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Calorie restriction modulates mitochondrial dynamics and autophagy in leukocytes of patients with obesity

Zaida Abad-Jiménez [a,1], Sandra López-Domènech [a,1], María Pelechá [a], Laura Perea-Galera [a], Susana Rovira-Llopis [b], Celia Bañuls [a], Ana Blas-García [c,d], Nadezda Apostolova [c,d], Carlos Morillas [a], Víctor Manuel Víctor [b,c,**], Milagros Rocha [a,c,*]

[a] Department of Endocrinology and Nutrition University Hospital Doctor Peset, Foundation for the Promotion of Health and Biomedical Research (FISABIO), 46017, Valencia, Spain
[b] Department of Physiology, Faculty of Medicine & Dentistry, University of Valencia, 46010, Valencia, Spain
[c] CIBEREHD (Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas), 28029, Madrid, Spain
[d] Department of Pharmacology, Faculty of Medicine & Dentistry, University of Valencia, 46010, Valencia, Spain

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ABSTRACT

Background: Although it is established that caloric restriction offers metabolic and clinical benefits, the molecular mechanisms underlying these effects remain unclear. Thus, this study aimed to investigate whether caloric restriction can modulate mitochondrial function and remodeling and stimulate autophagic flux in the PBMCs of patients with obesity.

Methods: This was an interventional study of 38 obese subjects (BMI >35 kg/m^2) who underwent 6 months of dietary therapy, including a 6-week very-low-calorie diet (VLCD) followed by an 18-week low-calorie diet (LCD). We determined clinical variables, mitochondrial function parameters (by fluorescence imaging of mitochondrial ROS and membrane potential), and protein expression of markers of mitochondrial dynamics (MNF1, MFN2, OPA, DRP1 and FIS1) and autophagy (LC3, Beclin, BCL2 and NBR1) by Western blot.

Results: Caloric restriction induced an improvement in metabolic outcomes that was accompanied by an increase in AMPK expression, a decrease of mitochondrial ROS and mitochondrial membrane potential, which was associated with increased markers of mitochondrial dynamics (MFN2, DRP1 and FIS1) and activation of autophagy as evidenced by augmented LC3 II/I, Beclin1 and NBR1, and a decrease in BCL2.

Conclusion: These findings shed light on the specific molecular mechanisms by which caloric restriction facilitates metabolic improvements, highlighting the relevance of pathways involving energy homeostasis and cell recovery, including mitochondrial function and dynamics and autophagy.

1. Introduction

Obesity is a chronic disease characterized by excessive body fat, defined according to the World Health Organization as a Body Mass Index (BMI) $\geq$ 30 kg/m^2. It is associated with an increased risk of mortality and the presence of a range of metabolic comorbidities including cardiovascular diseases (CVDs), non-alcoholic fatty liver disease and type 2 diabetes (T2D) [1].

It is known that obesity is closely related to oxidative stress, a harmful phenomenon involving an imbalance between the production of reactive oxygen species (ROS) and the organism's ability to neutralize them [2]. In particular, mitochondria play a crucial role in the development of oxidative stress, as mitochondrial dysfunction is characterized by altered mitochondrial membrane potential, increased electron leakage and an excess release of mitochondrial ROS (mtROS) [3]. Activated leukocytes in obesity present a prooxidant and proinflammatory phenotype that promotes mitochondrial dysfunction [4,5] and plays an essential role in adipose tissue dysfunction [6,7].

Mitochondria serve as pivotal organelles governing cellular energy

* Corresponding author. Department of Endocrinology and Nutrition University Hospital Doctor Peset, Foundation for the Promotion of Health and Biomedical Research (FISABIO), Juan de Garay nº21, 46017, Valencia, Spain.
** Corresponding author. Department of Physiology, Faculty of Medicine & Dentistry, University of Valencia, Av. Blasco Ibáñez nº15, 46010, Valencia, Spain.
E-mail addresses: victor.victor@uv.es (V.M. Víctor), milagros.rocha@fisabio.es (M. Rocha).
[1] These authors have contributed equally.

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Abbreviations

AMPK	AMP-Activated Protein Kinase
BCL2	B-cell lymphoma 2
Beclin1	BCL2-Interacting protein 1
BMI	Body Mass Index
CVDs	Cardiovascular Disease(s)
DBP	Diastolic Blood Pressure
DRP1	Dynamin-Related Protein 1
EWL	Excess Weight Loss
FIS1	Mitochondrial Fission Protein 1
HbA1c	Glycated Haemoglobin
HDLc	High Density Lipoprotein cholesterol
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance index
hsCRP	high-sensitivity C-Reactive Protein
IL6	Interleukin-6
LC3	Microtubule-associated proteins 1A/1B-light chain 3
LCD	Low-Calorie Diet
LDLc	Low Density Lipoprotein cholesterol
MFF	Mitochondrial Fission Factor
MFN	Mitofusin
mtROS	mitochondrial Reactive Oxygen Species
NBR1	Neighbor of BRCA1 gene 1
NF-κB	Nuclear Factor kappa B
OPA1	Optic Atrophy 1
PBMCs	Peripheral Blood Mononuclear Cell(s)
RFM	Relative Fat Mass
RFU	Relative Fluorescence Units
ROS	Reactive Oxygen Species
SBP	Systolic Blood Pressure
T2D	Type 2 Diabetes
TC	Total Cholesterol
TG	Triglycerides
TMRM	Tetramethylrhodamine methyl ester
TNFα	Tumor Necrosis Factor-alpha
VLCD	Very-Low-Calorie Diet
WHtR	Waist-to-Height Ratio

and homeostasis. Consequently, the removal of impaired mitochondria becomes imperative to preserve metabolic health. In this regard, mitochondrial dynamics modulate the intricate equilibrium between mitochondrial fusion and fission. Fusion, facilitated by proteins like mitofusins 1 and 2 (MFN1, MFN2) and optic atrophy 1 (OPA1), merges mitochondria to maintain a functional network. Conversely, fission, mediated by proteins such as dynamin-related protein 1 (DRP1), mitochondrial fission protein 1 (FIS1) and mitochondrial fission factor (MFF), divides mitochondria for diverse cellular functions [8]. This dynamic interplay enables adaptation to metabolic demands and cellular stress and ensures mitochondrial quality control. Notably, during periods of caloric restriction, mitochondria tend to fuse and share structural components, while they remain fragmented in a state of overfeeding [9].

