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The remarkable impact of *Opuntia Ficus Indica* fruit administration on metabolic syndrome: Correlations between cognitive functions, oxidative stress and lipid dysmetabolism in the high-fat, diet-fed rat model

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ABSTRACT

Background: A wealth of evidence underscores the bioactive properties of nutraceuticals and functional foods in addressing oxyinflammatory-based diseases with implications at both peripheral and central levels. Opuntia ficus-indica (OFI) is well-documented for its health-promoting attributes, though its fruit (OFIF) remains relatively understudied. Not only poses Metabolic Syndrome (MetS) cardiometabolic risks but also contributes significantly to cognitive impairment, especially in crucial brain areas such as hippocampus and hypothalamus.
Methods: Following 8 weeks of HFD to induce MetS, rats received OFIF oral supplementation for 4 weeks to evaluate cognitive and affective modifications using behavioural paradigms, i.e. open field, burrowing, white-dark box, novelty-suppressed feeding, and object recognition tests. Our investigation extended to biochemical evaluations of lipid homeostasis, central and peripheral oxidative stress and neurotrophic pathways, correlating these measures together with circulating leptin levels.
Results: Our data revealed that OFIF modulation of leptin positively correlates with systemic and brain oxidative stress, with markers of increased anxiety-like behaviour and impaired lipid homeostasis. On the other hand, leptin levels reduced by OFIF are associated with improved antioxidant barriers, declarative memory and neurotrophic signalling.
Discussion: This study underscores OFIF neuroactive potential in the context of MetS-associated cognitive impairment, offering insights into its mechanisms and implications for future therapeutic strategies.

1. Introduction

A wealth of evidence has surfaced regarding the bioactive properties of nutraceuticals in various physiopathological trajectories. Particularly intriguing is the involvement of functional food products in oxyinflammatory-based diseases that could trigger severe consequences at peripheral and central level [1].

Opuntia ficus-indica (OFI) is a plant extensively cultivated in many regions of the world for its pleasant-tasting fruits and cladodes. The plant, widely distributed in Mexico, America, Africa, Australia and in the Mediterranean area [2,3] has recently emerged as a potential source of valuable components for both human health and food industry [4–8]. Accordingly, the nutraceutical and health-promoting properties of cladodes and other vegetative parts of OFI are well established [9]. On the contrary, less attention has been paid through the years to the OFI fruit (OFIF) characterised by a peculiar phytochemical fingerprint hallmarked by the betalain pigments. Relevantly, nutritional studies in healthy humans have shown that OFIF consumption positively affects the body's redox balance, decreasing lipid oxidative damage and improving the antioxidant status [10]. Moreover, OFIF administration also ameliorates the inflammatory profile of healthy humans by reducing plasma levels of tumour necrosis factor-α (TNF-α), interleukin

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(IL)-1β, interferon-γ (IFN)-γ, IL-8, C-reactive protein, erythrocyte sedimentation rate, and increasing IL-10 levels. Remarkably, these anti-inflammatory effects significantly correlated with the anti-oxidative ones [11]. Interestingly, not only can OFIF supplementation positively affect the health status of peripheral systems, but also of the central nervous system (CNS). Indeed, OFIF administration has been shown to modulate corticospinal excitability together with glutamate-mediated intracortical excitability and homeostatic plasticity in healthy humans [12].

A mounting body of research suggests that Metabolic Syndrome (MetS) does not only constitute a risk factor for cardiovascular diseases, diabetes mellitus type 2 and obesity, but also plays a key role in the progression towards cognitive decline [13–15]. Indeed, various studies have identified two primary processes underlying cognitive alterations associated with MetS: widespread oxidative stress and inflammation. These arise from systemic hyperglycemia and central insulin resistance, both triggering oxidative stress and disrupting core pathways, thereby affecting several brain structures [16–18]. Within this context, it has been suggested that MetS may affect functional connectivity within brain networks, potentially leading to alterations in higher cognitive and affective functions [19]. Indeed, MetS-induced alterations boost inflammatory states that impair learning and memory, and the responses to both stress and anxiety that could be investigated *via* specific behavioural paradigms [20–22]. As mentioned above, the main cause could be the overgeneration of reactive oxygen species (ROS), the alteration of endogenous antioxidant systems and neuroinflammation [23–26]. This is particularly true in brain regions such as the hippocampus and the hypothalamus where the dysregulated conditions associated with MetS leads to modifications in the activation of several intracellular signalling cascades, including phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) [27,28]. In both human and animal studies, elevated insulin and glucose levels are associated with impaired brain insulin signalling, leading to reduced glucose transporter trafficking to neuronal membranes and alterations in the insulin/PI3K/Akt signalling pathway, ultimately impacting neuronal structure and function negatively [29–31]. As a matter of fact, alterations in brain regions like hippocampus, crucial for cognitive processing and hedonic responses to food, were found in obese individuals [32]. At the cortical level, the impairment in cognitive processing impacts weight management outcomes in dysmetabolic disorders, as early deficits in cognitive flexibility and increased impulsivity are associated with less successful weight loss efforts [33]. Conversely, individuals who have undergone weight loss demonstrate improvements in memory and executive functions [34]. Indeed, evidence from behavioural, functional imaging, and neurophysiological studies suggests for instance a compromised body representation in individuals suffering from obesity and MetS, underscoring the intricate neurocircuitry abnormalities involved that alter the integration of both internal and external information processing [32,35]. These insights suggest a reciprocal relationship between cognitive impairment and dysmetabolic diseases, where preventive interventions targeting either domain could potentially enhance outcomes in both areas.

Within this scenario, leptin, a peptide hormone produced primarily by differentiated and hypertrophic adipocytes, has been suggested to play an important role in the association between altered affective functions and MetS [36]. As a matter of fact, circulating levels of leptin have been shown to be involved in the up-regulation of the innate immune response by increasing the production of proinflammatory cytokines such as IL-6 and TNF-α [37]. Additionally, leptin seems also to be involved in the dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and of the mesolimbic dopaminergic pathway [37–39].

In this view, MetS induced by High-Fat Diet (HFD) in rats represents an invaluable model for exploring the eventual redox-dependent, neuroprotective role of novel health-enhancing foods [40]. Considering these factors, our aim was to investigate whether the inclusion of OFIF in the diet could mitigate cognitive impairment induced by MetS in HFD fed rats, focusing on central and systemic correlates. To achieve this goal, we examined the effects of OFIF supplementation on lipid dysmetabolism and redox homeostasis, aiming to correlate biochemical evidence, particularly circulating leptin levels, with cognitive impairment, in terms of behavioural, neuro-oxidative, and neurotrophic markers. Our comprehensive analysis involved the evaluation of various cognitive and affective functions, including exploratory behaviour, reactivity, response to stress and anxiety, depressive-like behaviour, and memory, using established behavioural tests. Additionally, we investigated whether OFIF modulated neurotrophic signalling pathways in key brain regions such as the hippocampus and hypothalamus, which are involved in energy homeostasis and behaviour, as well as neuro-oxidative pathways such as malondialdehyde, within our HFD-induced MetS model. This exploration of OFIF aligns with the broader objective of utilising food supplementation with neuro-nutraceuticals to alleviate cognitive deficits associated with MetS.

2. Materials and methods

2.1. *Opuntia ficus indica fruit preparation*

OFIF administered in the present work was prepared as follows. Briefly, OFIF (yellow cultivar from San Cono, Sicily) were peeled, finely chopped, weighed and 100 g pulp samples were homogenised and then freeze-dryed in the presence of 2.5 % β-Cyclodextrin for alimentary use. OFIF is characterised by a high percentage of moisture (> 80 %), minute amounts of proteins (13 % of dry mass) and lipids, significant amount of sugars (10 %), fibers (5 %), vitamins (A, B, C, E), betalains and minerals (Ca^{++}, Mg^{++}, Mn^{++}, K^{+}, Na^{+}, P, Fe^{++}) [41].

2.2. *Animals*

Male Wistar rats weighing 240–260 g were supplied by Envigo S.r.l (Indianapolis, Indiana). They were accommodated two per cage and sustained on a 12-hour light/dark cycle (8:00–20:00 h) at a consistent temperature (22–24 ∘C) and humidity (50 ± 10 %). Throughout the seven-days acclimatation phase, animals were initially nourished with a standard chow diet offering 3.94 kcal/g and subsequently separated into two uniform groups with balanced weight (7 week-old, n = 20, weighing 240±13.04 g). These experimental groups were supplied with standard laboratory food (code PF1609, certificate EN 4RF25, Mucedola, Milan, Italy), or provided with HFD food with 60 % of energy sourced from fats (code PF4215-PELLET, Mucedola, Milan, Italy), the specific attributes of which are detailed in our prior publications [42–44]. The control group (NPD: normal pelletized diet) received a low-fat regimen consisting of a standard rat chow (3.94 kcal/g, 55.50 % carbohydrate, 22 % protein, 3.50 % lipid, 4.50 % fiber). The second group (HFD: high-fat diet) was nourished with a hypercaloric pelletized diet (5.5 kcal/g) comprising of 34 % fat, 23 % protein, 38 % carbohydrates and 5 % fiber, until MetS was induced and evaluated based on criteria previously established in the literature [43,45]. The third group (HFD-OFIF) was fed an HFD for 8 weeks and then received OFIF at a calculated dose of 14 mg/kg, twice a day, as oral gavage, for the last 4 weeks. All animals had unrestricted access to food and water. During the experimental protocol, animal care and handling adhered to the ARRIVE guidelines and the European Directive (2010/63/EU). The experimental procedures received approval from the animal welfare committee of the University of Palermo and were authorised by the Ministry of Health (Rome, Italy; Authorization Number 376/2024-PR).

2.3. *Induction of metabolic syndrome*

The experimental timeline of the study has been divided into three phases: T0 (baseline), T1 (induction of MetS) and T2 (end of the experiment).

