Metformin induces weight loss associated with gut microbiota alteration in non-diabetic obese women: a randomized double-blind clinical trial

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Abstract

Objective: The increasing prevalence of obesity over the past few decades constitutes a global health challenge. Pharmacological therapy is recommended to accompany life-style modification for obesity management. Here, we perform a clinical trial to investigate the effects of metformin on anthropometric indices and gut microbiota composition in non-diabetic, treatment-naive obese women with a low-calorie diet (LCD).
Design: Randomized double-blind parallel-group clinical trial
Methods: Forty-six obese women were randomly assigned to the metformin (500 mg/tab) or placebo groups using computer-generated random numbers. Subjects in both groups took two tablets per day for 2 months. Anthropometric measurements and collection of blood and fecal samples were done at the baseline and at the end of the trial. Gut microbiota composition was assessed using 16S rRNA amplicon sequencing.
Results: Twenty-four and twenty-two subjects were included in the metformin + LCD and placebo + LCD groups, respectively; at the end of trial, 20 and 16 subjects were analyzed. The metformin + LCD and placebo + LCD caused a 4.5 and 2.6% decrease in BMI from the baseline values, respectively ($P < 0.01$). Insulin concentration decreased in the metformin + LCD group ($P = 0.046$). The overall fecal microbiota composition and diversity were unaffected in the metformin + LCD group. However, a significant specific increase in *Escherichia/Shigella* abundance was observed after metformin + LCD intervention ($P = 0.026$). Fecal acetate concentration, but not producers, was significantly higher in the placebo + LCD group, adjusted for baseline values and BMI ($P = 0.002$).
Conclusions: Despite the weight reduction after metformin intake, the overall fecal microbiota composition remained largely unchanged in obese women, with exception of changes in specific proteobacterial groups.

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Introduction

The obesity epidemic is an increasing public health and financial burden across the world (1, 2). It is estimated that the prevalence of obesity increased 33% by 2030 (3). Obesity is often associated with insulin resistance, making obese individuals susceptible to metabolic syndrome and its related cardiometabolic disorders (4). Because obesity management with life-style modification is an important challenge for most individuals, adjunct treatments like pharmacological therapy are frequently applied (5). Given the modest yet durable weight loss observed following metformin consumption in diabetics, women suffering from polycystic ovary syndrome and healthy obese adults, its administration in combination with lifestyle modification might be helpful for combating obesity (6), although further investigations are needed to confirm its efficacy in non-diabetic obese individuals.

Metformin, the first-line drug for treating type 2 diabetes, mediates its glucose-lowering property and its welcome weight-reducing side effect by inhibition of liver glucose production, appetite suppression, improvement of insulin sensitivity and regulation of fat oxidation and storage (7). Moreover, recent evidence suggests that gut microbiota alterations may contribute to these effects (8, 9). Composition of the gut microbiota, a complex and dynamic population of microorganisms living in the intestine, is shaped by several environmental factors such as diet and drugs (10).

Previous studies investigated the effect of metformin on gut microbiota composition in diabetic subjects (11, 12, 13, 14, 15). However, considering that the effect of metformin on gut microbiota composition differs under healthy and diabetes conditions (16, 17), it should be assessed in healthy obese subjects too. Obesity is accompanied by an altered gut microbiota, suggested to be linked to increased energy harvest (18, 19, 20, 21). Gut bacteria-released metabolites have the effective roles in weight control by stimulating gut satietogenic hormones, controlling lipid metabolism in adipose tissue, influencing insulin signaling and improving gut barrier function (20).

To assess gut microbial mediation of weight loss effects of metformin, we conducted a randomized double-blind parallel-group clinical trial investigating the effects of metformin on anthropometric indices and gut microbiota composition in obese women with a low-calorie diet (LCD).

Subjects and methods

Subjects

This study was a single site, randomized, double-blind, placebo-controlled parallel group clinical trial, which was conducted at the Obesity Clinic of Shariati Hospital in Tehran, Iran from October 2017 to March 2018. Subjects were recruited via advertising at the clinic and Shariati Hospital website and social media channels. Participants were eligible for the study if they met the following inclusion criteria: women aged between 20 and 45 years, BMI in the range of 30–40 kg/m^2, willingness to adhere to the study protocol. Exclusion criteria were pregnancy and lactation, smoking, having cardiovascular disease, kidney and liver disorders, inflammatory bowel diseases, diabetes and cancer, history of digestive tract surgery, history of acute and chronic diarrhea over the last month before the start of the study, antibiotic therapy within the 2 months prior to or during the study period, multivitamin supplementation during the study, routine use of probiotics and prebiotics products over the last month before the start of the study, routine use of anti-inflammatory drugs within 1 month prior to the start of the study, taking weight loss drugs over the last 3 months, history of specific weight loss diet over the last 3 months and history of mental illness.

