatory response (M1 isotype switch, testified by the upmodulation of IBA-1, iNOS and IL-1β), significantly differing from the control population as shown in the PCA analysis.

The data show that in the case of pre-treatment there is a marked decrease in IL-1β compared to LPS treatment alone, but this is not accompanied by the increase in IL-10 (ratio IL-1β/IL-10: 1.00) that occurs in post-treatment (ratio IL-1β/IL-10: 0.5). Although it may seem contradictory, one must consider that the IL-1β expression in LPS-treated population (average fold change = 39±6) was halved by the pre-treatment with OOEP (average fold change = 20±4). A low inflammatory response doesn't activate such strong negative regulators of the inflammation as normal inflammation does (Maitra et al., 2012; Morris et al., 2015) and, indeed, the IL-10 expression is reduced in the pre-treatment with OOEP.

Instead IL-10 is approximately 2-fold increased in post-treatment in comparison with LPS/CTR, which may indicate that the IL-10 produced as a result of LPS was enhanced by IL-10 transcription stimulated by OOEP.

Further studies will be done by varying the timepoints, in any case this experiment shows that OOEP attenuates the inflammatory response of microglia regardless of when they are administered.

		pre-treatment	post-treatment
	LPS/CTR	OOE/LPS	LPS/OOE
IL-1B	↑	↓	↓
IL-10	↑	↓	↑↑
IL1B/IL10	0.65	1.00	0.50
IBA-1	↑	↓	=
iNOS	↑	↓	= / ↓
PPAR-G	↓	↑	↓
ARG-1	↓	↓	↓
HO-1	↓	=	=
SOD	=	↑	=
CAT	↓	↓	↓
GPX	=	↑	=

Fig. 27 ***Summary of markers studied on LPS-stimulated BV2 cell line in pre-/post- treatment with OOEP****. The increase/decrease of markers in LPS/CTR column are related to the CTR/CTR cells (data not shown), whereas the other two columns are related to the LPS/CTR column.*

Concomitantly with the increase of IL-1β/IL-10 ratio in pre-treatment, we can observe an increase in PPAR-γ and a decrease in iNOS expression with respect to the LPS-treatment. In the case of post-treatment, PPAR-γ is lower than LPS-treated and iNOS is quite similar to the LPS-treated.
One possible explanation for the observed phenomenon is that the pre-treatment with polyphenols activated the microglia in an M2 (anti-inflammatory) direction, as shown by the increase in Arg-1 at 24 and 48 h when the cells were treated with polyphenols alone (Fig. 7). Subsequently, treatment with LPS activated the NF-kB pathway (up-modulation of IBA-1, IL-1β), but to a lesser extent than with LPS alone, and this would explain the concomitant increase in PPAR-γ (negative regulator of NF-kB) and the decrease in iNOS (switch M1 indicator).
On the other hand, post-treatment with polyphenols reduced IL-1β expression, but also increased IL-10 levels (better than treatment with LPS alone), but this does not affect IBA-1 (M1 marker) and there is a concomitant decrease in PPAR-γ and iNOS. This could mean that while in the pre-treatment the microglial cell is in an anti-inflammatory state (thanks to OOEP) and after stimulation switches to a weaker pro-inflammatory phenotype than in the LPS-treated by positively impacting oxidative stress (increase in GPX and SOD), the OOEP post-treatment only impacts the inflammatory pathway by reducing its effects, but too late to affect the expression of anti-oxidant genes. Nevertheless, the polyphenols given in the post-treatment could neutralise the ROS present in the medium through their intrinsic scavenging function.

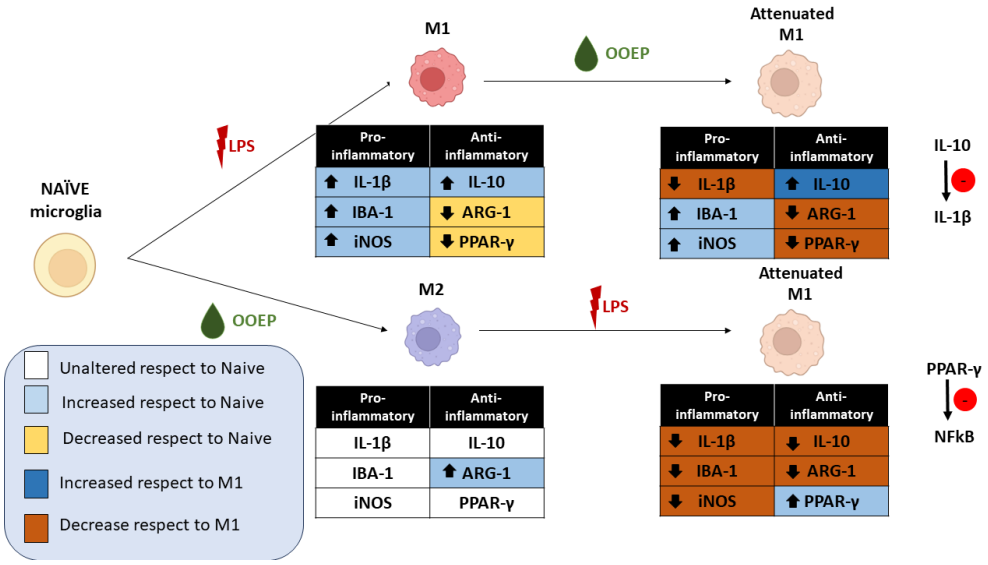


Fig. 28 ***Proposed mechanism to explain BV2 cell behaviour after pre-stimulation with LPS or with OOEP****. Light blue square indicates increase of the marker (respect to the control), white indicates no change respect to the control, yellow indicates decrease respect to the control, blue indicates higher increase (respect to LPS treatment, M1), red indicates decrease (respect to LPS treatment, M1).*