1) At the baseline phase (T0), following the acclimatisation period, animals were divided into NPD or HFD groups based on the diet provided for 8 weeks until MetS induction.
2) At T1, MetS induction was assessed by criteria commonly used in literature [42,45], including increased triglyceride levels, body weight and glucose tolerance compared to normally-fed rats. Subsequently, the animals were subdivided into 3 groups according to the type of diet (NPD and HFD) and treatment administered (opuntia ficus indica extract or vehicle) from T1 until T2, which was reached 4 weeks after T1. Specifically, the normal control group continued to receive a normal diet (NPD) until T2 and the second one (HFD group), serving as the MetS control, received HFD diet throughout the study (from T0 to T2). The NPD (n=6) and HFD (n=6) groups underwent identical stress conditions to the treated group, as they were orally administered with a standard volume of vehicle (water) during the last month of the trial, from T1 to T2. Finally, the third group (HFD-OFIF, n=8) received daily OFIF extract treatment during the last month of the experiment (T1-T2).
3) T2 represents the conclusion of the experimental study, taking place 12 weeks following the baseline phase (T0).

2.4. Nutritional supplementation with OFI fruit

OFIF was administered by oral gavage twice a day, for one month at a volume of 1 ml/kg body weight during the last month of the study (T1-T2).

2.5. Biometric, lipid and oxidative homeostasis parameters induced by MetS

At T2, the impact of OFIF on MetS was assessed focusing on changes in biometric, biochemical, and oxidative homeostasis parameters. Plasma samples were obtained for subsequent analyses aimed at assessing lipid homeostasis, oxidative stress parameters, and plasma antioxidant status.

2.5.1. Biometric parameters: body weight and food intake

Rats were weighed weekly from T0 to T2. Additionally, the food intake was assessed in the experimental groups. This involved weighing the chow before and after delivery to the cage twice a week. The remaining chow was then weighed, and food intake was established by the difference between the weight of the chow that was given to the animals and the weight of the remaining chow.

2.5.2. Lipid homeostasis

To evaluate the impact of OFIF supplementation on lipid homeostasis in MetS, following sacrifice, blood samples from each animal were obtained via cardiac puncture. Comprehensive methodologies are outlined in our previous publications [42]. Triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL), and high-density lipoprotein cholesterol (HDL) concentrations were measured in plasma samples using commercially available kits employing the Free Carpe Diem device (FREE® Carpe Diem; Diacron International, Grosseto, Italy). Data are presented in mg/dL.

2.5.3. Oxidative stress parameters and plasma antioxidant status

Plasma redox balance was evaluated by Diacron kits, as previously reported [42,43]. To evaluate the prooxidant status, assessments were conducted using the dROM (Reactive Oxygen Metabolites, mainly hydroperoxides) and the LP-CHOLOX test. The former measures levels of hydroperoxyl free radicals, while the latter determines levels of circulating lipid peroxides and, specifically, oxidised cholesterol. Hydroperoxides, lipoperoxides, and oxidised cholesterol levels in plasma samples were quantified using commercial kits with the Free Carpe Diem device (FREE® Carpe Diem; Diacron International, Grosseto, Italy). Data from dROM tests are reported in arbitrary units, namely Carratelli units (UCARR). The standard range for the test results was 250–300 U.CARR (Carratelli Units), with 1 U.CARR corresponding to 0.08 mg/dL of H_2O_2 [46]. In the LP-CHOLOX test, levels of LP-CHOLOX (lipoperoxides and oxidised cholesterol) are identified through the peroxides' capacity to catalyse the oxidation of Fe^{2+} to Fe^{3+}, leading to the formation of a coloured complex with an indicator mixture, detected by a spectrophotometer at 505 nm [47,48]. The results are reported in mEq/L.

Regarding plasma antioxidant capacity, the "BAP" test (Biological Antioxidant Potential) evaluates exogenous substances such as ascorbate, tocopherols, carotenoids, and bioflavonoids, along with endogenous substances like bilirubin, uric acid, and proteins, which in plasma possesses antioxidant properties and can neutralise radical species. The analysis was conducted using the Diacron kit by spectrophotometric measurements at a wavelength of 505 nm, as documented in prior research and expressing the results as mmol/L [49]. Moreover, the SHp test was employed to assess thiol groups, evaluating the reducing properties of OFIF extracts counteracting thiol groups oxidation and promoting a shift towards reduced forms.

2.6. Evaluation of leptin levels

Analysis of plasma leptin levels was performed on plasma samples obtained from blood taken from the animals after sacrifice at time T2. The blood was subjected to centrifugation at 2000 rpm, room temperature for 15 min excluding the brake from the centrifuge to avoid haemolysis. Analysis was performed by enzyme immunoassay using the ELISA kit from Millipore, Saint Louis, MO 63103 USA. Plasma samples were diluted 3-fold and Leptin concentration was assayed by calibration line prepared in the range of 8000–10.97 pg/ml by spectrophotometric readings at 450 nm wavelength. The kit has a sensitivity of 30 pg/ml.

2.7. Behavioural assessment

All the protocols, with the exception of burrowing and saccharin preference, were evaluated using a behavioural tracking software (AnyMaze, Version 7.20) and analysed by a trained scientist who was unaware of the experimental grouping.

2.7.1. Exploratory behaviour and reactivity

2.7.1.1. Open Field Test (OFT). The open field test (OFT) was employed to assess locomotor and exploratory behaviour of the animals. Subjects were introduced into a square open field (70x70cm) positioned facing the wall from a distance of 10 cm and were subsequently recorded for a duration of 10 minutes. Recorded activity parameters included total distance covered, immobility time, time spent in the centre zone and frequency of entries in the zone.

2.7.1.2. Burrowing test. The burrowing test was conducted following previously established procedures [50]. Plastic burrowing tubes (32 cm long x 10 cm Ø, elevated on one site by 6 cm) were filled with 2.5 kg gravel (quartz-light, grain size 2–4 mm) and positioned within the cage. Each rat underwent testing individually. Prior to evaluating burrowing performance, all rats underwent a training session at T1. During the training phase, on day 1, rats were provided with an empty burrowing tube for 60 minutes; on day 2–4, rats were given a gravel-filled tube for 60 minutes following a 30-minutes habituation period in an empty cage. Burrowing behaviour was then assessed at T2. Upon placement of the burrowing tube in the cage, the latency period before initiating burrowing was recorded. The remaining gravel in the tubes was weighed and the difference from the initial weight was calculated to determine the amount of burrowed gravel.

2.7.2. Anxiety-associated and anhedonia-associated behaviour

2.7.2.1. Novelty-Suppressed Feeding Test (NSFT). In the novelty-suppressed feeding test (NSFT), following a 24-hour period of food deprivation, rats were introduced in an open-field maze where a food pellet (2 g of HFD or NPD chow) was positioned on a white rounded disc at the centre of the arena. Animals had a 10-minute cut-off to reach and consume the food pellet, only rats that completed the test were included in the analysis [51]. The feeding duration was monitored, and once rats completed the test, they were promptly removed from the arena and returned to their home cage. In their cage the amount of food consumed by animals in the subsequent 10 minutes was measured to confirm completion of the test under deprived conditions.

2.7.2.2. Saccharin preference test. The saccharin preference test was conducted following protocol outlined in [52]. Two water bottles were filled with 500 g of the solutions. Initially, water intake from both bottles were assessed over a 24-hour period to determine any initial preference for one side. Subsequently, one of the two bottles was filled with 0.1 % saccharin solution (Saccharin $\geq$ 98 %, Sigma-Aldrich) and the intake was measured over another 24 hours. On the third day, both bottles were refilled again with regular water, and on the fourth day the saccharin-containing solution was provided in the bottle on the opposite side.

2.7.2.3. Black-white box test (BWB). For the black-white box test (BWB), a plastic box (60 cm length $\times$ 30 cm height $\times$ 30 cm width) partitioned into two equal-size compartments connected through a dimly lighted tunnel was used. One of the compartments was painted black and was covered with a lid and the other compartment was painted white and illuminated with a 45 W light bulb positioned 40 cm above the box [53]. The animals were placed in the white compartment facing the tunnel. The parameters evaluated for five minutes were number of entries and the time spent in the light zone.

2.7.3. Learning and memory: object recognition test

This Object Recognition test (ORT) is recognised as a reliable and widely employed method for evaluating learning and memory by assessing the declarative memory system in experimental models [54,55]. Initially, rodents were habituated to the arena (open field maze with the same characteristics as in 2.7.1.1). On the test day, animals were presented with two identical objects placed at a fixed distance from each other. Following a 1-hour retention period, one of the two objects was replaced with a novel one, while the other remained unchanged ("familiar object"). The objects were selected and 3D printed based on specific literature pertaining to this paradigm [56]. In assessing the ORT, we considered the retention index (RI%), representing the time spent exploring the novel object relative to the total time spent investigating both the familiar and novel one. This parameter enables the evaluation of the animal's ability to discriminate between a novel and a familiar stimulus one hour after exposure to the familiar ones, serving as the primary indicator of retention [57].

2.8. Biochemical analyses on brain tissues

2.8.1. Tissue preparation and protein extraction

Following the behavioural procedures, all animals underwent cervical dislocation and sacrifice using 2 % isoflurane anaesthesia in accordance with authorised procedures to facilitate blood collection and to quickly brain removal. The left hemisphere was promptly frozen for MDA quantification, while the right hemisphere was dissected under stereomicroscopy with refrigerate support to extract samples of the hippocampus and hypothalamus. Sampled tissues were processed for protein extraction, as previously outlined [58]. In brief, tissues were homogenised at 4°C in cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 % Triton, SDS 0.1 %), supplemented with protease inhibitor cocktail (Sigma-Aldrich P8340) and phosphatase inhibitor cocktail (Sigma-Aldrich P5726). Samples were sonicated (30 pulsations/min), quantified by the Lowry method [59], and stored at −80°C for western blotting analyses.

