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Original article

Obesity-related osteopontin protein and methylation blood levels are differentially modulated by a very low-calorie ketogenic diet or bariatric surgery

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SUMMARY

Background & aim: Osteopontin (OPN) was proposed to play a role in the pathophysiology of obesity and related disease, such as cancer. The aims were to evaluate the expression of OPN after caloric restriction-induced weight loss in adipose tissue (AT) from an animal model of diet-induced obesity (DIO) and to reflect these results on circulating OPN levels in patients with obesity (PWO); and to explore the effect of a very low-calorie ketogenic diet (VLCKD) on the circulating protein and DNA methylation levels of OPN, compared with a balanced hypocaloric diet (LCD) or bariatric surgery (BS) in PWO.
Methods: OPN/SPP1 expression was evaluated in subcutaneous (SAT) and visceral (VAT) AT derived from diet-induced obesity (DIO) mice and after a 4-week weight-loss protocol of calorie restriction (CR). Plasmatic OPN was also evaluated in 32 normal-weight volunteers (20 women) and 79 PWO (59 women) and after a 4–6 months follow up of a VLCKD (n = 20), BS (n = 39) or LCD (n = 20). DNA methylation levels of OPN were extracted from our Infinium HumanMethylation450 BeadChips data sets.
Results: OPN levels were higher in VAT of DIO mice and plasma of PWO than in normal-weight individuals and changed after weight loss. Particularly, circulating OPN increased 2 months after BS while it decreased at maximum ketosis-induced by VLCKD. A statistically significant decrease was also observed in methylation levels at cg11226901 after VLCKD.
Conclusions: OPN levels were reduced after VLCKD and severely increased after BS. Therefore, it could be a biomarker of the obesity-related metabolic stress and could be epigenetically regulated.
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1. Introduction

Osteopontin (OPN) is a cytokine that presents high values in adipose tissue. It is a secreted matricellular protein that was first

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identified in 1985 by Heingard et al. as sialoprotein derived from bovine bone matrix [1]. This cytokine has been found physiologically in bone cells, as well as in adipocytes, cardiomyocytes, renal and vascular cells [2]. There is abundant evidence to suggest that OPN plays a critical role in liver pathologies [3–5] chronic inflammatory diseases, cardiovascular disease, and various types of cancer [1]. Relevantly, OPN is involved in the pathophysiology of obesity-related adipose tissue [6].

Obesity is a state of low-grade chronic inflammation, where adipose tissue, mainly visceral fat, secretes several pro-inflammatory cytokines [7–10]. This status was proposed as a relevant link between obesity and other diseases with cancer among then. In fact, it was proposed that weight loss therapies could be a good strategy to reduce the risk and improve the prognosis of obesity-related cancer [11]. In this regard, previous studies have shown that a very low-calorie ketogenic diet (VLCKD), also named very low-energy ketogenic therapy (VLEKT) [12], is a nutritional therapy effective for the management of obesity and for the preservation of muscle mass [13]. In addition, this nutritional intervention is able to remodel oxidative and inflammatory state [14–16], and improves the immune function in patients with obesity [17]. These effects could be correlated with a potential efficacy to counteract the risk of obesity-related diseases, such as cancer [11]. VLCKDs have demonstrated to induce also beneficial effects in several diseases such as cardiovascular [18] and liver [19] disease, fibromyalgia [20], acne severity [21], migraine [22] and even it improves the sleep quality [23,24]. This type of diet is characterized by the restriction of carbohydrate and fat intake, with a slight increase in protein intake to the point of inducing a change in metabolism and the generation of plasma ketone bodies [25]. It was proposed that the beneficial effect of this type of nutritional intervention could be promoted by ketone bodies synergistically with the induction of body weight loss [14,26]. In fact, recently, ketone bodies have been found to possess anti-tumor potential [27], and they can act as signaling molecules with antioxidant, anti-inflammatory and epigenetic [28–30], as well as neuroprotective properties [31].

Considering the role of OPN in carcinogenesis and adipose tissue disfunction, the hypothesis of this work is that the expression of this protein in obesity could be modulated by nutritional therapies to lose weight based on the induction of nutritional ketosis. This modulation could be induced by the ketone bodies by means of epigenetic mechanisms. In fact, there is evidence on the epigenetic regulation of *OPN* gene expression by DNA methylation [32,33]. However, there are no studies evidencing the association between adiposity or nutritional ketosis on DNA methylation profile in *OPN/SPP1*.

Therefore, the aim of this study was: (1) to evaluate the expression of *OPN* in adipose tissue from an animal model with obesity and weight loss induced after caloric restriction and to reflect these results on the levels of OPN in blood of patients with obesity; (2) to explore the effect of a VLCKD on the protein and DNA methylation levels of *OPN*, compared with a standard, balanced hypocaloric diet (LCD) or bariatric surgery (BS) in blood of patients with obesity.

2. Materials and methods

2.1. Animal models

The present study was conducted on diet-induced obesity (DIO) female mice (n = 14) and their control counterparts, lean female mice (n = 10), which were purchased from Charles River Laboratories (Barcelona, Spain). For the experiment, 24 adult female mice were obtained at 8 weeks of age and euthanized 20 weeks later. After the acclimatization period (1 week), mice were randomized into groups (obese group, n = 14; lean group, n = 10). The obese group were fed *ad libitum* with a high-fat diet (HFD) (Open Source Diets, Research Diets; Brogaarden, Denmark, Reference D 12492) that consisted of 60 % lipid, 20 % protein, and 20 % carbohydrate. After 12 weeks, part of the animals in the obese group (n = 4) were subjected to a weight loss protocol based on caloric restriction (CR) (a diet of 30 % less quantity than their individual intake food during the last 4 weeks) for 7 weeks, while the remaining obese mice were maintained on an HFD diet (DIO, n = 10). Data were compared with a group of lean female mice (n = 10) that were fed *ad libitum* with a standard laboratory diet (SAFE; Panlab, Spain), containing 5.5 % lipid, 23 % protein, and 70 % carbohydrate, during the total experimental period of 19 weeks (Supplementary Fig. 1).