Selective autophagy plays a vital role in maintaining cellular health by targeting and removing damaged organelles, including mitochondria. It is activated, not only in response to damaged cellular components, but also by nutrient deprivation or an altered AMP/ATP ratio, which triggers AMP-activated protein kinase (AMPK) activation. This initiates a cascade involving membrane elongation by the phosphatidyl inositol 3-kinase (PI3K)/coiled-coil moesin-like, BCL2-interacting protein 1 (Beclin1) complex and autophagy-related proteins. These proteins facilitate the conversion of microtubule-associated protein 1A/1B-light chain 3 (LC3) I into LC3II, crucial for cargo binding by sequestosome 1 (p62) or neighbor of the BRCA1 gene (NBR1) proteins, thereby directing them to the autophagosome for degradation [10,11]. Induction of autophagy has emerged as a protective mechanism against proinflammatory [12] and oxidative stress responses [13] in peripheral blood mononuclear cells (PBMCs) of patients with T2D, suggesting that this pathway is implicated in obesity.

Calorie restriction and intermittent fasting have gained scientific and public interest in recent years as effective strategies for the treatment of obesity. More specifically, hypocaloric diets offer a non-pharmacological and non-surgical approach that triggers molecular changes related to the expansion of adipose tissue, the regulation of adipokines, and metabolic adaptations [14,15]. Indeed, accumulated evidence suggests that calorie deprivation can lead to the induction of adaptive autophagy in several tissues [16]. However, the precise molecular mechanisms associated with this phenomenon remain unclear.

On the basis of the aforementioned research, we aimed to determine whether, beyond its known metabolic and clinical benefits, caloric restriction can modulate mitochondrial function and remodeling and stimulate autophagic flux in PBMCs of patients with obesity.

2. Materials and methods

2.1. Study population

The patients enrolled in this interventional study were eligible to undergo a dietary weight loss intervention. Recruitment and follow-up were carried out in the Department of Endocrinology and Nutrition in the Outpatient's Clinic of University Hospital Dr. Peset (Valencia, Spain). The study protocol was approved by the Hospital's Human Research Ethics Committee (code 96/16) and conducted according to the guidelines of the Declaration of Helsinki. Each participant received information about the study and provided their written consent to participate.

To be eligible for the study, patients had to be between 18 and 60 years old, with a BMI $\geq$35 kg/m^2 and needed to have maintained a stable weight ($\pm$2 kg) for the three months prior to the study. Exclusion criteria were pregnancy or lactation, active infectious disease, severe diseaseor chronic inflammatory disease, and secondary obesity (Cushing's syndrome). All patients underwent a baseline examination, and the study variables were recorded at baseline and six months after the nutritional intervention.

The dietary weight loss intervention was designed in accordance with the guidelines of the Spanish Society for the Study of Obesity (SEEDO) [17]. Following an initial assessment, patients underwent a treatment protocol comprised of a 6-week Very-Low-Calorie Diet (VLCD) followed by an 18-week Low-Calorie Diet (LCD). The VLCD, in a liquid formula (Optisource Plus®, Nestlé S.A., Switzerland), consisted of 52.8 g of proteins, 75.0 g of carbohydrates, 13.5 g of fat, and 11.4 g of fibre, along with essential vitamins and minerals based on the Recommended Dietary Allowances. This formula provided a total energy of 2738 kJ/day (654 kcal/day). As part of the study protocol, the participants replaced their three regular daily meals with this VLCD formula, which was provided by the National Public Healthcare System following prescription by the endocrinologist. After the initial 6-week period, the patients met the Nutrition Service to receive dietary guidance regarding the LCD. The caloric requirements of each participant were calculated taking into account their sex, height, weight and physical activity level. Based on these calculations, participants were prescribed a personalized 18-week LCD based on the Mediterranean diet and with an average daily energy intake ranging from 5023 to 7535 kJ (1200–1800 kcal). No food was provided at this stage, although the participants received comprehensive written and verbal guidance regarding their dietary intake, which included precise quantities and cooking techniques. They were

advised to ensure a minimum daily intake of 2 L of calorie-free fluids, preferably water, and were motivated to continue with their regular physical routines. In addition, patients were encouraged to call (when necessary) for dietary counselling when they encountered difficulties following the diet (type of food, etc …), and were also encouraged to take photos of their meals so as to keep a record of the size of the meals. Adherence to the diet was monitored by means of an interview with the dietician after the VLCD and at the end of the experimental period. Patients were re-evaluated following completion of the experimental period. No changes were made to their medicine prescriptions throughout the duration of the study.

2.2. Clinical and biochemical determinations

During the physical examination, weight, height, systolic and diastolic blood pressure (SBP, DBP) and waist circumference were measured with anelectronic scale, a stadiometer, a sphygmomanometer and a metric measuring tape, respectively. BMI was calculated by dividing weight (kg) by height (in meters squared). The percentage of Excess Weight Loss (EWL) was determined using the formula: [(baseline weight − final weight)/(baseline weight − ideal weight (considering BMI = 25 kg/m^2))] × 100. The Relative Fat Mass (RFM) was calculated according to the formula described by Woolcott & Bergman [18]. The Waist-to-Height Ratio (WHtR) was calculated by dividing the waist circumference by the height (both in cm).

Blood samples were collected from the antecubital vein in fasting conditions at baseline and after the weight loss intervention. Biochemical determinations were performed at the Hospital's Clinical Analysis Department, as follows: glucose, total cholesterol (TC), and triglyceride (TG) serum levels were determined by enzymatic assay; HDL cholesterol (HDLc) concentration was measured using a Beckman LX20 analyser (Beckman Coulter Inc., Brea, CA, USA); and LDL cholesterol (LDLc) was calculated by Friedewald's formula when circulating levels of TG did not exceed 300 mg/dL. The percentage of glycated haemoglobin (HbA1c) was obtained with a glycohaemoglobin analyser (Arkray Inc., Kyoto, Japan). Insulin was determined by an immunochemiluminescence assay and the homeostatic model assessment for insulin resistance index (HOMA-IR), which was calculated by the formula ([fasting insulin (μUI/mL) × fasting glucose (mg/dL)]/405). Systemic levels of C3 fraction of the complement (C3c) were analysed using an immunonephelometric assay (Behring Nephelometer II, Dade Behring, Inc., Newark, DE, USA) with an intra-assay coefficient of <5.5 % variation). The remaining serum aliquots were immediately stored at −80 °C for subsequent analysis.