Sample size was determined for BMI. For an expected change of 2 kg/m^2 between metformin and placebo groups and by considering an alpha value equal to 0.05 and a power of 80%, the sample size was computed as 16 subjects in each group. Considering a 20% drop-out rate, the sample size has been increased up to 20 subjects per group.

The written informed consent was obtained from all participants. All procedures involving human subjects were approved by ethical committee of Endocrinology and Metabolism Research Institute of Tehran University of Medical Sciences (ID number: IR.TUMS.EMRI.REC.1395.0090) and the trial was registered in the Iranian Registry of Clinical Trials (IRCT) with code of IRCT20090420001825N2.

Study design

In this study, 46 subjects were randomly assigned to the metformin (n=24) or placebo (n=22) group by a balanced block randomization procedure using computer-generated random numbers. For a period of 2 months, patients had

daily intake of two tablets of metformin (each tablet: 500 mg, Gly-once, Koushanpharmed Co., Iran) or placebo (containing lactose and starch) with main meals. Verbal and written instructions on how to take the tablets were provided at the initial visit. Besides, both groups were instructed to maintain a reduction in daily caloric intake of 500 kcal during the study. The composition of the prescribed diet was 55% carbohydrate, 30% fat and 15% protein. Subjects in both groups of the study received physical activity advice, encouraging physical activity by walking fast for 30 min a day. Necessary recommendations regarding not changing the medications and avoiding other supplement intake during the study period were given to the participants.

Both participants and investigators were blinded to the treatment allocation. The metformin and placebo tablets were packed identically and separated by code. To implement the allocation concealment, sequentially numbered, opaque sealed containers with identical appearance were used. Participants were asked to bring the remaining tablets at the end of study for assessing the compliance. Participants were defined as non-compliant if they had taken less than 80% of the tablets. Adverse events and compliance were monitored for each participant by investigators during weekly phone calls.

Anthropometric, dietary and biochemical measurements

Demographic questionnaire was completed for each participant at the screening visit and medical history, prescribed and non-prescribed medications and dietary intakes were recorded. Anthropometric measurements were done at baseline and after 2 months. Height was measured without shoes using a stadiometer with a precision of 0.5 cm. Participants were weighed with light clothes without shoes using a digital scale (Seca, Germany) with an accuracy of 0.1 kg. BMI was calculated as body weight (kg) divided by the square of the height (m^2). Waist circumference was measured at the midpoint between the last rib and the iliac crest and hip circumference was measured at the widest portion of the buttocks with a precision of 0.1 cm. Waist-to-hip ratio (WHR) is calculated as waist measurement divided by hip measurement and waist to height ratio (WHtR) is the ratio of the circumference of the waist to the height measurement. Assessment of body composition was done using Dual-energy X-ray absorptiometry method (DEXA) by Lunar DPX-MD device (Lunar Corporation, USA).

The validated 147-item semi-quantitative food frequency questionnaire (FFQ) was completed at baseline to assess energy and macronutrients intakes. The frequency of food intake over the past year was interviewed by a trained nutritionist. The reliability and validity of the FFQ have been evaluated as acceptable (22, 23). Due to the incompletion and limitations of Iranian Food Composition Table, the Food Composition Database of United States Department of Agriculture (USDA) has been used to analyze foods and beverages.

Blood samples were taken after 12–14 h of overnight fasting before and after the intervention. Serum was immediately separated by centrifuging samples at 1300 ***g*** for 10 min at the room temperature and stored at −80°C freezer until analysis. Serum glucose, total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and high-sensitivity C-reactive protein (hsCRP) concentrations were measured by Roche kits using auto-analyzer instrument (Hitachi, Cobas C 311, Roche Diagnostics GmbH). Serum insulin concentration was measured by an enzyme immunoassay kit (Monobind Inc., Lake Forest, CA, USA). Insulin resistance index was calculated by the homeostasis model assessment of insulin resistance (HOMA-IR) equation: HOMA-IR = [FBS (mg/dL) × fasting inulin concentration (mU/L)/405]. Lipoprotein lipase (LPL), glucagon-like peptide 1 (GLP-1) (ZellBio GmbH, Germany) and fasting-induced adipose factor (FIAF) (BioVendor, Germany) were determined by ELISA kits.

