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Randomized Control Trials

Intermittent fasting, calorie restriction, and a ketogenic diet improve mitochondrial function by reducing lipopolysaccharide signaling in monocytes during obesity: A randomized clinical trial

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SUMMARY

Background: Mitochondrial dysfunction occurs in monocytes during obesity and contributes to a low-grade inflammatory state; therefore, maintaining good mitochondrial conditions is a key aspect of maintaining health. Dietary interventions are primary strategies for treating obesity, but little is known about their impact on monocyte bioenergetics. Thus, the aim of this study was to evaluate the effects of calorie restriction (CR), intermittent fasting (IF), a ketogenic diet (KD), and an ad libitum habitual diet (AL) on mitochondrial function in monocytes and its modulation by the gut microbiota.

Methods and findings: A randomized controlled clinical trial was conducted in which individuals with obesity were assigned to one of the 4 groups for 1 month. Subsequently, the subjects received rifaximin and continued with the assigned diet for another month. The oxygen consumption rate (OCR) was evaluated in isolated monocytes, as was the gut microbiota composition in feces and anthropometric and biochemical parameters. Forty-four subjects completed the study, and those who underwent CR, IF and KD interventions had an increase in the maximal respiration OCR ($p = 0.025$, $n^2p = 0.159$ [0.05, 0.27] 95% confidence interval) in monocytes compared to that in the AL group. The improvement in mitochondrial function was associated with a decrease in monocyte dependence on glycolysis after the IF and KD interventions. Together, diet and rifaximin increased the gut microbiota diversity in the IF and KD groups ($p = 0.0001$), enriched the abundance of *Phascolarctobacterium faecium* ($p = 0.019$) in the CR group and *Ruminococcus bromii* ($p = 0.020$) in the CR and KD groups, and reduced the abundance of

Abbreviations: AL, ad libitum habitual diet; ASVs, amplicon sequence variants; ATP, adenosine triphosphate; BHI, bioenergetic health index; BMH, body mass index; CI, confidence interval; CR, caloric restriction; 2-DG, 2-deoxy-glucose; DNA, deoxyribonucleic acid; ECAR, extracellular acidification rate; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; HADS, Hospital Anxiety and Depression Scale; HDL, high-density lipoprotein; IF, intermittent fasting; KD, ketogenic diet; LDL, low-density lipoprotein; LPS, lipopolysaccharide; OCR, oxygen consumption rate.

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lipopolysaccharide (LPS)-producing bacteria after CR, IF and KD interventions compared to the AL group at the end of the study according to ANCOVA with covariate adjustment. Spearman's correlation between the variables measured highlighted LPS as a potential modulator of the observed effects. In line with this findings, serum LPS and intracellular signaling in monocytes decreased with the three interventions (CR, $p = 0.002$; IF, $p = 0.001$; and KD, $p = 0.001$) compared to those in the AL group at the end of the study.
Conclusions: We conclude that these dietary interventions positively regulate mitochondrial bioenergetic health and improve the metabolic profile of monocytes in individuals with obesity via modulation of the gut microbiota. Moreover, the evaluation of mitochondrial function in monocytes could be used as an indicator of metabolic and inflammatory status, with potential applications in future clinical trials.
Trial registration: This trial was registered with ClinicalTrials.gov (NCT05200468).

1. Introduction

Dietary treatments have emerged to improve the metabolic profile of individuals with obesity. Among them, calorie restriction (CR), intermittent fasting (IF), and ketogenic diet (KD) are highly popular methods for promoting body weight loss and for conferring multiple metabolic benefits in animal and human studies [1–3], particularly associated with the improvement and preservation of mitochondrial function [4–6]. Mitochondria are the main source of energy in cells and generate this energy in the form of adenosine triphosphate (ATP) via oxidative phosphorylation. Mitochondrial dysfunction in individuals with obesity is implicated in the pathogenesis of multiple chronic diseases [7] since mitochondria are responsible for detecting and integrating signals from the environment to trigger adaptive cellular responses related to energy utilization. In immune cells, including monocytes, mitochondrial dysfunction can increase inflammation by contributing to alterations in several metabolic tissues [8]. Monocytes were previously thought to have a primarily glycolytic demand; however, it was recently reported that they positively regulate fatty acid oxidation as a mechanism to decrease inflammatory events in low-glucose environments [9], suggesting that dietary restrictions such as CR and IF and/or the consumption of a KD could have an impact on monocyte bioenergetics.

In addition to the observed effects on mitochondrial function, CR, IF and KD generate changes in the gut microbiota [10–13]. The gut microbiome impacts nearly all aspects of physiology, including energy metabolism, and can influence mitochondrial functions related to energy production, redox balance, and inflammatory cascades [14]. From this perspective, the monocyte mitochondria-gut microbiota axis represents a fundamental facet of this symbiosis, and proper functioning and communication are key aspects for maintaining good health. Antibiotic treatments have been used to evaluate the participation of the gut microbiota in a particular observed effect, since they decrease the diversity and produce changes in microbiota taxonomy [15]. In this context, rifaximin was chosen as a non-absorbable antibiotic [16], to study the importance of the gut microbiota to modulate the response to dietary interventions, especially, its indirect influence on mitochondrial function in monocytes.

Analysis of mitochondrial function in monocytes isolated from human blood samples has been used as an indicator of the metabolic status of subjects [17–19]. However, very few studies have reported the use of this analysis in intervention studies to evaluate the metabolic effect of dietary treatment, making this study novel and highly relevant. Therefore, our objective was to evaluate the effects of dietary interventions involving CR, IF and KD on mitochondrial function in monocytes and its association with changes in the composition of the gut microbiota caused by the dietary interventions and after rifaximin treatment. Long term rifaximin administration has shown beneficial changes in gut microbiota composition and reduction in plasma lipopolysaccharide (LPS) levels in patients with cirrhosis and encephalopathy [20–23]. However, the effects of this antibiotic on patients with obesity are scarce, recent evidence showed that in subjects with metabolic syndrome rifaximin by itself did not improve glucose or lipid homeostasis or reduced LPS plasma levels [24]. In the present work, we describe an adaptive mechanism of substrate utilization in monocytes modulated by CR, IF and KD dietary interventions and by LPS from the gut microbiota, showing that the combination of dietary and rifaximin treatment reversed the mitochondrial dysfunction observed in these cells in patients with obesity.

2. Methods

2.1. Participants

This study was conducted at the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ) in Mexico City from July 2022 to March 2023. The selection criteria were age 18–60 years, a body mass index (BMI) greater than 30 kg/m^2 and less than 50 kg/m^2 and signed the letter of informed consent. The exclusion criteria were patients with any other chronic disease diagnosed by a physician, who had a weight loss >3 kg in the last 3 months, who were pregnant, who smoke, or had drug treatment of any kind, who had any acute or chronic gastrointestinal disease, history of major surgery, consumption of pro-/pre/symbiotics, or diagnosis of anxiety and depression as determined by the Hospital Anxiety and Depression Scale (HADS).

2.2. Study approval

This study was conducted following the guidelines of the Declaration of Helsinki, and all human subject procedures were approved by the INCMNSZ Ethics Committee (REF 3728). The study was registered in Clinical Trials under number NCT05200468 and can be accessed at ClinicalTrials.gov.

2.3. Study design and outcomes

The study was an open randomized controlled clinical trial. Subjects were invited to participate through open advertising in hospital centers and social networks. Subjects who met the selection criteria were randomly assigned to one of three dietary interventions, CR, IF or KD, or to the fourth group, which consumed an ad libitum habitual diet (AL) for one month. Subsequently, rifaximin was administered (550 mg twice daily for 7 days), while continuing with the assigned dietary intervention for another

month. The study consisted of 4 follow-up visits. The primary outcome was to evaluate mitochondrial function determined by the oxygen consumption rate (OCR) in isolated peripheral blood monocytes, and the secondary outcomes were to evaluate anthropometric and biochemical parameters in the serum and the composition of the gut microbiota in the feces. All variables were assessed at the four visits during the study (Fig. 1a).

2.4. Dietary interventions

Three of the dietary interventions had a restriction of 500 kcal from the caloric calculation of their usual diet, and each participant received a menu for fifteen days that served as a guide to comply with the assigned treatment: a) Diet with CR had a macronutrient distribution of 25–35% protein, 45–55% carbohydrates, and 20–30% fats of the total caloric value. b) Diet with IF was the same as the CR diet, with a 16:8-fold restriction model; 16 h of fasting and 8 h to comply with the food intake. c) KD had a macronutrient distribution of 15–25% protein, 5–10% carbohydrate, and 70–80% fat of the total caloric value. The fourth group was the d) AL diet, in which there was no nutritional indication, and the subjects followed their habitual diet until the end of the study.

Compliance to the diet was evaluated using the 24-hour diet recall and the 3-day food record (food log) and was monitored by weekly telephone calls by the nutritionists. The % of adherence to the dietary interventions was obtained from the analysis of the feeding logs using Food Processor software (ESHA Research, USA) according to the kilocalories. The urine ketone concentration was determined to measure adherence to the KD. A logbook was used to record the consumption of rifaximin, which was completed daily by the participants.

2.5. Randomization

The assignments were completed using blocked randomization. The subjects were divided into 4 groups using fixed blocks of four cells supported by a table of random numbers. Once the number was assigned for each block, the treatment combinations were used. Randomization was carried out by a person outside the study. This person kept the randomization in a locked cabinet.

2.6. Biochemical parameters

Blood samples were taken from the subjects after a 12-h fast and the serum was kept at −80 °C until analysis. For the serum concentrations of glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides and C-reactive protein, the Cobas C111 autoanalyzer (Roche Diagnostic, Indianapolis, IN) was used. ELISA kits were used to measure insulin (ALPCO, Salem, NH) and lipopolysaccharide (LPS) (Cloud_clone Corp., USA) levels according to the manufacturer's instructions. HOMA-IR was calculated using the following formula (fasting glucose (mg/dL) * fasting insulin (μU/mL))/405 [25].

2.7. Anthropometric, clinical and body composition parameters

Body weight and body composition were measured with a multifrequency bioimpedance body composition analyzer (Inbody 720; Inbody Co, LTD, South Korea). The visceral fat area values were estimated with a built-in regression equation by InBody 720 equipment. This estimation significantly correlates with computed tomography data ($R = 0.759$) according to a previous study [26]. The measurements of the body weight, height, and waist circumference were performed as described in the Lohman reference manual [27].