2.3. Isolation of PBMCs

Blood samples were collected using BD Vacutainer® citrated tubes. Blood samples were carefully layered over Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) and centrifuged at 650g for 25 min at RT. The resulting halo of PBMCs was collected and centrifuged for 10 min at 650g. After centrifugation, the remaining erythrocytes were lysed (RBC Lysis Buffer, Sigma-Aldrich, Inc., St. Louis, MO, USA). The resulting pellet was then washed and suspended in HBSS (Capricorn Scientific, Ebsdorfergrund, Germany).The LUNA-FL system (Logos Biosystems Inc., Annandale, VA, USA) was used to determine cell count and viability by means of the acridine orange and propidium iodide double stain method.

2.4. Fluorescence microscopy

To determine mitochondrial superoxide production levels and mitochondrial membrane potential, a fluorometric method was applied using an IX81 Olympus fluorescence microscope (Olympus, Hamburg, Germany). PBMCs were seeded in a 48-well plaque and incubated for 30 min at 37 °C with MitoSOX red (5 μM) and Tetramethylrhodamine methyl ester (TMRM, 5 μM) to determine mitochondrial superoxide production levels and mitochondrial membrane potential, respectively. Cell nuclei were visualized using the fluorescent probeHoechst 33342. All fluorescent dyes were purchased from Life Technologies (Thermo Fisher Scientific, Waltham, MA, USA). Static cytometry software ScanR version 2.03.2 (Olympus, Hamburg, Germany) was employed to analyze a total of 20 images per well.

2.5. Western blotting

Whole-cell proteins were extracted from PBMCs, as described by Abad-Jiménez et al. [19]. A total of 25 μg of protein were resolved by electrophoresis in a SDS-polyacrylamide gel and then transferred onto a nitrocellulose membrane. The membranes were blocked for 1h with 5 % skimmed milk in TBS-T and then incubated overnight at 4 °C with the following primary antibodies: monoclonal anti-AMPK, monoclonal anti-AMPKα1 (phospho T183) α2 (phospho T172) and monoclonal anti-Beclin1 (all three from Abcam, Cambridge, UK), polyclonal anti-LC3A/B from Cell Signaling Technology (Danvers, MA, USA), monoclonal anti-B-cell lymphoma 2 (BCL2) from BD Biosciences, polyclonal anti-NBR1 from Proteintech (Rosemont, IL, USA), monoclonal anti-OPA1, polyclonal anti-MFN1, polyclonal anti-MFN2, polyclonal anti-DRP1 and polyclonal anti-FIS1from Merck-Millipore (Burlington, MA, USA). Mouse monoclonal anti-actin (Cell Signaling Technology, Danvers, MA, USA) or rabbit polyclonal anti-actin (Sigma-Aldrich, San Luis, MO, USA) were used as loading controls.

The chemiluminescence signal was detected with the ECL plus reagent (GE Healthcare, Little Chalfont, UK) after a binding step with the appropriate secondary antibodies: goat anti-rabbit from Vector Laboratories (Burlingame, CA, USA) or goat anti-mouse from Thermo Fisher Scientific (Waltham, MA, USA). The Fusion FX5 Acquisition System permitted visualization and the software Bio1D version 15.03a (Vilbert Lourmat, Marne-la-Vallée, France) was employed to quantify the band signal by densitometry.

2.6. Statistical analysis

This study was designed to achieve a power of 80 % and detect significant ($p < 0.05$) differences of 20 % in relation to the primary efficacy criterion –protein detection by Western blot– assuming a common standard deviation (SD) of 25 units. To ensure a loss-to-follow-up rate of 0 %, a minimum of 13 patients were required based on these premises. Statistical analysis was performed using SPSS v20.0 (IBM SPSS Statistic, Chicago, IL, USA) and GraphpadPrism v9.0.0 (GraphPad Software, Boston, MA, USA) softwares. Parametric data are expressed as the mean ± standard deviation (SD) or mean +standard error (SE), while non-parametric data are presented as median and interquartile range (25 % and 75 % percentile). A paired Student's t-test was used to compare parametric variables, whereas a Wilcoxon test was used for non-parametric data. The strength of the association between variables was measured with Pearson's or Spearman's correlation coefficient. All the tests were conducted at a confidence interval of 95 %, and the significance threshold for all the analyses was set at $p < 0.05$.

3. Results

This study consisted of a total of 38 patients, 74 % of whom were women. Mean age was 45.7 ± 9.0 years and BMI was 45.0 ± 6.3 at baseline. The percentage of patients prescribed medications for common comorbidities in obesity including arterial hypertension, hyperlipidemia and T2D was recorded (Table 1).

After 6 months of the intervention, the study subjects exhibited a moderate weight loss of 10.5 ± 4.2 %with a significant decrease in BMI levels ($p < 0.001$), waist ($p < 0.001$), and DBP ($p < 0.01$). The RFM and the WHtR were reduced in 4.9 % and 9.3 % respectively, suggesting an improvement in body mass composition. Regarding glucose metabolism,

Table 1
Clinical and biochemical parameters of the cohort population before and after the dietary intervention.

	Before	After	Normal Range
n (females %)	38 (74)		
Age (years)	45.7 ± 9.0		
Weight (kg)	121.8 ± 20.9	108.7 ± 17.8 ***	
BMI (kg/m^2)	45.0 ± 6.3	40.1 ± 5.1 ***	
EWL (%)		24.1 ± 8.3	
Waist (cm)	122.5 ± 15.1	111.1 ± 12.7 ***	
RFM	45.7 ± 5.8	43.0 ± 5.8***	
WHtR	0.74 ± 0.08	0.67 ± 0.06***	
SBP (mmHg)	132.4 ± 17.2	127.6 ± 15.9	
DBP (mmHg)	83.4 ± 9.6	77.6 ± 8.6 **	
Glucose (mg/dL)	99.2 ± 20.7	92.5 ± 13.2 **	(70–105)
Insulin (μU/mL)	15.8 ± 7.9	12.7 ± 6.8 ***	(1.7–31.0)
HOMA-IR	4.0 ± 2.2	2.9 ± 1.6 ***	
HbA1c (%)	5.7 ± 0.6	5.4 ± 0.4 ***	(4.0–6.0)
TC (mg/dL)	183.5 ± 36.2	189.9 ± 42.8	(80–200)
HDLc (mg/dL)	43.0 ± 10.5	45.8 ± 11.2 *	(45–70)
LDLc (mg/dL)	115.7 ± 32.2	121.5 ± 37.0	(40–150)
TG (mg/dL)	125 (88, 149)	103 (79, 130) *	(30–150)
C3c (mg/L)	136.7 ± 36.3	121.2 ± 19.5 **	(90–180)
hsCRP (mg/L)	11.54 ± 10.95	9.33 ± 6.93	(0.0–1.69)
Treatment			
Hypertension % (n)	21 (8)		
Hyperlipidemia % (n)	26 (10)		
T2D % (n)	8 (3)		