2.8.2. Western blotting

Western blotting analysis was conducted following previously established procedures [60]. Protein samples (50 μg) along with molecular weight marker (PageRulerTM Plus Prestained Protein Ladder, 26619 ThermoFisher Scientific), were separated on a 10 % polyacrylamide gel and electrophoretically transferred onto a nitrocellulose membrane (RPN303E, Hybond-C-extra, GE Healthcare Europe GmbH). The membranes were incubated in a blocking buffer (1× TBS, 0.1 % Tween−20, 5 % w/v nonfat dry milk) for 1-hour, followed by overnight incubation with anti-pAkt antibody (rabbit phospho-Akt (Ser473) antibody, 1:1000; 9271 Cell Signalling) in blocking buffer at −4°C with gentle shaking. The day after, membranes were washed three times for 5 min with TBS-T, and then incubated for 1 h at room temperature with goat anti-rabbit IgG (sc-2357 Santa Cruz Biotechnology) horseradish peroxidase-conjugated diluted 1:10,000. To normalise the densitometric signal, membranes were exposed to horseradish peroxidase-conjugated β-Actin primary antibody (sc-2005 Santa Cruz Biotechnology), diluted 1:10,000, for 1 h. After three washes with TBS-T, immunocomplexes were visualised using chemiluminescence reagent (SuperSignalTM West Pico PLUS, ThermoFisher Scientific) with an iBright FL1500 Imaging System (ThermoFisher Scientific). Densitometric analysis of bands was conducted by measuring the optical density (O.D.) using iBright FL1500 software.

2.8.3. Malondialdehyde (MDA) evaluation

Brain sagittal sections were washed in ice-cold 0.9 % NaCl and weighted. A 10 % (w/v) homogenate was prepared in ice-cold 40 mM Tris-HCl by using a micro homogeniser (OMNI International, Tissue Master TM 125) as previously described [61]. Evaluation of MDA levels in brain homogenates was performed as previously reported [62]. Briefly, a reaction mixture containing 0.2 ml of whole homogenate, 0.2 ml of 8.1 % sodium dodecyl sulphate (SDS), 1.5 ml of acetic acid solution adjusted at pH 3.5 with NaOH and 1.5 ml of 1 % thiobarbituric acid (TBA) aqueous solution was prepared for each sample. The reaction mixture was brought up to a final volume of 4.0 ml with distilled water and heated at 95°C for 60 min. After briefly cooling on ice, 1.0 ml of distilled water and 5.0 ml of a solution of a n-butanol/pyridine solution (15/1, v/v ratio) were added, and the resulting mixture was thoroughly vortexed. After centrifugation at 3200 RCF for 10 min, the absorbance of the organic layer was measured at 532 nm. MDA levels were expressed as nmol MDA/g tissue, using 1,1,3,3, tetramethoxypropane as an external standard. The assay was repeated for a total of three times for each sample.

2.9. Statistical analyses

Statistical analysis was conducted using GraphPad Prism 9.02 (San Diego, CA, USA). Behavioural and biochemical parameters were compared using a one-way ANOVA test, followed by Bonferroni post hoc evaluations to assess the differences between means. The results were shown using a box and whiskers graph depicting the minimum, 25th percentile, median, 75th percentile, and the maximum. Body Weight and food intake were assessed through a two-way repeated measures (RM) ANOVA, followed by Bonferroni post hoc tests for significant differences in both within- and between-subject comparisons, considering the effect of "time", "OFIF supplementation", and their interaction in the experimental groups. Statistical significance was established at $p<0.05$. Additionally, the statistical power (g-power) was considered if >0.75 and the effect size was evaluated if >0.40. The results are presented as the mean ± standard error of the mean (S.E.M.). Correlation matrix was

computed using a non-parametric Spearman's correlation test and visualised through a heatmap. Missing values for correlation matrix were handled with 'pairwise complete observation'. Principal component analysis (PCA) was calculated and visualised by computing loadings, scores and vectors. For each variable in the PCA the mean of the remaining animals (regardless of group) was added instead of missing values.

3. Results

3.1. Effects of OFI fruit supplementation on body weight in MetS

We evaluated body weight per week from T1 to T2 in the experimental groups using two-way repeated measures ANOVA, revealing significant differences in time ($F_{(3,17)}$ =54.22, p<0.0001), nutritional treatment ($F_{(2,17)}$ =24.96, p<0.0001) and their interaction ($F_{(6,51)}$ =6.07, p<0.0001), as in Fig. 1**A**. Furthermore, two-way ANOVA was conducted on food intake at T1 and T2 in the experimental groups and outlined significant differences in time ($F_{(1,17)}$ =187.0, p<0.0001), nutritional treatment ($F_{(2,17)}$ =39.25, p<0.0001) and their interaction ($F_{(2,17)}$ =9.85, p=0.0014), as in Fig. 1**B**.

3.2. Effects of OFI fruit treatment on lipid homeostasis in MetS

Lipid homeostasis was evaluated at T2 comparing plasma concentrations of TG, TC, LDL, and HDL in HFD-OFIF group versus HFD and NPD. The one-way ANOVA performed on the plasma levels of TG pointed out differences between groups, with a marked decrease in HFD-OFIF versus HFD alone, though HFD-OFIF is still significantly higher than NPD groups ($F_{(2,17)}$=40.69, p<0.0001, Fig. 2**A**).

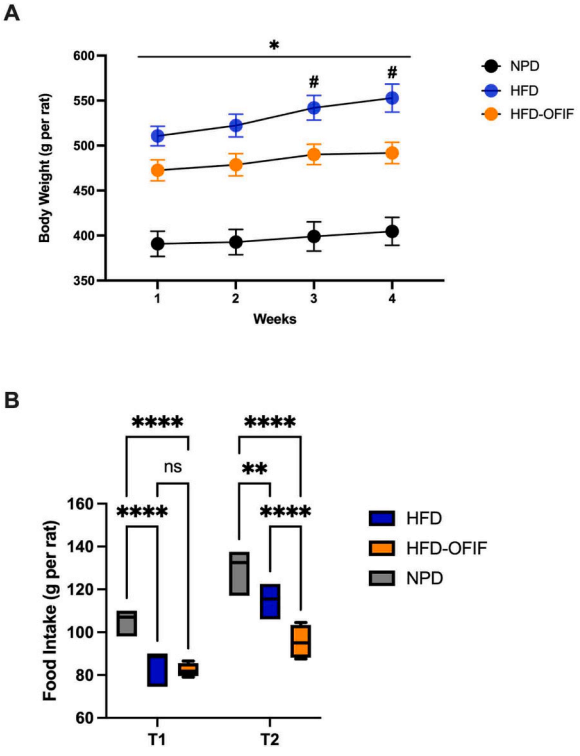

Fig. 1. Variation of body weight from T1 to T2 (g per rat) and of food intake (g per rat) at T1 and T2, in HFD-OFIF, HFD, and NPD experimental groups. **A.** Statistical significance by two-way ANOVA followed by post hoc Bonferroni is indicated as (*) p<0.05 for HFD and HFD-OFIF vs NPD group and (#) p<0.01 for HFD vs HFD-OFIF. **B**. Statistical significance by ANOVA followed by post hoc Bonferroni is indicated as (*) p<0.05, (**) p<0.001, (***) p<0.0001 and (****) p<0.0001.

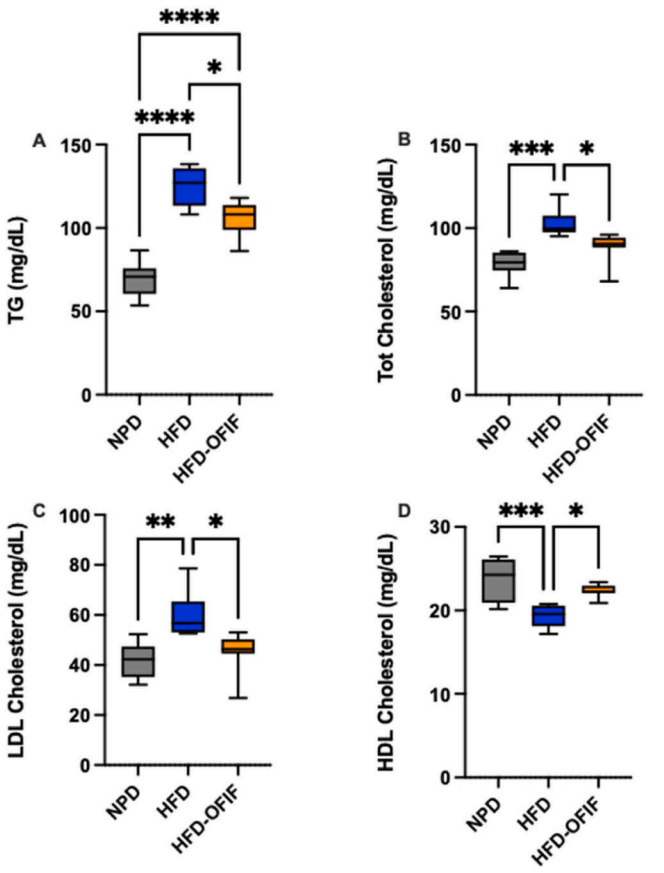

Fig. 2. Biochemical parameters of lipid homeostasis. (**A**) Triglycerides (TG), (**B**) Total Cholesterol (TC), (**C**) LDL Cholesterol, and (**D**) HDL Cholesterol (mg/dL) in HFD-OFIF, HFD, and NPD experimental groups. Statistical significance by one-way ANOVA followed by post hoc Bonferroni is indicated as (*) p<0.05, (**) p<0.001, (***) p<0.0001 and (****) p<0.0001.