2.8. Determination of mitochondrial function in monocytes

The person determining the primary outcome was blinded after randomization. Peripheral blood mononuclear cells were obtained as previously described [19]. Subsequently, monocytes were isolated using a monocyte isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. A total of 150,000 monocytes per well were seeded on a Seahorse plate, and the OCR and the extracellular acidification rate (ECAR) were measured. Four injections were performed with oligomycin (1.5 μM), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (2 μM), a mixture of rotenone and antimycin A (1.0 μM), and 2-deoxy-glucose (2-DG). Mitochondrial function parameters, such as nonmitochondrial respiration (rotenone and antimycin A OCR), basal respiration (basal OCR − nonmitochondrial OCR), ATP-linked respiration (ALR) (basal OCR − oligomycin OCR), proton leakage (oligomycin OCR − nonmitochondrial OCR), maximal respiration (FCCP OCR − nonmitochondrial OCR), and spare capacity (FCCP OCR − basal OCR), were calculated. The bioenergetic health index (BHI) was calculated with the following formula (ATP-linked respiration × spare capacity)/(proton leakage × nonmitochondrial respiration) as previously reported [28]. We calculated the basal acidification (basal ECAR), glycolytic reserve (oligomycin ECAR − basal ECAR) and compensatory glycolysis (rotenone and antimycin A ECAR − basal ECAR) as glycolytic parameters.

2.9. Gut microbiota analysis

Fresh stool samples from each patient were frozen and stored at −70 °C until use. The bacterial deoxyribonucleic acid (DNA) extraction and sequencing was performed as previously described [29]. For the analysis, reads were processed and filtered for quality in DADA2 (Divisive Amplicon Denoising Algorithm 2) version 1.26 implemented in the RStudio package to correct amplicon errors, identify chimeras, and merge paired-end reads. Amplicon sequencing data were processed into a table of exact amplicon sequence variants (ASVs) present in each sample. A taxonomy assignment lookup table was constructed for each ASV obtained using the SILVA ribosomal RNA gene database version 138.1, and singletons and ASVs classified as mitochondria were removed. The generated ASV matrix was normalized with the trimmed mean M-value method in edgeR-TMM to transform the data and allow accurate statistical comparison. The data were also imputed by MBimpute to correct for false zeros prevalent in the normalized data. Fastq files from all our samples were included in the Sequence Read Archive (SRA-gene bank) repository with project identification number: PRJNA1108598.

2.10. Protein determination

Monocytes were lysed in RIPA lysis buffer. Proteins were separated by SDS–PAGE and transferred to polyvinylidene difluoride membranes. Blocking and antibody incubation were performed in 5% skim milk. Primary antibodies anti-TLR4 (sc-293,072) from Santa Cruz Biotechnology (Dallas, Texas, USA) and anti-ERK 1/2 (#4695), anti-phospho-ERK 1/2 (#4376), anti-DRP1 (#5391) and anti-phospho-DRP1 Ser616 (#4494) from Cell Signaling (Danvers, Massachusetts, USA) were incubated overnight at 4 °C. GAPDH (ab181602, Abcam, Cambridge, UK) was used as loading control. HRP-conjugated secondary antibodies anti-rabbit (ab6721) and anti-mouse (ab6789) from Abcam (Cambridge, UK) were incubated

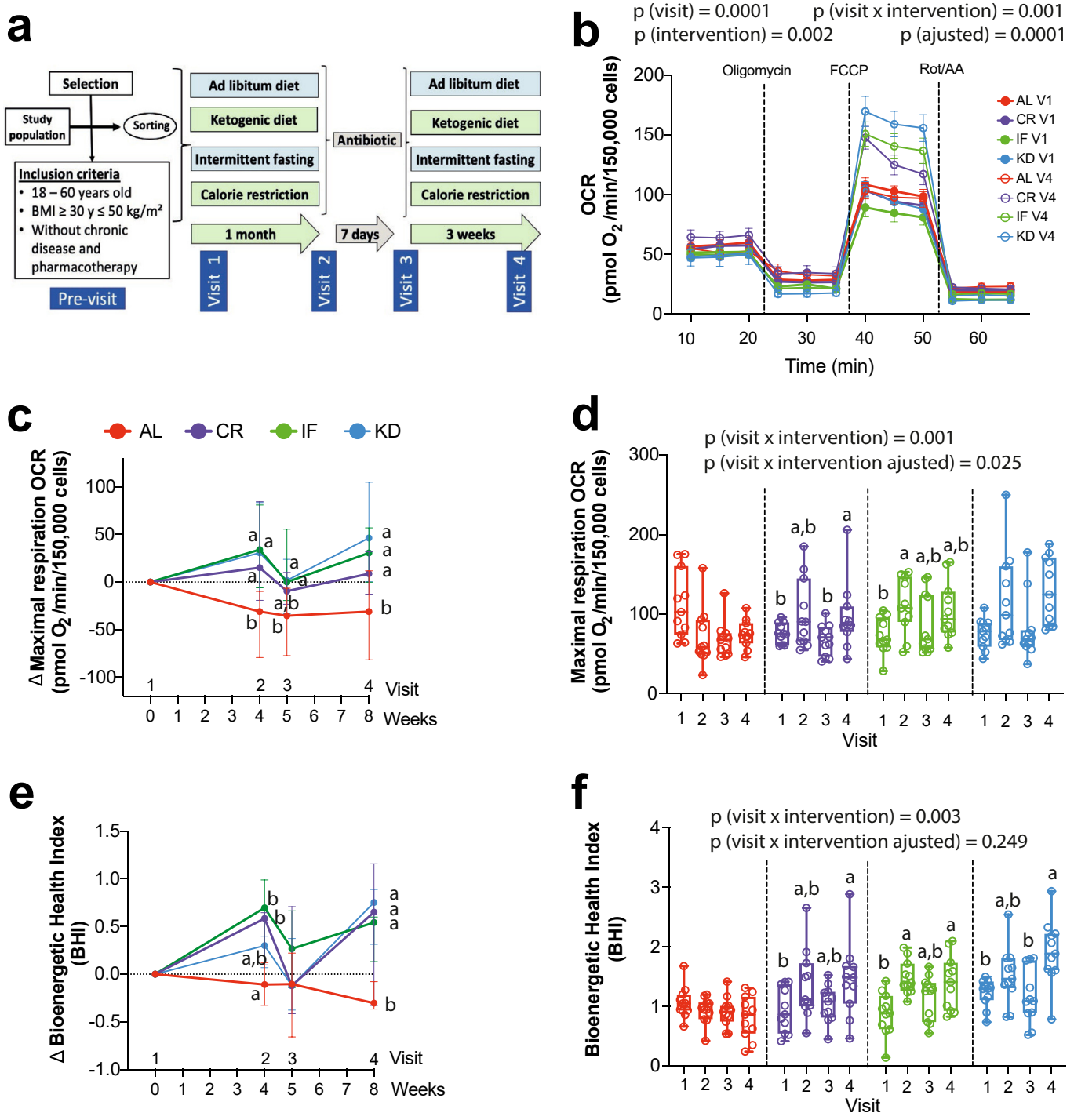

Fig. 1. Study design and mitochondrial respiration in monocytes. a) The experimental design of the study consisted of a pre-visit for the selection and randomization of patients who met the inclusion criteria, and 4 follow-up visits after being assigned to their intervention groups. b) Oxygen consumption rate (OCR) in the different states of mitochondrial respiration at visits 1 and 4 in subjects with obesity who were receiving calorie restriction (CR), intermittent fasting (IF) or a ketogenic diet (KD) or in subjects without intervention who continued with an ad libitum habitual diet (AL). c) Difference (deltas) in the OCR at the maximal respiration state at the different study visits of the subjects described in b. d) Maximal respiration OCR at the different study visits of the subjects described in b. e) Difference (deltas) in the bioenergetic health index (BHI) at the different study visits of the subjects described in b. f) BHI at the different study visits of the subjects described in b. N = 11 per group. In Panel b, we present the mean ± SEM. Statistical analysis was performed by repeated-measures ANOVA and adjustment for age, sex, and baseline weight. In Panels c and e, we show the medians with 95% confidence intervals. Statistical analysis of the deltas was performed at each visit, and each dietary intervention was compared with one-way ANOVA and the Bonferroni post hoc correction. In Panels c to f, the statistical analysis was performed with repeated-measures ANOVA and adjustment for age, sex, and baseline body weight. Additionally, post hoc analysis was performed with the Bonferroni correction. Different letters indicate a significant difference ($p < 0.05$) where a>b > c. ALR: ATP-linked respiration, PL: proton leakage (PF).

for 1 h at room temperature. Antibody detection reactions were developed by chemiluminescence, and images were analyzed using ChemiDocTM XRS + System Image LabTM software (Bio-Rad, Hercules, CA, USA). The assays were performed six times using different subjects' samples. Quantification was performed using ImageJ software.

2.11. Statistics

The sample size was calculated according to the methods of a previous study of isolated mitochondria before and after dietary intervention combined with physical training, and the change in mitochondrial function determined by myocyte OCR was 4.5 ± 5 nmol/mg/min [30]. Considering a type 1 error (α) = 0.05 and a type II error (β) of 0.20, using the difference of two means formula, we obtained a sample size of 9.67. Adding a loss of 20%, 12 participants per group were considered. Continuous variables are expressed as the mean and standard deviation, and dichotomous variables are expressed as frequencies and percentages. The distribution of continuous variables was evaluated using the Kolmogorov–Smirnov test. One-way ANOVA was used to compare baseline continuous variables and differences between groups at a single time, while the chi-square (Xi^2) test was used for trend comparisons for dichotomous variables. To analyze the interaction between time and treatment repeated-measures ANOVA was used for continuous variables. Friedman test was used to calculate p-values x visit, and p values x intervention were calculated with Kruskal Wallis test. The size effect was calculated by the partial eta squared (n^2p) test with a 95% confidence interval (CI). Adjustments were made for age, sex, and baseline weight with the Bonferroni post hoc correction. To evaluate the continuous variables before and after the intervention (Visit 1 vs. Visit 4), paired t tests were used for each of the groups. The delta (Δ) were calculated as follows at each visit: data from the actual visit - basal data. Linear model ANCOVA was used to obtain featured bacterial species between dietary interventions at visit 4. Adjustments were made for sex, age and BMI with the Bonferroni post hoc correction. Mixed-effects analysis with the Bonferroni post hoc correction were used to compare bacterial total abundance between visits 2, 3, and 4 for all the experimental groups. Beta diversity was determined using Adonis with Bray–Curtis test. The abundance of bacterial genera was correlated between the AL group and the other experimental intervention groups at the final visit of the study using Spearman correlation coefficients (r^2). Spearman correlations between the different variables of the study were performed with RStudio version 4.3.2 using the psych and corrplot packages. The data were analyzed in a blinded manner by an independent researcher with SPSS software version 24.0 for Windows, for which a significance value of $P < 0.05$ was established.