Data are expressed as mean ± SD except for TG which was are represented as median and IQ range (25 % and 75 % percentile) or n (percentage %). Values were statistically compared with a paired Student's t-test or Wilcoxon test and were considered significant when * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. The normal ranges cited correspond with data provided by the Clinical Analysis Department of University Hospital Dr. Peset (Valencia, Spain). BMI: Body Mass Index, C3c: Complement component 3, DBP: Diastolic Blood Pressure, EWL: Excess Weight Loss; HbA1c: glycated haemoglobin, HDLc: High Density Lipoprotein cholesterol, hsCRP: high-sensitivity C-Reactive Protein, LDLc: Low Density Lipoprotein cholesterol, RFM: Relative Fat Mass, SBP: Systolic Blood Pressure, TC: Total Cholesterol, TG: Triglycerides, WHtR: Waist-to-Height Ratio.

a significant improvement in systemic levels of glucose ($p < 0.01$), insulin, HbA1c and HOMA-IR ($p < 0.001$ for all) was observed after treatment. Similarly, we detected a slight improvement in lipid profile, with a significant increase of HDLc and a decrease in TG content post-intervention ($p < 0.05$ for both), although no significant changes were found in TC or LDLc levels. Moreover, there was a significant decrease in the levels of inflammatory reactant C3c after 6 months ($p < 0.01$).

Changes in an individual's nutritional status can modulate intracellular responses to energy expenditure adaptation. In this sense, we observed a significant increase in the activation of the central energy sensor AMPK (Fig. 1a-b, $p < 0.05$) after moderate weight loss, represented by an increase in the Phosphorylated:Total AMPK protein ratio. In parallel, we detected changes in the mitochondrial function of PBMCs; fluorescence microscopy analysis revealed a significant decrease in mtROS production (Fig. 1c) and mitochondrial membrane potential (Fig. 1d) after 6 months of weight (both $p < 0.05$). These results suggest the ability of calorie restriction to induce a nutritional shift through the activation of AMPK and modulation of mitochondrial function in immune cells.

Since changes in mitochondrial function are closely related to mitochondrial network remodeling, we subsequently investigated whether the dietary weight loss intervention could modulate mitochondrial dynamics processes (Fig. 2).

In this regard, we observed that the expression of the mitochondrial fusion-related proteins OPA1 and MFN1 was not modified (Fig. 2a and b, respectively), whereas a significant increase in MFN2 protein levels was detected (Fig. 2c, $p < 0.05$).When we focused on mitochondrial fission, we observed that the intervention modulated the response to this mitochondrial process through a significant increase in the expression of FIS1 (Fig. 2d, $p < 0.01$) and DRP1 (Fig. 2e, $p < 0.05$), suggesting that the intervention stimulated mitochondrial network remodeling in PBMCs of patients with obesity.

A key function of mitochondrial fission is the segregation of damaged mitochondria for degradation by autophagy. Our results revealed a significant increase in the ratio of LC3II/I (Fig. 3a, $p < 0.05$)– an indicator of autophagosome maturation –and in the expression of Beclin1 (Fig. 3b) – a key protein in the initiation of autophagosome formation – after the nutritional intervention, though the latter marker did not reach statistical significance. In parallel, following the intervention, we observed a significant decrease in the expression of BCL2 (Fig. 3c, $p < 0.05$), a downregulator of autophagy, and augmented levels of the autophagy receptor NBR1 (Fig. 3d, $p < 0.01$). These changes suggest that diet-induced weight loss could stimulate autophagy in PBMCs of patients with obesity, as an adaptive cellular response to nutrient deprivation.

Afterward, we sought to explore the relationship between the biochemical-clinical parameters related to obesity and metabolic disease and the intracellular markers evaluated in the PBMCs of the study participants. The main correlations found between variables at both baseline and post-treatment evaluation time points are shown in Fig. 4.

As expected, we observed positive correlations between indices related to body weight and composition (weight, RFM, WHtR) and parameters associated with metabolic syndrome, including blood pressure, glucose metabolism, and inflammation (SBP, fasting glucose, HbA1c, hsCRP), both before and after the intervention. Interestingly, we also found a negative association between some of these obesity-related clinical features and the protein expression levels of MFN2 and LC3II/I at baseline, suggesting that the detrimental metabolic status associated with obesity may be related to impaired mitochondrial network fusion and autophagy. Furthermore, the degree of activation of AMPK was found to be positively correlated with LC3 II/I and Beclin1 at baseline and with MFN2 after treatment, which may confirm the capability of this nutrient sensor to regulate autophagy and mitochondrial dynamics.

4. Discussion

Obesity is a growing concern worldwide. Calorie restriction is a recognized effective strategy for the treatment of obesity, especially in terms of weight loss and mitigation of cardiometabolic risk, though the molecular mechanisms underlying its effects are not fully elucidated. Our results indicate that nutritional intervention not only promotes the activation of intracellular pathways such as AMPK, mitochondrial dynamics and autophagy, but also facilitates the partial restoration of mitochondrial function. It is plausible that these intricate cellular mechanisms contribute significantly to the systemic health benefits observed after such interventions.

The relationship between obesity and high-calorie diets andmetabolic disorders such as insulin resistance, T2D and dyslipidemia is well demonstrated [20]. However, there is a growing need to investigate the advantages of dietary restrictionin addressing obesity-related comorbidities. In our study, we observed notable reductions in BMI, waist and blood pressure aftermoderate weight loss, in line with similar findings reported by others [21,22]. Collectively, these parameters are linked to a decrease in abdominal adiposity [23], suggesting that weight loss mitigates the associated cardiometabolic risk.Furthermore, our analysis of biochemical profilesrevealed a significantimprovement inparameters related to glucoseand lipid metabolism - in accordance with previous research [24,25]- and a significant decrease in C3c levels, a marker previously associated with insulin resistance [26,27].