The mice were housed in air-conditioned rooms (22–24 °C) under a controlled (12:12 h) light–dark cycle with free access to food and water. For all animals from the three groups, the weekly food intake was recorded and the body weight (BW) and composition were assessed by nuclear magnetic resonance using an EchoMRI-700 system (Echo Medical Systems, Houston, TX, USA). Finally, the mice were euthanized by decapitation, and their trunk blood was collected and immediately centrifuged. The plasma was stored at −80 °C for the biochemical measurements. The adipose tissue depots and biceps femoris muscle were excised, immediately frozen on dry ice, and then stored at −80 °C until assay.

All animal experiments and procedures used in this study were approved by the Animal Care Committee of the University of Santiago de Compostela (Ref. 15005/2015/003) in accordance with our institutional guidelines and the European Union standards for the care and use of experimental animals.

2.2. Patients

The study was performed in 79 patients (59 women) with overweight or obesity (PWO) and 32 normal-weight volunteers (20 women) who served as the control group. The control group was recruited from companions of patients that agreed to participate in this study; individuals in the control group reported no history of obesity, diabetes mellitus, high blood pressure, or dyslipidemia and were in good health. PWO underwent a weight reduction therapy based on one of two different energy-restriction programs or bariatric surgery (Supplementary Fig. 2). Thus, a group of patients completed treatment with a VLCKD (PnK method®). The other group was a subsample of the study for the Reduction of the Metabolic Syndrome in Navarra (RESMENA), Spain, a randomized control trial based on an LCD (30). A third group underwent BS treatment.

The inclusion criteria were, age 18–65 years, body mass index (BMI) ≥30 kg/m^2, stable body weight in the previous 3 months, desire to lose weight, and history of failed dietary efforts. The main exclusion criteria were diabetes mellitus, obesity induced by other endocrine disorders or by drugs, and participation in any active weight loss program in the previous 3 months. In addition, those patients with previous bariatric surgery, known or suspected abuse of narcotics or alcohol, severe depression or any other psychiatric disease, severe hepatic insufficiency, any type of renal insufficiency or gouts episodes, nephrolithiasis, neoplasia, previous events of cardiovascular or cerebrovascular disease, uncontrolled hypertension, orthostatic hypotension, and hydroelectrolytic or electrocardiographic (EcG) alterations, were excluded. Females who were pregnant, breast-feeding, or intending to become pregnant, and those with child-bearing potential and not using adequate contraceptive methods, were also excluded. Apart from obesity and metabolic syndrome, participants were generally healthy individuals [13].

Written informed consent to participate in the study was obtained before the start of the study in agreement with the Helsinki

Declaration; the study followed national and European Union guidelines. The study was approved by the respective Institutional Ethics Committee for clinical research of Galicia (ref 2009/076), University of Navarra (ref. 065/2009) and Hospital Clinico Virgen de la Victoria from Malaga (ref CP1600163).

2.3. Weight loss therapies

2.3.1. Very low-calorie ketogenic diet (VLCKD)

A cohort of patients subjected to the VLCKD were enrolled in this study. This cohort included 20 patients with obesity (BMI 35.5 ± 4.4 kg/m^2; n = 12 women; 47.1 ± 10.2 years) attending the Obesity Unit at the Complejo Hospitalario Universitario de Santiago de Compostela, Spain. The VLCKD was designed according to a commercial weight-loss program (PnK method®), that includes lifestyle and behavioural modification support as described elsewhere [13,34–36]. This method is based on high-biological-value protein preparations obtained from cow's milk, soya, avian eggs, green peas and cereals. Each preparation contained 15 g protein, 4 g carbohydrates, 3 g fat, and 50 mg docosahexaenoic acid, which provided 90–100 kcal of energy [13]. The weight loss program had three phases. The first phase consisted of a VLCKD (600–800 kcal/d) that was low in carbohydrates (<50 g/d from vegetables) and lipids (10 g/d from olive oil) pertaining to 0.8–1.2 g/kg of ideal body weight. Throughout this ketogenic phase, supplements of vitamins and minerals such as K, Na, Mg, Ca, and omega-3 fatty acids were provided. When the target amount of weight was lost, the ketogenic phase ended, and patients started a low-calorie diet (800–1500 kcal/d) followed by a maintenance diet of 1500–2000 kcal/d. The weight loss program had five steps [13] and adhered to the 2015 guidelines of the European Food Safety Authority regarding total carbohydrate intake [37].

Patients followed the different steps of the program for up to a maximum period of 4–6 months, although patients remained under medical supervision for the following 12 months [13,34–36]. The intervention included an evaluation by the specialist physician conducting the study and an assessment by an expert dietician. All patients underwent a structured program of physical exercise, with external supervision [13].

Throughout the study, the patients completed a maximum of 10 visits with the research team (every 15 ± 2 days); four visits involved a complete physical, anthropometric, and biochemical assessment, while the remaining visits involved control of adherence to the program and evaluation of potential side effects. The four assessment visits were scheduled according to the development of ketosis for each patient as follows: normal level of ketone bodies (baseline), maximum ketosis (1–2 months), reduction of the ketogenic approach because of partial reintroduction of normal nutrition (approximately 3 months), and no ketosis (4–6 months, end of the study) [13]. The total ketosis state lasted for 60–90 days. In all visits, patients received dietary instructions, individual supportive counseling, and encouragement to exercise on a regular basis using a formal exercise program. The compliance of this advice was not registered. The body weight, body composition, and circulating levels of OPN were evaluated at baseline (0 months), at maximum ketosis (2 months), and at no ketosis (4 months).

