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Original article

Epigenetic landscape in blood leukocytes following ketosis and weight loss induced by a very low calorie ketogenic diet (VLCKD) in patients with obesity

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SUMMARY

Background: The molecular mechanisms underlying the potential health benefits of a ketogenic diet are unknown and could be mediated by epigenetic mechanisms.
Objective: To identify the changes in the obesity-related methylome that are mediated by the induced weight loss or are dependent on ketosis in subjects with obesity underwent a very-low calorie ketogenic diet (VLCKD).
Methods: Twenty-one patients with obesity (n = 12 women, 47.9 ± 1.02 yr, 33.0 ± 0.2 kg/m^2) after 6 months on a VLCKD and 12 normal weight volunteers (n = 6 women, 50.3 ± 6.2 yrs, 22.7 ± 1.5 kg/m^2) were studied. Data from the Infinium MethylationEPIC BeadChip methylomes of blood leukocytes were obtained at time points of ketotic phases (basal, maximum ketosis, and out of ketosis) during VLCKD (n = 10) and at baseline in volunteers (n = 12). Results were further validated by pyrosequencing in representative cohort of patients on a VLCKD (n = 18) and correlated with gene expression.
Results: After weight reduction by VLCKD, differences were found at 988 CpG sites (786 unique genes). The VLCKD altered methylation levels in patients with obesity had high resemblance with those from normal weight volunteers and was concomitant with a downregulation of DNA methyltransferases (DNMT)1, 3a and 3b. Most of the encoded genes were involved in metabolic processes, protein metabolism, and muscle, organ, and skeletal system development. Novel genes representing the top scoring associated events were identified, including *ZNF331*, *FGFRL1* (VLCKD-induced weight loss) and *CBFA2T3*, *C3orf38*, *JSRP1*, and *LRFN4* (VLCKD-induced ketosis). Interestingly, *ZNF331* and *FGFRL1* were validated in an independent cohort and inversely correlated with gene expression.
Conclusions: The beneficial effects of VLCKD therapy on obesity involve a methylome more suggestive of normal weight that could be mainly mediated by the VLCKD-induced ketosis rather than weight loss.

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Abbreviations list

ACACB	Acetyl-CoA Carboxylase Beta
AEBP1	AE binding protein 1
ANOVA	analysis of variance
BMI	body mass index
C3orf38	chromosome 3 open reading frame 38
CACNA1H	Calcium Voltage-Gated Channel Subunit Alpha1 H
CAMKK1	Calcium/Calmodulin Dependent Protein Kinase Kinase 1
CBFA2T3	CBFA2/RUNX1 Partner Transcriptional Co-Repressor 3
cDNA	complementary DNA
CENPF	Centromere Protein F
CHAT	choline O-acetyltransferase
CHUK	Component Of Inhibitor Of Nuclear Factor Kappa B Kinase Complex
COL1A1	collagen, type I, alpha 1
COL5A1	collagen type V alpha 1 chain
COL9A2	collagen, type IX, alpha 2
CV	coefficient of variation
DMCpGs	differentially methylated CpGs
DNMTs	DNA methyltransferases
DNA	deoxyribonucleic acid
EWAS	epigenome-wide association study
FDR	false discovery rate
FF	fresh-frozen
FGFRL1	Fibroblast growth factor receptor (FGFR)-like protein 1
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GO	gene ontology
HRAS	HRas proto-oncogene, GTPase
INSR	insulin receptor
JSRP1	Junctional Sarcoplasmic Reticulum Protein 1
LAMA2	Laminin Subunit Alpha 2
LRFN4	Leucine Rich Repeat And Fibronectin Type III Domain Containing 4
MKNK2	MAPK Interacting Serine/Threonine Kinase 2
PRKAG2	Protein Kinase AMP-Activated Non-Catalytic Subunit Gamma 2
PRKCZ	Protein Kinase C Zeta
qRT-PCR	Real-time quantitative polymerase chain reaction
RELA	v-rel reticuloendotheliosis viral oncogene homolog A
RNA	Ribonucleic acid
RPTOR	Regulatory Associated Protein Of MTOR Complex 1
SD	standard deviation
SEM	standard error of the mean
SGCB	Beta-sarcoglycan
SMTN	Smoothelin
TRAF2	TNF Receptor Associated Factor 2
TSC2	Tuberous Sclerosis Complex 2
TSS 200	transcription start sites 200
VLCKD	very-low-calorie ketogenic diet
WC	Waist circumference
ZFHX3	zinc finger Homeobox 3
ZNF331	zinc finger protein 331
β-OHB	beta-hydroxy-butyrate

1. Introduction

Ketosis has gained interest over recent years due to its induced benefits that it imparts on several health conditions [1,2]. Ketosis is associated with a delay in the onset of diseases and increased longevity [3]. Similarly, ketosis is suggested to have an extensive range of health benefits, from increased physical endurance in athletes [4,5] to delayed aging [6]. Also to improve conditions such as neurodegenerative disease [7–9] cancer [10–12], cardiovascular disease [13], and obesity [14]. Some of these studies involved high fat ketogenic diets and even though the main characteristic of ketogenic diets is the carbohydrates restriction, the specific composition in macronutrients and calories should be taken into consideration for the impact in clinical practice [15].

A very-low-calorie-ketogenic diet (VLCKD) was demonstrated to be an effective strategy in managing obesity [16], including weight loss and maintenance [17], increased preservation of muscle mass [18], and enhanced resting metabolic rate [19]. Moreover, it is able to improve metabolic parameters in patients with obesity [20,21] and type 2 diabetes [22]. Additionally, it was demonstrated that a VLCKD is able to reduce food craving and improve psychobiological parameters to help improve quality of life in patients with obesity [23]. However, the molecular mechanisms underlying these benefits of ketogenic diet remain unknown.

The main molecular mechanism that links the effect of environmental factors, such as nutrition, with the regulation of the genes function is epigenetics [24]. Indeed, dietary factors or dietary patterns were identified as modulators of epigenetic mechanisms [25–28].

Recently, it was suggested that ketone bodies orchestrate gene expression via epigenomic mechanisms [29]. This molecular effect of nutritional ketosis has been reported in neurologic disorders such as epilepsy [30,31] and Kabuki syndrome and related disorders [32]. The effect of ketone bodies on epigenetic regulation was also proposed as a potential opportunity for anticancer therapies [33]. Moreover, ketogenic diets, including dietary restriction, delay aging through epigenetic effects [34].

On the other hand, obesity is associated with a specific methylation profile in several tissues [35–38] and the obesity-related methylome is related to complications such as insulin resistance [39] and cancer [40,41]. Moreover, several methylation marks were identified as potential biomarkers for predicting the success of weight loss therapies during the active phase of treatment [42–46] or during the period of weight loss maintenance [47,48]. Therefore, DNA methylation was proposed as a target for preventing and managing obesity [48–51].

In light of the above evidence, the aim of the current study was to evaluate how a VLCKD might affect the obesity methylome. Furthermore, this study aimed to identify the changes in the obesity-related methylome that are mediated by the induced weight loss or are dependent on ketosis.

2. Materials and methods

2.1. Patient cohort

The DNA and RNA for methylation and gene expression assays were isolated from blood samples of patients from a 6-month nutritional intervention study performed at the Endocrinology and Nutrition Department of the Hospital Clinico, Universitario of Valladolid; the patients were receiving treatment for obesity. In

addition, samples from a group of healthy volunteers were also analyzed.

The inclusion criteria were: age between 18 and 65 years, body mass index (BMI) $\geq$ 30 kg/m^2, stable body weight over the previous 3 months, a desire to lose weight, and a history of failed dietary efforts. The main exclusion criteria were thyroid alteration, diabetes mellitus, obesity induced by other endocrine disorders or drugs, and participation in any active weight-loss program in the previous 3 months. In addition, patients with previous bariatric surgery, reported or suspected abuse of narcotics or alcohol, severe depression or any other psychiatric disease, severe hepatic insufficiency, any type of renal insufficiency or gout episodes, nephrolithiasis, neoplasia, previous instances of cardiovascular or cerebrovascular disease, uncontrolled hypertension, orthostatic hypotension, and hydroelectrolytic or electrocardiographic alterations were excluded. Females who were pregnant, breastfeeding, or intending to become pregnant and those with child-bearing potential who were not using adequate contraceptive methods were also excluded. Apart from obesity and metabolic syndrome, participants were generally healthy individuals. Under these criteria, 21 patients with obesity and 12 volunteers with normal weight were included in this study.

The study protocol was in accordance with the Declaration of Helsinki and was approved by the Ethics Committee for Clinical Research of Hospital Clinico Universitario de Valladolid, Spain (C.I: 40/13, PNK-DHA2013-01). Participants provided written informed consent before any intervention related to the study. Participants received no monetary incentives.