Adipose tissue and the liver are recognized as pivotal players in driving metabolic adaptations in obesity. Unfortunately, gaining access to these tissues for human research presents significant challenges, primarily of an ethical nature. On the contrary, the use of PBMCs in nutritional studies has facilitated research due to their easy accessibility and high capacity to respond to even minimum changes in metabolic

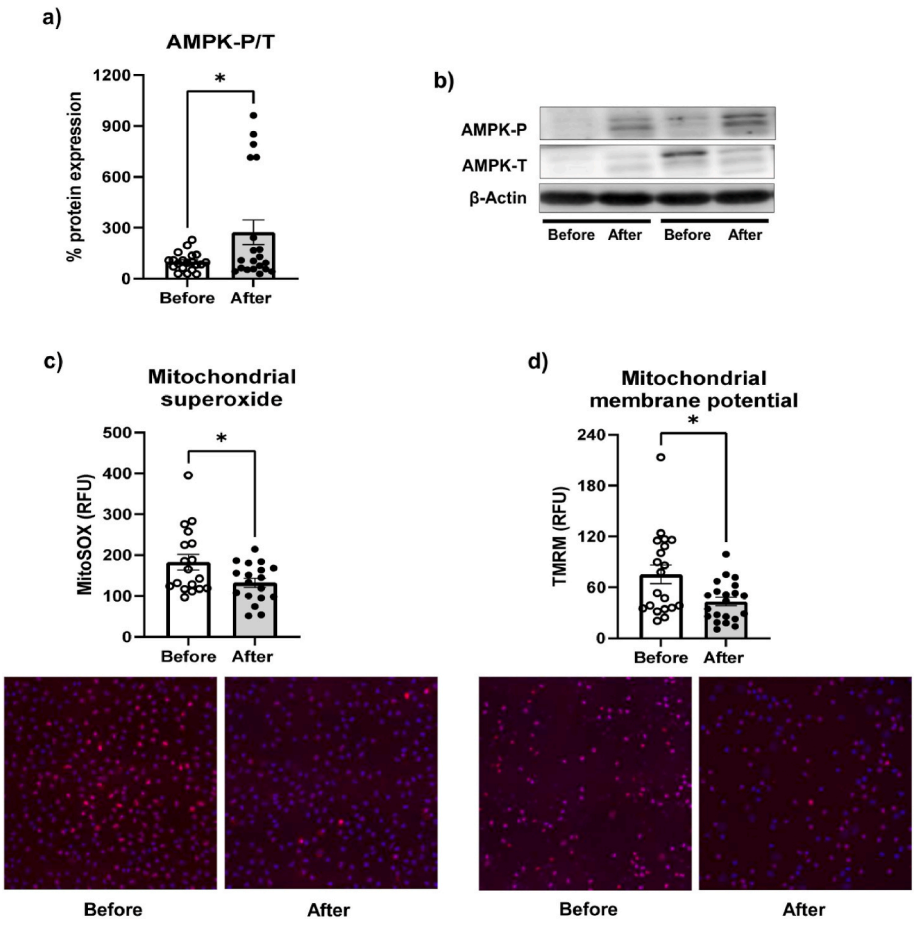

Fig. 1. AMPK activation and mitochondrial function in PBMCs from patients with obesity before and after dietary intervention.
Relative protein expression levels of AMPK-Phosphorylated (P)/Total (T) ratio (n = 20)(a) and representative Western blot images (b). Mean fluorescence intensity of MitoSOX (n = 18) (c) and TMRM (n = 20) (d) probes measuring mitochondrial superoxide production and mitochondrial membrane potential, respectively. Under the respective bar graph a representative fluorescence image of PBMCs stained with MitoSOX or TMRM (red signal) and Hoechst 33342 (blue signal, for nuclei visualization) are shown. Fluorescence data are expressed as arbitrary units of fluorescence. Data are represented as mean +SE. *$p < 0.05$ when compared using a paired Student's t-test. AMPK: AMP-activated Protein Kinase, RFU: Relative Fluorescence Units, TMRM: Tetramethylrhodamine Methyl ester.

conditions, which reflects the expression profiles of internal tissues [28,29]. For this reason, we explored important molecular mechanisms, including mitochondrial function and autophagy, in PBMCs from patients with obesity, as they are likely to reflect the metabolic adaptations of internal tissues to nutrient deprivation.

It is known that levels of AMPK, a master regulator of cellular energy homeostasis, are reduced in states of overnutrition such as obesity [30], while under caloric restriction, AMPK promotes glucose uptake, lipolysis, and fatty acid oxidation [31,32].We detected an increase in the phosphorylation of AMPK in PBMCs following the hypocaloric diet, which is in line with the findings of other studies [33–35]. From a cellular perspective, AMPK is a primary regulator that oversees various processes associated with nutrient sensing, such as oxidative stress [36], mitochondrial dynamics [37] and autophagy [38]. In the present study, we found positive correlations between AMPK activation and markers of mitochondrial fusion and autophagy in patients with obesity, which are in line with the previous findings.

Obesity is linked to chronic low-grade inflammation, which is key to the development of metabolic disorders such as insulin resistance and CVD. Mitochondrial dysfunction in PBMCs is known to influence the inflammatory response and their interaction with endothelial cells, both of which contribute to obesity-related complications. In this sense, we have previously reported that rising BMI levels in patients with obesity are related to increased mitochondrial dysfunction in immune cells, which is characterized by excess ROS production and altered mitochondrial membrane potential, and leads to increased endothelial dysfunction and adhesion of leukocytes to the endothelium [5]. As previously discussed, oxidative stress is one of the main mechanisms underlying the physiopathology of obesity. Excess ROS production promotes the expression of pro-inflammatory cytokines such as Tumor Necrosis Factor-alpha (TNFα) and Interleukin-6 (IL6) by PBMCs, mainly through the activation of the NF-κB (Nuclear Factor kappa B) pathway. In addition, other products released from damaged mitochondria (such as mitochondrial DNA and cardiolipin) can act as damage-associated molecular patterns (DAMPs) stimulating immune responses [39]. Ultimately, the mitochondrial dysfunction in PBMCs promotes a pro-inflammatory state and the expression of cellular adhesion molecules on endothelial cells, thus facilitating monocytes adhesion and migration and subsequent plaque formation in atherosclerosis. In this sense, previous research has shown how short-term calorie restriction reduces superoxide production in murine models and humans [25,40]. However, the role of fluctuations in the mitochondrial membrane potential in the context of obesity is poorly understood. Some preclinical studies have reported that an excess nutrient supply enhances mitochondrial membrane potential, which may be linked to mitochondrial function impairment and a rise in mtROS production [41,42], while others have described a decrease in mitochondrial membrane potential associated to obesity [43,44]. In the present study we observed that a 6-month hypocaloric diet notably reduced mitochondrial membrane potential in the PBMCs of patients with obesity, and this was associated