2.3.2. Low-calorie diet (LCD)

A group of patients with obesity (n = 20, BMI 35.8 ± 4.5 kg/m^2; n = 10 women; 49.9 ± 9.3 years) followed a therapy program based on a nutritional intervention controlled by trained dieticians from the Department of Nutrition, Food Sciences and Physiology of the University of Navarra, Spain, according to the RESMENA study protocol [38]. Briefly, the study lasted six months in two sequential periods; one intervention period of two months (30 % energy restriction, i.e., a reduction of 600–800 kcal/d; 40–55 % of energy intake from carbohydrates, 30 % from lipids and 15–30 % from proteins), during which patients received nutritional assessment every 15 days, followed by a self-control period of four months, during which individuals were advised to follow the lifestyle adopted in the first period. The energy-restricted diets prescribed were based on the American Heart Association guidelines and included 3–5 meals per day and a macronutrient distribution of 50–55 % of the total caloric value from carbohydrates, 15 % from proteins and 30 % from lipids. Moreover, all participants were asked to maintain their normal physical activity during the study, although adherence to recommendation was not recorded. Venous blood samples and anthropometric and body composition measurements were collected at baseline (week 0), at the end of the diet intervention (endpoint, 2 months), and 4 months after the end of the intervention (follow-up, 6 months).

2.3.3. Bariatric surgery (BS)

A group of patients with morbidly obesity (n = 39, BMI 45.6 ± 6.2 kg/m^2; n = 37 women; 40.8 ± 10.4 years) underwent bariatric surgery by laparoscopic techniques at the Hospital Clínico Virgen de la Victoria from Malaga and Complejo Hospitalario Universitario de Santiago de Compostela, Spain.

Before surgical intervention, patients were advised to follow a special diet that consisted of liquid diet for one-month, soft diet for one month, and subsequently a normal consistency diet providing about 800 kcal/d. Patients subjected to bariatric surgery were provided with a daily vitamin-mineral supplement, beginning on the day of the surgical procedure, to reduce the risk of developing nutritional deficiencies [39]. Patients involved in this study returned to the clinic for all follow-up visits. General recommendations about mobility and exercise were prescribed although adherence to recommendations was not recorded. The follow-up regimen included visits at three and 6 months at which time anthropometric and body composition measurements were performed, and venous blood samples were collected. Body composition data were available from n = 24 patients included in this group.

2.4. Anthropometric and body composition measurements in patients

All anthropometric measurements were undertaken with patients wearing only underwear and after an overnight fast (8–12 h) following validated procedures. Body weight and height measurements were performed using a wall-mounted stadiometer (Seca 220 scale, Medical Resources, EPI Inc OH, USA). The BMI was calculated by dividing body weight by the square of height (kg/m^2). Total body composition was measured by dual-energy X-ray Absortiometry (DXA; GE Healthcare Lunar, Madison, USA) as described elsewhere [13].

2.5. Biochemical analysis

Venous blood samples were drawn after a 12-h overnight fast, and EDTA-plasma and serum were separated from whole blood and immediately frozen at −80 °C until assay. The quantitative measurement of total human circulating OPN was performed using a commercial multiplex enzyme-linked immunosorbent assay (ELISA) kit (Bio-Plex custom assay, Bio-Rad Laboratories, Marnes-la-Coquette, France) according to the manufacturer's instructions. Circulating OPN levels were evaluated in plasma samples for all the studies. Circulating ketone bodies, specifically β-hydroxybutyrate (β-OHB), were measured using a portable meter (GlucoMen LX Sensor, A. Menarini Diagnostics, Neuss, Germany). Glucose and total cholesterol serum concentrations were measured in an

autoanalyzer Pentra C-200 (HORIBA ABX, Madrid, Spain) with specific kits. Insulin concentrations were assessed using an ELISA kit available from Mercodia, AB (Uppsala, Sweden) in a Triturus autoanalyzer (Grifols, Barcelona, Spain). Insulin resistance was indirectly determined by the homeostatic model assessment index (HOMA-IR), which was calculated following the formula (fasting plasma glucose [mg/mL] x fasting plasma insulin [mU/l]/405).

2.6. Data collection and DNA methylation analysis

The DNA methylation profile of OPN-secreted phosphoprotein 1 (*SPP1)* was evaluated by analysing different methylome datasets of blood leukocytes from our research group. Methylome data were obtained through hybridization of blood samples in Illumina Infinium HumanMethylation450 BeadChip (Illumina, San Diego, AC). These data were assembled and commonly detected CpGs with a valid signal ($p < 0.01$) in all samples included in this study were selected to evaluate the differential methylation levels based on obesity phenotypes, obesity-related comorbidities and after weight loss therapies.

For all samples, DNA extraction, bisulfite conversion and hybridization, as well as data normalization, quality filtering and statistical calculations were carried out following protocols according to manufacturer's instructions as described previously [40].

2.7. Gene expression assessment

RNA was isolated from mice adipose tissue according to the manufacturer's recommendations. Extracted total RNA was purified with DNase treatment using a DNA-free kit as a template to generate first-strand cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The expression of *SPP1* was assessed using TaqMan real-time PCR and a Step One Plus system (Applied Biosystems, USA) with specific primers and probes for the *SPP1* gene that were obtained from inventoried TaqMan Gene Expression Assays (Applied Biosystems, USA). All reactions were performed using the following cycling parameters: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. All experiments were performed in duplicate and gene expression levels were normalized using *GAPDH* as an internal control. The fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ relative quantitation method according to the manufacturer's guidelines (Applied Biosystems), and data are reported as mean ± standard error of the mean (SEM). RT-qPCR experiments were performed in compliance with the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (http://www.rdml.org/miqe). The commercially available and pre-validated TaqMan primer/probe sets used were as follows: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, Hs02758991_g1, Applied Biosystems) and secreted phosphoprotein 1 *SPP1* (*SPP1*, Hs00959010_m1 for human and Mm00436767_m1 for mice; Applied Biosystems).

2.8. Statistical analysis

The sample size of the current study was calculated to detect differences for OPN considering published values of circulating OPN and standard deviation [6]. The normal distribution of variables was explored using the Kolmogorov–Smirnov and Shapiro–Wilk tests. An analysis of covariance (ANCOVA) was used to study differences between groups, adjusted for gender and age when it was opportune. A repeated-measures ANCOVA was used to study the effects of time-course of the nutritional therapy program and groupings of body composition, biochemical parameters, and OPN levels in obese patients. The potential association between body composition and biochemical parameters with OPN levels was evaluated using the Spearman coefficient test. The analysis of the difference in methylation levels of *SPP1* between the different groups was performed with the CpG sites separately and also with the mean value of the CpG sites. Statistical analyses were performed using SPSS version 22.0 software (SPSS Inc., Chicago, IL) for Windows XP (Microsoft, Redmond, WA). $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Differences in OPN/SPP1 gene expression in visceral and subcutaneous adipose tissue from DIO and after energy restriction-induced weight loss in mice

In order to evaluate if the association between OPN circulating levels and adiposity could be a reflect of its expression in adipose tissue, an animal model of diet induced obesity (DIO) and weight loss by energy restriction was performed (Supplementary Fig. 1 and Supplementary Table 1).