These data are in line with the results obtained by (Leri et al., 2023), that shows that pretreatment with hydroxytyrosol or oleuropein aglycon on BV2 is able to attenuate proinflammatory factors before LPS treatment and allows an M2 polarization. Moreover, (Y.-B. Yu et al., 2020) had shown that treatment with hydroxytyrosol is able to mitigate the inflammatory response by switching from M1 to M2. Interestingly, similar results have been reported with other non-olive oil polyphenols such as resveratrol (Yang et al., 2017), salvianolic acid (D.-C. Ma et al., 2021), curcumin liposomes (Nasra et al., 2023) and polyphenols contained in pomegranate juice (Aharoni et al., 2015). It would appear that pre-treatment or direct treatment with polyphenols are ameliorative in oxidative/inflammatory models (Maiuolo et al., 2021).
Other evidence to be commented is the PCA analysis conducted on these data which shows in a blind manner (i.e. the server did not know metabolites and possible biological links) how activation of the NF-kB pathway (IL-1β, IL-10, iNOS) and phenotypic switch M1 is opposed by PPAR-γ as a negative regulator of NF-kB and ARG-1 as a modulator of the M2 phenotypic switch. In a very intriguing way, this PCA graph is recalling the PCA analysis of the microbiota in the subchronic inflammatory model (fig. 14). There too, the LPS-treated populations (sham/LPS and OOE/LPS) formed a unique cluster

compared to their respective control populations, and polyphenol treatment had little impact.
Finally, data on the enzymes regulating oxidative stress (CAT, SOD, GPX) indicate an orchestrated response with HO-1 for the purpose of cell survival (Tossetta & Marzioni, 2022), which has been reported previously in the literature (Karković Marković et al., 2019). Our hypothesis is that OOEP could increase the mitochondrial metabolism, leading to an increase of ROS. The increased level of superoxide ion can be effectively depleted by transcription and translation of SOD-1 enzyme and the subsequent increase in H_2O_2 level could be mitigated by the presence of GSH. GPX-4 will be transcribed and translated in order to convert the oxidized form in the reduced form of GSH, maintaining the redox balance (Fig. 9). More experiments will be conducted in this sense.

Use of NaDES in drug delivery

NaDES represent a new tool for carrying drug molecules and seem to have a promising future in the biomedical field. Moreover, NaDES, being biocompatible and able to increase solubility of low-soluble compounds, attract enormous scientific interest in drug delivery (Huang et al., 2021; Y. Liu et al., 2022).
In recent years, our laboratory has implemented polyphenol extraction systems (Francioso et al., 2020). This green extraction system ensures that polyphenols are extracted from the oily matrix and concentrated while maintaining their structure and biological activity unaltered, eliminating the use of potentially harmful organic solvents (Montedoro et al., 1992), thus reducing the environmental impact and risk for the operator.
Data show that oleacein and oleocantal, which represent about 70% of polyphenol content of Coratina oil used in our experiments, remain stable for up to 6 months at 25 and 40 °C when extracted in NaDES and, in any case, their degration produces hydroxytyrosol and tyrosol that have their own biological activities, comparable to those of the other polyphenols in olive oil (Karković Marković et al., 2019).
From a toxicity point of view, NaDES showed no obvious toxicity on cell culture, except for concentrations =/> 250 mM betaine (and 550 mM glycerol) in the case of BV2 cells, corresponding to a concentration 25 times higher than that used in our cell experiments. These data indicate that in cellular experiments high amount of polyphenols could be administered in a small volume of NaDES without causing any harmful effects to the cells. As regards the use of NaDES in *in vivo* experiments on mice models, it must be considered that the oral administration of NaDES may further reduce the potential toxic

effect of the high concentation, due to endogenous metabolism of glycerol and betain.
Of note, (Benlebna et al., 2018) reported toxicity related to a betaine-glycerol (1:2) NaDES when administered to rats. It must be pointed out that the way NaDES were administered in the DS mouse model in our experiment was completely different. In fact, whereas in our work we administered approximately 7.5 µL of DES in 10 ml of water per day to each mouse, which drank freely, in (Benlebna et al., 2018) the rats were given a total of 2 ml of DES (6 ml of DES, diluted 1:3) per day via gavage. Their work also lacks the presence of a true control group (the control group used was coffee polyphenols in 70% hydro-alcoholic solution administered via gavage) and we suggest that the observed toxicity could be due to the osmotic pressure generated in the animals' stomach following gavage of a hyperosmotic NaDES solution, which in fact led to stomach swelling, belly enlargement and death of two animals. In fact, EFSA had already reported that high amounts of glycerol are able to cause hygroscopic and osmotic effects, and set a limit of 2,800 mg/kg/day in rats (Mortensen et al., 2017).
NaDES are highly adaptable/tunable in composition and component ratios, which means that we are dealing with a tool that can be improved and could guarantee an even better extraction of polyphenols. RT-PCR data also showed that NaDES, as a vehicle in the experiments comparing OOEP and OOE-DES, did not alter the polyphenol response in either microglial or neuronal cells.
From the point of view of a possible medical/nutraceutical application, food-grade glycerol (also known as E422) does not yet have a maximum limit according to the FDA and EFSA, whereas for betaine the maximum permitted daily dose is 6 grams according to the FDA and 6 mg/kg/day per body weight according to EFSA, so the OOE-DES that we have formulated for the present study could have a similar application in human. In addition, betaine has been shown to have positive effects both at the cellular osmotic level, as a methyl group donor, and at the brain level (Bhatt et al., 2023).

Section 2: experiments on a mouse model of subchronic inflammation

Polyphenols partially restore LPS-induced cognitive impairment

Cognitive level of mice was assessed by means of two different parameters: on the one hand the NOR aimed at highlighting long-term memory, on the other hand the OF was used to assess various aspects such as the animal's desire to explore, anxiety and possible locomotor problems.