As for total cholesterol, it was noticeably decreased in HFD-OFIF rats versus HFD and showed similar levels between HFD-OFIF and NPD without any significant differences($F_{(2,17)}$=11.73, p=0.0006, Fig. 2**B**). Moreover, the plasma levels of LDL analysed by one-way ANOVA were remarkably decreased following OFIF treatment in the HFD-OFIF group with respect to HFD, and returned to basal values that were not statistically different than the NPD group ($F_{(2,17)}$=8.05, p=0.0035, Fig. 2**C**). Importantly, HDL cholesterol was increased by OFIF supplementation in HFD rats reaching significantly higher levels than HFD and no significant differences versus NPD ($F_{(2,17)}$=11.01, p=0.0009, Fig. 2**D**).

3.3. Effects of OFI fruit on circulating leptin levels in MetS

Systemic levels of leptin were assessed through ELISA assay in the experimental groups. One-way ANOVA followed by Bonferroni Post-hoc revealed that leptin was increased in HFD group versus NPD and that OFIF supplementation ameliorated leptin levels versus HFD recovering basal control levels ($F_{(2,17)}$ =21.40; p<0.0001, Fig. 3).

3.4. Effects of OFI fruit on plasma redox homeostasis biomarkers in MetS

The assessment of antioxidant and prooxidant status occurred at time T2, following nutritional intervention with OFIF, utilising plasma samples from all experimental groups to investigate plasma redox balance in MetS. Statistical analyses demonstrated significant modulation of the antioxidant capacity in MetS animals by OFIF. Specifically, a one-way ANOVA followed by post hoc testing revealed higher mean SHp values in the HFD-OFIF group compared to the HFD group, that were not-

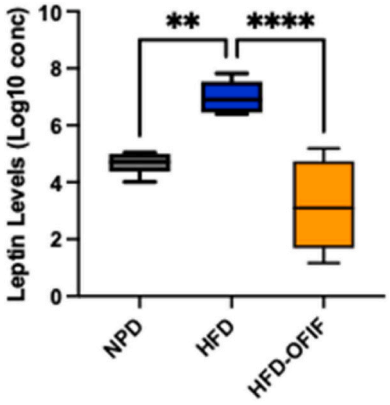

Fig. 3. Leptin levels in HFD-OFIF, NPD and HFD groups. Statistical significance by one-way ANOVA followed by post hoc Bonferroni is indicated as (****) $p<0.0001$ and (***) $p<0.001$.

significantly different than controls ($F_{(2,17)}=10.85$, p=0.0009; Fig. 4**A**). As well, BAP values were increased in HFD-OFIF versus HFD group but still significantly lower compared to the NPD group ($F_{(2,17)}=16.56$, p=0.0001; Fig. 4**B**).

Moreover, significant differences were observed in the prooxidant status, specifically in dROM and LP-CHOLOX levels showing that OFIF supplementation protected from the alterations induced by HFD and recovered basal values. In particular, dROM levels exhibited alterations among the experimental groups, with post hoc analysis revealing a significant reduction induced by OFIF supplementation compared to the HFD group ($F_{(2,17)}=7.85$, p=0.0038; Fig. 4**C**). Additionally, LP-CHOLOX levels were significantly diminished in the HFD-OFIF group compared to the HFD group, approaching baseline levels as no significant differences were observed compared to the NPD group ($F_{(2,17)}=10.66$, p=0.0010; Fig. 4**D**)

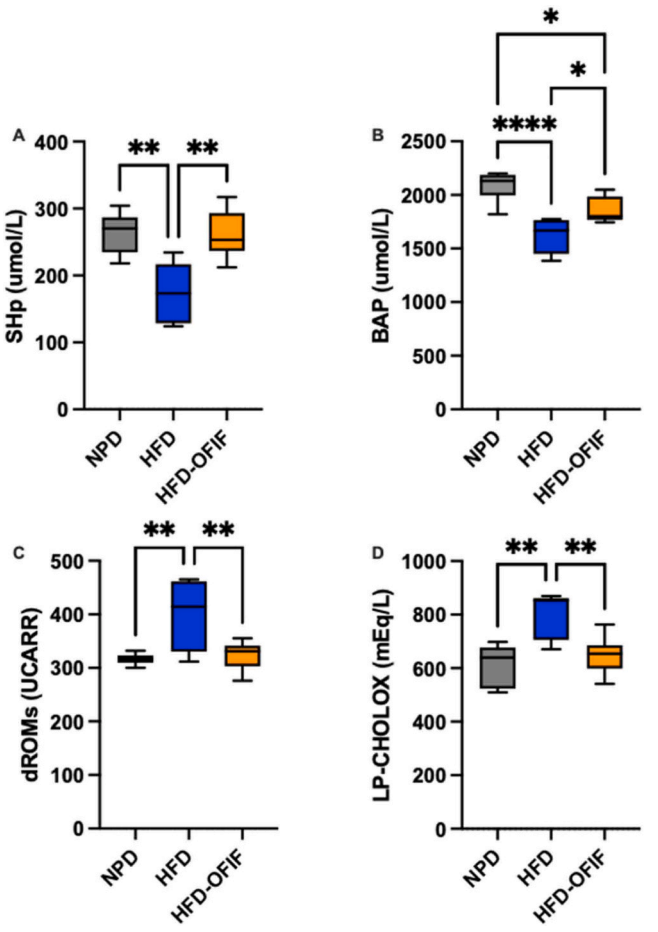

Fig. 4. Plasma redox homeostasis biomarkers between HFD-OFIF, NPD, and HFD groups at the end of experimental procedures. Antioxidant status evaluated by (**A**) SHp test for thiol group levels (mmol/L) and (**B**) Biological Antioxidant Potential (BAP test) levels (mmol/L). Prooxidant status evaluated by (**C**) dROM test for differences in ROM (primarily hydroperoxide) levels (UCARR) and (**D**) LP-CHOLOX test for differences in LP-CHOLOX (lipoperoxides and oxidised cholesterol) levels (mEq/L). Statistical significance of Bonferroni post hoc tests are indicated for (*) $p<0.05$, (**) $p<0.01$ and (****) $p<0.0001$, as represented in the graphs.

3.5. *Exploratory behaviour and reactivity*

3.5.1. *Open field test*

Animals were tested in an open field to assess their behavioural reactivity and exploratory behaviour. Parameters were recorded and evaluated via one-way ANOVA followed by Bonferroni post-hoc test. Total distance travelled was significantly increased in HFD-OFIF group vs HFD, and not different versus NPD ($F_{(2,17)}=7.45$, p=0.0047, Fig. 5**A**). The immobility time was markedly reduced after supplementation with OFIF in HFD rats, recovering basal values of NPD ($F_{(2,17)}=10.35$, p=0.0011, Fig. 5**B**). As for time spent at the centre of the maze and number of entries they were higher in HFD-OFIF group versus HFD and

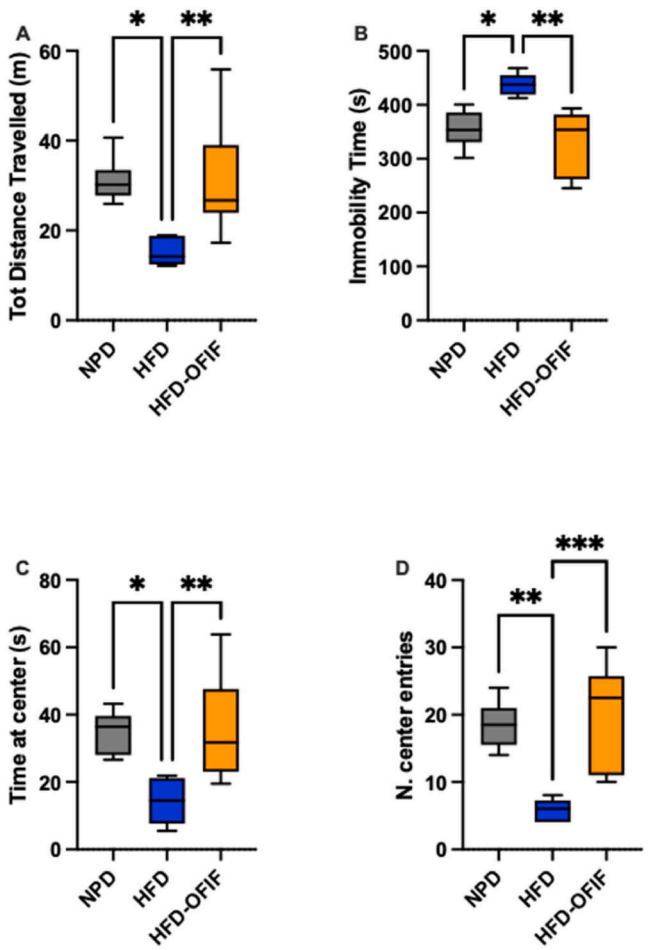

Fig. 5. Behavioural parameters assessed in the Open Field Test in NPD, HFD and HFD-OFIF groups. **A.** Total Distance Travelled (m). **B.** Immobility time (s). **C.** Time spent in the centre zone (s). **D.** Number of entries in the centre zone. Statistical significance of Bonferroni post hoc tests are indicated for (*) $p<0.05$, (**) $p<0.001$ and (***) $p<0.0001$.

not different versus NPD (respectively: $F_{(2,17)}$=7.508, p=0.0046, Fig. 5**C** and $F_{(2,17)}$=12.34, p=0.0005, Fig. 5**D**).

3.5.2. Burrowing test

OFIF supplementation recovered natural rodent burrowing behaviour in HFD rats. Indeed, HFD-OFIF rats started to first burrow significantly sooner when compared to HFD and resulted not significantly different from the NPD group ($F_{(2,17)}$=9.65, p=0.0016, Fig. 6**A**). However, the amount of gravel burrowed proved to be not significantly modified after OFIF supplementation in HFD animals, but this amount was reduced by both HFD and HFD-OFIF groups versus NPD ($F_{(2,17)}$ =5.99, p=0.0107, Fig. 6**B**).