DIO mice showed higher total body (Fig. 1A), and VAT (Fig. 1B) weight than lean mice or CR mice. Relevantly, compared with control group, DIO mice showed the significantly higher expression of *SPP1* in visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) and lower in muscle. In CR mice, no statistically significant differences were found in the VAT, SAT or muscle expression of *SPP1* compared to lean control mice. However, this group shows a statistically significant decrease in *SPP1* transcript levels of VAT compared with DIO mice (Fig. 1C).

3.2. Differences in circulating baseline levels of OPN according to adiposity in human

A total of 111 subjects (32 normal-weight and 79 patients with overweighty/obesity) were included in this study. Participants of both genders were evaluated for the baseline body weight, body composition, ketone bodies, HOMA-IR and levels of circulating OPN. Thus, the cohort of PWO were classified according to their BMI as overweight (25–29.9), obesity (30–39.9) and severe obesity (≥40) and compared with a group of normal-weight (BMI <25) individuals (Fig. 2A). In relation to body composition, statistically significant differences were observed between the groups (Fig. 2A). The baseline characteristics of the participants are shown in Table 1.

To evaluate the effect of the weight loss treatments on OPN, the baseline levels of OPN were first evaluated relative to excess adiposity (Fig. 2B). OPN baseline levels were significantly ($p = <0.001$) higher in patients with obesity than in normal-weight and overweight subjects, being higher in morbidly obesity than in obesity (Fig. 2B).

3.3. Changes in circulating levels of OPN after weight loss treatments in human

All patients included in this study reached a statistically significant weight loss (Fig. 3A) following the three weight loss treatments. This loss in total body weight induced after weight loss therapies was due to both, a loss of fat mass and fat-free mass, even though on VLCKD treatment the weight loss was mainly at the expense of fat mass, with a slight loss of fat-free mass (Fig. 3A). The β-OHB levels also differed between weight loss therapies. As expected by design, the VLCKD induced an increase in β-OHB levels, whereas no ketosis was observed after the LC diet. On the other hand, bariatric surgery also induced a mild but statistically significant increase in β-OHB levels. In addition, the three therapeutic

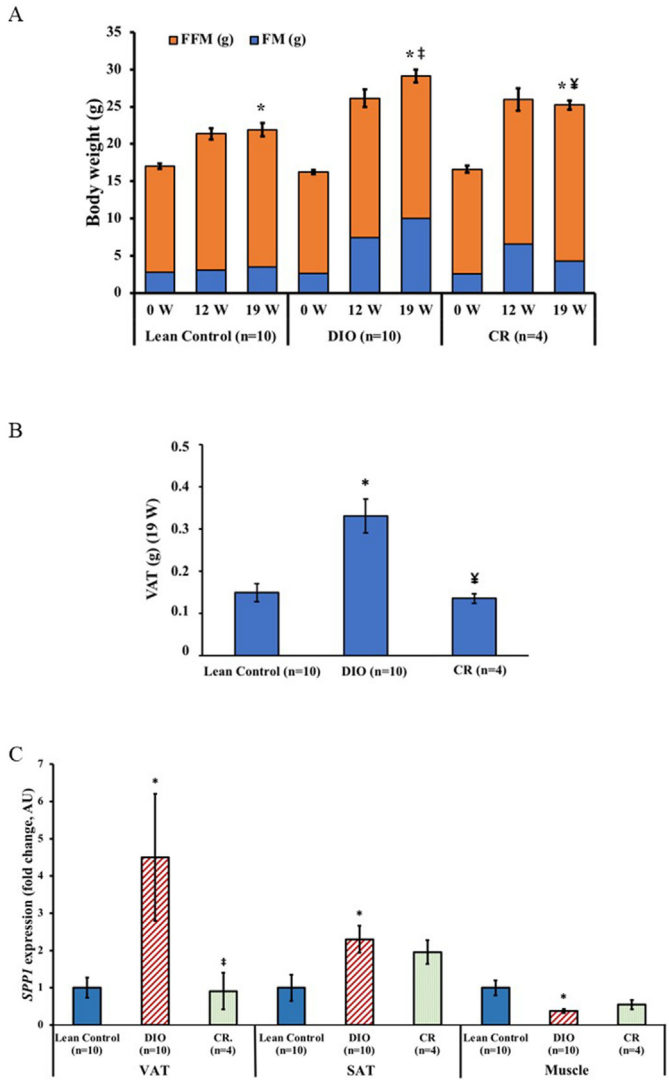

Fig. 1. Differences in body composition and Osteopontin gene expression in mice. (**A**) Comparison in body weight and body composition between Lean Control and diet-induced obesity (DIO) mice and after caloric restriction-induced weight loss during follow-up period. (**B**) Differences in visceral adipose tissue (VAT) between Lean Control, DIO mice and after caloric restriction-induced weight loss at 19 weeks. (**C**) Differences in *SPP1*/Osteopontin gene expression in visceral, subcutaneous adipose tissue and muscle from Lean Control, DIO mice and after caloric restriction-induced weight loss during follow-up period. Data are presented as the mean; error bars represent the standard error. *P*-value is calculated by Student's t-test and ANOVA for (A), by Student's t-test for (B) and (C). Asterisk (*) denotes statistically significant ($p < 0.05$) differences in relation to 0 weeks in (A) and to Lean Control mice in (B) and (C). ‡ denotes statistically significant ($p < 0.05$) differences in relation to Lean Control mice at 19 weeks. ¥ denotes statistically significant ($p < 0.05$) differences in relation to DIO mice at 19 weeks. CR, calorie restriction; DIO, diet-induced obesity.

approaches induced a significant improvement of HOMA-IR with greatest decrease induced by the VLCKD.