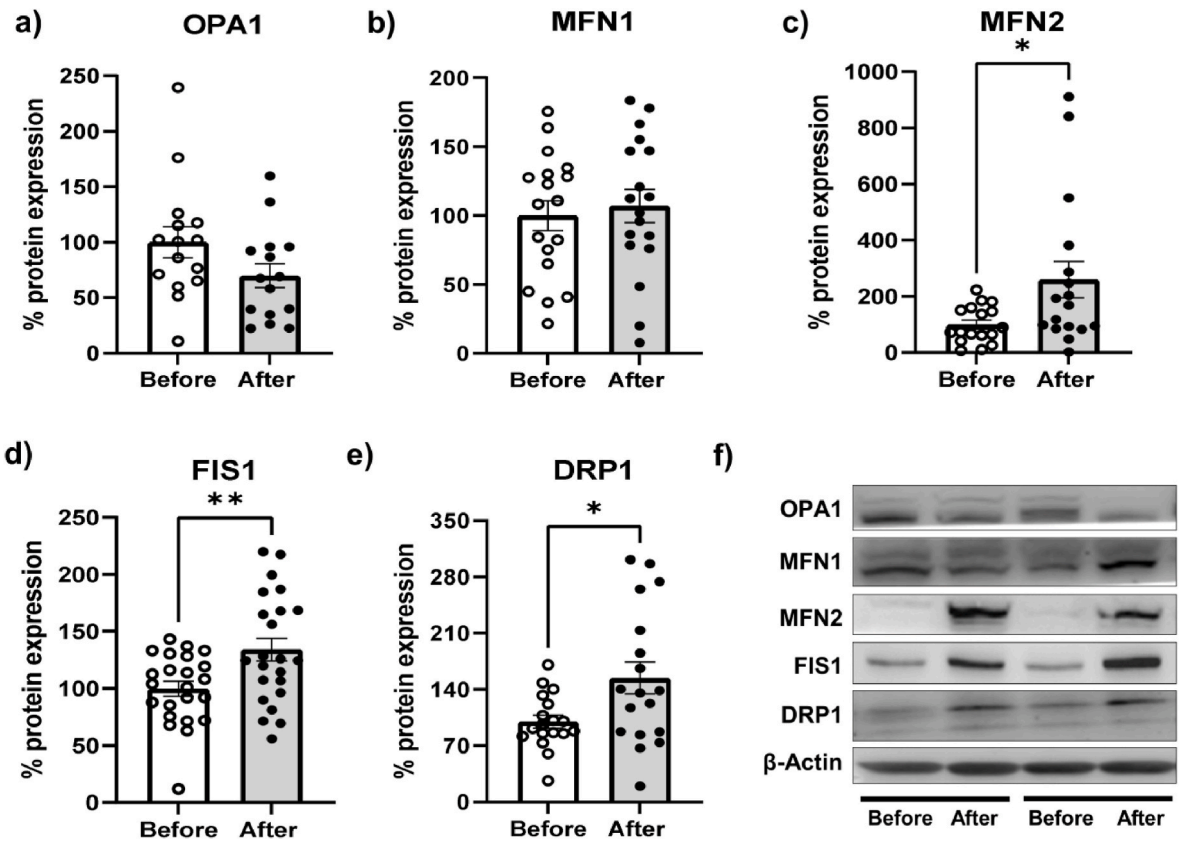

Fig. 2. Mitochondrial dynamics in PBMCs of patients with obesity before and after moderate weight loss.
Relative protein expression of (a) OPA1 (n = 15), (b) MFN1 (n = 18), (c) MFN2 (n = 17), (d) FIS1 (n = 23) and (e) DRP1 (n = 18) and representative Western blot images (f). Data are represented as mean +SE. *$p < 0.05$ and **$p < 0.01$ when compared using a paired Student's t-test. DRP1: Dynamin-Related Protein 1, FIS1: Mitochondrial Fission protein 1, MNF1: Mitofusin 1, MFN2: Mitofusin 2, OPA1: Optic Atrophy 1.

with a decrease in mtROS production. Similarly, leukocytes of patients with obesity undergoing bariatric surgery displayed a decrease in mitochondrial membrane potential accompanied by a reduction in superoxide production after the intervention [19], which is in line with the present findings. Moreover, in a sub-cohort of the present interventional study, we previously demonstrated that moderate weight loss reduced excess ROS production in immune cells, and that this was accompanied by an attenuation of systemic inflammation, endothelial dysfunction and leukocyte recruitment to the vessel wall [25]. Considered together, these results suggest that strategies aimed at improving mitochondrial biogenesis, reducing ROS, and enhancing metabolic flexibility in PBMCs could attenuate inflammation and prevent vascular complications in obesity.