3.6. Anxiety-associated and anhedonia-associated behaviour

3.6.1. Novelty-suppressed feeding test

Upon completion of the NSFT behavioural parameters were collected for evaluation of depression and anxiety-like traits. The evaluation of the latency to eat revealed that OFIF supplementation reduced the feeding time versus HFD and was not different from NPD ($F_{(2,16)}$=26.20, p<0.0001, Fig. 7**A**). Lastly, the evaluation of in-cage food intake in the following 10 min showed that no differences were encountered between HFD and NPD, and only HFD-OFIF group ate more once back in the cage ($F_{(2,17)}$=26.81, p<0.0001, Fig. 7**B**).

3.6.2. Saccharin preference test

No side preference emerged from the evaluation of water intake. Statistical differences in the consumption of saccharin were revealed in the experimental groups, supporting the idea of anhedonia-associated behaviour induced by HFD. In detail, both HFD and HFD-OFIF assumed less saccharin with respect to NPD, suggesting that OFIF does not manage to rescue depressive-like behaviour ($F_{(2,17)}$=6.76, p=0.0069, Fig. 7**C**).

3.6.3. Black-white box test

OFIF nutritional supplementation improved the anxiety-associated behaviour of HFD rats in the BWB test. Indeed, both the time spent and the number of entries in the light zone were notably higher in HFD-OFIF vs HFD (respectively: $F_{(2,17)}$=21.76, p<0.0001, Fig. 8**A** and $F_{(2,17)}$=7.85, p=0.0038, Fig. 8**B**). In particular, OFIF supplementation recovered the basal behaviour in terms of number of entries versus NPD, as shown by Bonferroni post-hoc test.

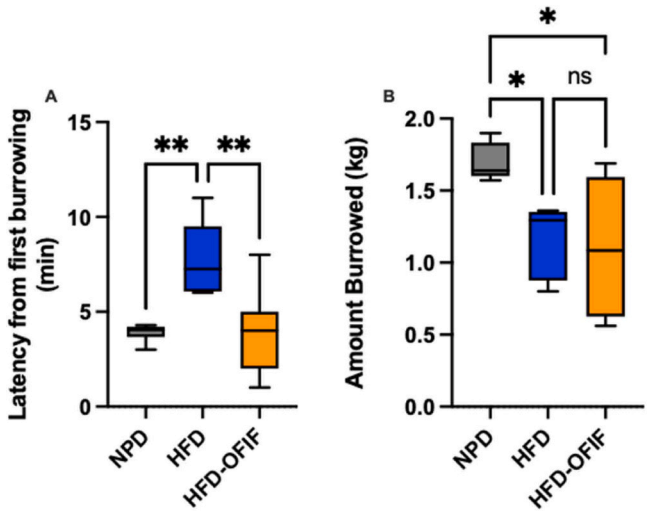

Fig. 6. Burrowing behaviour assessed in the NPD, HFD and HFD-OFIF rats. **A.** Latency from first burrowing (min) **B.** Amount of gravel burrowed (Kg). Statistical significance of Bonferroni post hoc tests are indicated for (*) $p<0.05$ and (**) $p<0.001$.

3.7. Learning and memory: object recognition test

The Object Recognition Test was utilised to evaluate declarative memory via the recognition of new objects compared to familiar ones. Initially, rats underwent training with two identical objects, and subsequent statistical analysis revealed no significant variation in the recognition index. This index indeed remained consistently around 50 % across all experimental groups (see Fig. 9**A**). After 1-hour retention, animals were exposed to a novel object and a familiar one. One-way ANOVA followed by Bonferroni post-hoc test showed that RI% of HFD-OFIF rats was higher than HFD alone and not significantly different from NPD ($F_{(2,17)}$=6.95, p=0.0062, Fig. 9**B**). Indeed, HFD rats did not manage to recognize the novel stimulus and spent more time in the familiar one (RI%<50 %) with respect to NPD (p<0.05).

3.8. Modulation of PI3K/Akt signalling in the hippocampus and in the hypothalamus

At hippocampal level, we observed a significant modulation of the PI3K/Akt signalling. One-way ANOVA followed by post-hoc test revealed a significant increase in p-Akt levels in HFD-OFIF group versus HFD alone, that was not different when compared to NPD control values ($F_{(2,17)}$ =7.91, p=0.0037, Fig. 10**A**).

In hypothalamic tissue, significant changes emerged in PI3K/Akt levels between the different experimental groups, with a particular increase after OFIF administration versus HFD alone and control NPD group ($F_{(2,17)}$ =9.18, p =0.002, Fig. 10**B**). Uncropped images of the original western blots are shown in Supplementary Fig. 1 and 2.

3.9. Effects of OFIF fruit on neuro-oxidative stress

As shown in Fig. 11, the statistical analyses performed by one-way ANOVA on MDA levels in the brain tissue showed significant differences between groups. In particular, OFIF administration significantly reduced the HFD-induced increase in MDA levels in comparison with the HFD group, though basal NPD values were not restored ($F_{(2,17)}$ =49.89, $p<0.0001$).

3.10. Correlation of biochemical, behavioural and biometric parameters

3.10.1. Spearman's correlation matrix of circulating leptin levels and MetS-induced alterations

A Spearman's correlation matrix was performed to evaluate the link between systemic leptin levels and the metabolic, cognitive, neuro-oxidative and redox homeostasis variables altered in MetS. Correlations emerged between different MetS parameters and leptin as indicated in Fig. 12. In particular, Spearman's correlation analysis showed a positive correlation between the altered leptin levels in the condition of induced MetS and the biomarkers of dysmetabolism, of prooxidant status and of cognitive impairment revealed by biochemical, behavioural and molecular assays. On the contrary, leptin levels are discorrelated with parameters related to antioxidant status and to improved cognitive and affective states.

Namely, a significant high-intensity positive correlation was found between leptin with total cholesterol (p=0.002; ρ=0.78), with the change in body weight (p=0.013; ρ=0.65), with food intake (p=0.046; ρ=0.68), with LDL cholesterol (p=0.005; ρ=0.72), with LP-cholox (p=0.002; ρ=0.77), with NSFT feeding time (p=0.001; ρ=0.81) and with brain MDA levels (p=0.005; ρ=0.83). Additionally, a medium-intensity positive correlation can be observed with pro-oxidant status assessed by the dROM test (p=0.028; ρ=0.59) and with triglycerides level though non-significant in this case (p=0.051; ρ=0.53).

On the other hand, leptin levels showed a significant strong negative correlation with the antioxidant status assessed by SH test (p=0.001; ρ=-0.81), with HDL cholesterol (p=0.014; ρ=-0.65), with hypothalamic marker of neurotrophic pathways (hypothalamic p-Akt p=0.009;

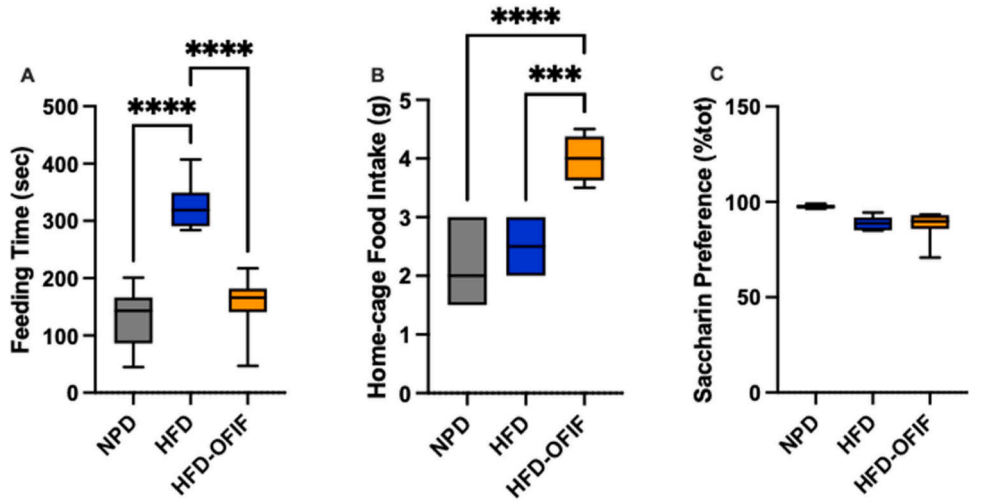

Fig. 7. Parameters of the novelty-suppressed feeding test (NSFT) and of the Saccharin preference test in the NPD, HFD and HFD-OFIF rats. **A.** Latency from feeding (feeding time, sec) **B.** Amount of food consumed back in the home-cage (food-intake, g). Statistical significance of Bonferroni post hoc tests are indicated for (***) $p<0.0001$ and (****) $p<0.0001$. **C.** The percentage of preference for saccharin solution out of total.

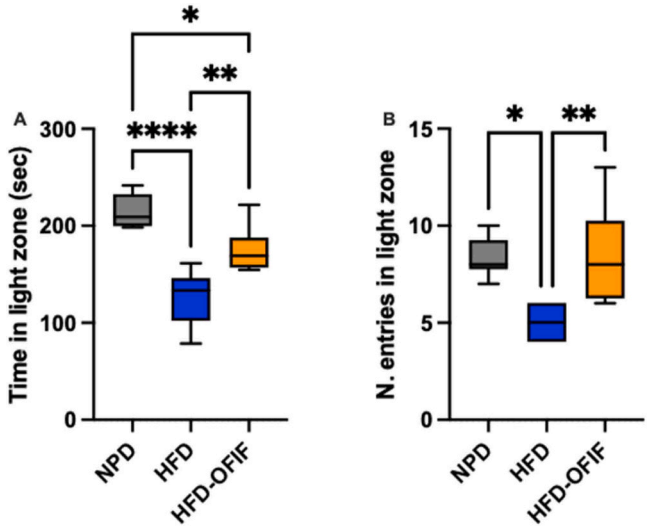

Fig. 8. Parameters of the dark-white box (DWB) in the NPD, HFD and HFD-OFIF rats. **A.** Time spent in the light zone (sec) **B.** Number of entries in the light zone. Statistical significance of Bonferroni post hoc tests are indicated for (*) $p<0.05$, (**) $p<0.001$ and (***) $p<0.0001$.