In relation to circulating OPN time-course during the three weight loss treatments, there were changes in its levels, with statistically significant differences being observed during VLCKD ($p = 0.024$) and bariatric surgery ($p = 0.005$). In VLCKD, OPN levels decreased at the point of maximum ketosis (2 months). On the contrary, in BS group, OPN levels increased significantly at 3 months (Fig. 3B). No statistically significant differences were found in OPN levels at the different LC diet follow-up points.

Notably, circulating OPN levels in PWO were associated with body composition parameters (Table 2). Baseline OPN levels were positively correlated with baseline fat mass ($r = 0.405$; $p = 0.003$) (Fig. 3C) and follow-up fat mass ($r = 0.361$; $p = 0.010$) (Fig. 3D). Follow-up OPN levels were positively correlated with baseline fat mass ($r = 0.610$; $p = <0.001$) (Fig. 3C) and follow-up fat mass ($r = 0.542$; $p \leq 0.001$) (Fig. 3D). Thus, endpoint OPN levels were positively correlated with baseline fat mass ($r = 0.585$; $p \leq 0.001$) (Fig. 3C) and follow-up fat mass ($r = 0.542$; $p \leq 0.001$) (Fig. 3D).

3.4. Differences in DNA methylation of OPN/SPP1 associated to nutritional ketosis in patients with obesity

When OPN methylation was analyzed in PWO compared to normal-weight subjects, no significant differences in DNA methylation levels were observed (Fig. 4A). After weight loss induced by bariatric surgery or VLCKD, DNA methylation was not statistically different through the evaluated CpG sites (Fig. 4B). Only, a slight significant decrease in cg11226901 methylation was observed after VLCKD (0.928 ± 0.005, $p = 0.041$).

4. Discussion

This study demonstrates that patients with obesity treated to lose weight with the induction of nutritional ketosis based on a VLCKD showed a decrease in the enhanced levels of OPN circulating levels while patients underwent bariatric surgery exhibited an increase in OPN circulating levels from baseline, without no changes observed after LCD. Considering that both weight loss therapies induced a reduction in fat mass, the differential effect could be associated to the improvement in fat free mass [13,25] or promoted by the previously demonstrated anti-inflammatory and antioxidant properties of nutritional ketosis induced by the VLCKD [14]. The effects of adiposity changes on *OPN* expression and secretion could be epigenetically regulated by changes in DNA methylation.

OPN is an adipokine whose plasma levels increase in obesity, and is involved in several pathophysiological processes that include bone remodeling, immunity, inflammation or neoplastic transformation [6]. We observed that plasmatic OPN levels were higher in individuals with obesity compared to normal and overweight. These increase in OPN circulating levels associated to obesity could be produced by its secretion from adipose tissue. In fact, a similar pattern was observed in the expression of mice adipose tissue *OPN*. Increased transcript levels of OPN were observed in DIO mice compared to lean mice in VAT and SAT, while they decreased in VAT from CR mice respect to the DIO mice. These results are in line with previous research showing that OPN circulating levels and expression in visceral adipose tissue are elevated in obesity [6,41].

Interestingly, circulating levels of OPN showed differential changes after the follow-up of three weight loss therapies, VLCKD, LCD and bariatric surgery in patients with obesity. As for VLCKD, OPN levels decreased at the point of maximum ketosis, similar to previous studies where weight loss is accompanied by a decrease in plasma OPN levels in patients with obesity [6], as well as in response to acute exercise [42]. By contrast, a pronounced increase in OPN levels was observed after bariatric surgery. Several hypotheses could explain the opposite effect of the weight loss therapies on OPN circulating levels. On the one hand, both therapies are able to induce an important reduction in fat mass and it would be expected a concomitant reduction in OPN that was not the case. Among differences between both therapies is that VLCKD is able to preserve muscle mass and function [25], contrary to what occurs in other weight loss strategies, such as bariatric surgery, where significant loss of muscle mass occurs [43,44]. Under physiological conditions OPN is found at very low or undetectable levels in skeletal muscle; however, when muscle damage is induced, OPN gene transcription increase up to 100 times [45]. In addition, OPN