3. Results

3.1. Characteristics of the study subjects

Fifty-eight participants were selected from 63 evaluated subjects and were randomly assigned to the different experimental groups. Forty-four subjects completed the four visits of the study, leaving a total of 11 subjects per group. Recruitment and follow-up were performed from July 2022 to March 2023. The participants analyzed in the different dietary interventions (CR, IF and KD) had an adherence to treatment greater than 80%. Figure 1a shows the experimental design and Supplementary Fig. 1 shows the flow chart with the follow-up of the study subjects and the causes of the losses during the assignment, follow-up, or analysis stages. The demographic, anthropometric, clinical, and biochemical variables of the participants at baseline are described in Table 1. There were no significant differences in the initial demographic variables or anthropometric or biochemical parameters between the different experimental groups. In addition, there were no reported adverse effects during the study.

3.2. Monocytes isolated from subjects who underwent CR, IF or KD significantly increased mitochondrial function

As the primary outcome of this study, we evaluated mitochondrial function in monocytes by measuring the OCR in different respiration states. Supplementary Fig. 2a schematizes the variables obtained from the bioenergetic profile and the values of mitochondrial respiration states are presented in Table 2. We observed a significant increase (p = 0.0001) in the maximal respiration OCR between the final and initial visits in the CR, IF and KD intervention groups (Fig. 1b). Compared with those in the nonintervention group, the maximal respiration OCR in monocytes in the intervention groups was significantly greater at visit 2 (p = 0.002) and

Table 1
Demographic, anthropometric, biochemical, and clinical characteristics of participants in the intervention group.

Variable	Total (n = 44)	Ad libitum habitual diet (n = 11)	Ketogenic diet (n = 11)	Intermittent fasting (n = 11)	Calorie restriction (n = 11)	P value
Sex						
Female, n (%)[a]	36 (82)	9 (82)	8 (73)	11 (100)	8 (73)	0.48
Age, years	36.9 ± 10.7	41.0 ± 9.63	37.0 ± 9.52	32.5 ± 12.3	37.2 ± 10.9	0.33
Body weight, kg	89.2 ± 13.8	93.7 ± 10.4	86.6 ± 17.6	83.3 ± 10.4	93.2 ± 14.5	0.21
BMI, kg/m^2	34.8 ± 4.20	35.6 ± 3.98	33.3 ± 4.49	33.7 ± 3.09	36.6 ± 4.71	0.20
Visceral fat area, cm^2 [b]	149 ± 25.3	158 ± 23.8	139 ± 31.4	143 ± 23.1	156 ± 20.4	0.23
Fat mass, %	45.3 ± 5.18	45.5 ± 5.29	43.1 ± 6.10	46.6 ± 4.09	46.2 ± 5.05	0.39
Skeletal muscle mass, %	30.1 ± 3.21	30.2 ± 3.22	31.3 ± 4.01	29.2 ± 2.48	29.9 ± 3.01	0.48
Waist circumference, cm	105 ± 11.0	107 ± 9.29	104 ± 12.7	101 ± 11.0	109 ± 10.7	0.33
Glucose, mg/dL	100 ± 13.8	106 ± 19.4	99.0 ± 7.48	93.0 ± 9.15	102 ± 14.0	0.13
Total cholesterol, mg/dL	189 ± 35.9	186 ± 29.0	196 ± 45.8	177 ± 32.7	197 ± 35.0	0.52
Triglycerides, mg/dL	157 ± 69.4	174 ± 91.5	165 ± 63.9	145 ± 63.3	145 ± 59.1	0.72
HDL cholesterol, mg/dL	46.4 ± 8.48	49.3 ± 9.15	44.2 ± 11.0	45.4 ± 7.96	46.7 ± 4.87	0.55
LDL cholesterol, mg/dL	117 ± 32.7	116 ± 28.9	121 ± 22.1	112 ± 32.1	119 ± 46.8	0.92
C-Reactive Protein, mg/dL	4.43 ± 3.57	4.88 ± 3.98	3.13 ± 2.65	5.22 ± 4.61	4.36 ± 2.65	0.58
Insulin, IU/mL	17.3 ± 12.1	23.9 ± 21.5	15.1 ± 6.89	14.7 ± 7.05	16.1 ± 6.48	0.27
Lipopolysaccharide, pg/mL	2982 ± 1223	2469 ± 1377	2921 ± 922	3298 ± 647	3351 ± 1789	0.42

BMI: Body mass index; Data are presented as the mean ± SD. Statistical analysis for continuous variables was performed with one-way ANOVA.
[a] Statistical analysis was performed with the chi-square test for trend.
[b] Visceral fat area values were estimated by the bioelectrical impedance equipment.

Table 2
Changes in mitochondrial respiration and anaerobic glycolysis parameters in the intervention group.

	Ad libitum habitual diet	Ketogenic diet	Intermittent fasting	Calorie restriction	P value x intervention	P[a] value	P[b] value	n^2 p	CI 95%	n^2 p[b]	CI 95%[b]
Basal respiration, pmol O_2/min											
Visit 1	42.10 ± 12.51	33.84 ± 6.94	37.93 ± 12.61	36.26 ± 14.17	0.410	**0.015**	0.358	0.170	[0.06, 0.28]	0.086	[-0.03,0.20]
Visit 2	27.82 ± 12.58	44.11 ± 23.92	41.54 ± 12.17	38.98 ± 18.81	0.107						
Visit 3	23.67 ± 9.46	34.22 ± 25.35	39.31 ± 20.90	30.18 ± 16.52	0.319						
Visit 4	26.85 ± 4.35	36.70 ± 20.88	35.35 ± 10.57	47.21 ± 20.84	0.117						
P value x visit	0.006	0.145	0.896	0.525							
Proton leak, pmol O_2/min											
Visit 1	10.58 ± 6.16	5.78 ± 3.18	10.84 ± 6.21	6.83 ± 5.69	0.065	0.204	0.879	0.104	[-0.01,0.22]	0.040	[-0.07,0.15]
Visit 2	4.61 ± 2.95	4.52 ± 3.84	4.50 ± 3.41	5.65 ± 5.3	0.971						
Visit 3	4.36 ± 1.60	4.16 ± 3.40	6.63 ± 6.78	5.79 ± 5.12	0.853						
Visit 4	7.15 ± 3.54	2.67 ± 2.00	4.87 ± 4.18	7.95 ± 5.91	0.023						
P value x visit	0.026	0.145	0.099	0.569							
ATP-linked respiration, pmol O_2/min											
Visit 1	31.52 ± 8.50	28.06 ± 7.77	27.09 ± 10.03	29.43 ± 13.13	0.665	**0.024**	0.256	0.159	[0.05,0.27]	0.097	[-0.02,0.21]
Visit 2	23.33 ± 10.14	39.59 ± 22.88	37.04 ± 12.42	33.33 ± 15.52	0.044						
Visit 3	19.40 ± 8.69	30.06 ± 25.39	32.68 ± 15.93	24.39 ± 14.82	0.176						
Visit 4	18.71 ± 6.64	31.01 ± 16.57	30.53 ± 8.56	38.9 ± 16.51	0.005						
P value x visit	0.007	0.029	0.183	0.288							
Maximal respiration, pmol O_2/min											
Visit 1	112.30 ± 44.15	79.91 ± 16.26	72.89 ± 22.52	76.25 ± 12.68	0.113	**0.0001**	**0.025**	**0.278**	**[0.16,0.39]**	**0.159**	**[0.05,0.27]**
Visit 2	61.75 ± 22.74	115.67 ± 58.84	110.40 ± 34.78	100.14 ± 43.83	0.008						
Visit 3	69.10 ± 23.30	80.78 ± 40.73	87.09 ± 38.55	67.68 ± 19.31	0.861						
Visit 4	76.81 ± 16.83	102.33 ± 37.93	80.58 ± 29.18	155.48 ± 90.18	0.004						
P value x visit	0.064	0.039	0.012	0.001							
Spare capacity, pmol O_2/min											
Visit 1	70.20 ± 42.65	46.07 ± 17.63	34.96 ± 19.56	39.99 ± 18.98	0.195	**0.0001**	**0.023**	**0.263**	**[0.15,0.38]**	**0.160**	**[0.05,0.27]**
Visit 2	33.93 ± 18.44	71.57 ± 39.79	68.86 ± 28.21	61.16 ± 38.29	0.028						
Visit 3	45.43 ± 19.46	46.55 ± 22.26	47.78 ± 24.14	37.5 ± 17.82	0.530						
Visit 4	49.96 ± 18.37	65.62 ± 27.80	45.22 ± 22.68	108.27 ± 72.17	0.003						
P value x visit	0.014	0.241	0.21	0.003							
Nonmitochondrial respiration, pmol O_2/min											
Visit 1	17.57 ± 5.87	15.55 ± 5.07	12.11 ± 5.13	20.04 ± 4.38	0.009	**0.002**	0.357	0.210	[0.10,0.32]	0.086	[-0.03,0.20]
Visit 2	20.43 ± 7.43	25.08 ± 9.86	22.86 ± 9.62	21.64 ± 7.28	0.682						
Visit 3	21.73 ± 7.86	22.52 ± 10.83	21.37 ± 4.01	17.54 ± 7.07	0.462						
Visit 4	22.13 ± 10.38	11.44 ± 4.90	16.76 ± 8.84	28.78 ± 16.06	0.006						
P value x visit	0.43	0.001	0.001	0.058							
BHI											
Visit 1	1.09 ± 0.27	1.20 ± 0.26	0.88 ± 0.36	0.94 ± 0.38	0.112	**0.003**	0.249	**0.203**	**[0.09,0.32]**	0.098	[-0.02,0.21]
Visit 2	0.92 ± 0.22	1.52 ± 0.48	1.49 ± 0.28	1.36 ± 0.58	0.003						
Visit 3	0.95 ± 0.24	1.17 ± 0.46	1.15 ± 0.37	1.04 ± 0.31	0.536						
Visit 4	0.79 ± 0.36	1.91 ± 0.55	1.40 ± 0.45	1.45 ± 0.63	0.0003						
P x visit	0.077	0.001	0.01	0.036							
Basal glycolysis, mpH/min											
Visit 1	54.7 ± 18.76	58.62 ± 17.30	51.86 ± 12.21	54.39 ± 23.02	0.852	**0.021**	0.148	0.158	[0.04,0.27]	0.111	[0.00,0.22]
Visit 2	67.22 ± 19.29	38.03 ± 11.21	36.47 ± 6.96	46.14 ± 19.91	0.001						
Visit 3	52.08 ± 13.33	41.28 ± 9.91	52.18 ± 11.54	53.50 ± 16.67	0.113						
Visit 4	57.54 ± 13.27	35.07 ± 9.13	43.26 ± 12.51	45.50 ± 8.23	0.001						
P x visit	0.288	0.005	0.043	0.664							
Glycolytic reserve, mpH/min											
Visit 1	52.19 ± 17.44	52.89 ± 15.14	46.67 ± 12.41	42.94 ± 12.99	0.540	**0.001**	0.526	**0.223**	**[0.11,0.34]**	0.069	[-0.04,0.18]
Visit 2	55.6 ± 27.77	25.69 ± 8.34	39.47 ± 20.51	41.26 ± 22.68	0.014						
Visit 3	45.37 ± 10.69	30.02 ± 13.91	49.89 ± 18.40	43.08 ± 12.23	0.030						
Visit 4	52.11 ± 20.99	25.27 ± 7.95	29.07 ± 7.98	38.29 ± 11.26	0.001						
P x visit	0.529	0.0001	0.178	0.529							
Compensatory glycolysis, mpH/min											
Visit 1	74.13 ± 16.84	82.53 ± 22.88	70.79 ± 20.34	69.93 ± 23.75	0.555	**0.005**	0.477	**0.189**	**[0.08,0.30]**	0.073	[-0.04,0.19]
Visit 2	76.46 ± 21.34	39.36 ± 13.21	56.36 ± 24.90	55.22 ± 24.69	0.003						
Visit 3	70.49 ± 25.68	48.60 ± 15.03	71.23 ± 20.07	68.07 ± 15.14	0.011						
Visit 4	74.17± 26.70	36.69 ± 9.14	36.69 ± 9.14	58.37 ± 13.48	0.0001						
P x visit	0.336	0.001	0.148	0.148							