2.2. Very-low-calorie ketogenic diet protocol

Patients included in this study derived from a randomized clinical trial investigating the effect of docosahexaenoic acid (DHA) supplementation in a very low-calorie ketogenic diet. The clinical trial consisted in two arms: one arm where patients follow a VLCKD and other arms where patients followed a VLCKD + DHA [21].

Nutritional intervention was based on a commercial weight-loss program (PNK method ®), as described elsewhere [18,21]. Briefly, the intervention included an evaluation by the specialist physician conducting the study, an assessment by an expert dietician, and exercise recommendations. This method is based on high-biological-value protein preparations obtained from cow's milk, soy, avian eggs, green peas, and cereals. Each protein preparation contained 15g protein, 4g carbohydrates, 3g fat, and provided 90–100 kcal. The VLCKD + DHA arm was supplemented with 500 mg/day DHA [21].

The weight-loss program has five steps and adheres to the most recent guidelines of the EFSA (2015) on total carbohydrate intake [52]. The first three steps consist of a VLCKD (600–800 kcal/day) that is low in carbohydrates (<50g daily from vegetables) and lipids (only 10g of olive oil per day). The amount of high biological-value proteins ranged between 0.8 and 1.2g per kg of ideal body weight to ensure that patients were meeting their minimum bodily requirements and to prevent the loss of lean mass. In step 1, the patients ate high-biological-value protein preparations five times a day and vegetables with low glycemic indices. In step 2, one of the protein servings was substituted with a natural protein (e.g., meat or fish) either at lunch or at dinner. In step 3, a second serving of low-fat natural protein was substituted for the second serving of biological protein preparation. Throughout these ketogenic phases, supplements of vitamins and minerals, such as K, Na, Mg, Ca, and omega-3 fatty acids, were provided in accordance with international recommendations [53]. These three steps were maintained until the patient lost the target amount of weight, ideally 80%. Because of this, the ketogenic steps varied in time depending on the individual and the weight-loss target. The total ketosis state lasted for a maximum of 60 days.

In either step 4 or 5, ketosis was ended by the physician in charge of the patient based on the amount of weight lost, and the patient began a low-calorie diet (800–1500 kcal/day). At this point, the patients underwent a progressive incorporation of different food groups and participated in a program of alimentary re-education to guarantee long-term maintenance of the weight loss. The maintenance diet consisted of an eating plan balanced for carbohydrates, protein, and fat. Depending on the individual, calories consumed ranged between 1500 and 2000 kcal/day, with the goal of maintaining the weight loss and promoting a healthy lifestyle.

During this study, patients followed the steps of the method until they reached the target weight, or up to a maximum of 4 months of follow-up, although patients remained under medical supervision for the following months. Patients visited the research team every 15 $\pm$ 2 days to evaluate adherence and potential side effects. Complete anthropometry, body composition, and biochemical assessments were performed at four of the visits, which were determined according to the evolution of each patient through the steps of ketosis and weight loss: Visit 1 (Baseline), visit 2 (Maximum Ketosis), visit 3 (Reduced Ketosis) and visit 4 (Endpoint). DNA methylation and gene expression were performed at visits 1, 2, and 4.

In all visits, patients received dietary instructions, individual supportive counsel, and encouragement to exercise on a regular basis using a formal exercise program. Additionally, a program of reinforcement telephone calls was instituted, and a phone number was provided to all participants to address any concerns.

2.3. Anthropometric assessment

All anthropometric measurements were performed after an overnight fast (8–10 h) under resting conditions in duplicate and performed by well-trained health workers. At each visit, patients were weighed on the same calibrated scale (Seca 200 scale, Medical Resources, EPI Inc OH, USA). BMI was calculated as body weight in kg, divided by the square of body height in meters (BMI = weight (kg)/height^2 (m)). Waist circumference (WC) was measured using a standard flexible non-elastic metric tape placed over the midpoint between the last rib and the iliac crest, with the patient standing and exhaling.

2.4. Determining levels of ketone bodies

Ketosis was determined by measuring ketone bodies, specifically β-hydroxy-butyrate (β-OHB), in capillary blood using a portable meter (GlucoMen LX Sensor, A. Menarini Diagnostics, Neuss, Germany; sensitivity <0.2 mmol/l). As with anthropometric assessments, all determinations of capillary ketonemia were made after an overnight fast of 8–10 h. These measurements were performed daily by each patient during the entire VLCKD, and the corresponding values were reviewed using machine memory by the research team to control adherence. Additionally, β-OHB levels were determined at each visit by the physician in charge of the patient.

2.5. DNA methylation analysis

2.5.1. DNA preparation and bisulphite conversion

DNA from fresh-frozen (FF) blood samples was isolated using a standard phenol-chloroform/proteinase-k protocol according to the manufacturer's instructions, with slight modifications. Genomic DNA was isolated from leukocytes using the MasturPure™ DNA purification kit (Epicentre Biotechnologies,

Madison, WI, USA). The isolated DNA was treated with RNase A for 1 h at 45 °C. All DNA samples were quantified using the fluorometric method (Quan-iT PicoGreen DsDNA Assay, Life Technologies) and were assessed for purity using a NanoDrop (Thermo Scientific) to determine 260/280 and 260/230 ratio measurements. The integrity of the FF DNA was verified by electrophoresis in 1.3% agarose gel. DNA (500 ng) was bisulfite converted using the EZ DNA methylation kit Methylation-Gold (Zymo Research, CA, USA) according to the manufacturer's instructions, which converts non-methylated cytosine into uracil.

2.5.2. Infinium MethylationEPIC BeadChip

High-quality DNA samples (500 ng) obtained from blood leukocytes of patients included in the VLCKD + DHA arm of the clinical trial (discovery cohort; n = 10 patients, 3 paired samples/patient) were selected for bisulfite conversion (Zymo Research; EZ-96 DNA Methylation™ Kit) and hybridization to Infinium MethylationEPIC BeadChip (Illumina) following the Illumina Infinium HD methylation protocol. DNA quality checks, bisulfite modification, hybridization, data normalization, statistical filtering, and value calculations were performed as previously described [54,55].

Whole-genome amplification and hybridization were then performed on a BeadChip followed by single-base extension and analysis on a HiScan SQ module (Illumina) to assess cytosine methylation states. The annotation of CG islands (CGIs) used the following categorization: 1) shore, for each of the 2-kb sequences flanking a CGI; 2) shelf, for each of the 2-kb sequences next to a shore; and 3) open sea, for DNA not included in any of the previous sequences or in CGIs [54,55]. The transcription start site 200 and the transcription start site 1500 indicate regions either 200 or 1500 bp from the transcription start site, respectively.

2.5.3. Pyrosequencing analysis

Pyrosequencing was used to assess selected markers in 18 patients (validation cohort: (n = 7 derived from the discovery cohort and n = 11 from an independent cohort of patients; 3 paired samples/patient)). DNA samples analyzed in the validation cohort were derived from patients included in the two arms of the clinical trial and merged for the statistical analysis (VLCKD: n = 10; VLCKD + DHA: n = 8). The primer sequences used in this analysis were designed using Qiagen's PyroMark Assay Design 2.0 software to hybridize to CpG-free sites to ensure methylation-independent amplification (details and primer sequences are available in Supplementary Table S1). Genomic DNA was isolated from FF blood leukocytes using the MasturPure™ DNA purification kit (Epicentre Biotechnologies, Madison, WI, USA), according to the manufacturer's instructions. DNA methylation analyses were performed using bisulfite-treated DNA (Zymo Research; EZ-96 DNA Methylation™ Kit) followed by a highly quantitative analysis based on PCR-based pyrosequencing using the PyroMark Q24 System version 2.0.7 (Qiagen). Methylation level was expressed as the percentage of methylated cytosine over the sum of methylated and unmethylated cytosines. Non-CpG cytosine residues were used as built-in controls to verify bisulfite conversion. The values are expressed as the mean for all sites. We also included human non-methylated and methylated DNA set as controls in each run (Zymo Research). The inter-assay precision (%CV) was <2.5%, intra-assay (%CV) was <1.0%.

2.6. Expression assay by qRT-PCR

RNA from blood leukocytes (n = 18 patients) was extracted using Trizol (Invitrogen) according to the manufacturer's recommendations. The RNA concentrations were measured with a Nanodrop 2000 spectrophotometer (Thermo Scientific). From total extracted RNA, 2 μg were DNase treated using a DNA-free kit as a template (Ambion) to generate first-strand cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative polymerase chain reaction (qRT-PCR) was performed using TaqMan Universal PCR Master Mix, TaqMan Probes (Applied Biosystems) (details and primer sequences are available in Supplementary Table S1), and the Step OnePlus Real-Time PCR System (Applied Biosystems). All experiments were performed in duplicate, and gene expression levels were normalized to the levels of housekeeping gene GAPDH. 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 the geometric mean (SEM). qRT-PCR experiments were performed in compliance with the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (http://www.rdml.org/miqe).