With regard to the NOR, it is known from the literature that when LPS is administered both centrally and peripherally, it leads to cognitive decline (Nazem et al., 2015). As explained in the results, data are not significant, but the trend is clear: mice treated with a low dose of LPS, albeit lower than that recognised as toxic (Tateda et al., 1996), show a much reduced performance, which is only partly reverted by treatment with olive oil polyphenols. However, previous work by our collaborators showed administration of LPS led to a significant reduction in NOR in mice under similar experimental conditions (12 weeks of treatment) (Hoyles et al., 2021). It is not clear why in this work we did not observed a significant effect on cognition, however, it could be somehow linked to higher variability of the animals performance.
As far as the OF is concerned, we cannot show any consequences in the desire to explore or in locomotor capacities, but there is a clear tendency for LPS-treated animals to preferentially stand at the edge of the field, thus demonstrating an anxious state, as already demonstrated in the literature (Dantzer et al., 2008). This behaviour can be restored by treatment with olive oil polyphenols. This result is in line with that already seen in rats administered with other polyphenols, such as saffranin (Pontifex et al., 2022), curcumin (Zhang et al., 2022) and in various clinical trials with various polyphenols (Pizarro Meléndez et al., 2022). On the contrary, (Kimizoğlu et al., 2023) studied the impact of a polyphenol-rich and polyphenol-poor olive oil supplementation in old female rats, and although he recorded a proliferation of neuronal cells in the dentate gyrus following treatment, this did not result in a reduction of the anxious state, nor in an increase in spatial memory.

LPS treatment given intraperitoneally modified the microbiota

The dogma behind the gut-brain axis is that changes in the microbiota lead to consequences at the brain level. In the present work, the opposite occurs, and a change in a stimulus at the systemic level leads to a modulation at the microbiota level which in turn releases substances that are potentially harmful to the body already affected. This phenomenon is recorded in other publications (illustrative, but not exhaustive) where changes are shown following infection with *Toxoplasma gondii* (Lv et al., 2022), following treatment with azoxymethane and dextran sulfate sodium to generate a mouse model of colitis and colorectal cancer (Ibrahim et al., 2019), by injection of endometrial segments to generate a mouse model of endometriosis (Yuan et al., 2018) and by immobilisation stress in a mouse model of depression (Kim et al., 2021).
It is known in the literature that many insults are able to modify the microbiota directly and indirectly (Gomaa, 2020; Hasan & Yang, 2019; Weiss & Hennet,

2017).The gut is the hollow organ in which the microbiota thrives and creates a reciprocal coexistence relationship with the host, through continuous crosstalk with enterocyte and immune cells, as well as through modulation of mucus production and luminal pH (Perez-Lopez et al., 2016).
Excluding environmental contaminations, each used treatment (sham/OOE , LPS/water , LPS/OOE) is capable of modifying the beta-diversity of the microbiota in comparison with sham/water group, demonstrating a great susceptibility of the ecological macro-cluster.
Another fact to consider is the importance of the cage effect on this 'organ' that closely shapes and clusters the microbiota of mice sharing the same cage.
Treatment with LPS changed bacteria belonging to different bacterial families compared to the control group. Of these, there was a decrease in Lachnospiraceae and an increase in OTU 76 (eggertelhaceae) and OTU 66 (muribaculaceae), which were previously correlated with an inflammatory bowel disease mouse model (Gao et al., 2018). On the other hand, treatment with OOEP did lead to an increase in OTU_66, but on the other hand it led to an increase in several OTUs belonging to the Lachnospiraceae family (OTU 68, 126, 145, 157), a SCFA-producing family, already reported by (Q. Wang et al., 2022). Finally, OOE/LPS-treatment led to a decrease in OTUs belonging to this family (OTU 109, 222) and an increase in OTU 10, 117. The positive action of Lachnospiraceae is debated, as although they produce SCFA and tryptophan metabolites, reduce pro-inflammatory cytokines and give radioprotection (Gao et al., 2018; Vacca et al., 2020), their increase has been correlated with various diseases (Vacca et al., 2020). Probably, some OTUs are more harmful than others and unfortunately this work was not aimed at discriminating each of them, but we believe that further studies on this family will produce important data in the study of the gut-brain axis.

Metabolites modified by LPS

Treatment with LPS leads to a decrease in the faecal concentration of 4-hydroxyphenylpyruvate (4HP) and citrulline.
4-HP is produced during tyrosine catabolism (Deutsch, 1997). The metORIGIN server positively correlated the change in this metabolite to the families Deferribacteriaceae, Coriobacteriaceae and Flavobacteriaceae, which in turn belong to 3 different phyla (Deferribacterota, Actinobacteriota and Bacteroidota, respectively). This finding could be very interesting in hepato-protection. Indeed, serum levels of 3-(4-hydroxyphenyl)lactate, the decarboxylated form of 4-hydroxyphenylpyruvate that is produced by the gut microbiota, has been correlated with an increased risk of NAFLD in humans (Caussy et al., 2018). Unfortunately, this metabolite has not been analysed in

plasma, so further studies will be needed to prove this theory. In any case, histology of murine livers showed no fibrotic presence (data not shown).

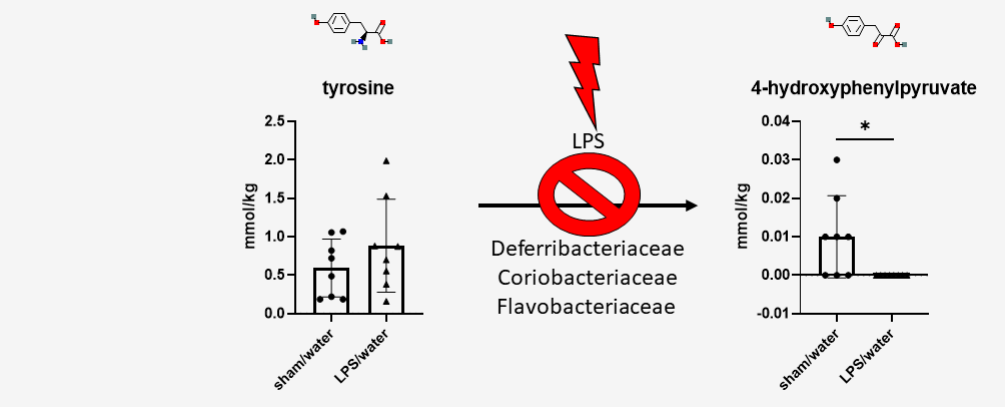


Fig. 29 ***Proposed mechanism behind the decrease of 4-hydroxyphenylpyruvate in the caecum of LPS/water mice in comparison with sham/water mice****. Statistical significance is expresed as *0.05, according to Student's T-test.*

Plasma citrulline has been shown to be a good marker of intestinal function (Crenn et al., 2000; Maric et al., 2021), whereas faecal citrulline has been negatively correlated with gout (Shao et al., 2017) and positively correlated with ulcerative colitis and Chron's disease in paediatric patients (Kolho et al., 2017).