n^2p: Effect size. Partial Eta Squared.
95% CI: 95% confidence interval.
BHI: bioenergetic health index. The data are presented as the mean ± SD. P values x visit were calculated with Friedman test and P values x intervention were calculated with Kruskal Wallis test.
Visit 1 corresponds to week 0 (basal), visit 2 is after 4 weeks of intervention, visit 3 is at week 5 (after 7 days of rifaximin administration), visit 4 is after 8 weeks of intervention.
[a] P values x intervention x visit were performed with ANOVA for repeated measures.
[b] Statistical analysis adjusted for age, sex, and baseline weight.

visit 4 ($p = 0.0001$) (Fig. 1c), with a n^2p of 0.159 (95% CI 0.05, 0.27) (Table 2). When evaluating the mitochondrial respiration pattern of each experimental group at each follow-up visit, we observed that at visit 3, the maximal respiration OCR was no longer significant like that at the basal visit in all three dietary interventions (Fig. 1d and Supplementary Fig. 2 b-e and Table 2). Since rifaximin is an antibiotic virtually unabsorbed after oral administration [16], these results suggest that rifaximin administration exerts an indirect effect in mitochondrial respiration in monocytes probably trough modulation of gut microbiota and its metabolites. In Fig. 1e we observed that subjects with any of the dietary interventions had an increase in the BHI in monocytes compared to the nonintervention group which was significant for the CR and IF groups at the second visit 2 ($p = 0.003$) and for the three dietary interventions at visit 4 ($p = 0.0003$). When analyzing the differences between each visit by experimental diet, we observed that this index significantly increased at the end of the study in subjects who continued with their assigned dietary intervention (Fig. 1f and Table 2). Non-mitochondrial respiration was significantly reduced in the CR, IF and KD groups at the end of the study, an effect not observed in the AL group (Table 2).

3.3. Subjects in the IF and KD intervention groups exhibited a significant decrease in anaerobic glycolysis in monocytes

We evaluated anaerobic glycolysis by measuring the ECAR. Supplementary Fig. 3a shows the variables measured by this assay, and the results of these parameters are presented in Table 2. No changes in the ECAR were observed for the AL (Supplementary Fig. 3b) or CR (Supplementary Fig. 3c) groups. However, monocytes from subjects treated with IF or KD had a significant decrease in basal glycolysis at visit 2 compared to those from the AL and CR groups; this difference remained significant only for the KD group at the end of the study (Fig. 2a). Similar results were observed when comparing the effect of the interventions at each study visit (Fig. 2b and Supplementary Fig. 3d and e and Table 2). The change in basal glycolysis had an effect size of 0.111 (95% CI 0.002, 0.22), indicating that these interventions helped to decrease monocyte dependence on glycolysis (Table 2). The glycolytic reserve (Fig. 2c and d) and compensatory glycolysis (Fig. 2e and f) were significantly lower in the KD group than in the other experimental groups and lower than the values before the start of the study (Supplementary Fig. 3e and Table 2), suggesting that this intervention was highly effective in achieving the metabolic switch of substrate utilization in monocytes.

3.4. Subjects who underwent intervention with CR, IF or KD had a significant decrease in body weight and visceral fat area

CR, IF and KD interventions are effective at reducing body weight and fat mass. Therefore, we determined several anthropometric parameters of the subjects at the different follow-up visits, the results of which are shown in Table 3. Subjects assigned to the CR, IF or KD intervention groups had a significant decrease in body weight compared to patients in the AL group, with a n^2p of 0.174 (95% CI 0.06.0.29) (Table 3 and Fig. 3a). This effect progressed after the first month of treatment (V2) and persisted until the conclusion of the study (V4). In all three dietary intervention groups, a significant decrease in body weight was observed between the initial and final follow-up visits, while patients without intervention did not experience significant weight loss (Fig. 3b). A significant decrease in visceral fat area, estimated by the bioelectrical impedance equipment, was also observed with a n^2p of 0.204 (95% CI 0.09.0.32) (Table 3), where the main changes were observed between the initial and final visits of the study for subjects with CR, IF or KD but not for subjects in the AL group (Fig. 3c). Analysis performed between the study visits in each intervention group with Friedman test revealed that fat mass and waist circumference were significantly decreased, while skeletal muscle mass was significantly increased in CR, IF and KD groups an effect nor observed for the untreated AL group (Table 3).

3.5. Subjects with dietary interventions had a significant increase in gut microbiota diversity and modifications in taxonomy at the genus and species levels

Compared with untreated subjects, patients treated with CR, IF or KD had significantly greater alpha diversity according to the Shannon index (Supplementary Fig. 4a). The effect of CR was observed only at visit 2 ($p = 0.0001$), while for the IF ($p = 0.0001$) and KD ($p = 0.0001$) interventions, the strongest effect was observed at the final study visit compared to their baseline values. In contrast, subjects from the AL group had no increase in alpha diversity. Rifaximin treatment decreased the bacterial diversity after 7 days of administration in all the experimental groups (Supplementary Fig. 4a). Beta diversity performed with Adonis Bray–Curtis test analysis revealed no differences between groups at the initial study visit (Supplementary Fig. 4b) and after acute rifaximin treatment (V3) (Supplementary Fig. 4c), while at the end of the study, the microbial community of subjects from each dietary intervention; CR (Fig. 4a), IF (Fig. 4b) and KD (Fig. 4c) was significantly different from that of the untreated AL group.

Taxonomic analysis at the genus level revealed a positive correlation between dietary treatment with CR (Supplementary Fig. 4d), with IF (Supplementary Fig. 4e), and with KD (Supplementary Fig. 4f) and the abundance of gram-positive bacterial genera at the final study visit. In contrast, no intervention in the AL group was correlated with gram-negative bacterial genera at visit 4 (Supplementary Fig. 4 d, e and f). Linear model ANCOVA performed at visit 4 with adjustment correction made for sex, age and BMI revealed that in the CR group there was a significant enrichment of *Phascolarctobacterium faecium* compared to the AL and KD groups (Fig. 4d), and of *Ruminococcus bromii* compared to the AL and IF groups (Fig. 4e). *R. bromii* was also enriched in the KD group compared to the AL and IF groups (Fig. 4e). ANCOVA performed at visit 4 with adjustment correction made for age and BMI showed a significant increase in *Akkermansia muciniphila* in the IF group (Supplementary Fig. 4g), and *Faecalibacterium prausnitzii* in the KD group (Supplementary Fig. 4h) compared to the other interventions and the AL group after dietary and rifaximin treatment.

Total abundance analysis between visit 2 and visit 3 revealed that acute rifaximin treatment significantly decreased bacterial number and alfa diversity (Supplementary Fig. 4a) but increased the abundance of some gram-negative species such as *Bacteroides caccae* in AL, CR and IF groups (Fig. 4f), *Bacteroides eggerthii* in the CR, IF and KD groups (Fig. 4g) and *Dialister succinatiphilus* in the IF and KD groups (Fig. 4h). Notably, these bacterial species that were transiently increased after acute rifaximin administration had a significant reduction between visit 3 and visit 4 in the subjects that continued with one of the dietary interventions, but not in the subjects from the untreated AL group (Fig. 4g-i). Comparison between interventions at visit 4 showed that *Bacteroides eggertii* (Supplementary Fig. 4i) and *B. caccae* (Supplementary Fig. 4j) remained significantly enriched in the AL group compared to the three groups treated with a dietary intervention. These results indicate that the gut microbiota was modulated by both, the dietary intervention and the administration of rifaximin, with changes in taxonomy at the genus and specie levels.