2.7. Statistical analysis

The sample size of the current study was calculated to detect differences for methylation levels taking into account published values of epigenome-wide analysis in the field of obesity [35,40,41]. The interventional differences were examined in two independent cohorts. Microarray-based DNA methylation analysis was performed in the discovery cohort (n = 10 patients; 3 paired samples/patient), and then the identified genes were validated in an independent cohort of patients (validation cohort; n = 18 patients; 3 paired samples/patient). Finally, the association between DNA methylation level of the identified CpG sites and the anthropometric or biochemical parameters was assessed in the global cohort of patients included in this study (n = 28).

The methylation level of each cytosine was expressed as a β value, which was calculated as the fluorescence intensity ratio of the methylated to the unmethylated version of the probe. β values ranged between 0 (unmethylated) and 1 (completely methylated) according to the combination of the Cy3 and Cy5 fluorescence intensities. Color balance adjustment and normalization were performed to normalize the samples between the two-color channels using Genome Studio Illumina software (V2010.3). Genome Studio normalizes data using different internal controls that are present on the Infinium MethylationEPIC BeadChip. This software also normalized data depending on internal background probes [55]. β values with detected p-values > 0.01 were considered to fall below the minimum intensity and threshold, and these CpGs were consequently removed from further analysis. Additionally, probes that contained single nucleotide polymorphisms (SNPs) at the 10 bp 3′ end of the interrogating probe were filtered out. To identify consistent patterns of DMCpGs due to the nutritional intervention, a linear model was fitted using a B-spline approximation [56]. Three linear models were fitted: Model 1 was fitted by including the three points of the nutritional intervention to evaluate the general effect of VLCKD; Model 2 including baseline and maximum ketosis to evaluate the effect of ketosis and weight loss; Model 3 including methylation levels at maximum ketosis and endpoint to evaluate the effect of only weight loss, without ketosis. P values were adjusted for multiple comparisons using the false discovery rate (FDR) procedure of Benjamini and Hochberg, and results were considered statistically significant when FDR < 0.10. Additionally, we applied a threshold for the significant sites based on the mean difference between visits with a minimum β value change of ±0.02. Euclidean cluster analysis of significant CpGs was performed using a heatmap function. The global methylation level was compared between the nutritional intervention visits by univariant ANOVA and a Bonferroni post-hoc analysis. All of the aforementioned statistical analyses were performed using R software (version 3.2.0).

To estimate enrichment in biological processes, a hypergeometric test was performed using the GOstats package on the biological processes defined by gene ontology (GO) [57]. This analysis detected significant over-representation of GO terms in one set (i.e., list of identified genes) with respect to the entire genome. GO terms with an adjusted p-value < 0.05 were considered significant.

With SPSS version 21.0 software (SPSS Inc., Chicago, IL) for Windows XP (Microsoft, Redmond, WA), the genomic distribution of the differentially methylated CpGs was compared with the distribution of the CpGs from all analyzed sites on the Infinium MethylationEPIC BeadChip. P values were computed using the chi-square test to determine over- or under-representation of the CpGs. The potential association between anthropometric or biochemical parameters and DNA methylation levels (β-values) was evaluated using the Spearman coefficient test. Differences in DNA methylation levels and expression of the identified genes during the time-course of the intervention and between the nutritional intervention visits were assessed by the non-parametric tests, Kruskal Wallis and Mann–Whitney U, respectively. $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Patient characteristics

Samples from a total of 21 patients who followed the nutritional intervention based on a VLCKD were compared with samples from 12 healthy volunteers with normal weight and evaluated in this study (Supplemental Fig. 1). We first evaluated the discovery cohort (n = 10 participants with obesity who followed a VLCKD + DHA (n = 5 women) and n = 12 (6 women) volunteers with normal weight). An extended validation cohort composed of 11 patients (7 women) with obesity that followed a VLCKD + DHA or a VLCKD-DHA was also analyzed. Statistically significant differences were not observed between either cohort in age, gender, height, body weight, BMI, waist circumference, ketosis, or the response to weight loss treatment (Table 1). Differences statistically significant were only detected in body weight, BMI and waist circumference between patients with obesity and subjects with normal weight (Table 1). All patients lost weight after nutritional intervention (21.8 ± 4.9%), together with reductions in BMI (21.9 ± 5.1%) and waist circumference (19.3 ± 4.4%).

3.2. DNA methylation changes during the global VLCKD intervention

DNA methylation profiles of blood leukocytes involving approximately 850 thousand CpGs were analyzed after VLCKD intervention. This analysis revealed statistically significant differences (cut-off point $\Delta \geq 0.02$; FDR ≤ 0.10) at 988 CpG sites, from a total of 739,222 valid CpGs (see detailed list in Supplementary Table S2). The differentially methylated CpGs were mostly characterized as changes towards CpG hypomethylation occurring after nutritional intervention, in both total DMCpGs (Fig. 1A) and in the DMCpGs located in promoters, and islands or shores (Fig. 1B). This result of global hypomethylation was correlated with the down-regulation in the expression of DNA methyltransferase (DNMT) 1 and the *de novo* methyltransferases DNMT3A and DNMT3B (Fig. 1C and Supplemental Fig. 2).

The identified CpG sites mapped to 786 unique genes and we were able to separate the samples according to nutritional intervention visits using a hierarchical cluster approach (Fig. 1D). It should be noted that the methylation levels of the 988 CpG sites differentially methylated after nutritional intervention were altered to resemble methylation levels observed in samples from subjects with normal weight (Fig. 1C). The 20 DMCpGs with the highest difference with respect to baseline among genes that are represented by more than 2 CpGs are represented in Table 2.

Regarding the functional distribution (Fig. 2), the differences in DNA methylation were mainly observed in open sea regions (Fig. 2A), with 43.2% of the DMCpG sites located in promoter regions and the majority of DMCpGs in the body (Fig. 2B). Moreover, the DMCpGs were mainly found in chromosomes 2, 9, 16, 17, 19, and 22 when compared with all CpGs analyzed (Fig. 2C).

Among the 988 DMCpGs, we found 886 (89.7%) with decreased and 102 (10.32%) with increased levels of DNA methylation after nutritional intervention. Moreover, the DMCpGs that lost methylation with respect to baseline were located mainly in the open sea (Fig. 2D) and body (Fig. 2E). In contrast, the DMCpGs that gained methylation after the nutritional intervention were mostly located in promoters (TSS 200 and 1st exon) (Fig. 2D) and in CpG islands (Fig. 2E). Regarding chromosome distribution, the DMCpGs with decreased methylation levels after nutritional treatment were found on chromosomes 1, 4, 6 7, 8, 9, 14, and 16, whereas DMCpGs with increased

Table 1
Clinical characteristics of patients at baseline and during the intervention with a VLCKD.

	Discovery cohort				Validation cohort			P value			
	Normal weight	Obesity			Obesity						
		Baseline (day 0)	Maximum ketosis (day 30)	Endpoint (day 180)	Baseline (day 0)	Maximum ketosis (day 30)	Endpoint (day 180)	Adiposity	Time	Cohort	Time x Cohort
N	12	10	10	10	18*	18	18	–	–	–	–
Age (years)	50.3 ± 6.2	48.8 ± 9.20	–	–	47.1 ± 9.8	–	–	0.654	–	0.678	–
Gender (men/women)	6/6	5/5	–	–	7/11	–	–	–	–	–	–
Height (m)	1.67 ± 0.08	1.68 ± 0.08	–	–	1.65 ± 0.10	–	–	0.773	–	0.446	–
Body weight (Kg)	63.8 ± 8.7	93.4 ± 9.9[a]	84.3 ± 8.4	73.9 ± 6.7[b]	90.8 ± 11.6	81.9 ± 10.4	70.4 ± 10.4[b]	<0.0001	<0.001	0.488	0.649
BMI (Kg/m^2)	22.7 ± 1.49	32.9 ± 1.4[a]	29.8 ± 1.7	26.1 ± 1.8[b]	33.2 ± 1.7	30.06 ± 1.7	25.7 ± 1.7[b]	<0.0001	<0.001	0.986	0.334
Waist circumference (cm)	77.4 ± 7.2	111.1 ± 6.6[a]	102.3 ± 7.4	90.2 ± 3.1[b]	108.3 ± 8.5	100.1 ± 7.3	86.9 ± 7.4[b]	<0.0001	<0.001	0.325	0.836
Ketonemia (mM)	–	0.15 ± 0.07	2.11 ± 1.11[b]	0.15 ± 0.07	0.18 ± 0.08	1.71 ± 1.08[b]	0.18 ± 0.08	–	<0.0001	0.445	0.374
Weight loss (%)	–	–	9.69 ± 2.51	20.58 ± 5.17[c]	–	9.80 ± 1.99	22.52 ± 4.75[c]	–	<0.0001	0.336	0.455

Data shown are mean ± SD (standard deviation).* The sample size of the validation cohort was completed with n = 7 patients from the discovery cohort and 11 patients from an independent cohort.
Abbreviations: VLCKD, very-low-calorie ketogenic diet.
[a] Statistically significant differences compared with control Normal weight in discovery cohort.
[b] Statistically significant differences compared with Baseline in both cohorts.
[c] Statistically significant differences compared with Maximum Ketosis in both cohorts.