In our study, mice treated with LPS for 8 weeks showed a change in the microbiota that led to an increase in citrulline concentration at the caecal level. Assuming proper function of the enterocyte amino acid-transporters, one can assume that there is a parallel increase in the concentration at the serum level (Maric et al., 2021). This is a very important finding because it would represent a protective feedback triggered at the level of the microbiota to defeat the harmful effects of LPS. In fact, (Rajcic et al., 2021) demonstrated that dietary L-citrulline supplementation was able to stimulate zonulin and occludin synthesis at the enterocyte level and thus reduce the increase in endotoxemia, while (Ouelaa et al., 2017) showed that L-citrulline administration prevented TLR4-linked inflammation, at the liver level, as assessed *ex vivo*.

Effects due to OOEP in the gut

OOEPs led to increased values of GABA, creatinine and 3-methyl-oxo-valerate compared to water/sham population.

GABA is the main inhibitory neurotransmitter of the central nervous system and many bacteria are able to produce and consume it (Doifode et al., 2021; Strandwitz, 2018). It is a metabolite that is not able to cross the BBB (Boonstra et al., 2015; Hinton & Johnston, 2020), yet a great deal of work shows a possible beneficial effect on stress (summarised in (Hepsomali et al., 2020)), through direct communication between the vagus nerve and the CNS (Bravo et al., 2011; Dicks, 2022). GABA binds GABAergic receptors on interneurons causing activation of chlorine channels and iperpolarisation (Tang et al., 2021). This inhibitory capacity on the CNS counterbalances the excitatory activity exerted by glutamate, thus creating a very important balance in both the development and adult life of the brain, termed excitatory/inhibitory balance, the dysregulation of which in an excitatory sense has been correlated with neurodevelopmental disorders (Tang et al., 2021).
In this case, there was an increase in the amount of GABA in the gut, probably due either to a GABA-producing bacterial population, which we were unable to identify, or to inhibition of the growth of bacterial populations capable of using GABA as a nutritional source (Strandwitz, 2018). As reported by (Awad et al., 2009), it is also possible to inhibit the enzyme GABAtransaminase via the polyphenol rosmarinic acid (Awad et al., 2009) and consecutively obtain higher concentrations of GABA in the gut. Unfortunately, the same result has not been reported in the OOE/LPS population.
Creatinine is the waste product from the exoergonic utilisation of creatine at the muscle level and is one of the gold standards for studying renal function as it is almost completely eliminated by the kidney (Kashani et al., 2020; Krstić et al., 2016). This metabolite may also be present in the intestine and its absorption at systemic level as such can be observed (Kashani et al., 2020; Krstić et al., 2016) (Pappenheimer, 1990; Yun et al., 2016). We did not find a possible physiological explanation for this increase, so we assumed that it was the bacterial populations present that produced it in greater quantities under stimulation of OOEP.
There are contrasting opininons about 3-methyl-2-oxovalerate from a biological point of view. Indeed, while it has been seen to be associated with lacunar stroke (Guo et al., 2023), chronic kidney disease (D. Su et al., 2023), caries risk (Musalem-Dominguez et al., 2023), type II diabetes (Menni et al., 2013), HIV infection (Fulcher et al., 2022), inflammatory bowel disease (Schicho et al., 2012), on the other hand it is related to the growth of beneficial ascomycota members (Ząbek et al., 2017) and exercise (Pechlivanis et al., 2015) and is an important inhibitor of the FOXO-1 complex in endothelial cells (Andrade et al., 2021). In contrast, 3-methyl-2-oxovalerate was found in trials in which other types of polyphenols were taken, such as chamomile (Madrid-

Gambin et al., 2019), wine (Vázquez-Fresno et al., 2016) and coffee (Madrid-Gambin et al., 2016). Thus, one could speculate either that 3-methyl-2-oxovalerate is an intermediate in the degradation of polyphenols or that polyphenols stimulate an increase in the intestinal concentration of this metabolite or of its precursor isoleucine.
Indeed, isoleucine can be converted to succinic acid by passing through the reaction intermediate 3-methyl-2-oxovalerate. At this point, succinic acid can be converted to GABA via the reactions succinate semialdehyde dehydrogenase and GABA aminotransferase (Ansari et al., 2021). As an alternative fate, it can be converted after passing through the TCA into oxaloacetate, then phosphoenolpyruvate and into pyruvate, then finally creatine can be converted into creatinine.
This could explain the coordinated behaviour of GABA, 3-methyl-2-oxovalerate and creatinine. Very interestingly, these 3 metabolites do not change in a statistically significant manner in OOE/LPS-treated population.

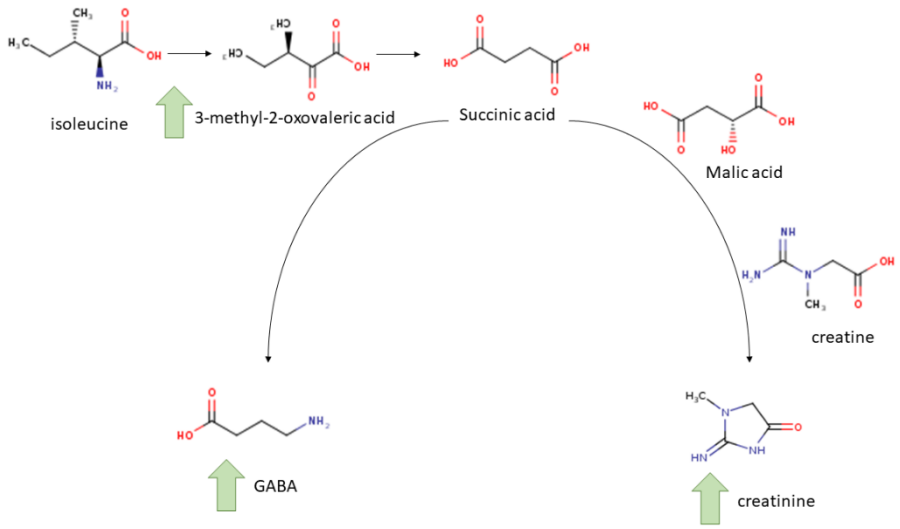


Fig. 30 ***Proposed mechanism behind the synchronical increase of 3-methyl-2oxovalerate, GABA and creatinine in the caecum of sham/OOE mice in comparison with sham/water mice***.