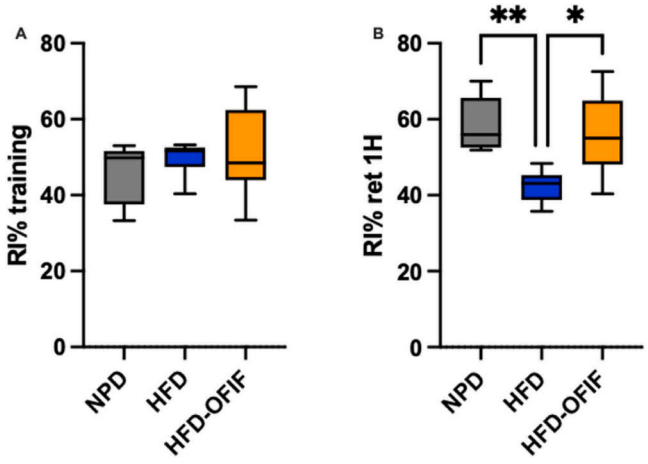

Fig. 9. Object recognition test evaluated in the NPD, HFD and HFD-OFIF groups. **A.** Recognition index (RI%) during training phase **B.** RI% after 1-hour retention (ret 1 h). Statistical significance of Bonferroni post hoc tests are indicated for (*) $p<0.05$ and (**) $p<0.001$.

ρ=-0.68) and with behavioural markers of cognitive and affective improvements (respectively: light-dark entries p<0.0001, ρ=-0.85; light-dark time p=0.02, ρ=-0.62; OFT centre entries p=0.001, ρ=-0.81; OFT time centre p=0.007, ρ=-0.70). Furthermore, leptin levels showed medium-intensity negative correlation with the antioxidant status assessed by BAP test (p=0.037 and ρ=-0.57), with hippocampal marker of neuroinflammation (hippocampal p-Akt p=0.046; ρ= −0.55) and with behavioural marker of declarative memory improvement (RI% retention p=0.048; ρ=-0.54).

3.10.2. Principal component analysis

PCA was carried out on the centred and scaled data of altered variables in MetS (Fig. 13). The first principal components (PC1) account for 58.12 % of the variance in the data. On the one hand, the variables contributing more markedly to PC1 with a highly positive loading are leptin (loading 0.928), NSFT feeding time (loading 0.883), LP-cholox (loading 0.837), body weight (0.773), LDL cholesterol (loading 0.738) and total cholesterol (loading 0.732). On the other hand, the variables with a highly negative loading along PC1 are OFT centre entries (loading −0.889), SHp values (loading −0.851), dark-white entries (loading −0.721) and OFT centre time (loading −0.713).

4. Discussion

Our research delves into the nuanced realm of cognitive alterations in MetS by examining the correlation of multi-dimensional biomarkers and the protective influence of OFIF administration in a HFD rat model. We explored multi-faceted parameters unravelling behavioural, molecular, cognitive, and metabolic alterations.

The *in vivo* supplementation for one month with OFIF twice a day to HFD rats induced beneficial effects on dysmetabolism and on the related alterations induced at systemic and central level. Relevantly, the dose of OFIF employed in the present study (14 mg/Kg) has been chosen in the perspective of a nutraceutical supplementation in humans. Indeed, the equivalent of the chosen dose to be administered to a 75 Kg human, could be contained into two capsules (1050 g) as a nutraceutical supplement.

Our findings unveil for the first time that OFIF effectively curbed the

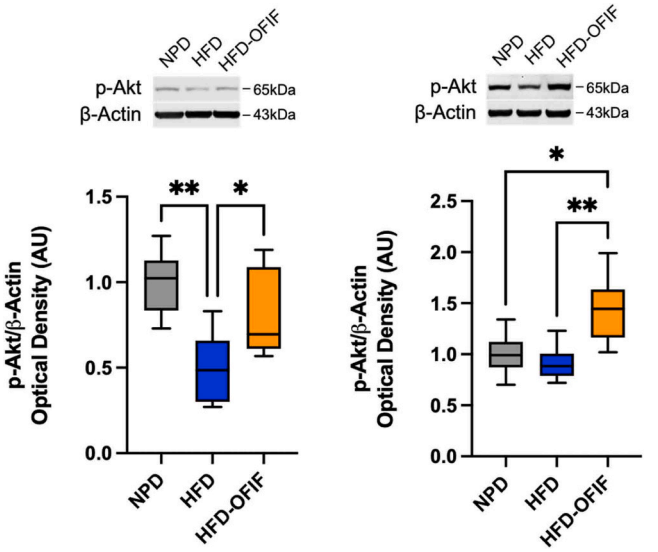

Fig. 10. Modulation of p-Akt activation by OFIF supplementation in the hippocampus and hypothalamus. **A.** Representative images of phosphorylated (p)-Akt and β-Actin Western blotting bands and histogram of p-Akt normalised to β-Actin Optical density in NPD, HFD and HFD-OFIF hippocampus **B.** Representative images of phosphorylated (p)-Akt and β-Actin Western blotting bands and histogram of p-Akt normalised to β-Actin Optical density in NPD, HFD and HFD-OFIF hypothalamus. Statistical significance of Bonferroni post hoc tests are indicated for (*) $p<0.05$ and (**) $p<0.001$.

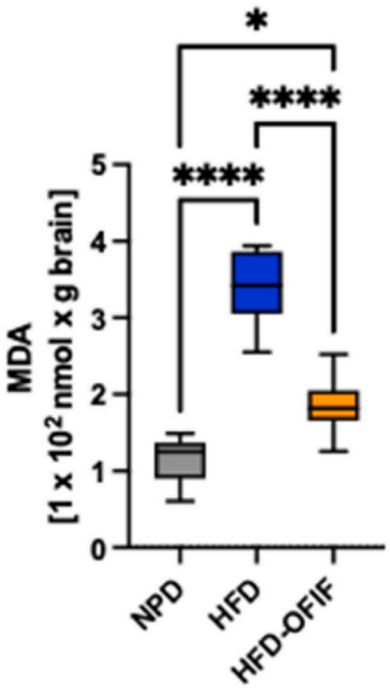

Fig. 11. Malondialdehyde (MDA) levels in the brain of HFD-OFIF, NPD and HFD groups. Statistical significance by one-way ANOVA followed by post hoc Bonferroni is indicated as (****) $p<0.0001$ and (*) $p<0.05$.

increase in body weight and concomitantly reduced the food-intake with respect to HFD rats. This action of OFIF on food intake could be due to multifaceted and synergic mechanisms, involving modulation of both leptin resistance, which will be discussed below, and modification of incretin pathways and appetite regulatory hormones like ghrelin, orexin and glucagon-like peptide 1 [63], as already emerged for prickly pear blades [64,65] that could explain food reward-related behaviours, including motivational components of eating [66,67]. In support of this hypothesis, it has been shown that OFIF fibers attenuate ghrelin-induced feeding behaviour [68], while anthocyanins, by modulating short-chain fatty acids (SCFAs) production, can be directly involved in the appetite suppression via a central homeostatic mechanism [69].

In addition to restoring biometric parameters in MetS, OFIF administration effectively modulates lipid dysmetabolism in a positive manner, significantly lowering plasma TG levels with respect to the HFD group. Remarkably, the OFIF treatment also restores total cholesterol, LDL and HDL plasma levels to control values.

While the lipid lowering effects of *OFI* cladodes and flowers are well-documented [70,71], this is the first report demonstrating the ability of OFIF, from the yellow cultivar, to exert such a complete and harmonic positive modulation of plasma lipid profile, with a remarkable effect on HDL levels and a reduction of TG, total cholesterol and LDL. We can speculate that, among others, the capability of OFIF active substances to regulate gut microbiota composition and SCFAs production [69], together with the content of fibers [41], can likely diminish lipid digestion and absorption and exert a trophic effect on gut epithelium though further research will be necessary to fully uncover OFIF gastrointestinal effects. Besides that, the presence of essential fat acids and flavonoids in OFIF, may trigger molecular mechanisms that activate protein kinase C (PKC) and peroxisome proliferator-activated receptors (PPAR), leading to the enhancement of plasma HDL levels and increased LDL clearance [9,72].

In addition to the evaluation of lipid profile, our study encompassed OFIF's impact on peripheral redox homeostasis employing robust plasma biomarkers. Antioxidant defences in terms of concentration of sulfhydryl proteins (SHp) were enhanced by OFIF consumption in HFD, recovering basal NPD values, indicating that OFIF supplementation restores the balance toward thiolic groups, which is disrupted by MetS. Additionally, the plasma antioxidant barriers were ameliorated by OFIF versus HFD, though still markedly lower than controls. This could be attributed to a compensatory mechanism mediated by OFIF, bolstering antioxidant defences to counteract excessive free radicals. OFIF has an antioxidant capacity for capturing free radicals, interfering with radicals' production and reducing oxidative stress [73–75] thanks to its remarkable amount and variety of anti-oxidative and redox-modulating molecules [76,77]. Flavonoids are the most abundant secondary metabolite class, with isorhamnetin the major flavonoid aglycone in most flavonoid conjugates. Organic and phenolic acids, such as (iso) citric acid, piscidic acid, ferulic acid and gluconic acid are also represented. In addition betaxanthins, with indicaxanthin as the major compound, coumarins with dihydroxy psoralen-O-hexoside and terpenes with hydroxy-megastigmen-one-hexoside complete the phytochemical profile of OFIF, that is also enriched by significant amounts of ascorbic acid, beta-carotene and tocopherols.