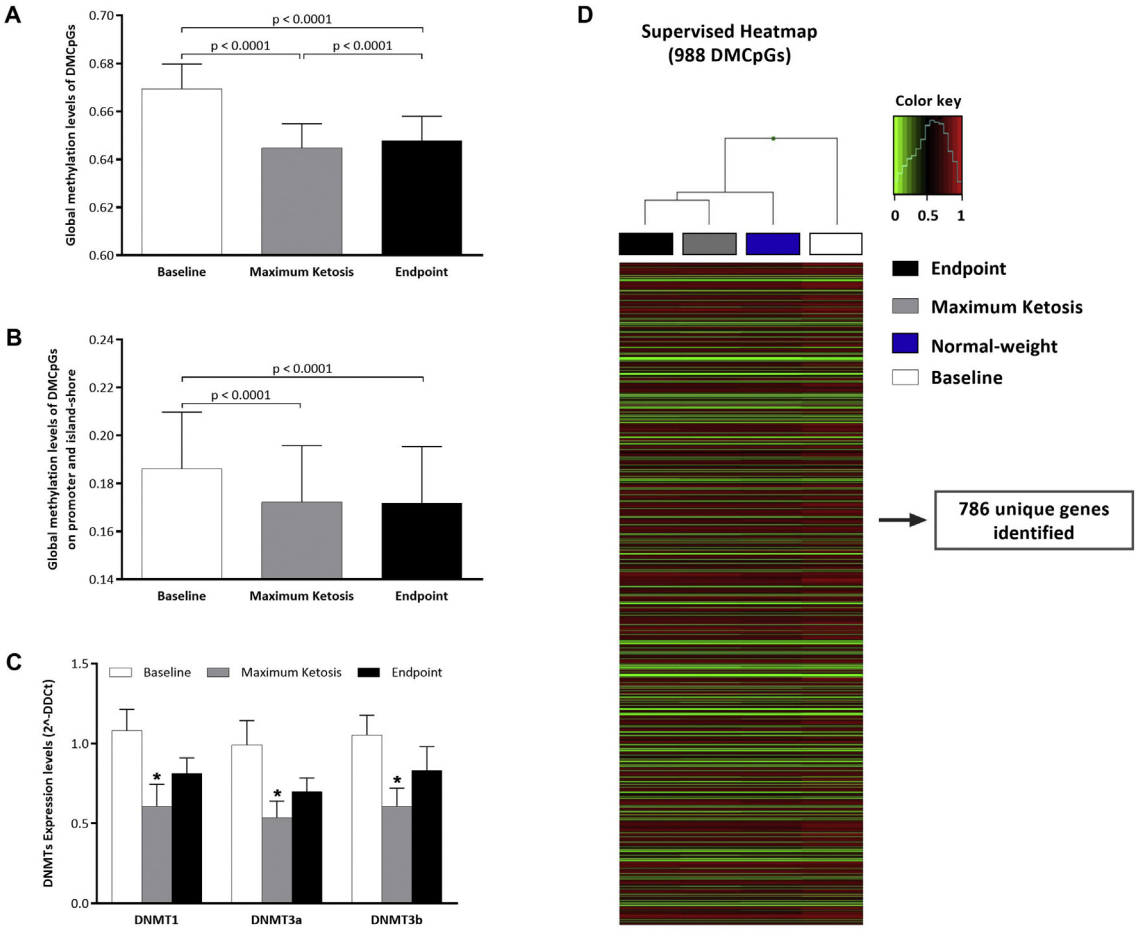

Fig. 1. Profile of DNA methylation following the VLCKD (n = 10; Discovery cohort). (A). Global differences in methylation levels of 988 DMCpGs identified by Infinium MethylationEPIC BeadChip analysis. (B). Global differences in methylation levels of DMCpGs located in the promoter and island/shore. (C). Expression levels of DNA methyltransferase (DNMT) 1 and the de novo methyltransferases DNMT3A and DNMT3B (D). Supervised clustering of the 988 CpGs that were found to be differentially methylated between patients with obesity and healthy volunteers with normal weight (n = 12) groups. The identified CpG sites mapped to 786 unique genes. Differences statistically significant detected (P < 0.0001). DMCpGs, differentially methylated CpGs; VLCKD, very-low calorie ketogenic diet.

methylation levels were mainly found in chromosomes 3, 5, 10, 11, 12, 17, 21, and 22 (Fig. 2F).

3.3. Biological significance of the dietary intervention-related DMCpG sites and associated genes

Interestingly, most differentially methylated genes belonged to a network significantly enriched in protein interactions ($p < 0.001$) according to STRING analysis (Fig. 3A). GO analysis was performed to test whether certain molecular functions or biological processes were significantly enriched within the 786 genes associated with the 988 DMCpGs discovered through VLCKD intervention (Fig. 3B). Among the categories of functional processes that exhibited statistical significance (FDR < 0.05), we found processes related to regulation of transcription, signal transduction, cell differentiation, proliferation and apoptosis, metabolic processes, response to hypoxia, protein processing, muscle organ and skeletal system development, nervous system development and axon guidance (Fig. 3B). Among these, we highlighted relevant pathways in the field of obesity physiopathology such as insulin signaling, protein digestion and absorption, adipocytokine signaling and muscle development (Fig. 4A–D). To investigate biological relevance, the CpG sites representing promoter regions (TSS1500, TSS200, 5′ UTR and 1st Exon) at CpG islands/shores were selected. This selection yielded 141 CpGs representing 150 unique genes. Based on this filter we identified CpG sites that could be genetic targets whose methylation is associated with VLCKD responses. Among these, the most representative gene was *ZNF331,* which was represented by 2 CpG sites located in the promoter and island with the highest difference with respect to baseline. Furthermore, *FGFRL1* was also selected from among the DMCpG sites because of its biological relevance in metabolic pathways and obesity pathogenesis and because the methylation level of this gene in leukocytes was previously proposed as an episignature that mirrors methylation levels of dysfunctional adipose tissue in obesity [35].

3.4. DNA methylation changes associated with the effects of dietary-induced ketosis

An analysis comparing baseline (day 0) with maximum ketosis (day 30) yielded 1365 DMCpGs. With respect to all CpGs analyzed, these CpGs were found mainly in the open sea (Fig. 5A) and body (Fig. 5B) and in chromosomes 1, 10, 11, 17, 19, and 22 (Fig. 5C). The DMCpGs that gained methylation were found in the island and

Table 2
List of DMCpGs with the highest differences for Baseline - Maximum Ketosis - Endpoint between genes that are represented by more than 2 CpGs.