In the LPS/water mice, the only metabolite whose change is statistically significant is citrate, which decrease with respect to water/sham population. It's quite difficult to speculte on this datum, due to the scarce literature, but we have found out that the decrease of this metabolite has been seen to be a characteristic feature of inflammatory bowel disease in humans (Astorga et al.,

2022), which could indicates a direct link between endotoxemia and intestinal bowel disease. More studies will be conducted to validate this hypothesis.

Impact of polyphenols on liver metabolites in the mouse model of sub-chronic inflammation

Compared to the water/sham population, the sham/LPS population shows a change in the beta-alanine pathway. In fact, there is a coordinated reduction of the three metabolites beta-alanine, carnosine and L-histidine. This change is modified by the impact of polyphenols in OOE/LPS group, raising the levels of these metabolites as shown in the table:

WATER/LPS	metabolite	OOE/LPS
↑	L-aspartate	↑
↓	Beta-alanine	↑
↓	carnosine	↑
↓	L-histidine	↑

The beta-alanine metabolism pathway is not the only one affected by polyphenol treatment. In fact, pathway analysis conducted on the metabolic data coming from the liver shows that four further pathways are involved (fig. 31). These changes lead to an increase in the amino acids threonine, glycine and glutamate, with a coordinated reduction in glutamine. The pathways appear to converge in the increased synthesis of histidine, which in addition to its role as a proteinogenic amino acid is also an important antioxidant and iron-chelating molecule (Holeček, 2020). Moreover, (Holeček & Vodeničarovová, 2019) had already shown that intravenous injection of histidine was able to lead to an increase in many amino acids, carnosine and a reduction in ATP, similar to what we observe in our experiments. In contrast to the article cited above, we did not observe an increase in urea (data not shown), nor in glutamine (which, on the contrary, decreased), demonstrating that the body has achieved an excellent capacity in the management of increased concentrations of amino acids and nitrogen compounds, and reductions in isoleucine and glycine.

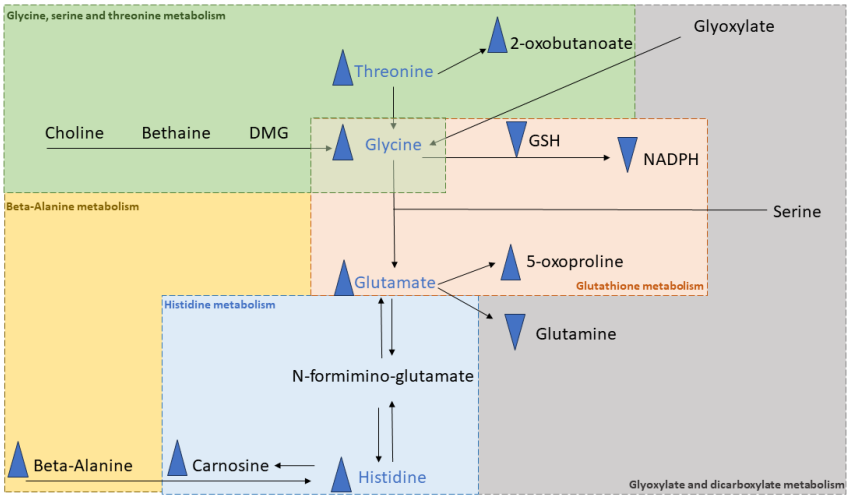


Fig. 31 ***OOEP impact on hepatic metabolites, analysis between OOE/LPS and water/LPS groups.*** *Proposed mechanism of the pathways that are divergent between LPS/OOE and LPS/water groups. In the graph, blue triangles (up and down) indicate the impact of OOEP on water/LPS population.*

Effect of polyphenols on inflammation (liver and brain)

Inflammation is one of the most complex processes within the body and there is still much to learn. As other researchers have already seen, the immune system is a huge balance of signals that is maintained by virtue of dynamic feedbacks and adjustments via adaptive molecules (Cicchese et al., 2018; Kowalski & Li, 2017). LPS is an extremely effective molecule in activating inflammation and continuous treatment with LPS, both centrally and systemically, has been shown to lead to cognitive impairment in mouse models even at very low concentrations (Brown, 2019; Hoyles et al., 2021; Marottoli et al., 2017). This association is reinforced by evidence in the literature which demonstrate an association between LPS and neurodegenerative diseases, such as AD and ALS (Brown, 2019; Keizman et al., 2009).

As brilliantly explained by (Kowalski & Li, 2017; Morris et al., 2015) a quantitatively low level of LPS (10-100 ng/ml) is present at the plasma level in the elderly and this unresolved inflammation favour the onset of new associated diseases (i.e. diabetes, CVD, etc.). Since low-grade inflammation does not lead to a strong activation of the immune system, there is no activation of the negative regulators of this process and non-resolving inflammation develops.

In previous work on this mouse model (Hoyles et al., 2021), a reduction in the cognitive capacity of the mice at the end of LPS treatment was found, although plasma levels of IL-1β and TNF-α were not statistically altered. Similarly, in our study, we found no major pro-inflammatory changes in the water/LPS population, but a reduction in the anti-inflammatory cytokines IL-10 and IL-13 in the liver. Polyphenols, on the other hand, seem to have modulated the inflammatory process to some extent, by raising the anti-inflammatory cytokines IL-10 and IL-13 and the pro-inflammatory cytokine IL-6 compared to the sham/LPS population. In addition, IL-13 is a very powerful activator of the M2 macrophage switch (Funes et al., 2018; Iwaszko et al., 2021), which is linked to repair processes in damaged tissues.