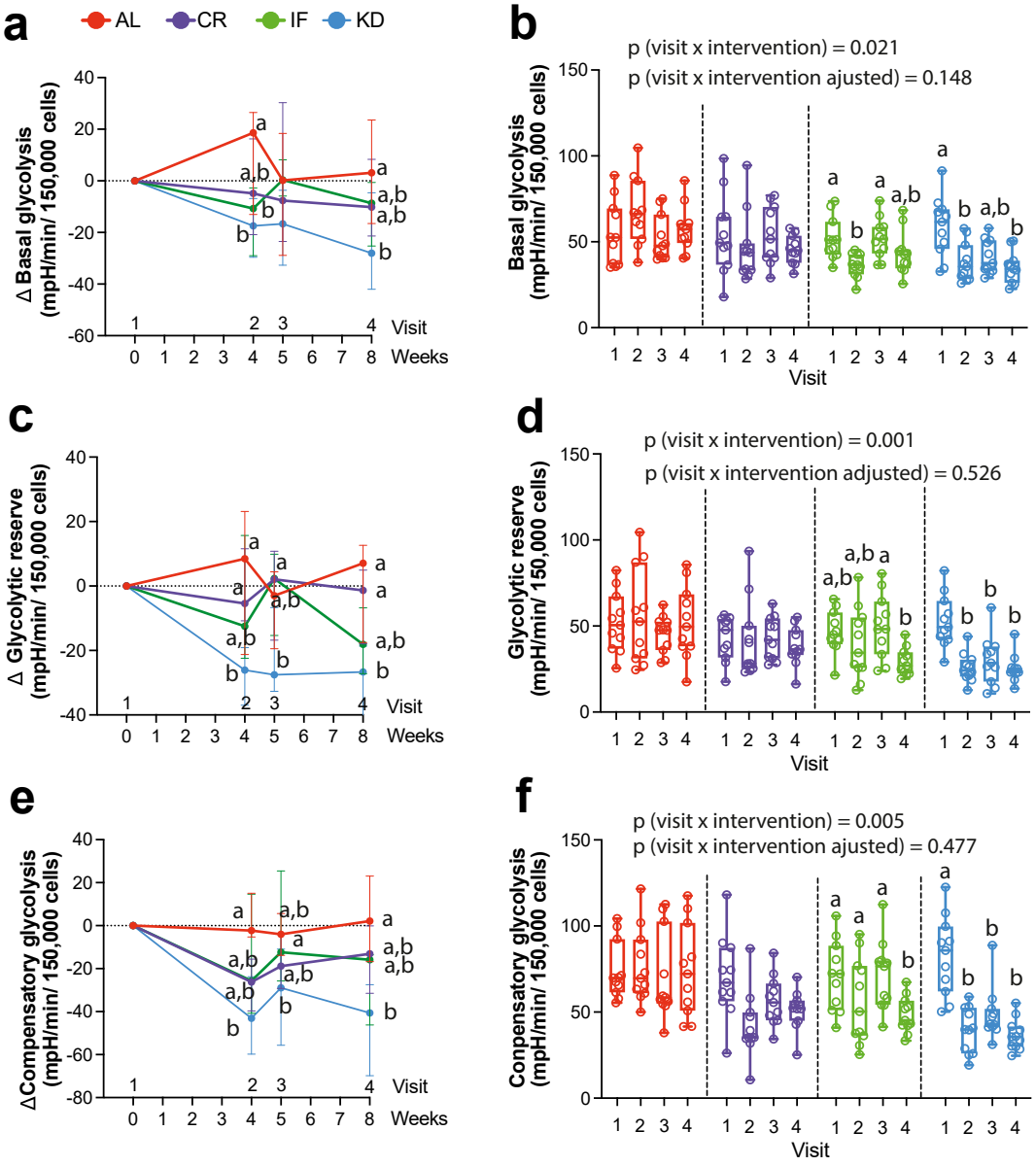

Fig. 2. Extracellular acidification rate (ECAR) for assessing glycolysis in monocytes. a) Differences (deltas) in basal glycolysis between the different study visits of subjects with obesity and those receiving dietary intervention involving calorie restriction (CR), intermittent fasting (IF) or a ketogenic diet (KD) or of subjects without intervention who continued with an ad libitum habitual diet (AL). b) Basal glycolysis in the different study visits of the subjects described in a. c) Difference (deltas) in the calculated glycolytic reserve values for each visit of the subjects described in a. d) Glycolytic reserve calculated for each visit of the study of the subjects described in a. e) Difference (deltas) in the compensatory glycolysis values calculated for each visit of the study of the subjects described in a. f) Compensatory glycolysis calculated for each visit of the subjects described in a. In Panels a, c, and e, statistical analysis of deltas at each visit was performed comparing each intervention with one-way ANOVA followed by Bonferroni post hoc correction. Medians with 95% confidence intervals. In Panels b, d and f, the statistical analysis was performed with repeated-measures ANOVA and adjustment for age, sex, and baseline weight. Additionally, post hoc analysis was performed with the Bonferroni correction. n = 11 per group. Different letters indicate a significant difference ($p < 0.05$) where a>b.

3.6. Correlation analysis highlighted serum LPS as a potential mediator of the changes observed in monocyte bioenergetics and gut microbiota composition

To determine if there was an association between the subject's phenotype or the bioenergetic profile of monocytes and changes in the gut microbiota, we performed cluster analysis using Spearman's correlation between the different variables measured in the study. In addition to the aforementioned results, we also determined some biochemical parameters at the different visits of all the subjects which are shown in Table 4. Correlations between the studied variables revealed the generation of four different clusters (Fig. 5a). Interestingly, we observed a positive correlation between nonmitochondrial respiration with *B. caccae* ($r^2 = 0.4$ $p = 0.0098$), and between *B. eggertii* with glycolytic reserve ($r^2 = 0.5$, $p = 0.00015$) and compensatory glycolysis ($r^2 = 0.6$, $p = 0.00067$). These bacterial species were significantly increased after acute rifaximin treatment (visit 3 vs visit 2) and remained enriched at the end of the study in the untreated AL group. The serum LPS concentration was inversely correlated with the maximal respiration OCR ($r^2 = -0.4$, $p = 0.02$), BHI ($r^2 = -0.3$, $p = 0.057$) and the bacterium *P. faecium* ($r^2 = -0.4$, $p = 0.0153$), which was particularly enriched in subjects with CR and IF. Conversely, LPS was positively correlated with BMI ($r^2 = 0.4$, $p = 0.024$), compensatory glycolysis

Table 3
Changes in anthropometric and clinical parameters according to intervention group.

Variable	Ad libitum habitual diet	Ketogenic diet	Intermittent fasting	Calorie restriction	P value x intervention	P[a] value	P[b] value	n^2 p	CI 95%	n^2 p[b]	CI 95%[b]
Body weight, kg[c]											
Visit 1	93.7 ± 10.4	86.6 ± 17.6	83.3 ± 10.4	93.2 ± 14.5	0.199	**0.0001**	**0.01**	**0.239**	**[0.13,0.35]**	**0.174**	**[0.06,0.29]**
Visit 2	93.7 ± 10.7	82.8 ± 16.5	80.8 ± 10.0	90.9 ± 14.0	0.099						
Visit 3	93.3 ± 10.9	82.7 ± 16.8	80.6 ± 9.68	90.8 ± 14.3	0.107						
Visit 4	93.4 ± 10.8	81.4 ± 16.9	79.5 ± 9.51	90.0 ± 14.1	0.069						
P value x visit	0.237	0.0001	0.0001	0.0001							
BMI, kg/m^2 [c]											
Visit 1	35.6 ± 3.98	33.3 ± 4.49	33.7 ± 3.09	36.6 ± 4.71	0.213	**0.0001**	0.24	**0.238**	**[0.12,0.35]**	0.097	[-0.02,0.21]
Visit 2	35.9 ± 4.17	31.9 ± 4.47	32.7 ± 2.94	35.8 ± 4.64	0.073						
Visit 3	35.8 ± 4.16	31.8 ± 4.55	32.6 ± 2.79	35.6 ± 4.17	0.089						
Visit 4	35.5 ± 4.00	31.3 ± 4.59	32.2 ± 3.01	35.3 ± 4.78	0.061						
P value x visit	0.266	0.0001	0.0001	0.0001							
Visceral fat area, cm^2 [d,c]											
Visit 1	158 ± 23.8	139 ± 31.4	143 ± 23.1	156 ± 20.4	0.154	**0.002**	**0.005**	**0.224**	**[0.11,0.34]**	**0.204**	**[0.09,0.32]**
Visit 2	156 ± 25.2	129 ± 29.6	136 ± 22.3	152 ± 20.9	0.074						
Visit 3	155 ± 24.0	129 ± 28.4	137 ± 21.3	149 ± 19.7	0.101						
Visit 4	155 ± 24.8	123 ± 29.6	134 ± 20.4	148 ± 21.0	0.049						
P value x visit	0.116	0.0001	0.0001	0.0001							
Fat mass, %[c]											
Visit 1	45.5 ± 5.29	43.1 ± 6.10	46.6 ± 4.09	46.2 ± 5.05	0.489	**0.013**	0.153	0.168	[0.05,0.28]	0.110	[0.00,0.22]
Visit 2	45.8 ± 5.45	41.4 ± 6.08	45.4 ± 4.20	46.1 ± 4.75	0.177						
Visit 3	45.6 ± 5.27	41.2 ± 5.93	45.4 ± 4.45	45.3 ± 5.27	0.219						
Visit 4	45.3 ± 5.48	39.9 ± 6.08	45.3 ± 4.58	44.4 ± 5.03	0.086						
P value x visit	0.894	0.0001	0.019	0.004							
Skeletal muscle mass, %[b]											
Visit 1	30.2 ± 3.22	31.3 ± 4.01	29.2 ± 2.48	29.9 ± 3.01	0.580	**0.020**	0.430	0.159	[0.05,0.27]	0.077	[-0.04,0.19]
Visit 2	30.0 ± 3.53	32.3± 3.89	29.8 ± 2.52	29.9 ± 2.79	0.274						
Visit 3	30.1 ± 3.27	32.3± 3.84	29.8 ± 2.66	32.6 ± 6.96	0.451						
Visit 4	30.2 ± 3.27	33.6± 3.82	29.9 ± 2.75	30.5 ± 3.76	0.058						
P value x visit	0.679	0.0001	0.031	0.002							
Waist circumference, cm											
Visit 1	107 ± 9.29	104 ± 12.7	101 ± 11.0	109 ± 10.7	0.401	**0.007**	0.180	**0.182**	**[0.07,0.30]**	0.105	[-0.01-0.22]
Visit 2	108 ± 9.55	100 ±11.3	97.4 ± 10.6	107 ± 9.68	0.036						
Visit 3	104 ± 9.73	98.6 ± 11.1	96.0 ± 10.6	107 ± 10.3	0.041						
Visit 4	106 ± 8.75	98.7 ± 11.3	94.6 ± 9.72	105 ± 10.8	0.028						
P value x visit	0.066	0.0001	0.0001	0.003							

n^2p: Effect size. Partial Eta Squared.
95% CI: 95% confidence interval.
BMI: body mass index.
The data are presented as the mean ± SD. P values x visit were calculated with Friedman test and P values x intervention were calculated with Kruskal Wallis test.
Visit 1 corresponds to week 0 (basal), visit 2 is after 4 weeks of intervention, visit 3 is at week 5 (after 7 days of rifaximin administration), visit 4 us after 8 weekis of intervention.
[a] P value x intervention x visit were performed with ANOVA for repeated measures.
[b] Statistical analysis adjusted for age, sex, and baseline weight.
[c] Statistical analysis adjusted for age and sex.
[d] Visceral fat area values were estimated by the bioelectrical impedance equipment.

($r^2 = 0.4$, $p = 0.048$) and *B. eggertii* ($r^2 = 0.6$, $p = 0.0000068$), all variables enriched before the dietary interventions and in the AL group. These results indicated that the modulation of the gut microbiota by dietary intervention and rifaximin administration had an impact on the phenotype and bioenergetic profile of the subjects in the monocyte population, highlighting the potential role of LPS as a modulator of the observed effects.