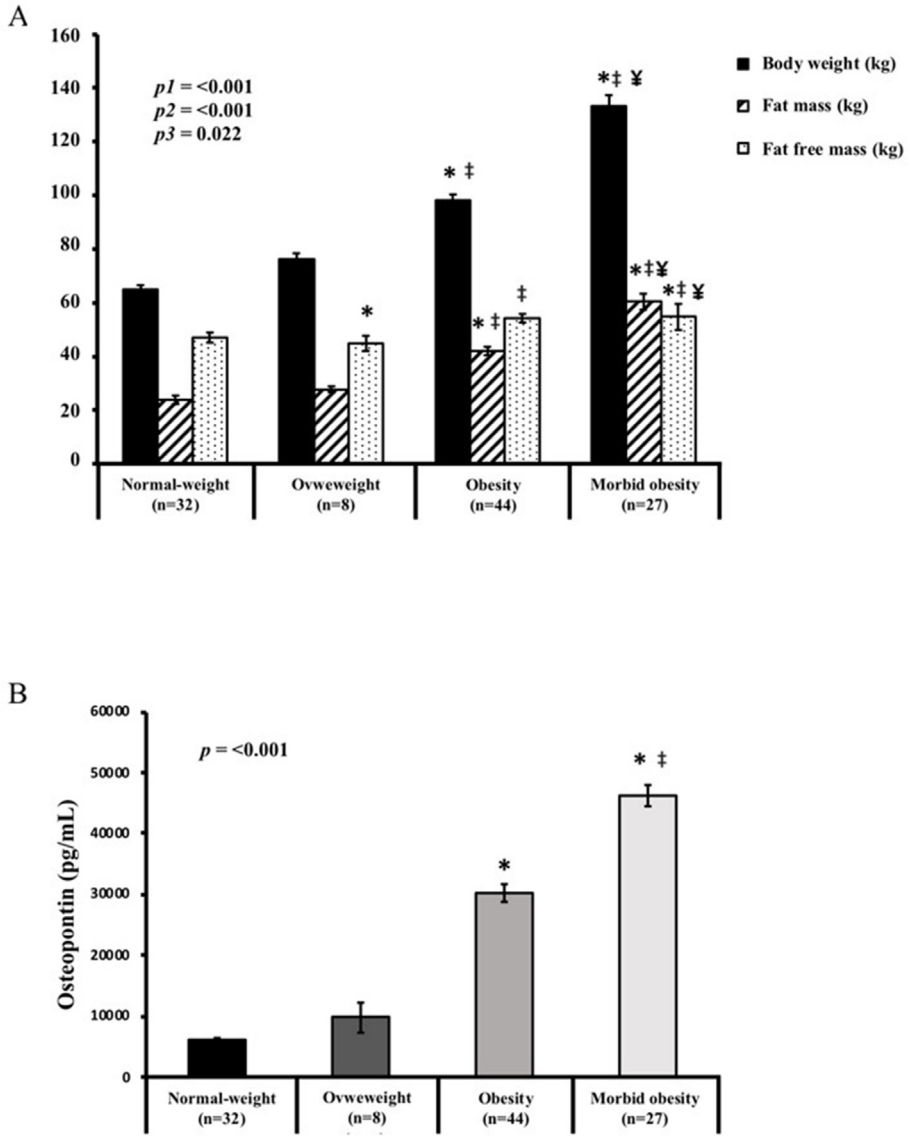

Fig. 2. Comparison of body composition and Osteopontin levels according to adiposity at baseline. (A) Differences in body weight and body composition between normal-weight, overweight, obese and severe obese individuals at baseline. **(B)** Differences in circulating levels of Osteopontin between normal-weight, overweight, obese and severe obese individuals at baseline. Data are presented as the mean; error bars represent the standard error. *P*-value is calculated by an analysis of covariance (ANCOVA) adjusted by gender and age for (A) ($p1$) body weight ($p2$) fat mass, and ($p3$) fat free mass and (B). Asterisk (*) denotes statistically significant ($p < 0.05$) differences in relation to normal-weight individuals evaluated using Student's t-test. ‡ denotes statistically significant ($p < 0.05$) differences in relation to overweight individuals evaluated using Student's t-test. ¥ denotes statistically significant ($p < 0.05$) differences in relation to individuals with obesity evaluated using Student's t-test.

Table 1
Basal characteristics and Osteopontin levels of study subjects according to adiposity.

	Normal-weight	Overweight	Obesity	Severe obesity	*P*-value
N	32	8	44	27	
Sex (male/female)	12/20	6/2	13/31	2/25	<0.001[a]
Age (years)	35.9 ± 9.7	37.9 ± 9.4	45.3 ± 9.9	41.5 ± 8.1	0.001
Height (m)	1.68 ± 0.09	1.66 ± 0.11	1.69 ± 0.09	1.63 ± 0.10	0.106
Body weight (kg)	64.7 ± 9.2	78.1 ± 8.2	96.6 ± 14.3	133.1 ± 21.5	<0.001
BMI (Kg/m^2)	22.7 ± 1.7	27.9 ± 1.5	34.8 ± 2.7	49.3 ± 9.0	<0.001
FM (kg)	24.0 ± 7.0	27.7 ± 2.5	41.2 ± 10.1	60.5 ± 7.8	<0.001
FFM (kg)	47.1 ± 9.0	44.8 ± 6.4	54.2 ± 11.0	54.8 ± 12.8	0.030
Osteopontin (pg/mL)	5986 ± 3037	5617 ± 7131	28,220 ± 28475	42,983 ± 40674	<0.001

Data show mean ± standard desviation. *P*-value was calculated with ANCOVA adjusted by gender and age.
[a] *P*-value was calculated with Chi-square. BMI, body mass index; FM, fat mass; FFM, fat free mass.