TargetID	CHR	Position	Gene Name	Gene region	CpG context	Methylation levels (mean)			Differences		p value	FDR
						B	MK	E	MK - B	E – B		
cg04254103	19	1794380	ATP8B3; ATP8B3; ATP8B3	Body; Body; Body	N_Shore	0.826	0.786	0.796	−0.039	−0.030	0.001	0.091
cg06643002	14	105735797	BRF1; BRF1; BRF1; BRF1;	Body; Body; Body; Body; Body		0.782	0.741	0.748	−0.040	−0.034	0.001	0.099
cg12063937	1	7731375	CAMTA1	Body		0.837	0.785	0.802	−0.051	−0.034	0.000	0.054
cg00035197	16	88962986	CBFA2T3; CBFA2T3	Body; Body	Island	0.803	0.748	0.763	−0.056	−0.040	0.001	0.082
cg09962824	21	44479417	CBS	Body	N_Shore	0.754	0.707	0.714	−0.047	−0.040	0.000	0.051
cg23790296	10	73233854	CDH23; CDH23; CDH23; CDH23; CDH23	Body; Body; Body; Body; Body		0.846	0.814	0.820	−0.032	−0.026	0.001	0.095
cg26493726	19	36508614	CLIP3	Body	S_Shelf	0.808	0.771	0.773	−0.037	−0.035	0.000	0.069
cg01818220	9	23821773	ELAVL2; ELAVL2; ELAVL2; ELAVL2	5′UTR; TSS1500	Island	0.092	0.148	0.134	0.056	0.042	0.000	0.008
cg01933710	5	132596864	FSTL4	Body		0.828	0.778	0.799	−0.049	−0.029	0.001	0.076
cg07390459	2	121582002	GLI2	Body		0.768	0.720	0.727	−0.048	−0.041	0.001	0.097
cg14777822	2	121728297	GLI2	Body		0.770	0.728	0.726	−0.042	−0.044	0.001	0.093
cg16120742	7	50345049	IKZF1	5′UTR	S_Shore	0.114	0.058	0.041	−0.057	−0.073	0.000	0.026
cg02999309	10	134502626	INPP5A	Body	N_Shore	0.836	0.799	0.807	−0.037	−0.029	0.000	0.024
cg14010696	19	5119250	KDM4B	Body	Island	0.853	0.812	0.819	−0.041	−0.034	0.000	0.008
cg16333587	14	101369826	MEG8	Body		0.857	0.817	0.828	−0.040	−0.029	0.000	0.051
cg20963002	14	101360088	MEG8	TSS1500		0.862	0.821	0.828	−0.041	−0.034	0.001	0.098
cg18026309	2	26683651	OTOF; OTOF; OTOF; OTOF; OTOF	Body; Body; Body; Body; Body		0.884	0.851	0.857	−0.033	−0.027	0.000	0.051
cg01764953	11	70814657	SHANK2	Body		0.914	0.887	0.887	−0.027	−0.026	0.000	0.067
cg19696891	19	54057705	ZNF331; ZNF331; ZNF331	5′UTR; 5′UTR; TSS1500	Island	0.379	0.431	0.447	0.052	0.068	0.000	0.015
cg03643149	19	54041519	ZNF331; ZNF331; ZNF331; ZNF331; ZNF331	TSS1500; 1stExon; TSS200; TSS200; 5′UTR; 5′UTR	Island	0.397	0.445	0.458	0.048	0.061	0.000	0.019

Data shown are mean.
Abbreviations: B, baseline; CHR, chromosome; E, endpoint; FDR, false discovery rate; MK, maximum ketosis.

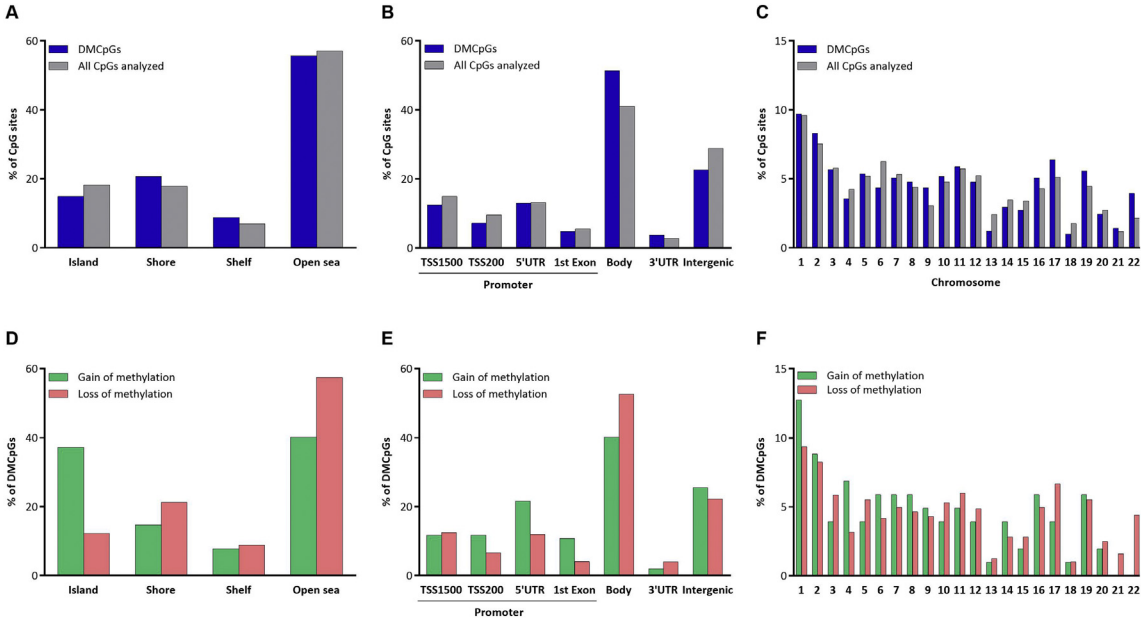

Fig. 2. Characterization of the DMCpGs after VLCKD in the discovery cohort (n = 10). (A) Genomic distribution of the DMCpGs and their respective locations regarding the broader CpG context, (B) gene region and (C) chromosome. (D) Genomic distribution of DMCpGs comparing those that exhibited an increase with those that exhibited a decrease in methylation levels following VLCKD, and their respective locations in the broader CpG context, (E) the gene region and (F) chromosome. DMCpGs, differentially methylated CpGs; VLCKD, very-low calorie ketogenic diet.

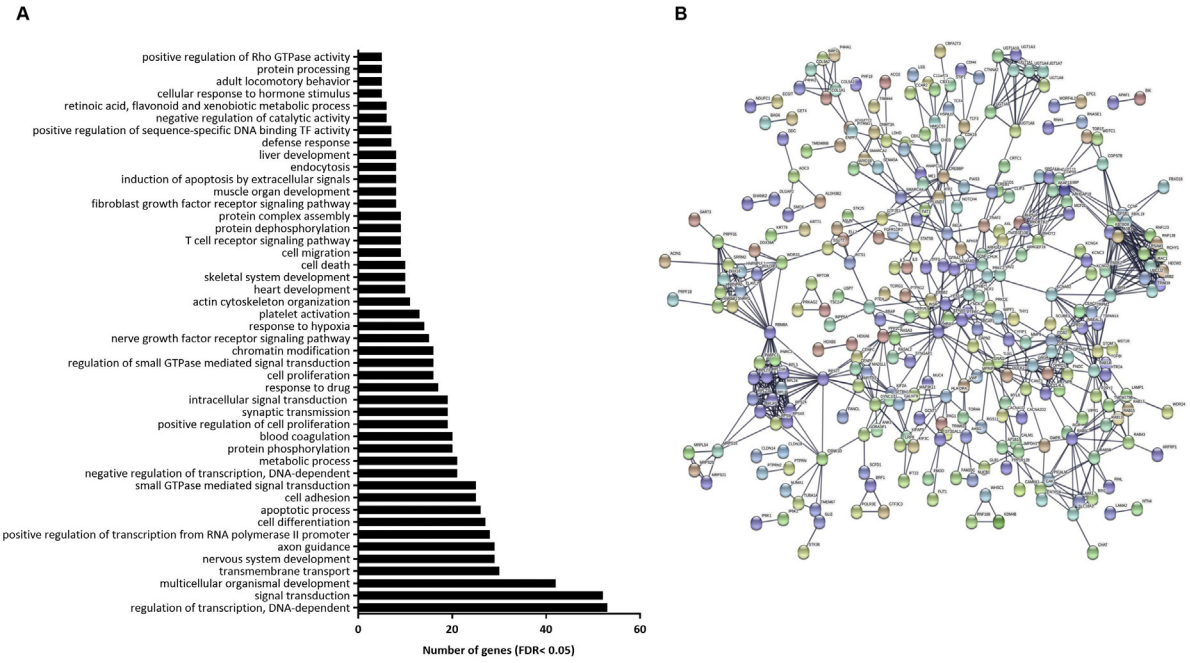

Fig. 3. Biological implications of the DMCpGs following a VLCKD. (A) Summary of the GO analysis of the biological process categories representing the differentially methylated genes associated with DMCpG sites. (B) Gene-protein interaction network-STRING analysis. Most of the genes regulated by methylation belonged to a network significantly enriched in protein interactions ($p < 0.001$) according to STRING analysis. DMCpGs, differentially methylated CpGs; GO, gene ontology; VLCKD, very-low calorie ketogenic diet.