Fecal water content and microbiota profiling

Stool samples were collected at baseline and 2 months after intervention using a stool specimen collection kit, brought to the clinic in ice packs. Samples were stored at −80°C for fecal microbial and metabolite profiling. Fecal water content was measured by weighing frozen stool samples before and after lyophilization (Christ Alpha 1-4 LSCbasic Freeze Dryer, Germany). The analyses of gut microbiota taxonomic composition, fecal DNA extraction, library preparation and 16S rRNA gene sequencing were performed as described in Tito *et al.* (24). In brief, we used PowerMicrobiome RNA Isolation Kit (MOBIO Laboratories Inc., Germany) for DNA extraction and sequencing was done using the Illumina MiSeq platform at Nucleomics core, KU Leuven. 16S data pre-processing was performed using LotuS (25) and DADA2 (26) pipelines and taxonomy assignment with

the RDP classifier v 2.12 (27), with default parameters. After removing reads annotated to the class Chloroplast, family mitochondria and unclassified bacteria, data were rarefied to 10 000 reads per sample, alpha-diversity and beta-diversity indices were generated using the *vegan* and *phyloseq* R packages. Richness and Simpson indices were used in microbial alpha-diversity measurements based on operational taxonomic units (OTUs). Richness is the number of different kinds of microorganisms present in a particular community and Simpson takes into account the relative abundance, as well as the Richness. All further analyses were performed at genus level. Enterotyping (or community typing) using the DMM approach was performed in R as described previously (28). To increase accuracy, enterotyping was performed on a combined genus–abundance matrix that included study and disease cohort samples, complemented with 1106 samples from the FGFP (29). Microbiome variation among individuals was visualized by PCoA using Bray–Curtis dissimilarity on the genus–abundance matrix.

Targeted metabolomics analysis of fecal samples

Fecal short-chain fatty acids (SCFAs) were measured using gas chromatography. Approximately 100 mg of fecal sample was suspended in 1 mL of saturated NaCl (36%) solution. An internal standard (50 µL of 10.7 µM 2-ethylbutyric acid by Merck (München, Germany) in MQ water) was added and the samples were homogenized with glass beads. After adding 150 µL of 96% H_2SO_4, SCFAs were extracted in 3 mL of ether. The ether layer was collected and dried with Na_2SO_4 (150 mg). The supernatant (0.5 µL) was analyzed by a gas chromatography–flame ionization detection (GC–FID) method (Agilent). The system was equipped with a DB FFAP analytical column (30 m × 0.53 mm ID, 1.0 µm; Agilent) and helium GC grade (5.6) was used as carrier gas with a constant flow of 4.2 mL/min. The initial oven temperature was held at 100°C for 3 min, ramped with 4°C/min to 140°C (isothermal for 5 min) and further with 40°C/min to 235°C (isothermal for 15 min). The resulting chromatograms were processed using ChemStation (Agilent Technologies). Acetate, propionate and butyrate were quantified with appropriate calibration curves obtained from internal standard quantitation.

Statistical analysis

All statistical analyses were performed using R version 3.5.0. A P value <0.05 was defined as the level of significance. The primary outcome was change in BMI during the 8 weeks of the trial. Secondary outcomes included waist and hip circumferences, WHR, WHtR, serum FBS, insulin, lipid profile, ALT, AST, GLP-1, hsCRP, FIAF and LPL concentrations, HOMA-IR, faecal SCFAs and gut microbiota composition. Data were expressed as mean ± standard deviation or frequency (%) for quantitative and qualitative variables. The normality distribution for variables was tested by the Kolmogorov–Smirnov test. For insulin, HOMA, TG, GLP1, LPL, AST, ALT and hsCRP, which did not follow normal distribution, analyses were performed after log transformation. Chi-square test was used to determine associations between categorical variables. Comparison of quantitative baseline variables between two groups was done using independent-samples t tests. The changes in biochemical variables and anthropometric measurements of the subjects between the beginning and end of the trial were compared by a paired-sample t test. Primary and secondary outcomes were compared between two groups using generalized linear models adjusting for baseline measurements and BMI. For microbiota-related variables with non-normal distribution, Wilcoxon rank-sum or Wilcoxon signed-rank tests were used to compare two independent groups or repeated observations of the same group, respectively. Differences in alpha diversity indices and relative abundance of taxa within each group were determined using the Wilcoxon signed-rank test and the DESeq package. The association between biochemical and anthropometric variables and gut microbial diversity indices were determined using the Spearman correlation test. The Benjamini–Hochberg false discovery rate adjustment was used for multiple testing (30).