In an effort to identify the mechanisms linking caloric restriction with the mitochondrial improvements observed in our patients, we subsequently investigated the impact of weight loss on mitochondrial dynamics and recycling in obesity, which preserve mitochondrial health. In our cohort, we observed a significant increase in the protein expression of MNF2, FIS1 and DRP1 after dietary intervention. In patients with obesity, *MFN2* mRNA levels in muscle *in vivo* increased upon acute weight loss [45], and its deficiency has been related to impaired insulin signaling and glucose homeostasis in liver and muscle of rodents [46]. Moreover, deficiency of MFN2 in adipose tissue has been linked to its pathological expansion [47], and therefore might regulate adipocyte apoptosis and the subsequent progression to metabolic syndrome [48], which is supported by the negative correlations between MFN2 expression levels and the obesity-related clinical features found in the present study. The increase in MFN2 after dietary intervention is in line with an improvement in metabolic outcomes. In the specific case of mitochondrial fission, studies have indicated an increase in FIS1 and DRP1 in hepatocytes from calorie-restricted mice, while no alterations were observed in proteins associated with mitochondrial fusion (MFN1 and OPA1) [49], data that are in accordance with our current findings. Recently, chemicals targeting mitochondrial fusion and fission, such as mitochondrial division inhibitor-1 (Mdivi-1) and elamipretide (SS-31) have shown promising results in reducing proinflammatory cytokines and ROS levels in cardiac cells and leukocytes of patients with T2D [50,51]. In this sense, the regulation of mitochondrial architecture through a hypocaloric diet represents a potential therapy for metabolic diseases and cardiovascular protection in patients with obesity.

Given that calorie restriction promotes the partial restoration of mitochondrial function, we might expect damaged mitochondria to be degraded through an autophagic process, since autophagy is essential for maintaining mitochondrial number and health [52]. The impact of fasting or calorie restriction on the activation of the signaling pathways involved in mitochondrial autophagy stimulation is well-established [53]. In line with this, Egan et al., demonstrated how caloric restriction drives the initiation of autophagy processes, including mitochondrial autophagy, through the AMPK pathway in murine liver and primary hepatocytes [54]. In the present study, we have observed an increase in markers of autophagy, including LC3II/I ratio, Beclin1 and NBR1, and a significant decrease in BCL2 protein expression after weight loss in a human cohort living with obesity. Previous investigations in hepatocytes have shown that a high-fat diet induces defects in autophagy [55,56], while weight loss was found to increase the expression of the autophagosomal protein LC3II/I [57]. In this sense, emerging evidence suggests that polyphenol-induced caloric restriction triggers the upregulation of several proteins, including LC3, which play a key role in the mitochondrial autophagy pathway, as reviewed by Davinelli et al. [58]. Moreover, nutrient deprivation has been seen to elevate LC3II

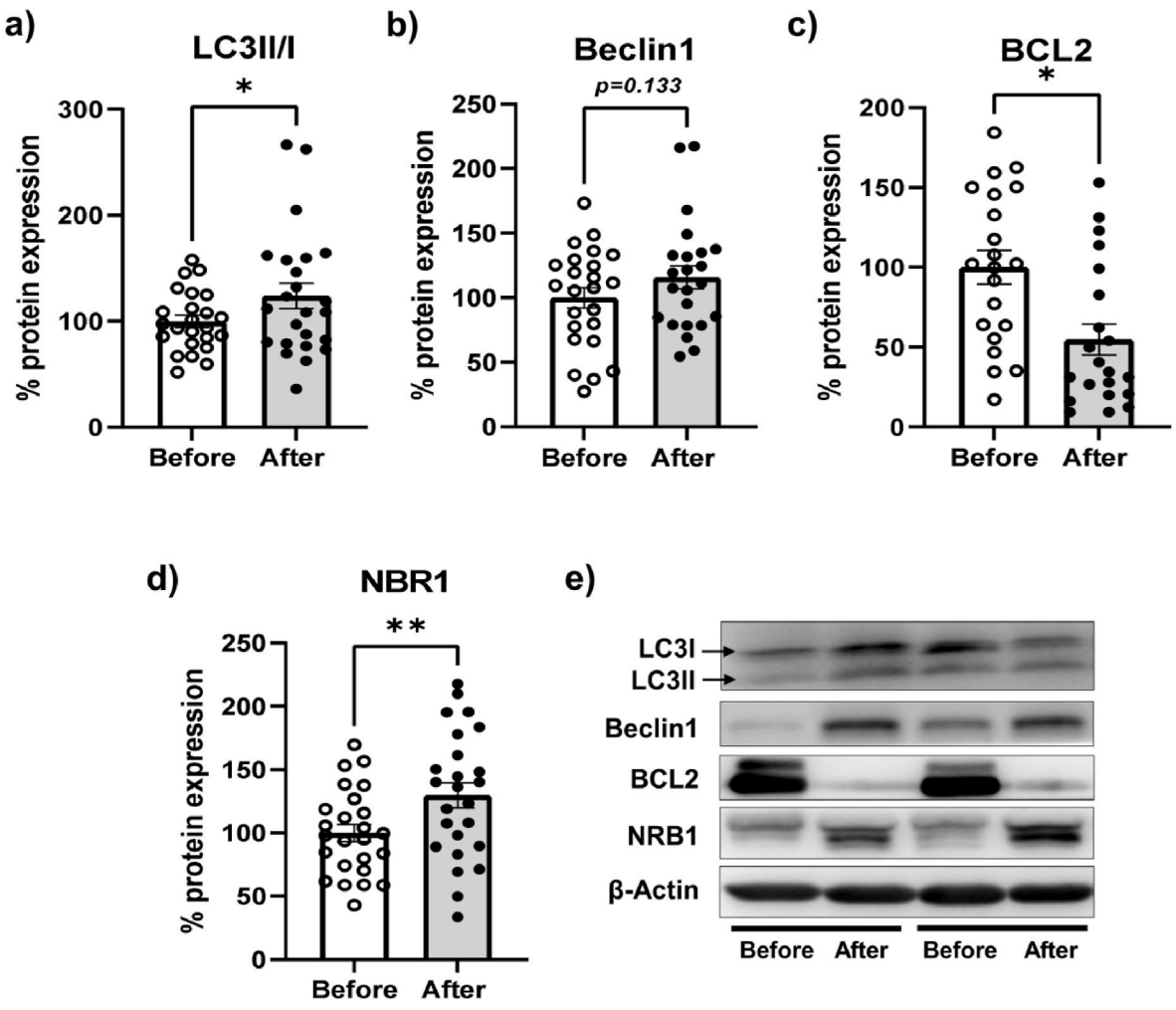

Fig. 3. Expression of autophagy-related markers in PBMCs from patients with obesity after dietary intervention.
Relative protein expression of (a) LC3 II/I (n = 24), (b) Beclin1 (n = 24), (c) BCL2 (n = 21), (d) NBR1 (n = 25) and representative Western blot images (e) before and after 6 months of dietary weight loss intervention. Data are represented as the mean +SE. *$p < 0.05$ and **$p < 0.01$ when compared using a paired Student's t-test. BCL2: B Cell Lymphoma 2, LC3: Microtubule-associated protein 1A/1B-light chain 3, NRB1: Neighbor of BRCA1 gene 1.