Similarly, in the brain there is a significant increase in TNF-α, followed more discreetly by an increase in IL-1β in both OOEP-treated populations. It is clear that what we are seeing may be the result of the direct effect of polyphenols on the animal, of metabolites produced in the gut that are able to modulate brain activity, or that it is the result of peripheral changes that echoed in the brain. Many authors have treated different mouse models with 50 mg/kg/day of purified polyphenols, such as hydroxytyrosol (Zheng et al., 2015), hydroxytyrosol-acetate (Qin et al., 2021), oleuropein (Grossi et al., 2013), tyrosol (X. Li et al., 2022) and total polyphenols (Abdallah et al., 2022; Farr et al., 2012), and much of this work assessed the antioxidant response, but not the inflammatory response, which was at best assessed by measuring circulating cytokines in plasma (Qin et al., 2021).

In the present work we present a particular phenomenon, showing that polyphenols are able to positively modulate inflammatory activity, increasing few pro-inflammatory markers.

As brilliantly explained by (Demirci-Çekiç et al., 2022), polyphenols in high concentration can facilitate Fenton reactions with transition metals resulting in the overproduction of ROS and moreover the physiological cellular metabolism is imprinted on an imbalance in favour of oxidation (rather than antioxidant capacity), precisely in order to stimulate the correct anti-inflammatory response. A great amount of polyphenols may break this important cellular feedback, as seen by (Rodríguez-García et al., 2022) and (Kouka et al., 2020). It should be noted, however, that only TNF-α shows a strong modulation in the brain, whereas the other two proinflammatory cytokines (IL-1β and IL-6) are only slightly modified and this would hardly fit into a profile of overt neuroinflammation. Instead, we believe that the elevation of TNF-α may have a different explanation.

TNF-α is an important regulator of inflammation (Ortí-Casañ et al., 2022) and, apart from binding to pro-inflammatory TNFR-1 receptors, in the brain it binds

the anti-inflammatory TNFR-2 receptors, which are expressed on neurons and microglia (Pozniak et al., 2014). The activation of this secondary pathway is able to activate the NF-kB pathway in order for neurons to survive during excitotoxic stress (Marchetti et al., 2004), to stimulate Aβ clearance (Ortí-Casañ et al., 2022) and to stimulate the release of NGF, BDNF and GDNF in astrocytes (Pozniak et al., 2014). In addition, TNF-α is very important in synaptic scaling, a fundamental negative feedback process to ensure proper expression of post-synaptic AMPA receptors on neurons (Stellwagen & Malenka, 2006).
Moreover, our findings are in line with the increased GABA in the gut, since it may induce a response at the CNS level; thus TNF-α may reduce GABA-receptor levels at the neuronal level leading to a reduction in the inhibitory capacity of GABA, thus inhibiting long-term potentiation (Pickering et al., 2005; Pribiag & Stellwagen, 2013). The decrease in long term potentiation could lead to analgesic effects at the level of dopaminergic neurons (Pati & Kash, 2021), enhances synaptic efficacy by increasing surface expression of AMPA receptors and thus facilitating the action of glutamate (Das, 2003; Pickering et al., 2005), protecting neurons from oxidative or excitotoxic insults (Pickering et al., 2005).

Lipid changes in the chronic stress model

PPAR-γ is a transcription factor involved in controlling the expression of genes involved in metabolic homeostasis, lipid, glucose and energy metabolism, adipogenesis and inflammation (Han et al., 2017; Strosznajder et al., 2021). Generally, PPAR-γ is poorly expressed in the liver unless there is a major alteration in homeostasis such as obesity and metabolic syndrome (Han et al., 2017); in fact, knock-out mouse models of PPAR-γ have demonstrated an alteration in lipid pattern and metabolic syndrome (Gray et al., 2005). In our model, PPAR-γ and 4-HNE increased synchronically in the liver in LPS/water population, suggesting that 4-HNE upregulated PPAR-γ, as allready seen by (Pizzimenti et al., 2002; Z. Wang et al., 2012).
Sphingomyelins are positively correlated with insulin resistance in obese women, although negatively with PPAR-γ (Zeghari et al., 2000). Nevertheless, sphingomyelins appear to be activators of PPAR-γ (Mazzei et al., 2011). High levels of sphingomyelins were associated with non-alcoholic fatty liver disease and were reduced by saroglitazar, an agonist of PPAR-γ (Kumar et al., 2020). Momchilova demonstrated that resveratrol, another polyphenol well studied in the literature, is able to reduce sphingomyelin concentrations (Momchilova et al., 2022). Similarly, oleic acid and hydroxytyrosol, alone or in combination,

are able to negatively modulate C6 glioma cells sphingomyelin production (Priore et al., 2017).
Moreover 4-HNE, PPAR-γ and the sphyngomyelins decreased after the OOEP treatment. Intriguingly, one could envisage that OOEP reduced lipid peroxidation (4-HNE) and in this manner downmodulated PPAR-γ and sphingomyelin expression. More studies will be conducted to validate this hypothesis.

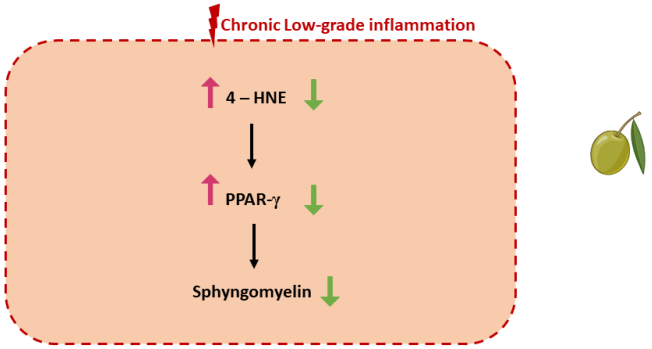


Fig. 32 ***Proposed mechanism for the 4-HNE, PPAR-γ and sphyngomyelin changes in the LPS/water and LPS/OOE groups****. The changes produced by LPS are highlighted in red, the changes produced by OOEP are in green.*