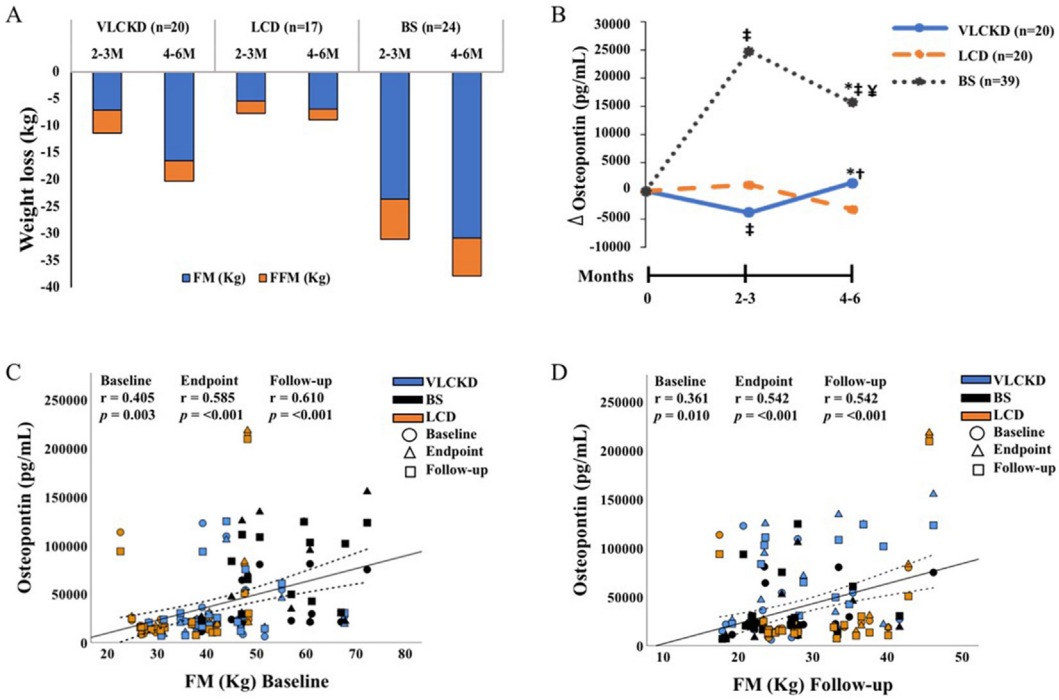

Fig. 3. Changes in body composition and Osteopontin levels and association between Osteopontin levels and fat mass during weight loss treatments. (A) Changes in body composition during very-low calorie ketogenic diet (VLCKD), a low-calorie diet (LCD) or bariatric surgery (BS). **(B)** Changes in circulating levels of Osteopontin during VLCKD, LCD or BS **(C)** Association between Osteopontin levels and baseline fat mass during intervention. **(D)** Association between Osteopontin levels and follow-up fat mass during intervention (A, B) Data show differences compared to baseline during the time-course of the intervention. Statistically significant differences were evaluated with repeated-measures ANOVA or ANCOVA adjusted for gender and age, as applicable. *Statistically significant ($p < 0.05$) differences over the duration of the nutritional program (from 0 to 4–6 months), ‡ statistically significant ($p < 0.05$) differences in relation to baseline, † statistically significant ($p < 0.05$) differences in relation to 2–3 months and ¥ statistically significant ($p < 0.05$) differences between treatments compared to baseline (C, D) Scatterplot representing the association between Osteopontin levels and fat mass. The center line represents the linear regression trendline. The lines above and below the center line represent the upper and lower bounds of the 95 % confidence interval around the trendline. r, correlation coefficient evaluated by the Spearman test; *p*, *p*-value.

Table 2
Association between osteopontin circulating levels and characteristics, body composition, anthropometric and biochemical parameters in participants with obesity at baseline.

	Spearman's r coeficient	*P*-value
Age (years)	−0.056	0.646
Height (m)	−0.044	0.722
Body weight (kg)	**0.348**	**0.003**
BMI (Kg/m^2)	**0.368**	**0.002**
FM (kg)	**0.405**	**0.003**
FFM (kg)	−0.134	0.349
β-OHB (mmol/L)	−0.060	0.726
HOMA-IR	0.006	0.962

BMI, body mass index; FM, fat mass; FFM, fat free mass.

levels are also elevated when there is bone destruction. The almost non-existent loss of fat-free mass that occurs in patients following a VLCKD could also explain the decrease in OPN levels. However, in the animal model we observed that the *OPN* expression is increased in adipose tissue, mainly visceral adipose tissue; while it was lower in muscle from DIO than in lean mice. It suggests that the OPN circulating levels observed in obesity status could be particularly secreted by adipose tissue.

Adipose tissue in obesity is dysfunctional and this situation creates a proinflammatory microenvironment that was proposed as the main promote of the physiopathology of obesity [46,47]. BS induced metabolic stress, loss of muscle and bone mass in the first months following the surgery [43,44,48], contrary to what occurs in patients following a VLCKD [14,48], which could explain the increase in OPN circulating levels observed in the patients underwent BS.

On the other hand, these results were particularly observed in the more acute phase of the treatment, showing that ketosis induced by the VLCKD could be a key aspect in the differential observed effect of both weight loss strategies. As far as we know, there are not evidence regarding to the effect of ketone bodies or nutritional ketosis on expression and secretion of OPN. It further reinforces the beneficial effect of this type of nutritional intervention which could suggest that patients with obesity after following a VLCKD are in a good health status. Similar pattern of OPN circulating levels was previously observed considering other proteins associated with obesity physiopathology, such as FGF21 [48].

Considering that epigenetic regulation, specifically DNA methylation, plays a role in obesity development and also *OPN/SPP1* gene expression is epigenetically regulated, we hypothesized that the effect of adiposity on OPN secretion, could be regulated by epigenetic mechanisms such as DNA methylation. In this study we observed no differences between normal weight volunteers and patients with obesity, nor after bariatric surgery in *OPN/SPP1* DNA methylation levels, but a slight change was observed after following VLCKD. These results suggest that the effect of adiposity or nutritional ketosis on OPN secretion could be epigenetically regulated by means of DNA methylation. However, further studies are needed to elucidate this hypothesis.

The combination of findings from animal experiments and human studies strengthen the validity of the conclusions drawn by providing a more comprehensive view of this topic. The longitudinal design is other relevant strength of this study, which allows the evaluation of the changes of OPN circulating levels in parallel with the metabolic and body composition distribution after a