3.7. Subjects with CR, IF and KD interventions had a significant decrease in LPS-mediated intracellular signaling in monocytes

Serum LPS concentrations were elevated in most patients with obesity before the initiation of dietary treatment (Table 4), indicating metabolic endotoxemia. The combination of antibiotic treatment together with dietary intervention significantly decreased the serum LPS concentration at the final visit in the CR and IF intervention groups, with a trend in the KD group compared to that in the AL group (Fig. 5b). However, there was a significant reduction when comparing the initial and final visits in the three dietary interventions of CR, IF and KD (Table 4). Gut microbiota analysis revealed that some gram-negative bacterial species such as *B. caccae* and *B. eggertii*, which were positively associated with serum LPS a glycolytic phenotype in monocytes, were transiently increased after acute rifaximin treatment, but decreased at the end of the study in the subjects that continued with the dietary treatments of CR, IF and KD. Interestingly, this effect was not observed in

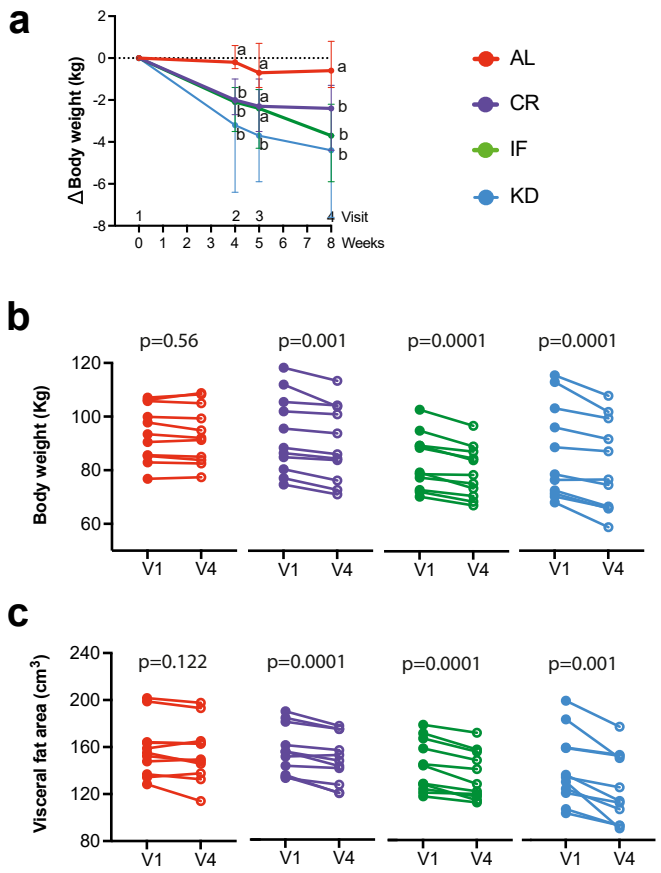

Fig. 3. Body weight and visceral fat area in the study subjects. a) Differences in body weight (kg) at different visits of the study of subjects with obesity with dietary interventions of calorie restriction (CR), intermittent fasting (IF) or ketogenic diet (KD) or without intervention who continued with an ad libitum habitual diet (AL). Median with 95% confidence interval. b) Body weight (kg) at visit 1 and 4 for the subjects described in a. c) Visceral fat area (cm3) estimated by the bioelectrical impedance equipment at visits 1 and 4 for the subjects described in a. In panel a statistical analysis of the deltas was performed at each visit, and each dietary intervention was compared with one-way ANOVA followed by the Bonferroni post hoc correction. For Panels b and c, statistical analysis was performed by paired t tests. n = 11 per group. Different letters indicate significant differences ($p < 0.05$) where a>b.

the AL group, since these subjects remained with high concentrations of these bacterial species and serum LPS, suggesting that this metabolite may be responsible of changes observed in the monocyte bioenergetic profile.

To determine the effect of the LPS concentration on mitochondrial function and substrate utilization in monocytes, several intracellular signaling proteins were measured. LPS induces inflammatory processes in monocytes by activating the mitogen-activated protein (MAP) kinase cascade via extracellular signal-regulated Factor 1/2 (ERK1/2) [31]. This effect is mediated by Toll-like receptor 4 (TLR4) [32]. No significant differences were detected in the TLR4 abundance in monocytes following dietary intervention or by rifaximin administration (Fig. 5c and d). However, when evaluating the effect of CR, IF and KD on LPS signaling, we observed a reduction in ERK 1/2 phosphorylation at the end of the study (p visit x intervention = 0.0001), and this effect was not observed for the AL group (Fig. 5c and e). To assess whether TLR4 activation might be related to the mitochondrial function observed in monocytes, we measured the total abundance and phosphorylation of dynamin-related protein 1 (DRP1), which can be activated by ERK1/2 signaling via its phosphorylation at serine residue 616 and whose activation is involved in the process of mitochondrial fission [33]. Interestingly, there was a significant decrease in DRP1 phosphorylation at serine residue 616 in monocytes from subjects who achieved CR ($p = 0.002$), IF ($p = 0.001$) or KD ($p = 0.001$) at the final study visit, but this effect was not observed in the AL group (Fig. 5c and f). These results suggest that the increase in mitochondrial respiration in monocytes observed in response to these interventions may be related to a decrease in the LPS concentration and signaling in these cells, resulting in decreased mitochondrial fragmentation. Thus, to determine whether the switch between glycolysis and mitochondrial respiration mediated by LPS was dependent on ERK1/2 signaling, we conducted an *in vitro* study in monocytes isolated from healthy subjects exposed to LPS with or without preincubation with the ERK1/2 inhibitor FR180204. Monocytes treated with LPS had a significant increase in the ECAR (Supplementary Fig. 5a), particularly in basal glycolysis (Fig. 5g), and a significant decrease in the basal, ATP-linked, maximal respiration OCR (Fig. 5h and Supplementary Fig. 5b) and spare capacity (Fig. 5h). Preincubation with the specific ERK1/2 inhibitor FR180204 ablated this effect, indicating that the LPS-mediated effect on the metabolic switch between anaerobic glycolysis and mitochondrial oxidative phosphorylation in monocytes is dependent on ERK1/2 signaling.

4. Discussion

In the present study, we found that dietary interventions involving CR, IF and KD improved mitochondrial bioenergetic marker levels in monocytes from individuals with obesity. Although no studies have reported the effect of these interventions on mitochondrial respiration in monocytes, there is evidence of changes in metabolic tissues. Short-term CR in mice significantly increased oxygen consumption in several tissues including white and brown adipose tissue, brain liver and hearth [34]. Similarly, compared with the control treatment, IF intervention increased the OCR of mitochondrial complexes I and II in the skeletal muscle of male Wistar rats [35]. Moreover, 12 weeks of KD intervention significantly increased ATP-dependent mitochondrial respiration from fatty acid utilization in skeletal muscle of healthy subjects [30]. Even though our findings show that these dietary treatments increased the monocyte dependence on mitochondrial respiration, suggesting an increase in fatty acid oxidation, there is still the doubt whether these changes may also occur in metabolic tissues as reported from animal studies.

In monocytes, mitochondrial dysfunction is related to the progression of inflammation during obesity [8,17]. An increase in glycolysis without activation of mitochondrial function favors lactate production in the cytoplasm, increasing the extrusion of protons and acidification of the extracellular medium. In this sense, the decrease in glycolysis observed in monocytes assessed by the ECAR following dietary intervention with IF and the KD suggested that these individuals may have a less inflammatory phenotype. In fact, Sharma and coworkers reported that activation of glycolysis in white adipose tissue macrophages sustains local and systemic production of inflammatory interleukin-1β (IL-1β) in obese mice [36].

Both mitochondrial respiration and anaerobic glycolysis were affected by acute (7 days) administration of rifaximin, indicating a potential role of the gut microbiota in this effect. Dietary interventions tended to increase the alpha diversity of the gut microbiota after one month of treatment, while acute administration of rifaximin (550 mg twice daily for 7 days) significantly decreased the alpha diversity in all the experimental groups. Previous studies reported that long-term rifaximin treatment at lower daily doses (8 weeks 550 mg/day) [22] or (12 weeks, 400 mg/day) [37] did not modify the gut microbiota diversity in patients with

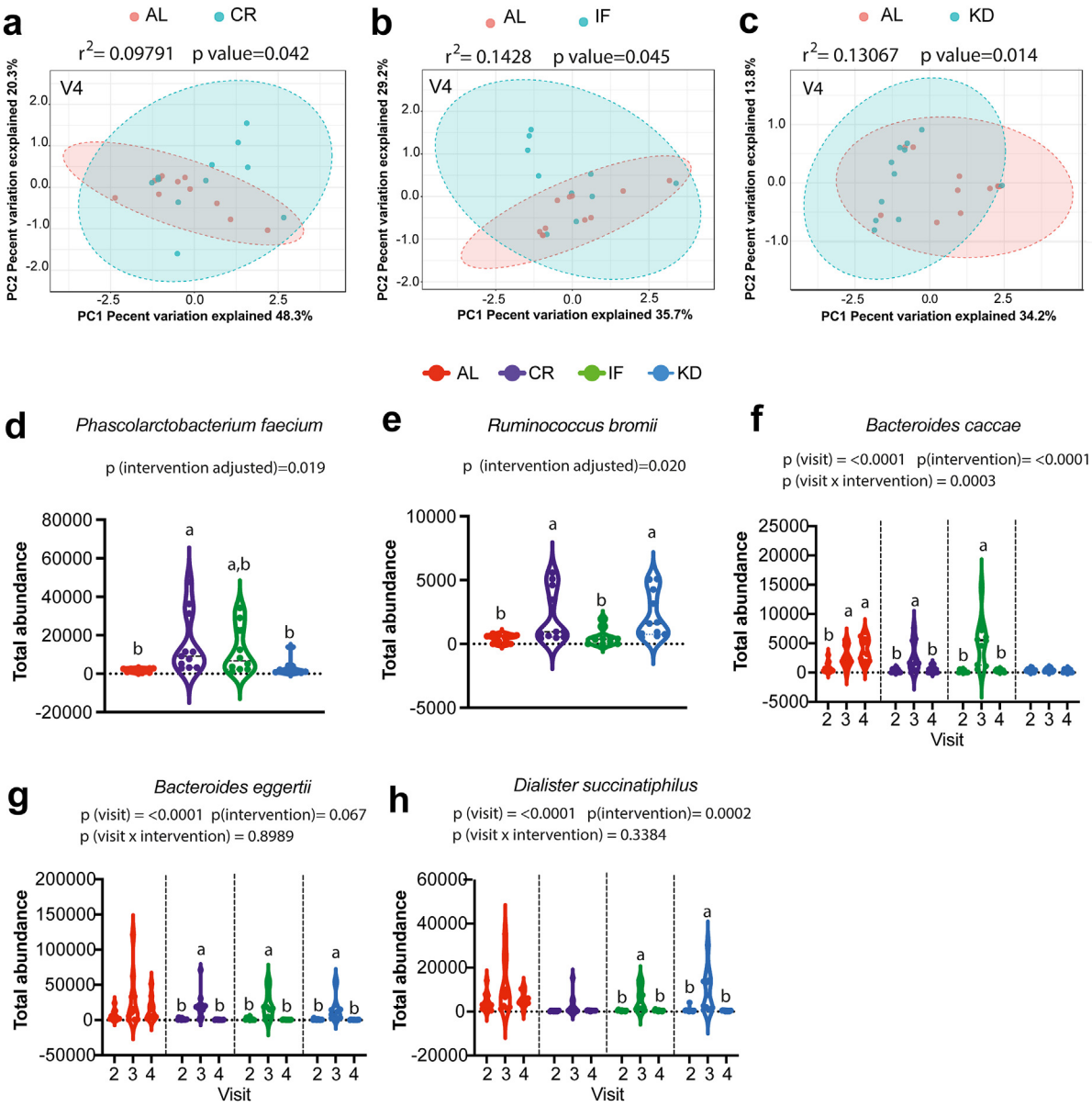

Fig. 4. Composition of the gut microbiota. a) Beta diversity at visit 4 in the patients who achieved calorie restriction (CR) compared to those in the ad libitum habitual diet (AL) group. b) Beta diversity at visit 4 in the patients with intermittent fasting (IF) compared to those in the AL group. c) Beta diversity at visit 4 in the subjects with a ketogenic diet (KD) compared to the AL group. Total abundance of bacterial species d) *Phascolarctobacterium faecium and* e) *Ruminococcus bromii* at visit 4 of subjects with obesity with dietary interventions of CR, IF or KD or without intervention AL. Total abundance of bacterial species f) *Bacteroides caccae*, g) *Bacteroides eggertii*, and h) *Dialister succinatiphilus*, at visits 2, 3 and 4 of subjects with obesity with dietary interventions of CR, IF or KD or in the AL group. N = 11 per group. In Panels a to c, beta-diversity was determined using Adonis with Bray–Curtis test. In Panels d and e ANCOVA was performed with adjustments made for sex, age, and BMI with the Bonferroni post hoc correction. In panels f to h statistical analysis was performed with mixed-effects analysis with the Bonferroni post hoc correction. Different letters indicate a significant difference ($p < 0.05$) where a>b.