Section 3: Mouse model of Down syndrome

Effects of polyphenols extracted in NaDES in the Down syndrome model

At the end of treatment (6 months of age), the trisomic mouse model shows overt levels of inflammation in the brain (as shown by the IL-1β/IL-10 ratio). This is in line with the common pathogenesis between AD and DS, characterised by high levels of neuroinflammation and oxidative stress in the brain (Hamlett et al., 2018). Treatment with polyphenols led to a reduction of inflammation in the brain, similar to the effect of oleocanthal in TGSwDI mice (Dinda et al., 2019; Qosa et al., 2015).
At the same time as the increased inflammation, higher levels of SOD-1 and CAT were observed, which are partly reduced by treatment with OOE-DES.
It should be noticed that higher levels of lipid peroxidation (Ishihara et al., 2009) and SOD-1 are present in DS models, precisely because of the trisomy (Hamlett et al., 2018; Vacano et al., 2012). Consistently, the H_2O_2 produced by this enzyme can be eliminated either through the CAT enzyme, which is in fact upmodulated, as also demonstrated by (Corrales et al., 2017), or through

the GPX, whose transcription, on the other hand, is not modified by the pathology.
From the point of view of the oxidative stress response, phosphorylated Nrf2 in the cortex is higher in trisomic mice than in euploids, and this is probably the result of a negative feedback due to the continuous pro-inflammatory stimulus present from birth in the trisomic model. Indeed, (Tramutola et al., 2023) reported that this model has higher levels of lipid peroxidation in the cortex. Very interestingly, the treatment with OOE-DES reduced phosphorylation of this protein in the trisomic cortex, probably due to the fact that inflammation was substantially reduced.
Finally, there were no major differences between euploid and trisomic control populations in terms of BDNF and SNAP25 expression. This could be due to the fact that at this age (6 months) the mice have not yet developed overt neurodegeneration due to their young age and because, as explained by several authors, it is assumed that the cognitive deficit may be mainly due to a problem in the electrical conduction linked to GABAergic signalling (Arima-Yoshida et al., 2020) and the kainate receptor, due to a genetic over-dosage of chromosome 16 (Valbuena et al., 2019). Moreover, (Chiotto et al., 2019) showed that on a morphological level, ts2cje-derived neurons would appear normal if there were no spine density reduction problem. Nevertheless, there are improvements in BDNF and SNAP25 expression (not statistically significant) in polyphenol-treated populations. This finding is in line with what has been observed by (Pantano et al., 2017), who demonstrated that the administration of 12.5 mg/kg oleuropein is able to give cognitive improvements in TgCRND8 AD mice. Very interestingly, polyphenols are able to increase GPX expression and decrease HO-1 expression in the euploid mouse model, but not in a statistically significant manner in the trisomic model. This finding is in line with the observation made on the BV2 microglial line (fig.9), and it is very interesting to see that in the mouse model the effect is much higher, perhaps by virtue of the fact that more cell lines in the brain participate in an orchestrated antioxidant response. Similarly, it can be seen that treatment with polyphenols did not impact SOD expression in mice (as in the case of BV2 where there was an increase), but did impact CAT (reduction) and GPX (increase) in the same way.
Another finding that is well worth commenting is the paradoxical effect between the euploid and trisomic populations as regards the expression of PGC-1α. In fact, while in the euploid population treated with polyphenols there is an increase compared to its own control, in the case of the trisomic mice treated with polyphenols there is a reduction that is not statistically significant, but nonetheless evident compared to the trisomic control, which brings the

values back to those of the euploid control. As noted by (Rodríguez-García et al., 2022) OOEP is able to upmodulate PGC-1α and UCP2 levels in muscle and brown adipose tissue, and this seems to be a common feature of several polyphenols (Silvester et al., 2019). (J. Wang et al., 2022) was able to demonstrate how this protein was downmodulated in APP/PS1 mice and how viral administration of a vector expressing this protein could be able to reduce behavioural dysfunction. Indeed, there is a direct connection between PGC-1α and BDNF (Wrann et al., 2013). It is very interesting to see that at the level of the cortex in trisomic control mice, increased levels of PGC-1α are parallel to the phosphorylated form of Nrf2, demonstrating that the antioxidant pathways are active simultaneously (Xiong et al., 2015).

Lipidomic analysis

The correlation between PGC-1α and lipid families in the cortex identified two negative correlations between PGC-1α and diacylglycerols (already reported at the liver level by (Aroor et al., 2015)) and hexocyl-ceramides (Fig. 33).

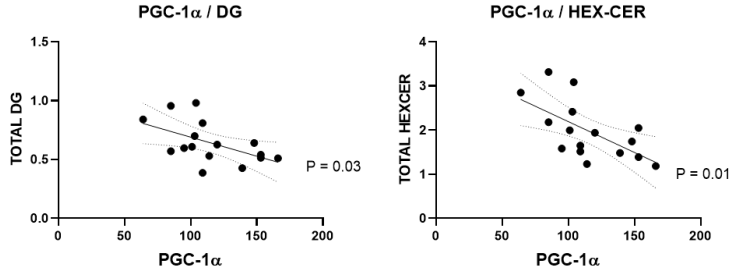


Fig. 33 ***Linear regression between PGC-1α and diacylglycerols and PGC-1a and hexocyl-ceramides in the cortices of 16 mice (n = 4 for group).***

From the point of view of global changes at the level of the brain, polyphenols reduced the expression of triglycerides only in the euploid population. Notably, in the trisomic population they were already low.

The reduction in triglycerides is in line with the effects seen by tyrosol on the 3T3-L1 pre-adipocyte model (Pacifici et al., 2020) and by oleic acid and hydroxytyrosol, alone and in combination, on C6 glioma cells (Priore et al., 2017).

In a very interesting and antithetical manner, the administration of olive oil as such led to the brain-wide increase of triglycerides in rats (Kurban et al., 2007; Rabiei et al., 2013). This could be due to the fatty acids in olive oil that clearly modulate the expression of other lipids. In our case, fatty acids weren't present.