Consistent with previous studies, the evaluation of the antioxidant activity indicates that the *OFI* juice studied contain substances which are able to inhibit the action of free radicals as 2,2-Diphenyl-1-Picrylhydrazyl Radical (DPPH$^{\bullet}$)[78]. While OFIF treatment did not fully restore basal levels of antioxidant capacity of normally fed rats, it wholly recovered oxidative biomarkers, namely diminishing both hydroperoxides and lipoperoxides in HFD rats. This suggests a potent protection against plasma lipid peroxidation products. The antioxidant capacity of the phytonutrients present in OFIF can be responsible for blocking the formation of primary oxidation products such as hydroperoxides and lipoperoxides, thus inhibiting ferroptosis, a theory discovered in 2012 that lipoperoxidation-driven programmed cell death is responsible for the pathophysiology of various degenerative diseases [79,80]. It can be hypothesised that the antioxidant molecules present in OFIF are capable of slowing down the propagation phases of radical chain reactions and activate gene expression of the inducible antioxidant response [81]. In line with this hypothesis, we evaluated the antioxidant capacity of the Opuntia fruit by the crocin bleaching method [82], unravelling a strong ability of the lyophilised fruit to counteract the formation of

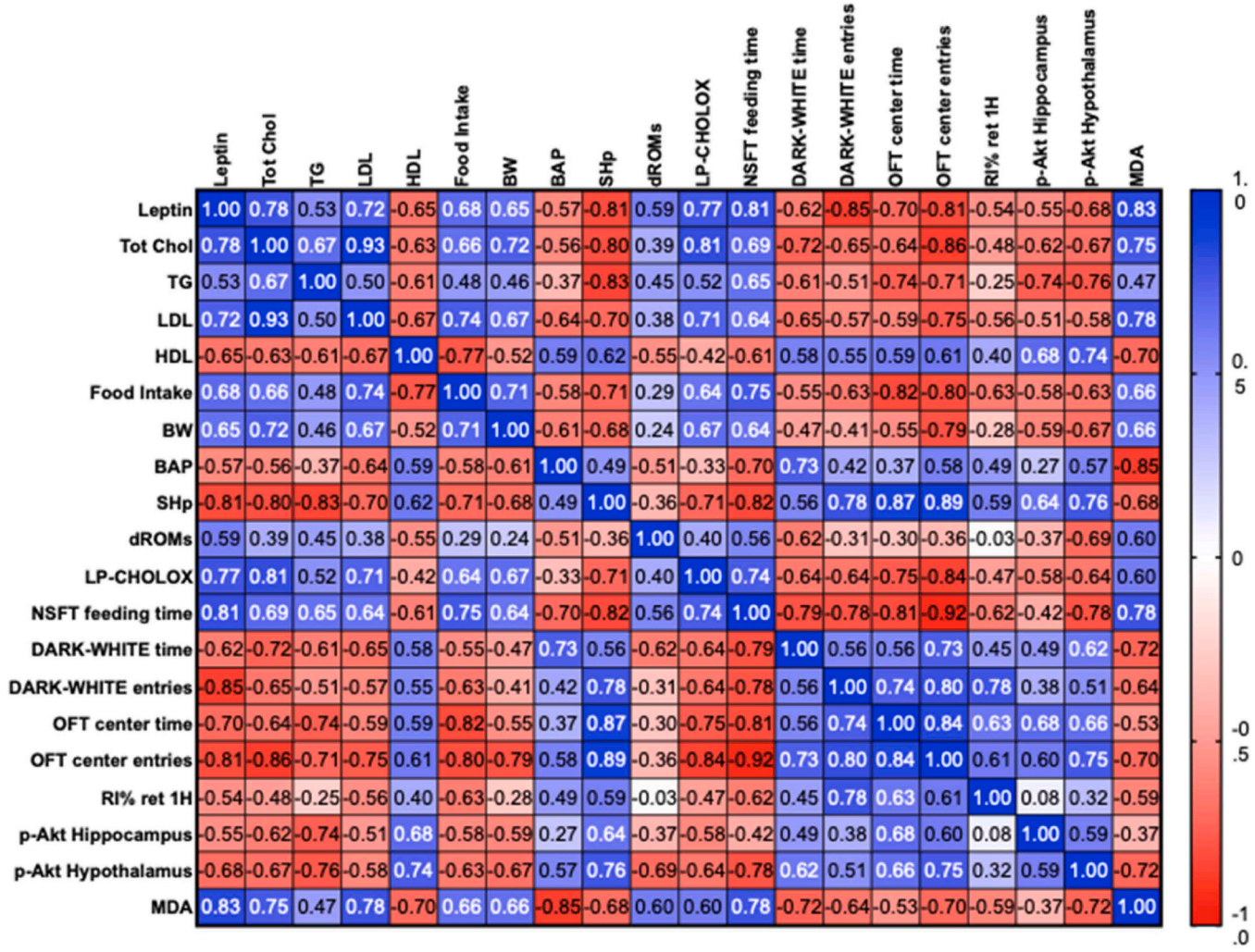

Fig. 12. Spearman's correlation matrix between leptin and different MetS parameters. The main parameters involved are total cholesterol (Tot Chol), triglycerides (TG), low density lipoprotein (LDL), high density lipoprotein (HDL), food intake, body weight (BW), thiol groups (SHp), biological antioxidant potential (BAP), hydroperoxide (dROM), lipoperoxides and oxidised cholesterol (LP-CHOLOX), feeding time in the novelty suppressed feeding test (NSFT), time and entries in the light zone during dark-white box test (dark-white time and dark-white entries), time and entries at the center during open field test (OFT center time and OFT center entries), recognition index after 1 hour retention (RI % ret 1H) of object recognition test (ORT), phosphorylated protein kinase B (p-Akt) in hippocampus and hypothalamus and brain malondialdehyde (MDA).

lipoperoxide radicals generated *in situ* by the radicalising agent (supplementary file).

Given the impact of OFIF on feeding behaviour, lipid homeostasis and oxidative stress, we investigated the potential involvement of circulating leptin. This hormone, produced by adipose tissue, governs feeding behaviour by acting on the hypothalamus, where it diminishes the perception of food reward and enhances the release of satiety signals [83]. Indeed, leptin is implicated in the modified oxy-inflammatory pattern that could account for numerous effects induced by MetS [84–86]. In accordance, our data obtained through ELISA staining revealed a notable reduction of systemic leptin levels in HFD rats supplemented with OFIF, that returned to baseline values. Excessive levels of leptin in the blood lead to reduced central sensitivity to leptin, namely leptin resistance. Several mechanisms underlying leptin resistance have been proposed, including the impairment of transport of circulating leptin across the blood-brain barrier and/or the deterioration in intracellular signalling downstream of the leptin receptor [87,88]. Leptin receptors are expressed widely throughout the brain, especially in the hypothalamus, involved in the regulation of feeding behaviour, and in regions associated with cognition, such as the hippocampus [87]. In the same brain regions, MetS leads to the development of leptin resistance, eliciting cognitive dysfunctions and depressive-like behaviours [89].

Leptin and insulin share common and strictly interconnected signal transduction pathways, utterly relevant in the central modulation of the eating behaviour. Along these lines, the currently observed effects exerted by OFIF on the reduction of food intake and on the lipid metabolism, may also be related to a reduction of brain insulin resistance by OFIF administration. Accordingly, a hallmark phytochemical of OFIF yellow cultivar, i.e. the betaxanthin indicaxanthin, has been recently shown to counteract HFD-induced peripheral insulin-resistance in murine model of metabolic syndrome [62].

We then investigated the cognitive and affective functions in the MetS experimental model and the putative effect exerted by OFIF consumption for 1 month. Rats initially were tested for their general locomotor activity and behavioural reactivity in the OFT maze. The OFIF supplementation in HFD rats enhanced exploration of the open field arena, also prolonging centre zone occupancy. Similarly, physiological burrowing behaviour was restored in the HFD-OFIF group, as evidenced by a shorter latency to initiate the test compared to HFD rats, although it did not alter the amount of gravel burrowed [90–92].

Brain impairments induced by MetS include alterations in anxiety-related and depressive-like behaviour [1,93–95], that OFIF

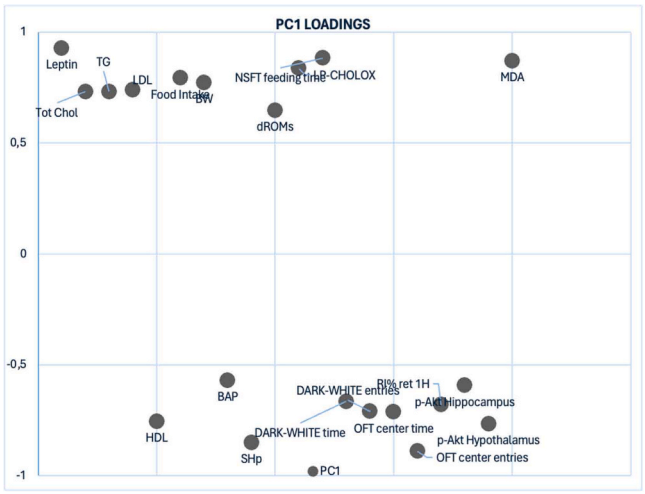

Fig. 13. Principal component analysis (PCA) of measured variables in MetS. The loadings of PCA with PC1 accounting for 58.12 % is shown on the x-axis, where the closer the parameters are to the borderlines the larger their impact is on the principal component individuated.