Results

Study participants

Twenty-four and 22 obese women were included in the metformin + LCD and placebo + LCD groups respectively. Twenty women in the metformin group and 16 women in the placebo group completed the 2-month trial and were included in the statistical analysis. In the metformin group, one participant was lost to follow-up. A total of eight participants, three from the metformin group and five from the placebo group, dropped out of the study, stating loss of interests as the reason for discontinuation. Moreover, one person in the placebo group was excluded from analysis because of lack of procured stool samples

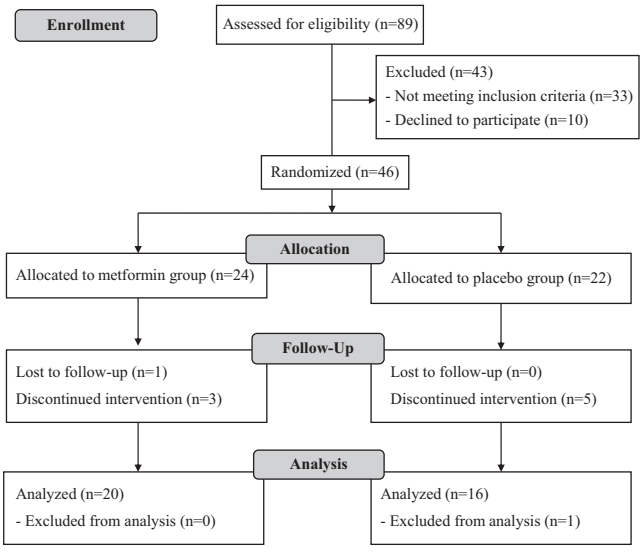

Figure 1
Flow diagram of study participants.

(Fig. 1). The completion rate of study was slightly higher in the metformin group (83% vs 73%). According to tablet counting, the compliance to treatments in both groups was above 80%.

The baseline characteristics of the participants in the two groups are shown in Table 1. The participants' mean age in the metformin and placebo groups was 37.4 and 34.2 years, respectively (P=0.17). The baseline BMI, hip circumference and WHtR in the metformin group were significantly higher than of those in the placebo group (P<0.05). No statistically significant differences in other anthropometric indices, body composition, bone mineral density (BMD) and dietary intakes were observed between the study groups at the beginning of the study.

The general rate of side effects from the interventions was 11%. Two patients in the intervention group (2 out of 20, 10%) complained about fatigue, flatulence and diarrhea; two in the placebo group (2 out of 16, 12.5%) reported flatulence and sleeplessness. The proportion of participants experiencing any adverse events was comparable between groups.

Effect of intervention on anthropometric indices

Both the metformin and control groups showed a decrease in BMI from the baseline values, 4.5 and 2.6%, respectively (P<0.01). The BMI reduction was higher in the metformin group than that in the placebo group, adjusted for baseline BMI (P=0.016). Weight, waist and hip circumferences decreased statistically significant in both groups and there were no significant differences between two groups after adjusting for baseline values. The WHR reduction was significant only in the metformin group (P=0.033). However, the WHtR was significantly decreased in both groups during the study (P<0.001 for both groups) (Table 2).

Table 1 Baseline characteristics and dietary intakes of the study participants. Data are presented as mean ± S.D. Comparisons were made with independent samples t test.

Variables	Metformin (n = 20)	Placebo (n = 16)	P value
Age (years)	37.4 ± 6.97	34.24 ± 6.82	0.17
Body composition			
BMI (kg/m^2)	35.0 ± 3.4	32.7 ± 2.2	0.02
Lean body mass (kg)	45.8 ± 7.1	44.1 ± 3.1	0.45
Fat mass (kg)	41.4 ± 10.2	38.1 ± 5.4	0.32
Body fat percentage (%)	45.9 ± 3.3	44.9 ± 3.9	0.46
BMD (g/cm^2)	1.15 ± 0.06	1.18 ± 0.07	0.28
Dietary intake			
Energy (kcal/day)	2955 ± 836.5	2614 ± 657.5	0.25
Carbohydrate (g/day)	465.3 ± 140.3	397.9 ± 117.6	0.19
Protein (g/day)	98.9 ± 33.5	93.9 ± 23.8	0.66
Fat (g/day)	91.2 ± 31.1	83.3 ± 23.7	0.46
Fiber (g/1000 kcal)	19.1 ± 4.3	19.8 ± 7.2	0.72

BMI, body mass index; BMD, bone mineral density; WHR, waist-to-hip ratio; WHtR, waist to height ratio.