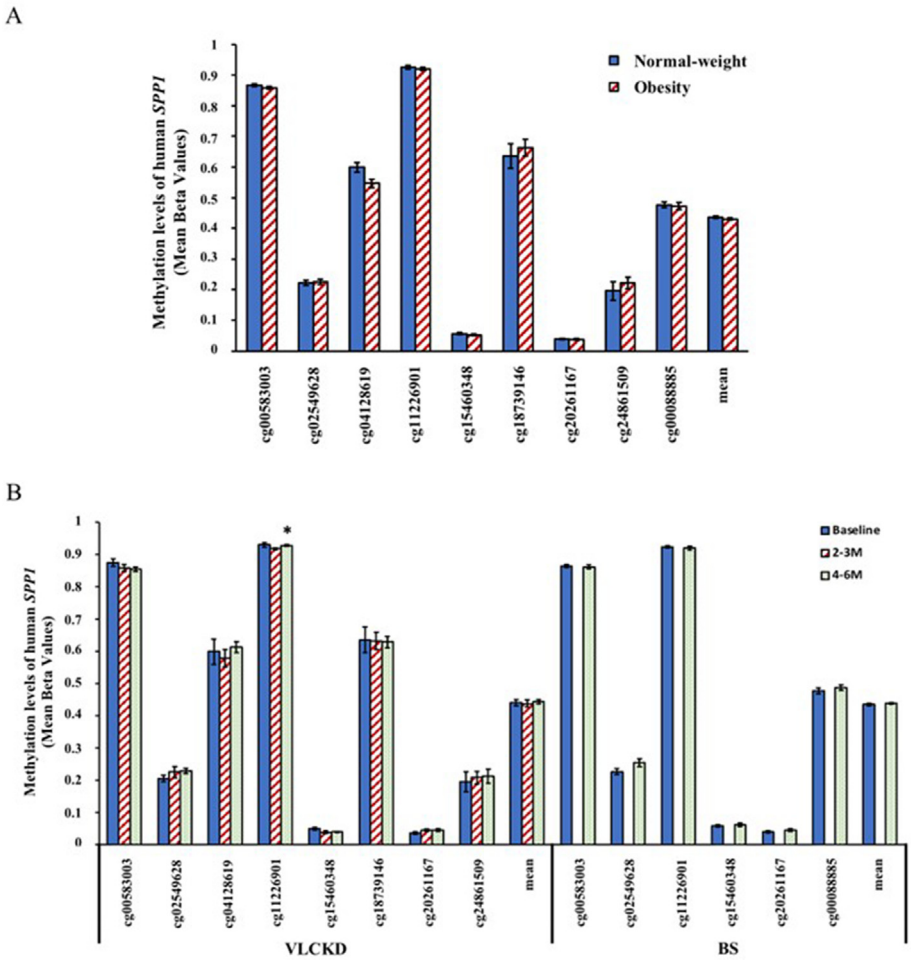

Fig. 4. Differences in DNA methylation of *SPP1*/Osteopontin. (A) Differences in DNA methylation of *SPP1*/Osteopontin according to adiposity at baseline **(B)** Differences in DNA methylation of *SPP1*/Osteopontin in patients with obesity during very-low calorie ketogenic diet (VLCKD) or bariatric surgery (BS). Data are presented as the mean; error bars represent the standard error. Asterisk (*) denotes statistically significant ($p < 0.05$) differences in relation to baseline evaluated using Student's t-test.

therapy to lose weight. The study examines both OPN expression in adipose tissue and its levels in blood, providing a holistic perspective on its role in obesity and weight loss. Comparing three different weight-loss strategies (VLCKD, LCD, and bariatric surgery) allows for evaluating the efficacy of each intervention and its specific impact on OPN levels. Including DNA methylation analysis of OPN is innovative, shedding light on how dietary and surgical interventions may influence its epigenetic regulation. The use of an animal model combined with human data strengthens the validation of findings and enhances their potential applicability in clinical settings. This study provides, for the first time, evidence of the differential profile of OPN depending on the type of weight loss treatment, suggesting that the circulating levels could be a biomarker of the health status of patients after an obesity therapy. Moreover, a potential effect of nutritional ketosis on OPN secretion is revealed for the first time in this study.

On the other hand, the observational association design of this study could be a limitation because it does not demonstrate causality but propose hypotheses and mechanisms potentially underlying the association between OPN secretion and changes in adiposity, metabolic stress and nutritional ketosis. A small sample size could limit the generalizability of the results and reduce the statistical power to detect significant differences, short study durations may not allow for evaluating the long-term effects of interventions, particularly VLCKD. Moreover, OPN is not exclusively associated with obesity and weight loss, as it is also linked to general inflammation and other diseases. This fact could complicate the interpretation of findings. Finally, lifestyle factors such as physical activity, sleep patterns, and behavioural influences were not considered as confounding variables in this study and this fact could compromise a comprehensive interpretation of the dietary interventions' effects. However, participants were instructed to maintain their usual physical activity throughout the study, ensuring that any observed metabolic changes could be attributed primarily to the dietary intervention. Additionally, the primary focus of our research was to investigate the direct changes in OPN levels associated with the weight loss interventions, rather than the broader lifestyle context. Thus, the study has notable strengths, such as its comprehensive approach, clinical relevance, and innovative epigenetic analysis. This study opens a door to performing further investigation to determine the underlying mechanisms and provide evidence of the potential use of OPN as a biomarker of obesity-related landscape, as previously suggested [49]. However, factors like sample size, study duration, and control of confounding variables may limit its impact. These strengths and limitations should be considered when interpreting the results and designing future research.

The findings from this study could have significant implications in the fields of obesity research, clinical nutrition, and therapeutic development. The evaluation of osteopontin (OPN) expression in

adipose tissue and its levels in blood could establish OPN as a reliable biomarker for tracking obesity progression, weight loss, and metabolic improvements. Future studies could explore the utility of OPN as a diagnostic or prognostic marker for obesity-related complications, such as cancer. In addition, the findings may enhance understanding of OPN's role in inflammation, immune modulation, and adipose tissue remodeling during obesity and weight loss. This could pave the way for targeting OPN-related pathways to develop new pharmacological or dietary interventions. Patients with specific OPN expression or methylation patterns could benefit more from one intervention over another, optimizing clinical outcomes.

In conclusion, this study evidences that nutritional ketosis and weight loss induced by a VLCKD modulates the increased circulating levels of OPN in patients with obesity. OPN expression could be induced in response to a metabolic stress and therefore, OPN circulating levels could be a biomarker of the obesity-related inflammation and metabolic stress resolution or health status after a weight loss treatment. Further studies are needed to elucidate if this effect is epigenetically regulated. The study's findings have potential clinical relevance, particularly in the context of obesity and obesity-related diseases, including cancer. This study has the potential to deepen our understanding of OPN's role in obesity and weight loss while providing actionable insights into the efficacy of different interventions like VLCKD, LCD, and BS. The findings could influence future research directions, clinical practice, and the development of novel therapies targeting obesity and its related complications. Understanding how nutritional ketosis affects OPN levels could have implications for improving patient outcomes.

Authorship contribution statement

PML and AGI, performed experiments and analysed data; PML and ABC wrote the manuscript; IS and DG-A, MAZ, IA, JB performed the recruitment and following of the patients; MN-G, DdL, FJT and JAM contributed to the interpretation of data and discussion; FFC and ABC obtained funding; ABC designed and coordinated research. ABC is the guarantor of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and accuracy of the data analysis. All authors red and approved the final manuscript.

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Conflict of Interest

D. G-A., D.dL., F.J.T., A.B.C. and F.F.C. received advisory board fees and/or research grants from PronoKal Group®, Spain. I.S. and M.N-G. belong to the Medical department of Pronokal, Spain.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2025.02.006.

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