supplementation managed to rescue in our study. For instance, the deleterious effects of HFD on normal feeding latency in the NSFT following a 24-hour deprivation was restored in HFD-OFIF group. Interestingly, upon returning to their home cage, HFD-OFIF rats consumed more food than both NPD and HFD groups, possibly due to decreased activation of satiety centres and enhanced reward circuits, both indicative of reduced anxiety responses. In accordance with these promising data, in the BWB test HFD-OFIF rats spent significantly more time in the light zone and entered it more frequently. Both NSFT and BWB tests are closely associated with anxiogenic-like symptoms, suggesting that OFIF nutritional supplementation effectively reverses the dysfunctional phenotype induced by HFD. However, outcomes from the saccharin preference test did not align with previous findings, as no significant differences were observed after OFIF consumption in HFD rats. The potential effect of OFIF on the saccharin preference test may have been obscured by alterations in the palatability of sweet solutions. Conceivably, OFIF oral administration influenced anxiogenic-related behaviours by modulating leptin secretion and circulating levels, since circulating leptin levels have been found to counterbalance glucocorticoid levels, considered as mediating factors for MetS-aggravated affective impairment via increased HPA axis activity [94,96,97]. Noticeably, HFD dysregulates mesolimbic circuit interfering not only directly with dopaminergic but also with serotoninergic and peptidergic transmission, such as orexinergic transmission [67,98]. It was found that HFD decreases plasmatic serotonin levels [99] and modulate orexin neurons in the lateral hypothalamus, which are required for the activation of mesolimbic ventral tegmental area (VTA) [66,67]. Hypothetically, OFIF could have interfered with neurotransmissions projecting not only to VTA to other centres of the mesolimbic pathway known to mediate motivated behaviours, such as the prefrontal cortex [100]. Indeed, the production of SCAs, boosted by OFIF supplementation, may enhance the concentration of serotonin, thus significantly improving behavioural responses in HFD rats [101]. Moreover, OFIF was found to increase monoamine neurotransmitters in the brain, dopamine included [102].

Additionally, our study aimed to determine whether OFIF could mitigate the detrimental effects of HFD on the memory system, particularly the declarative memory network, using an OR task commonly employed in dysmetabolism models [97]. The data revealed that OFIF supplementation improved recognition of novel stimuli after one hour of exposure to two familiar objects. Consistent with existing literature, the heightened preference for novelty observed in the HFD-OFIF group may be attributed to enhanced activity in the dorsal hippocampus and connected hierarchical networks, including the perirhinal cortex and medial prefrontal cortex [97,103–105]. This is particularly intriguing considering that studies on memory impairment in experimental models of hypercaloric diets have proposed that a receptorial deficiency in leptin at hippocampal level induces alterations of OR behavioural task and even of long-term potentiation of synaptic plasticity [106,107].

Leptin can act through the components of the insulin signalling machinery, including the PI3k-Akt cascade [108]. p-Akt is a serine/threonine kinase involved in the transduction of mitogenic and metabolic signals and the subsequent regulation of different cellular processes, including proliferation, migration and apoptosis. In the brain, p-Akt signalling plays a key role in the modulation of neuronal survival, development, maintenance, repair and synaptogenesis. Dysregulation of p-Akt signalling leads to harmful consequences, such as increased oxidative stress, neuroinflammation, and decreased ATP production, which in turn are linked to the development of severe brain pathologies, including neurodegenerative diseases [109].

Brain leptin and insulin-mediated p-Akt signalling is impaired in subjects with MetS [27,110]. Accordingly, here we observed a decrease in p-Akt activation in the hippocampus of HFD rats, in line with the hypothesis of behavioural alterations driven by leptin dysregulation and leptin resistance. Noteworthy, OFIF supplementation recovers p-Akt levels, suggesting an effect on leptin homeostasis and its downstream signalling cascades and an overall trophic effect in brain regions involved in behaviours and cognition. In the hypothalamus, OFIF supplementation significantly increases p-Akt activation, even when compared to NPD rats, arguing for a specific modulation of insulin/leptin signalling and the recovery of energy homeostasis. This finding underscores the intricate relationship between neurotrophic signalling pathways and cognitive function in the context of MetS. Importantly, it sets the stage for a deeper exploration of the underlying mechanisms linking dysmetabolic states to neuronal alteration. In this regard, alterations of the CNS redox homeostasis are strictly interconnected with neurological disorders and cognitive impairments[111, 112]. Within the by-products of the oxidative dysmetabolism in the CNS, MDA has emerged as a key molecule. Indeed, MDA levels increase during HFD-induced oxy-inflammation and the aldehyde irreversibly forms adducts with macromolecules, modifying brain functions and contributing to neurological disorders development [113]. Consistently with the amelioration of the peripheral oxidative status and with the modulation of brain p-Akt signalling, involved in the activation of the antioxidant defence system [114], our results clearly show how OFIF administration also reduced the HFD-induced increase of brain lipid peroxidation, significantly reducing MDA levels. Accordingly, the currently observed ability of OFIF treatment to remarkably counteract the HFD-triggered cognitive and affective dysfunctions could be, at least in part, due to its anti-oxidative potential [10,11]. Within this scenario, these results appear remarkable as they demonstrate, for the first time in an *in vivo* context, the ability of OFIF to counteract lipid peroxidation in the CNS.

As already suggested, a possible elucidation for the beneficial effects of OFIF on the impairment induced by MetS, especially concerning central mechanisms, might lie in the alteration of leptin homeostasis. To corroborate this hypothesis, in the present study we run a cross-correlation analysis and a PCA to identify eventual linking points between leptin, lipid dysmetabolism, oxidative stress and cognitive alterations. This approach uncovered for the first time, to the best of our knowledge, that OFIF modulation of circulating leptin levels positively correlates with markers related to dyslipidemia, with oxidative stress at systemic and brain level and with behavioural parameters related to anxiety-like behaviour. On the other hand, leptin levels reduced by OFIF are associated with improved antioxidant barriers, declarative memory, response to stress and anxiety, and ameliorated hippocampal and hypothalamic p-Akt pathways. In addition, the PCA revealed that detrimental biomarkers of MetS, leptin above all, show an integrated behaviour that negatively correlates with multifaceted parameters

related to cognitive impairment in MetS. Taken collectively, our research underscores the potential involvement of leptin homeostasis as a fundamental mechanistic pathway in linking impaired cognitive and affective functions with metabolic syndrome, beyond the influence of oxidative stress. Ultimately, leptin-mediated cascade results in deficits in neuroplasticity within brain regions like the hippocampus, thereby precipitating behaviours characteristic of depression and anxiety [115, 116]. Lack of functional leptin receptors was found to drive hyperphagia-induced obesity and other traits of MetS, hypothesizeably due to interaction with insulin to inhibit GSK3 and enhancement of the actions of NMDA receptors to facilitate the generation of LTP in the hippocampus [117–119]. Indeed, the decrease in plasma leptin levels determined by OFIF could improve hypothalamic sensitivity to leptin that in turn could restore glucose homeostasis enhancing hypothalamic glucose sensing in HFD-induced conditions [120,121]. This could also explain the reduction here encountered in body weight and food intake since glucose, insulin and leptin signalling could constitute an intertwined switch that affects satiety. Disrupted leptin signalling, especially at hypothalamic level, may stem from alterations in synaptic and intracellular trafficking, crucial physiopathological regulators of cellular processes [122,123]. Even though there is no concerted opinion on the direct influence of leptin on spatial memory in MetS traits [15], our findings corroborate a plausible involvement of leptin in both cognitive and affective symptoms modulated by OFIF in HFD model, thereby elucidating the association between metabolic and cognitive alterations. Notwithstanding the fact that our study provides valuable insights into the potential role of leptin homeostasis, it should be acknowledged as a pitfall of this research that further examination of leptin receptor activity within the central nervous system, together with a specific investigation on insulin resistance and glucoregulatory impact at hypothalamic level, will be essential for elucidating the precise mechanisms underlying the relationship here suggested.

5. Conclusion

Our study provides robust evidence that OFIF supplementation can effectively mitigate cognitive alterations and associated metabolic and biochemical dysfunctions in a HFD rat model. The significant findings of this research include the ability of OFIF to curb body weight, reduce food intake, and positively modulate plasma lipid profiles, particularly enhancing HDL levels. These results highlight OFIF's potential as a nutraceutical intervention for improving metabolic health. Furthermore, OFIF's antioxidant properties were shown to restore peripheral redox balance and significantly reduce biomarkers of oxidative stress, indicating its protective role against lipid peroxidation and associated oxidative damage in the brain. Importantly, OFIF supplementation normalised systemic leptin levels that were found to correlate with improved cognitive functions, including memory and anxiety-related behaviours. Our data suggest that OFIF influences the mesolimbic and prefrontal cortex pathways, potentially impacting motivational and hedonic components of feeding behaviour. Moreover, the antioxidative effects of OFIF point to its potential role in preventing oxidative stress-related neuronal damage, which could have broader implications for neurodegenerative diseases beyond MetS. Future research should delve deeper to provide critical insights into how dietary supplements like OFIF can restore metabolic and cognitive health. Additionally, exploring the potential of OFIF could be in future evaluated in healthy subjects to translate these findings into interventions for individuals with MetS. In conclusion, OFIF demonstrates by targeting leptin homeostasis and oxidative stress, promising potential as a nutraceutical intervention to counteract cognitive and metabolic impairments associated with MetS.

Informed consent statement

Not applicable.

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CRediT authorship contribution statement

Miriana Scordino: Writing – review & editing, Investigation. **Alessandro Massaro:** Writing – review & editing, Investigation. **Monica Frinchi:** Writing – review & editing, Methodology, Investigation. **Giulia Urone:** Writing – review & editing, Investigation. **Pierangelo Sardo:** Writing – review & editing, Supervision, Resources, Investigation. **Giuseppe Giglia:** Writing – review & editing, Resources, Methodology, Investigation, Funding acquisition. **Danila Di Majo:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Giuseppa Mudò:** Writing – review & editing, Supervision, Resources, Investigation. **Giuseppe Ferraro:** Writing – review & editing, Supervision, Resources, Investigation. **Mario Allegra:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Giuditta Gambino:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nicolò Ricciardi:** Writing – original draft, Methodology, Investigation, Data curation. **Valentina Di Liberto:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117028.

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