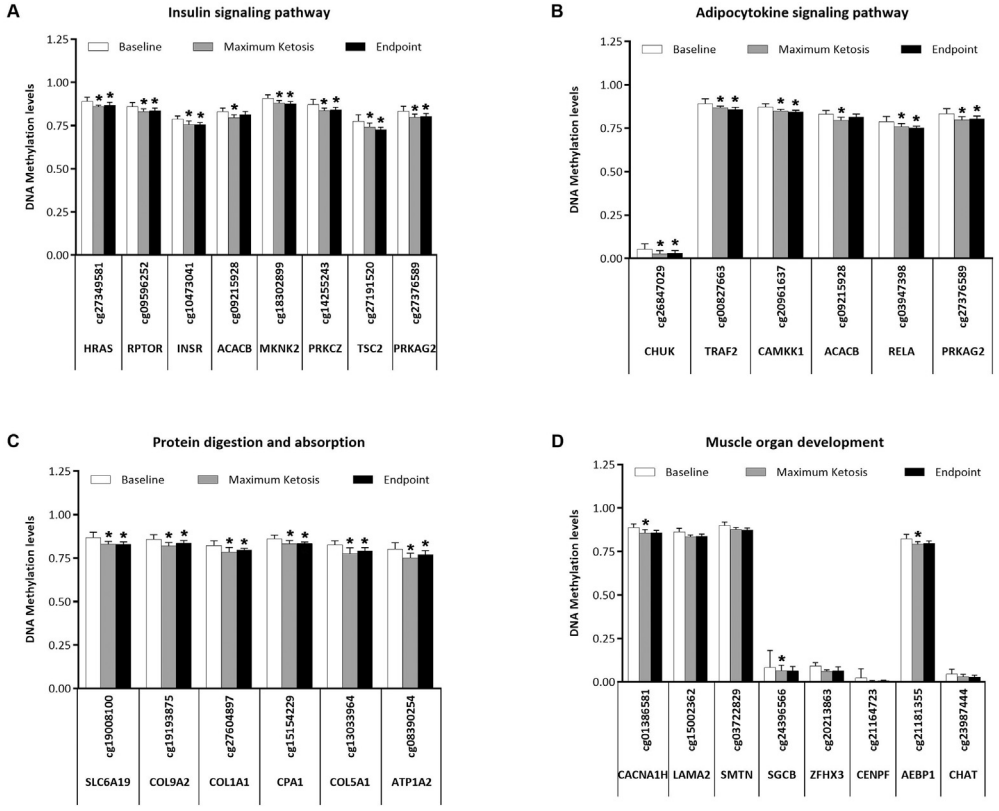

Fig. 4. Genes with known functions that present DMCpGs following a VLCKD. (A) Novel genes epigenetically regulated following VLCKD belonged to the insulin signaling pathway, (B) adipocytokine signaling pathway, (C) protein digestion and absorption functions, and (D) muscle organ development functions. DNA methylation values are expressed as b-values from the Infinium MethylationEPIC BeadChip. *Denotes differences statistically significant ($P < 0.05$). DMCpGs, differentially methylated CpGs; VLCKD, very-low calorie ketogenic diet.

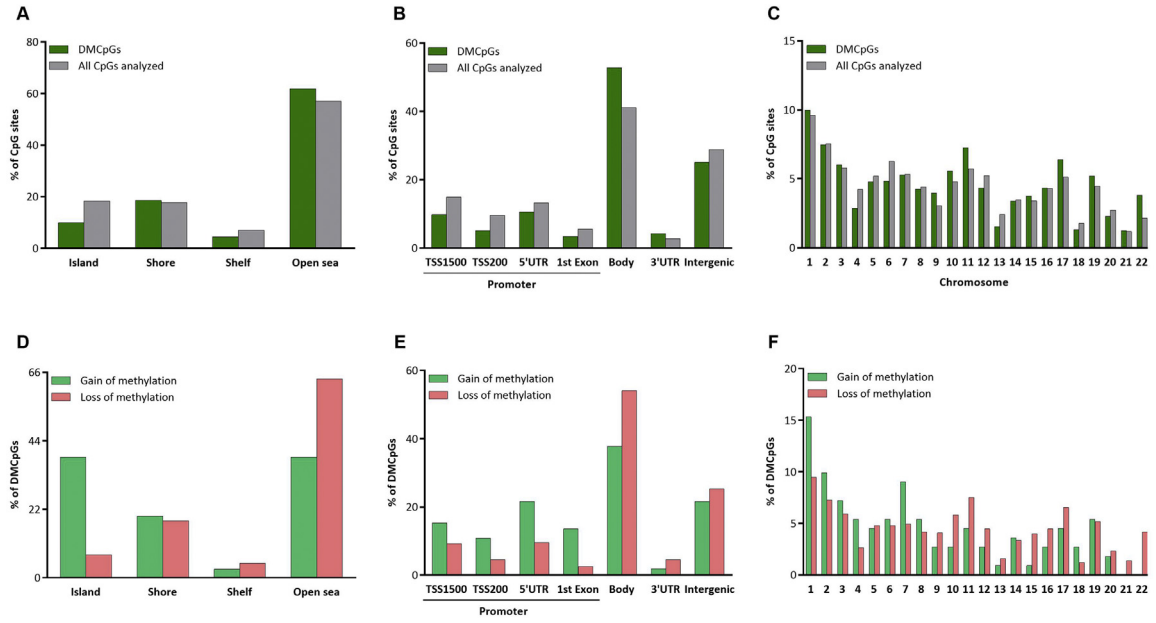

Fig. 5. Characterization of the DMCpGs during ketosis induced by a VLCKD from the discovery cohort (n = 10). (A) Genomic distribution of the DMCpGs and their respective locations regarding the broader CpG context, (B) gene region and (C) chromosome. (D) Genomic distribution of DMCpGs comparing those that exhibited an increase with those that exhibited a decrease in methylation levels during the VLCKD-induced ketosis and their respective locations in the broader CpG context, (E) the gene region and (F) chromosome. DMCpGs, differentially methylated CpGs; VLCKD, very-low calorie ketogenic diet.

promoter and in chromosomes 1, 2, 3, 4 and 7. The DMCpGs that lost methylation were found in the open sea and body and in chromosomes 9, 10, 11, 12, 17, 21, 22 (Fig. 5D–F).

With the aim of isolating the specific effects of ketosis on methylation profile, additional analysis was performed. A Venn diagram was created by including: DMCpGs Baseline – Maximum Ketosis = 1365, DMCpGs Baseline – Endpoint = 405, and DMCpGs Maximum Ketosis – Endpoint = 21. Using these conditions, we identified 280 CpGs whose methylation was affected by the induced weight loss *per se* (see detailed list in Supplementary Table S3) and 1239 CpGs were identified as VLCKD-induced ketosis-related DMCpGs (Fig. 6A). These VLCKD-induced ketosis-related DMCpGs corresponded to 966 annotated genes, and 161 of these were represented by 137 VLCKD-induced ketosis-related DMCpGs located in the promoter and island or shore (see detailed list in Supplementary Table S4). Among the categories of functional processes that exhibited statistical significance (FDR < 0.05), we found processes related to regulation of transcription, signal transduction, cell adhesion, cell differentiation, proliferation and apoptosis, as well as nervous system development and axon guidance. Moreover, these ketosis-related differentially methylated genes belonged to pathways involved in focal adhesion, insulin and adipocytokine signaling pathways, MAPK and P53 signaling pathways and in cancer and type II diabetes mellitus-related pathways (Fig. 6B). Overall pathways associated with obesity physiopathology.

Among these, we highlighted relevant pathways in the field of obesity physiopathology such as insulin signaling, protein digestion and absorption, adipocytokine signaling and muscle development.

To identify potentially novel signatures of DNA methylation associated with VLCKD-induced ketosis, those genes with more

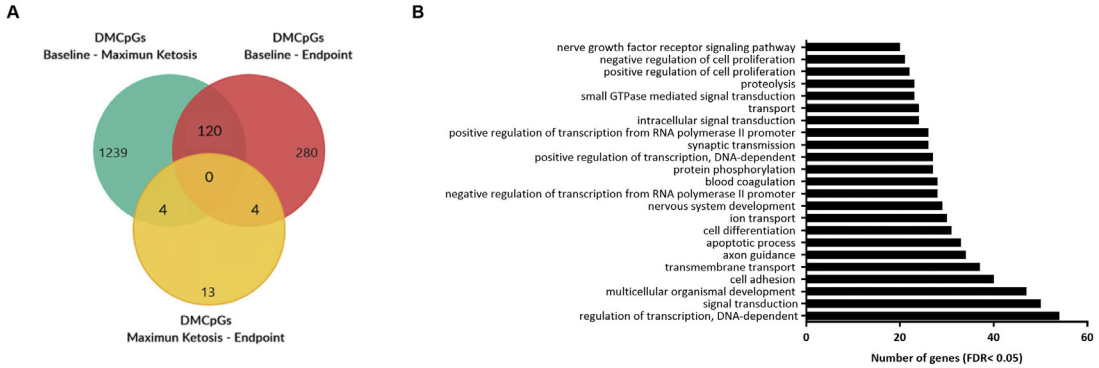

Fig. 6. Biological implications of the DMCpGs related to VLCKD-induced ketosis. (A) Venn diagram of the DMCpGs detected between baseline and maximum ketosis, between baseline and endpoint, and between maximum ketosis and endpoint. From this analysis, 1239 CpGs were identified as nutritional ketosis-related DMCpGs. (B) Summary of the GO analysis of the biological process categories representing the differentially methylated genes associated with nutritional ketosis-related DMCpG sites. DMCpGs, differentially methylated CpGs; GO, gene ontology; VLCKD, very-low calorie ketogenic diet.

than 2 CpG sites located within CpG islands and shores and with a difference in β-values ≥ |2|% were selected. Based on these criteria, 12 genes were identified and are listed in Table 3. When the list was further filtered by promoter region, the genes *CBFA2T3*, *C3orf38*, *JSRP1,* and *LRFN4* showed at least one VLCKD-induced ketosis-related DMCpG located in the promoter and island/shore.