cirrhosis. However, when analyzing the gut microbiota composition, Yu and coworkers showed that after 1 week of rifaximin *Klebsiella pneumoniae*, *Bacteroides thetaiotaomicron*, *Allestipes putredinis*, *Bacteroides fragilis*, all gram-negative bacteria, transiently increased while there was a decrease in gram-positive species such as *R. bromii*, *Roseburia intestinalis* and *Bifidobacterium breve* [37]. This result agrees with our findings where we observe an acute and transient increase in gram-negative species in all experimental groups after 7 days of rifaximin administration. In contrast, Bajaj and coworkers [22] reported that 8 weeks of rifaximin administration produce beneficial changes in gut microbiota composition by reducing Veillonellaceae family, enriched in cirrhotic patients [38], with a concomitant increase in *Eubacteriaceae*. In our study, patients that continued with good adherence to the dietary interventions of CR, IF and KD, had a positive change in gut microbiota diversity and composition after rifaximin administration, an effect that was not observed in patients without dietary intervention from the AL group. These results indicate that the combination of this drug with the dietary interventions, especially IF and KD, had synergistic effects on increasing the diversity of the gut microbiota, suggesting that the acute modulation of the intestinal microbiota caused by rifaximin could enhance the beneficial effects of these dietary interventions.

Taxonomy analysis revealed enrichment of *P. faecium* and *R. bromii* after dietary interventions, and both species are substantial producers of short-chain fatty acids, especially butyrate

Table 4
Changes in biochemical parameters in the intervention group.

Variable	Ad libitum habitual diet	Ketogenic diet	Intermittent fasting	Calorie restriction	P value x intervention	P[a] value	P[b] value	n^2 p	CI 95%	n^2 p[b]	CI 95%[ik]
Glucose, mg/dL											
Visit 1	104 ± 12.5	99.0 ± 7.48	93.0 ± 9.15	102 ± 14.0	0.400	0.207	0.985	0.101	[-0.01,0.21]	0.020	[-0-09,0.13]
Visit 2	104 ± 12.8	96.7 ± 9.05	95.6 ± 10.5	101 ± 13.5	0.317						
Visit 3	100 ± 14.6	104 ± 12.5	93.3 ± 8.29	98.7 ± 9.31	0.089						
Visit 4	106 ± 19.4	97.9 ± 5.79	93.1 ± 6.93	100 ± 9.15	0.301						
P value x visit	0.241	0.079	0.821	0.896							
Total cholesterol, mg/dL											
Visit 1	186 ± 29.0	196 ± 45.8	177 ± 32.7	197 ± 35.0	0.725	0.51	0.408	0.079	-0.04,0.18]	0.070	[-0.03,0.19]
Visit 2	174 ± 31.2	181 ± 34.8	161 ± 42.7	175 ± 30.6	0.497						
Visit 3	171 ± 32.6	186 ± 39.0	181 ± 36.4	183 ± 27.9	0.852						
Visit 4	159 ± 27.2	185 ± 34.3	172 ± 28.9	185 ± 33.8	0.130						
P value x visit	0.138	0.21	0.321	0.017							
Triglycerides, mg/dL											
Visit 1	174 ± 91.5	165 ± 63.9	145 ± 63.3	145 ± 59.1	0.600	0.306	0.0001		[-0.02,0.20]	0.252	[0.14,0.37]
Visit 2	185 ± 145	111 ± 25.8	144 ± 64.3	144 ± 44.0	0.178						
Visit 3	149 ± 100	126 ± 45.3	141 ± 56.6	143 ± 47.2	0.906						
Visit 4	175 ± 89.6	175 ± 116	159 ± 84.9	175 ± 101	0.859						
P value x visit	0.615	0.032	0.797	0.375							
HDL cholesterol, mg/dL											
Visit 1	49.3 ± 9.15	44.2 ± 11.0	45.4 ± 7.96	46.7 ± 4.87	0.375	0.11	0.987	0.119	[0.01,0.23]	0.020	[-0.09,0.13]
Visit 2	42.6 ± 9.12	46.5 ± 14.4	46.5 ± 14.5	41.6 ± 7.43	0.986						
Visit 3	43.3 ± 2.42	47.2 ± 15.6	45.7 ± 10.6	43.8 ± 11.8	0.975						
Visit 4	42.9 ± 10.0	46.8 ± 11.6	48.4 ± 11.4	43.8 ± 10.5	0.627						
P value x visit	0.078	0.965	0.81	0.086							
LDL cholesterol, mg/dL											
Visit 1	116 ± 28.9	121 ± 22.1	112 ± 32.1	119 ± 46.8	0.891	0.41	0.659	0.078	[-0.04,0.19]	0.058	[-0.06,0.17]
Visit 2	107 ± 23.4	120 ± 27.2	96.4 ± 32.1	116 ± 29.4	0.317						
Visit 3	110 ± 26.3	126 ± 33.1	119 ± 34.8	123 ± 24.5	0.763						
Visit 4	100 ± 22.3	125 ± 33.3	114 ± 24.5	122 ± 33.2	0.133						
P value x visit	0.484	0.819	0.116	0.484							
C-reactive protein, mg/dL											
Visit 1	4.88 ± 3.98	3.13 ± 2.65	5.22 ± 4.61	4.36 ± 2.65	0.934	**0.026**	0.995	0.153	[0.04,0.27]	0.015	[-0.10,0.13]
Visit 2	4.23 ± 3.35	4.29 ± 4.01	3.13 ± 3.89	5.07 ± 3.22	0.245						
Visit 3	5.93 ± 5.31	3.05 ± 3.98	4.41 ± 3.91	3.86 ± 3.34	0.487						
Visit 4	6.13 ± 4.95	2.86 ± 3.02	5.03 ± 5.31	4.33 ± 3.77	0.479						
P value x visit	0.173	0.009	0.229	0.058							
Insulin, IU/mL											
Visit 1	23.9 ± 21.5	15.1 ± 6.89	14.7 ± 7.05	16.1 ± 6.48	0.651	0.173	0.532	0.106	[-0.01,0.22]	0.068	[-0.05,0.18]
Visit 2	23.1 ± 14.6	12.7 ± 5.88	19.7 ± 16.0	23.8 ± 19.6	0.343						
Visit 3	17.4 ± 9.18	10.2 ± 4.29	18.6 ± 15.3	28.3 ± 25.4	0.112						
Visit 4	18.7 ± 16.4	10.5 ± 4.63	13.4 ± 6.40	26.2 ± 21.7	0.205						
P value x visit	0.071	0.103	0.445	0.418							
HOMA-IR											
Visit 1	9.52 ± 12.0	3.73 ± 1.73	3.79 ± 1.68	4.23 ± 2.30	0.480	0.357	0.539	0.122	[-0.008,0.23]	0.026	[-0.08,0.13]
Visit 2	6.12 ± 4.13	3.07 ± 1.44	4.90 ± 4.48	6.13 ± 5.10	0.260						
Visit 3	4.58 ± 2.65	2.62 ± 1.09	4.64 ± 4.60	7.30 ± 7.24	0.123						
Visit 4	5.09 ± 4.99	2.52 ± 1.09	3.07 ± 1.43	6.55 ± 5.47	0.257						
P value x visit	0.104	0.21	0.288	0.664							
Leptin, ng/mL											
Visit 1	53.1 ± 37.7	38.9 ± 31.5	55.6 ± 26.7	57.5 ± 39.7	0.392	0.862	0.961	0.018	[-0.01, 0.13]	0.007	[-0.11-0.12]
Visit 4	48.4 ± 27.4	28.6 ± 28.1	48.1 ± 30.7	56.3 ± 26.5	0.062						
P value x visit	0.366	0.035	0.366	0.366							
Adiponectin, μg/mL											
Visit 1	4.92 ± 2.37	6.01 ± 3.70	5.76 ± 3.62	6.95 ± 3.94	0.354	0.113	0.895	0.137	[0.02, 0.25]	0.015	[-0.01,0.13]
Visit 4	4.91 ± 2.39	5.76 ± 3.62	7.39 ± 4.98	6.20 ± 2.70	0.567						
P value x visit	0.763	1	0.035	0.132							
Lipopolysaccharides, pg/ml											
Visit 1	2469 ± 1377	2921 ± 922	3298 ± 647	3351 ± 1789	0.631	**0.0001**	0.863	**0.287**	**[0.17,0.40]**	0.044	[-0.07,0.16]

(*continued on next page*)

Table 4 (*continued*)

Variable	Ad libitum habitual diet	Ketogenic diet	Intermittent fasting	Calorie restriction	P value x intervention	P[a] value	P[b] value	n^2 p	CI 95%	n^2 p[b]	CI 95%[ik]
Visit 2	3494 ± 2672	2682 ± 1499	2760 ± 1023	2776 ± 2007	0.953						
Visit 3	3099 ± 1288	4572 ± 2677	4406 ± 3060	3315 ± 1443	0.440						
Visit 4	2663 ± 1478	1445 ± 1294	350 ± 456	399 ± 715	0.00018						
P value x visit	0.948	0.004	0.0001	0.001							

n^2p: Effect size. Partial Eta Squared.
95% CI: 95% confidence interval.
HOMA-IR= Homeostasis Model Assessment Score of Insulin resistance.
The data are presented as the mean ± SD. P values x visit were calculated with Friedman test and P values x intervention were calculated with Kruskal Wallis test.
Visit 1 corresponds to week 0 (basal), visit 2 is after 4 weeks of intervention, visit 3 is at week 5 (after 7 days of rifaximin administration), visit 4 is after 8 weeks of intervention.
[a] P value x intervention x visit were performed with ANOVA for repeated measures
[b] Statistical analysis adjusted for age, sex, and baseline weight.