With regard to lower triglyceride levels in TS-Veh, we have not found any references that could justify this finding. To our knowledge there are no lipidomic studies on the brain of DS animal models or post-mortem specimen from DS affected individuals. (de Almeida & Greguol, 2020) conducted a meta-analysis on lipid levels between normal and DS patients and found no statistically significant differences in plasma triglycerides.

Regarding the liver, there is an increase in Nrf2 and PPAR-γ activation in the trisomic population after treatment with polyphenols. This finding is similar to what (Pagnotta et al., 2022) had already seen in the hippocampus of the same trisomic model, and indeed treatment with another polyphenol such as Caffeic acid phenethyl ester CAPE had succeeded in activating Nrf2 translocation.
From a lipid family perspective, the polyphenols impacted the lysophosphatidylcholine and phosphatidylcholine populations, upmodulating both of them.
Lysophosphatidylcholines (LPC) are important components of cell membranes that can act as important pro- (P. Liu et al., 2020) or anti-inflammatory signals (Knuplez & Marsche, 2020). They can in fact be released outside the cell by the action of phospholipases on phosphatidylcholine together with other lipid mediators, such as arachidonic acid, which will become the substrate for cyclooxygenase (COX) and lipoxygenase (LOX) (Knuplez & Marsche, 2020). This is despite the fact that data on the significance of an increase in these lipids are conflicting, in fact LPC levels have been seen to be reduced in the liver in diseases such as hepatitis, hepatocarcinoma, NAFLD and NASH (Knuplez & Marsche, 2020; Paul et al., 2022).
Interestingly, (Wong et al., 2017) reports a decrease in LPC and phosphatidylcholines in blood and liquor of AD patients, maybe due to an accumulation of these lipids in the liver.
The only articles we have found that describe the regulation of LPC in the liver by polyphenols are (Rubio-Rodríguez et al., 2021) where functional beverages (based on 100 mg/dl of various polyphenols) were administered to rats for 19 weeks resulting in a reduction in LPC levels, while (Miranda et al., 2017) shows how heavy coffee drinkers have lower plasma levels of LPC; finally, Lonicerae Japonicae Flos, a typical herb medicine, led to increases in plasma LPC in normal rats (L. Li et al., 2020). Similarly (Priore et al., 2017) showed an increase in LPCs following treatment with oleic acid and hydroxytyrosol, which is in agreement with what we observed. Phosphatidylcholines, together with phosphatidylethanolamines, are the most abundant lipids in cell membranes and are indispensable in the formation of VLDL (van der Veen et al., 2017). (Hijazi et al., 2017) reports a decrease in Phosphatidylcholine at the

level of primary trisomic neurons, in line with (Murphy et al., 2000), which leads to a 20% reduction in sphingolipids in plasma of DS patients. We found no references that assessed phosphatidylcholine at the liver level after polyphenols administration.

At the level of the intestinal mucosa, there was an increase in lactosyl-ceramides in trisomic mice, which were readily lowered by treatment with polyphenols. Ceramides are bioactive lipids, derived from the degradation of sphingomyelin, which can be converted into ceramide-1-phosphate, sphingosine and sphingosine-1-phosphate, glucosylceramide and lactosylceramide (LacCer) and complex glycosphingolipids (i.e.gangliosides) (Won et al., 2007).
(Daniluk et al., 2019) shows that in the serum of children with ulcerative colitis and Chron's disease, LacCer levels are increased, as seen previously by (Stevens et al., 1988) in intestinal biopsies. Further studies will be conducted to identify possible intestinal inflammation.

In line with our data, (Moon et al., 2005) shows how epigallocatechin gallate is able to reduce the activation of NF-kB and consecutively the presence of these inflammation-associated glycolipids.

Conclusions

Although there are still many proposed hypothesis that we need to test, we believe that several conclusions can be derived from our study.

1. Polyphenols extracted from olive oil, and not olive oil itself, which is a mixture of compounds of various kinds (including fatty acids and vitamins), have important biological effects in the field of inflammation and oxidative stress, even at micromolar concentrations. Olive oil has been used for thousands of years by Mediterranean populations and perhaps it would be worth exporting this ingredient to the diets of other countries around the world.
2. From an inflammatory point of view, they are able to modulate inflammation by acting as a modulators on a chronicised inflammation model (which is a common characteristic among the elderly and metabolic syndrome population), and counteracting the activation of microglia in pro-inflammatory M1 state (which are the main characters in neuroinflammation), stimulating a weaker or perhaps better-managed response. Moreover, this modulation could be exerted either in pre-treatment than in post-treatment, so these molecules could also bring beneficial results in models where the neuroinflammatory process is already underway, paving the way for possible clinical trials.
3. Olive oil polyphenols manage oxidative stress activating the antioxidant pathways under the control of Nrf2 and PGC1-α and, in this way, they modulate the expression of CAT, SOD, GPX and HO-1 and probably some other important actors to balance the fragile equilibrium broken by neuroinflammation.
4. From a comprehensive point of view, olive oil polyphenols positively impact the brain directly (by modulating inflammation and oxidative stress) and indirectly (by modifying the microbiota and metabolites produced both in the gut and liver), promoting neurodevelopment and synaptogenesis. Thus, olive oil polyphenols are an excellent tool to alleviate neuroinflammation and in this way strike at the base of neurodegeneration.
5. Polyphenol extraction can be efficiently conducted through NaDES, which ensure reduced risks for the environment, operators, treated cells and animals. In addition, they preserve polyphenols effectively up to 40 °C and could represent an alternative method for polyphenol intake, even for countries where the availability of polyphenol-rich oils is

limited. Moreover, one could think of the idea of manufacturing NaDES that carry not only OOEP but also positive-bioactive molecules as already seen with betaine.

6. Lipidomic studies show how diseases associated with neurodegeneration completely affect cell and tissue biochemistry, altering not only protein expression, but also lipids, which are attracting increasing interest in the scientific community. Due to the backwardness of analysis techniques, these important molecules have been neglected for too long, but now more than ever we have the techniques and capabilities to study and interpret their changes and go deeper in the 'neurodegeneration' phenomenon, identifying new possible biomarkers and molecular targets to be pinpointed to mediate this difficult-to-predict but far-reaching disease.

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