3.5. Validation of candidate genes in a representative cohort

We used pyrosequencing, a technique that is most feasible for studies of patients in hospitals, to evaluate the DNA methylation levels of *ZNF331* and *FGFRL1* in an independent cohort of samples from leukocytes (validation cohort; n = 18 patients who follow a VLCKD + DHA or VLCKD-DHA merged Table 1).

Statistically significant differences were found in the methylation levels after nutritional intervention with respect to baseline (Fig. 7A). After weight loss treatment, a statistically significant increase with respect to baseline was observed during maximal ketosis in the 4 CpGs evaluated ($p = 0.049$). However, no statistically significant changes were observed in *FGFRL1* methylation levels after the weight loss treatment (Fig. 7B).

DNA methylation has previously been associated with transcriptional repression [58,59]. Accordingly, the expressions of *ZNF331* (Fig. 7C) and *FGFRL1* (Fig. 7D) were evaluated. A statistically significant downregulation in the expression of *ZNF331* was found after nutritional intervention, while the expression of *FGFRL1* was upregulated, especially at maximum ketosis compared with baseline. Even though the results for methylation level of *FGFRL1* were not validated in the independent cohort, the expression of this gene was inversely correlated with the methylation levels observed in the discovery cohort. Overall, these results suggest that epigenetic regulation of *ZNF331* and *FGFRL1* related to weight loss could have a relevant biological function.

The validation part of the current study was performed by merged the two arms of the clinical trial because the initial results revealed that both arms induced similar weight loss and ketosis [21], the main outcome used to evaluate changes in the methylome. Indeed, a further analysis in the validation cohort keeping the two arms separated, showed that the trend in the methylation levels through the intervention was similar to that observed in the merged analysis for the studied genes, ZNF331 and FGFRL1 (Supplemental Fig. 3). Differences through the intervention appeared to be higher in the VLCKD + DHA than in the VLCKD arm; however, a repeated measured ANOVA analysis depending on arm showed statistically significance only for the time-course of the intervention in ZNF331 methylation levels ($p < 0.01$), without statistically significance for the interaction between time x arm, nor between the two arms ($p > 0.05$).

4. Discussion

In this study, we carried out an epigenome-wide association study (EWAS) with a longitudinal approach to evaluate the effects of a ketogenic nutritional intervention for weight-loss (based on a very-low-calorie ketogenic diet (VLCKD)) on DNA methylation levels in patients with obesity. As far as we know, this is the first study performed that evaluated the effects of a ketogenic diet, particularly a low fat-low carbohydrate ketogenic diet, to treat obesity on the pattern of DNA methylation. This analysis was performed using an array-based platform that interrogates 850.000 specific CpG sites. Using this design, 988 CpG sites exhibited differential methylation after VLCKD intervention. These CpG sites encoded 786 genes belonging to biological processes involved in the function of adipose tissue, neurological function, muscle development, and others metabolic processes. In particular, the methylation profile of DMCpGs after a short-period of dieting was similar to the profile observed in normal-weight individuals. A global hypomethylation was observed in the DMCpGs identified correlated with a downregulation in the DNA methyltransferase (DNMT) family, which are DNA-modifying enzymes that play a crucial role in regulation of DNA methylation [60]. These effects were observed from the acute phase of this nutritional intervention characterized by an induced ketosis state. We identified 1239 CpG sites that were modified by a short-time and moderate ketosis state *per se*, independently of the magnitude of weight loss. Therefore, changes in DNA methylation profile after VLCKD could be related with the beneficial effects of this kind of nutritional therapy on body weight and body composition [18], biochemical and hormonal parameters [20,61], and quality of life [23] and promoted mainly by the induced ketosis state.

In our longitudinal study after weight reduction, there were changes in methylation levels of genes involved in the insulin signaling pathway, such as *HRAS, RPTOR, INSR, ACACB, MKNK2, PRKCZ, TSC2,* and *PRKAG2;* Collagen-related genes such as *COL9A2, COL1A1,* and *COL5A1* as well as, genes involved in adipocyte signaling such as *CHUK, TRAF2, CAMKK1, ACACB, RELA,* and *PRKAG2.* These results endorse the hypothesis of epigenetic regulation of adipose tissue dysfunction in obesity as it was previously observed in visceral adipose tissue [39].

The current study also revealed an epigenetic effect of treatment with a VLCKD on genes associated with muscle development such as *CACNA1H*, *LAMA2*, *SMTN*, *SGCB*, *ZFHX3*, *CENPF*, *AEBP1,* and *CHAT*. Muscle is an important tissue in the pathogenesis of obesity, which is directly linked with morbidity and mortality in humans [59,62,63]. It has been demonstrated that a VLCKD is able to preserve the mass and function of muscle [18], as well as modulate the secretion of specific myokines [61]. The current study demonstrates that these effects could be mediated by epigenetic mechanisms.

The most relevant characteristic of epigenetic regulation is plasticity [64]. However, a cross-sectional approach is unable to evaluate the potential reversibility of obesity-related epigenetic marks and causal interferences. With this study, we could identify that the methylation levels of most of the DMCpGS after VLCKD were similar to that observed in normal weight patients.

Importantly, the achieved results in this study after VLCKD were representative of the more acute phase, during a short period (<60 days) and moderate intensity VLCKD-induced ketosis state. In fact, a specific methylome was observed independent of weight loss induced by VLCKD intervention. Among the encoded genes, *CBFA2T3* (also called *MTG16*) was the most represented, with 4 CpGs differentially methylated and *JSRP1*, represented by 2 CpGs in the promoter region. Ketone bodies have previously been described as important signaling molecules [65] and were proposed to be able to modulate epigenetic mechanisms [66]. Molecular and cell biology assays showed *CBFA2T3* to have characteristics consistent with a tumor suppressor [67] that lead to decreased glycolysis and stimulated mitochondrial respiration, and therefore may induce a Warburg–like effect [68] which is consistent with ketosis. This finding further relevant considering that obesity is a risk factor for cancer development [69,70]. On The other hand, *JSRP1* was associated with pathways involving skeletal muscle excitation-contraction [71]. If we consider that often methylation in the gene promoter regions implies reductions in gene expression [58,59,72], the lower methylation levels observed for *CBFA2T3* and *JSRP1* could lead to higher expression of these genes, with consequent improvements in mitochondrial and muscle function, respectively. However, until now there have been no studies regarding the role of these genes in the context of energy

Table 3
List of DMCpGs with the highest differences for Baseline - Maximum Ketosis between genes that are represented by more than 2 CpGs.