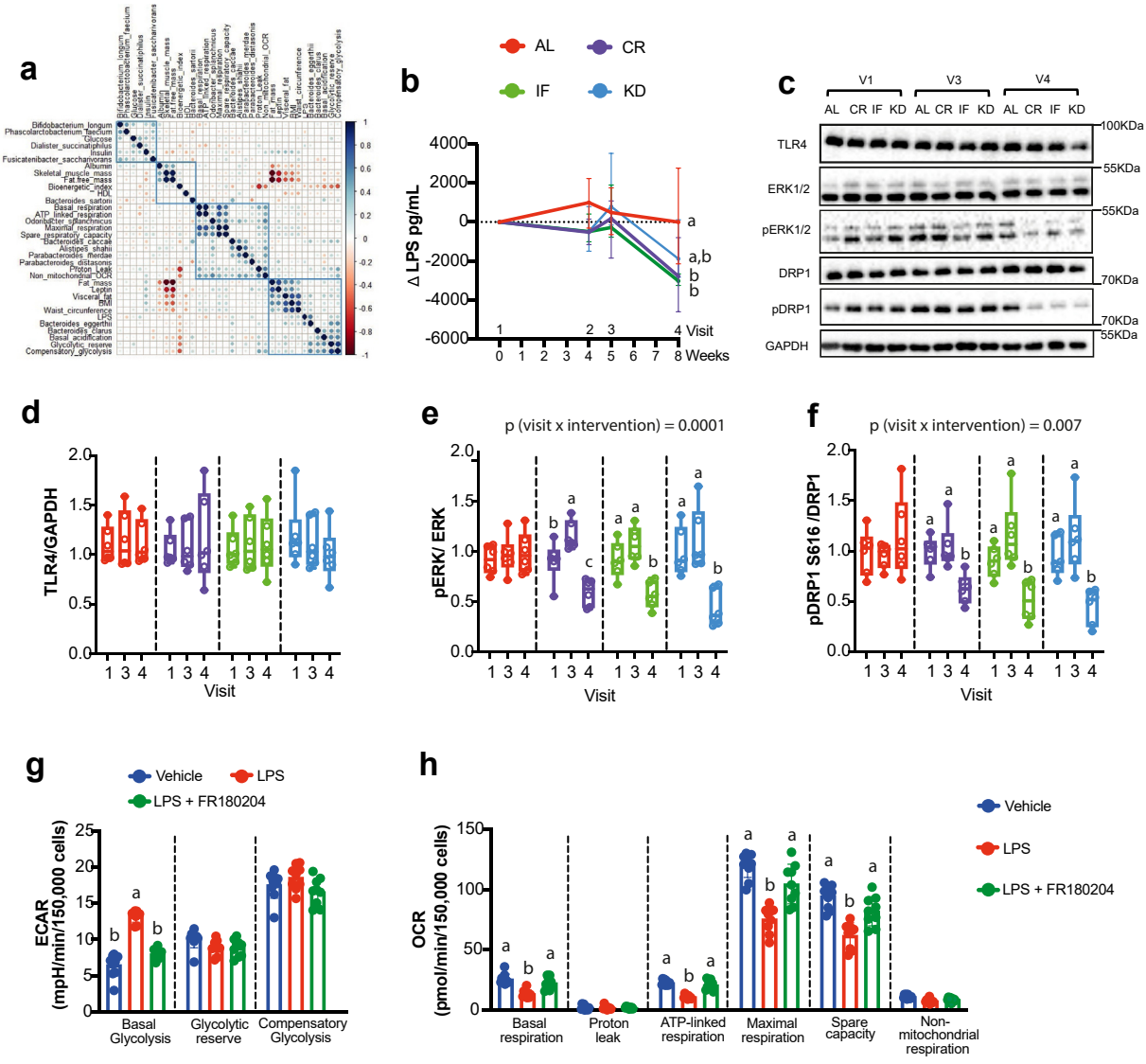

Fig. 5. Lipopolysaccharide as the modulator of mitochondrial bioenergetics in monocytes. a) Spearman correlation coefficients between the different variables measured in the study. b) Differences (deltas) in the serum LPS concentration at the different study visits in subjects with obesity with dietary intervention comprising calorie restriction (CR), intermittent fasting (IF) or a ketogenic diet (KD) or in subjects without intervention who continued with an ad libitum habitual diet (AL). n = 11 per group. Medians with 95% confidence intervals. c) Representative images of protein abundance: Toll-like receptor 4 (TLR4), extracellular signal-regulated Factor 1 and 2 (ERK1/2) and its phosphorylation (pERK1/2), dynamin-related protein 1 (DRP1) and its phosphorylation at serine residue 616 (pDRP1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (used as loading control) at visits 1, 3 and 4 of the subjects described in b. d) Densitometry analysis showing TLR4 protein quantification adjusted for GAPDH loading control concentration at visits 1, 3 and 4 in subjects described in b. e) Densitometric analysis showing the pERK1/2/ERK1/2 ratio adjusted for the GAPDH loading control concentration at visit 1, 3 and 4 of the subjects described in b. f) Densitometric analysis showing the ratio of pDRP1/DRP1 adjusted for the concentration of the GAPDH loading control at visit 1, 3 and 4 of the subjects described in b. n = 6 per group. g) Glycolytic parameters assessed in isolated monocytes incubated with LPS with or without the ERK1/2 inhibitor FR180204. h) Mitochondrial function parameters in monocytes described in g. Mean ± SEM. n = 10 per group. In Panels b, g and h, a statistical analysis comparing each intervention was performed with one-way ANOVA followed by the Bonferroni post hoc correction. In Panels d–f, statistical analysis was performed with repeated-measures ANOVA and adjustment for age, sex, and baseline weight, followed by the Bonferroni post hoc correction. Different letters indicate a significant difference ($p < 0.05$) where a>b > c.

[39,40], which has beneficial effects on human health and is associated with the suppression of inflammatory and oxidative stress processes [41]. In monocytes, butyrate reduces the expression and secretion of proinflammatory cytokines such as tumor necrosis factor-alpha [42] and interleukin 12 [43] and increases the secretion of interleukin 10 (IL-10) [42,43], which is an anti-inflammatory agent. The anti-inflammatory effects generated by the enrichment of the aforementioned bacteria could be related to changes in the bioenergetic profile of monocytes. In fact, Jyonouchi and coworkers reported that there is a positive correlation between the IL-1β/IL-10 ratio and mitochondrial dysfunction in mononuclear cells extracted from patients with autism [44]. Additionally, stimulation of bone marrow-derived macrophages with IL-10 restores maximal respiration OCR and spare capacity and significantly decreases glycolysis, as assessed by the ECAR [45].

Correlation analysis highlighted LPS as a putative mediator of the alterations in mitochondrial function observed in monocytes from patients with obesity before dietary intervention and in those from the AL group. This is supported by the fact that LPS stimulation has been used as a model for generating mitochondrial dysfunction in macrophages [45] and monocytes [9], and an increase in LPS is associated with favoring a glycolytic phenotype in immune cells while decreasing mitochondrial respiration. Moreover, LPS is involved in multiple proinflammatory effects [46]. When evaluating the effect of dietary treatments on LPS signaling, we observed that the serum concentrations of this endotoxin were inversely associated with ERK1/2 phosphorylation in monocytes. Consequently, after rifaximin administration, the CR, IF and KD interventions significantly decreased DRP1 phosphorylation, suggesting a reduction in mitochondrial fission, an effect not observed in the AL group. In line with these findings, fasting and CR have been associated with decreased ERK1/2 signaling in the livers of obese Zucker rats [47]. However, there is no evidence connecting this result with the mitochondrial phenotype.

Although LPS is a strong candidate for explaining the effects observed on mitochondrial function in monocytes, there may be other candidates, such as specific metabolites, involved in this process. In future studies, metabolomic analysis could be performed to identify other possible mediators. Parisi and coworkers reported that exposure of mononuclear cells to serum from subjects with obesity generates mitochondrial dysfunction and increases the secretion of proinflammatory cytokines related to premature senescence [48], suggesting that the serum of these subjects contains some metabolites that generate these effects, including a possible dysregulation of mitochondrial dynamics. CR intervention in rats attenuates mitochondrial fragmentation in skeletal muscle [49], and ketone bodies promote mitochondrial fusion in an *in vitro* model in human cells [50]. Therefore, it is possible that the increase in ketone bodies generated by fasting or by the consumption of a KD was associated with the phenotype observed in monocytes in our clinical trial.

Although we observed important and significant changes in monocyte's bioenergetics and body composition, the sample size in our study was an evident limitation and contributed to the large fluctuations we observed in some of the evaluated parameters. Biochemical parameters were the most affected by this variability since we did not observe improvement in glucose tolerance as previously reported for these dietary interventions [51]. Reporting differential abundance of bacterial species after covariate adjustment was quite a challenge, leaving us with very few bacterial species significantly modified by the dietary interventions. These results were mainly affected by the sample size and the difference in number between male and female participants, since more bacterial species were significant after age and BMI adjustment but not after including sex as a covariate. We should consider including equal number of men and women and to increase the sample size for future studies. Additionally, the study design does not allow us to unequivocally determine whether the observed effects can be attributed specifically to dietary interventions, the administration of rifaximin, or both; therefore, future studies could be designed to use rifaximin or a placebo together with dietary treatments to exclusively address these limitations and determine whether the administration of rifaximin in combination with dietary interventions is worthwhile for improving the metabolic profile of individuals with obesity.

5. Conclusion

This work demonstrated that dietary interventions involving CR, IF and KD in individuals with obesity positively modulate mitochondrial bioenergetic health in monocytes and proposed a potential mechanism to explain this effect and its relationship with the gut microbiota via decreased serum LPS levels and signaling. Additionally, we confirmed the close connection between host mitochondrial function and the gut microbiota and translated several mechanisms observed in animal models using these dietary interventions in a clinical trial, serving as a model for future intervention studies.

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Author contributions

LAV-V and MG-C conceived and designed the study. KGH-G, CC-H, AKP-F, LEG-S, EP-O, AL-B, IM and LAV-V acquired the data and performed the experiments. CC-H, LEG-S, AES-Z, IM-V, MS-T, OR-L, VO-S, MG-C and LAV-V analyzed the data. NT, ART, LGN, MG-C and LAV-V participated in critically analyzing and interpreting the data. MG-C and LAV-V wrote the manuscript. All the authors revised critically and approved the manuscript.

Declaration of conflict of interest

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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.2024.06.036.

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