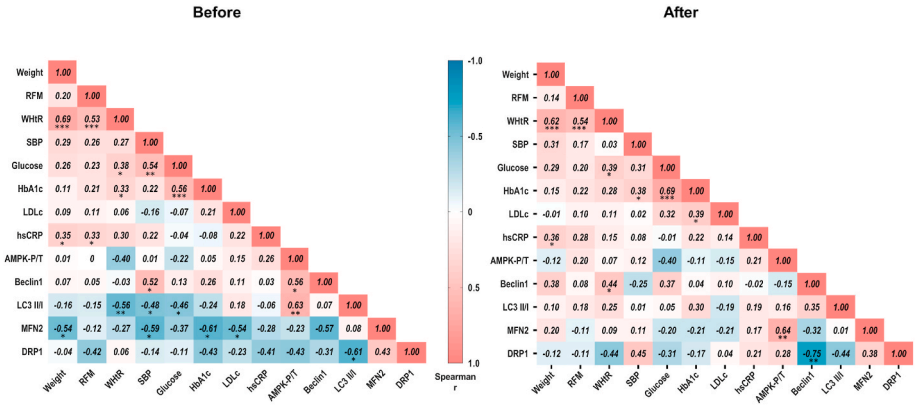

Fig. 4. Heatmap of Spearman's correlation coefficient matrix depicting the strength of relationships between obesity-related anthropometric indices, biochemical parameters, and the expression levels of proteins related to nutrient sensing (AMPK-P/T), autophagy (LC3II/I, Beclin1) and mitochondrial dynamics (MFN2, DRP1) before and after the intervention.
Numbers show the exact Spearman's r coefficient. Negative correlations are shown in blue while positive correlations are shown in red. The darker the color, the stronger the correlation. Statistically significant correlations are marked with *p < 0.05, **p < 0.01, ***p < 0.001. AMPK-P/T: AMP-activated Protein Kinase – Phosphorilated/Total, DRP1: Dynamin-Related Protein 1, HbA1c: glycated haemoglobin, hsCRP: high-sensitivity C-Reactive Protein,LC3: Microtubule-associated protein 1A/1B-light chain 3,LDLc: Low Density Lipoprotein cholesterol, MFN2: Mitofusin 2, RFM: Relative Fat Mass, SBP: Systolic Blood Pressure, WHtR: Waist-to-Height Ratio.

protein levels and accelerate mitochondrial autophagy in cultured GFP-LC3 transgenic mice hepatocytes [59]. In the same way, Yang et al. [60] found that LC3 and Beclin1 were significantly upregulated in skeletal muscle following calorie restriction. The same response, together with an increase in NBR1 expression, has been observed in PBMCs of patients with obesity undergoing bariatric surgery [35]. It is known that BCL2 inhibits autophagy by forming a complex with Beclin1 [61], so it is possible that a significant decrease in its levels after weight loss promotes autophagy induction. Furthermore, undermined autophagy in PBMCs may promote inflammatory macrophage polarization, causing inflammation at both systemic and hepatic levels, and exacerbating liver injury [62]. In addition, in the present study we found that the increase in the ratio LC3II/I levels was negatively associated with WHtR, SBP and fasting glucose, thus suggesting that undermined autophagy during obesity could be related to metabolic impairment. Therefore, it is plausible that, during nutrient deprivation, such as that which occurs with calorie restriction, autophagy acts as a defensive mechanism against obesity, providing cells with a safety mechanism through which damaged mitochondria and/or other cellular components are eliminated [63].

The metabolic switch that occurs during caloric restriction through increased AMPK, mitochondrial dynamics and autophagy and the partial restoration of mitochondrial function may underlie the systemic health benefits observed after such interventions. As suggested by Hofer et al. [64], this may be a result of an improvement of the cardiovascular system, adipose tissue, and other metabolic target organs, pointing to an abroad range of potential applications.

The major strength of this cohort study include its design, which facilitates a translational comparison of the implications of weight loss. We have evaluated intracellular responses to weight loss following a hypocaloric diet, which, as far as we are aware, makes this the first study to address changes in mitochondrial remodeling and the subsequent induction of autophagy in PBMCs of patients with obesity. However, this study has certain limitations. While our relatively small sample size aligns with the sample size calculations for this study and supports the validity of our data, it nonetheless limited our ability to conduct sex- and gender-based analyses. This limitation should be addressed in future studies. Furthermore, we cannot establish a causal relationship between weight loss and intracellular processes due to the study's observational design; in this sense we stress the need for future randomized studies to uncover mechanisms behind the metabolic improvements in obese subjects undergoing calorie restriction, which are sure to help to bring progress in obesity prevention and treatment strategies.

5. Conclusions

Our findings indicate that individuals with obesity who undergo a calorie restriction intervention display an improvement in their oxidant status and mitochondrial function in their PBMCs. This phenomenon would seem to occur through the stimulation of mitochondrial network remodeling followed by the elimination of damaged mitochondria by means of selective autophagy. These improvements are likely to contribute to the overall enhancement in clinical features observed. However, further investigations, including rigorous clinical trials, are crucial to gain full understanding of the mechanisms responsible for the benefits of dietary intervention to address obesity.

CRediT authorship contribution statement

Zaida Abad-Jiménez: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Sandra López-Domènech:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **María Pelechá:** Writing – review & editing, Visualization, Investigation, Formal analysis, Conceptualization. **Laura Perea-Galera:** Writing – review & editing, Visualization, Investigation, Conceptualization. **Susana Rovira-Llopis:** Writing – review & editing, Investigation, Conceptualization. **Celia Bañuls:** Writing – review & editing, Investigation, Conceptualization. **Ana Blas-García:** Writing – review & editing, Investigation, Conceptualization. **Nadezda Apostolova:** Writing – review & editing, Investigation, Conceptualization. **Carlos Morillas:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization. **Víctor Manuel Víctor:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Milagros Rocha:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Author's disclosure

Declarations of interest: none.

Research data for this article

The data that support the findings of this study are available from the corresponding author [MR], upon reasonable request.

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Declaration of competing interest

Declarations of interest: None.

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