Target Id	Gene name	Function	Disease Annotation	CHR	Position	Gene region	CpG Context	Methylation levels				p value	FDR
								B		MK			
								Mean	SD	Mean	SD		
cg10143842	C3orf38	No items found		3	88198517	TSS1500	Island	0.066	0.114	0.024	0.024	0.001	0.096
cg24029436				3	88199835	Body	S_Shore	0.113	0.105	0.059	0.038	0.000	0.018
cg00035197	CBFA2T3	response to		16	88962986	Body; Body	Island	0.803	0.041	0.748	0.036	0.000	0.051
cg01977582		hypoxia, negative		16	88976280	5′UTR; Body	S_Shore	0.812	0.031	0.782	0.012	0.001	0.094
cg07243576		regulation of cell		16	88973245	5′UTR; Body	N_Shelf	0.849	0.028	0.817	0.017	0.001	0.098
cg27102297		proliferation, granulocyte differentiation, negative regulation of glycolytic process, negative regulation of transcription, DNA-templated, regulation of aerobic respiration		16	88966903	Body; Body	S_Shore	0.767	0.032	0.731	0.023	0.001	0.088
cg01775970	CBS	L-serine metabolic	Obesity-related	21	44486298	Body	Island	0.873	0.012	0.852	0.014	0.000	0.077
cg09962824		process, L-serine catabolic process, cysteine biosynthetic process, L-cysteine catabolic process, DNA protection, homocysteine catabolic process, homocysteine metabolic process, hydrogen sulfide biosynthetic process	traits	21	44479417	Body	N_Shore	0.748	0.037	0.706	0.031	0.000	0.044
cg13033964	COL5A1	cell adhesion, cell	Crohn's disease	9	137634551	Body; Body		0.825	0.023	0.775	0.033	0.000	0.025
cg13714791		migration, collagen fibril organization, collagen biosynthetic process	Blood pressure Longevity	9	137694578	Body	S_Shore	0.839	0.025	0.813	0.024	0.001	0.096
cg00308560	FAM20C	protein serine/		7	216899	Body	N_Shore	0.857	0.014	0.836	0.013	0.000	0.046
cg11554153		threonine kinase activity, protein binding, manganese ion binding		7	209165	Body	S_Shore	0.821	0.020	0.788	0.017	0.000	0.052
cg17915125	INPP5A	protein binding and		10	134476336	Body	N_Shore	0.879	0.020	0.852	0.011	0.000	0.079
cg02999309		PH domain binding		10	134502626	Body	N_Shore	0.836	0.024	0.799	0.020	0.000	0.021
cg10356204	JSRP1	skeletal muscle		19	2255744	TSS1500	S_Shore	0.697	0.040	0.654	0.032	0.001	0.099
cg16098170		contraction		19	2255848	TSS1500	S_Shore	0.806	0.034	0.770	0.025	0.000	0.085
cg12010750	KCNC1	protein	Night sleep	11	17803160	Body	S_Shore	0.834	0.030	0.803	0.019	0.000	0.069
cg23787205		tetramerization	phenotypes	11	17801046	Body	Island	0.894	0.031	0.869	0.016	0.000	0.086
cg03214876	KIAA1875	No items found		8	145166631	Body	S_Shore	0.875	0.019	0.843	0.013	0.000	0.006
cg11202914				8	145172221	Body	S_Shore	0.905	0.017	0.886	0.010	0.000	0.010
cg12378449	LRFN4	protein binding		11	66627382	Body; Body; Body; Body	N_Shore	0.780	0.036	0.742	0.023	0.001	0.097
cg19391515				11	66626563	Body; Body; 1stExon; Body	Island	0.812	0.025	0.785	0.015	0.000	0.032
cg12378449	PC	protein binding	Pyruvate	11	66627382	Body; Body; Body; Body	N_Shore	0.780	0.036	0.742	0.023	0.001	0.097
cg19391515			carboxylase deficiency	11	66626563	Body; Body; 1stExon; Body	Island	0.812	0.025	0.785	0.015	0.000	0.032
cg16014906	STK32C	No items found		10	134095321	Body	N_Shore	0.841	0.023	0.819	0.019	0.000	0.070
cg24305451				10	134021178	3′UTR	S_Shore	0.843	0.035	0.810	0.030	0.001	0.091

Data shown are mean ± SD (standard deviation).
Abbreviations: B, baseline; CHR, chromosome; FDR, false discovery rate; MK, maximum ketosis.

metabolism related-diseases such as obesity, or regarding their regulation by ketone bodies-induced epigenetic regulation. The current results warrant further investigation on this topic.

To strengthen these findings and to explore the relationship between methylation levels and the expression of the identified genes, we analyzed the methylation and expression of *ZNF331* and *FGFRL1* in an independent cohort. These genes belong to a family of genes associated with adipogenesis [73], T2D, insulin resistance [74,75], and obesity-related cancer [40,41], and their methylation levels of its sequence could be part of an obesity-related epi-signature as previously proposed [35]. Even though the role of these genes in body weight homeostasis requires further elucidation, the

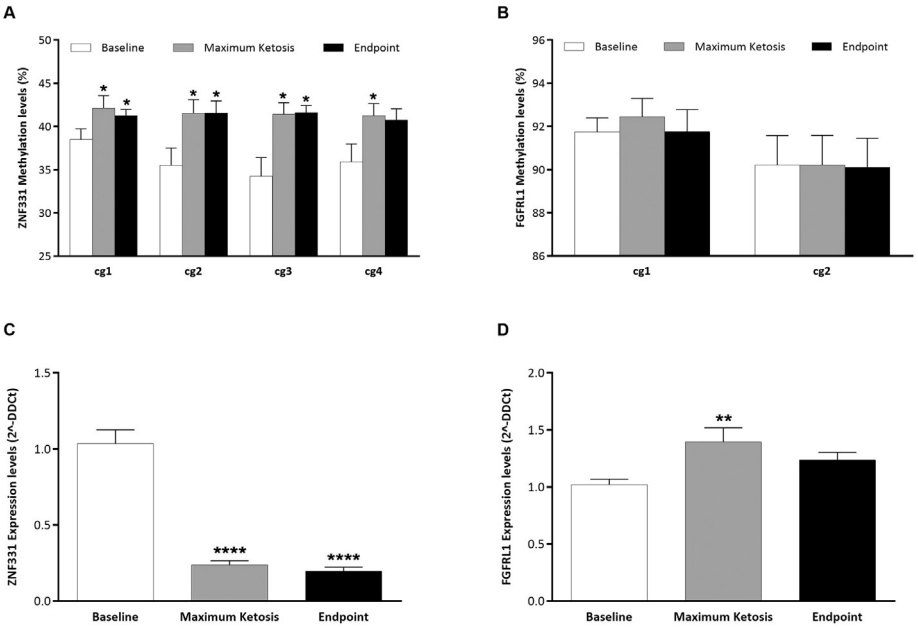

Fig. 7. DNA methylation of ZNF331 and FGFRL1 is inversely associated with gene expression. DNA methylation differences for ZNF331 (A) and FGFRL1 (B) after VLCKD in the validation cohort (n = 18) as measured by pyrosequencing. Differential gene expression of ZNF331 (C) and FGFRL1 (D) after VLCKD in the validation cohort (n = 18). *Denotes differences statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001). VLCKD, very-low calorie ketogenic diet.

results of this study support future exploration of this concept and suggest its epigenetic regulation may be a novel mechanism involved in the management obesity.

A strength of this study is its longitudinal design. With this design, we could evidence for the first time an association between ketosis and DNA methylation independent of weight loss. Despite the tissue specificity of DNA methylation, we were able to detect time-course changes in methylation levels of genes involved in adipose and muscle physiopathology using an easy to obtain tissue for serial sampling in a clinical setting. The relatively small sample size is one limitation of this study. However, as each subject underwent 3 evaluations and the results were subjected to paired analysis, statistical power was increased and real differences were observed between experimental points. Moreover, subsequent analyses were performed with highly stringent filters to produce consistent results, which were further validated in an independent cohort. In addition, although the magnitude of the DNA methylation differences after nutritional intervention could be considered small, these differences may have been due to the use of a mix of blood cells as opposed to single cell analysis, and also because of the short follow-up period used (6 months of weight loss and 30 days of ketosis state), which further increases the relevance of the findings as they were detected even with such a short clinical course. Longer-term prospective studies are needed to confirm the association between DNA methylation levels in blood leukocytes and weight loss response to a VLCKD, and more particularly, the role of ketosis in the regulation of epigenetic mechanisms such as DNA methylation. This study provides information that could be valuable and useful in the context of −omics and big data era to assess both potential benefits and adverse effects of therapeutic agents [76], such as ketone bodies to counteract obesity.

In conclusion, this study demonstrates for the first time that the beneficial effects of VLCKD therapy in reducing obesity involve epigenetic mechanisms. Because the effect was observed in the more acute phase of the nutritional intervention, this epigenetic effect could be mainly mediated by ketosis induced by the VLCKD. Further studies are needed to evaluate whether the DNA methylation markers identified could be useful in predicting the success of this intervention in the context of personalized medicine.

Author contributions

ABC and AGI designed and performed experiments, analyzed data and wrote the manuscript; DL, DP, IS and FIM performed the recruitment and following of the patients and helped with experiments; AJ helped with data analysis; AFQ, MPP, JAM and MAMO contributed to the interpretation of data and discussion; FFC and ABC obtained funding, designed and coordinated research. FFC and ABC are the guarantors 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 read and approved the final manuscript.

Conflict of Interest

A.B.C., D. dL. and F.F.C. received advisory board fees and/or research grants from Pronokal Protein Supplies, Spain. I.S. is the Medical Director 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.2021.05.010.

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