Effect of intervention on serum biochemical variables

Concentrations of biochemical variables before and after the intervention in obese women are shown in Table 2. Compared to the baseline value, fasting blood sugar (FBS) was significantly increased in the placebo group after the intervention (P=0.019). Insulin concentration was decreased in both groups during the study, although this reduction was significant only in the metformin group (P=0.046). HOMA-IR had a decreasing trend in both groups; however, it was statistically non-significant. TC and LDL-C were significantly decreased in comparison to the baseline values in the placebo group (P<0.05), and their concentrations were different between the two groups after adjusting for baseline values and BMI (P<0.05). In contrast, HDL-C value was significantly increased in the metformin group (P=0.023). GLP1 had an increasing trend in both groups, but it was only significant in the placebo group (P=0.009). There were no significant changes in ALT, AST, hsCRP, FIAF and LPL concentrations during the study.

Table 2 Effects of 2 months consumption of metformin and placebo on anthropometric indices and biochemical variables in obese women.

Variables*	Metformin (n = 20)		Placebo (n = 16)		*P* value**
	Baseline	After intervention	Baseline	After intervention	
Body composition					
Weight (kg)	90.9 ± 10.8	86.9 ± 11.2†	86.8 ± 6.3	84.5 ± 6.6‡	0.06
BMI (kg/m²)	35.0 ± 3.4	33.4 ± 3.5†	32.7 ± 2.2	31.8 ± 2.6‡	0.016
Waist (cm)	102.8 ± 7.3	97.5 ± 8.7†	98.9 ± 5.3	94.9 ± 5.0‡	0.34
Hip (cm)	121.3 ± 7.3	116.5 ± 8.3†	115.3 ± 7.1	112.2 ± 7.0†	0.08
WHR	0.85 ± 0.05	0.84 ± 0.04††	0.86 ± 0.06	0.85 ± 0.05	0.95
WHtR	0.64 ± 0.05	0.60 ± 0.05†	0.61 ± 0.02	0.58 ± 0.03†	0.23
Glucose metabolism					
FBS (mg/dL)	81.0 ± 9.13	84.55 ± 8.63	80.81 ± 11.93	84.31 ± 8.49††	0.60
Insulin (mU/L)***	15.78 ± 25.76	8.31 ± 7.97††	26.53 ± 54.92	6.10 ± 4.00	0.39
HOMA-IR***	3.30 ± 5.76	1.77 ± 1.77	5.13 ± 10.56	2.15 ± 3.65	0.74
Lipid profile					
TC (mg/dL)	173.95 ± 38.26	177.25 ± 40.34	183.12 ± 35.65	174.94 ± 32.28††	0.013
TG (mg/dL)	140.12 ± 59.27	135.15 ± 42.80	145.77 ± 46.06	162.87 ± 73.87	0.87
LDL (mg/dL)	105.45 ± 29.08	112.95 ± 34.57††	115.25 ± 28.84	107.75 ± 25.94††	0.002
HDL (mg/dL)	39.75 ± 10.90	42.40 ± 9.58††	38.87 ± 6.29	40.37 ± 5.61	0.46
Liver markers					
ALT (U/L)***	13.62 ± 7.36	12.85 ± 6.41	11.36 ± 4.66	13.25 ± 5.81	0.33
AST (U/L)***	22.00 ± 8.92	19.45 ± 4.55	18.95 ± 3.99	19.19 ± 5.94	0.96
Satiety hormone					
GLP1 (pg/mL)***	40.41 ± 35.93	97.28 ± 120.88	28.42 ± 10.80	164.36 ± 143.66‡	0.51
Inflammatory marker					
hsCRP (mg/L)***	4.15 ± 3.69	7.89 ± 16.73	3.13 ± 2.97	5.08 ± 5.11	0.32
Adipose factors					
FIAF (ng/mL)	82.55 ± 20.34	78.35 ± 25.82	72.06 ± 19.79	69.18 ± 15.72	0.93
LPL (pg/mL)***	362.06 ± 142.88	577.89 ± 779.32	392.51 ± 131.12	851.22 ± 789.16	0.12

Significant difference within group throughout the study (†$P < 0.001$, ‡$P < 0.01$, ††$P < 0.05$, paired samples t test).
*Data are presented as mean ± S.D; **comparisons between 2 groups were made with generalized linear models adjusting for baseline measurements and BMI; ***statistical test performed on log-transformed data.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FBS, fasting blood sugar; FIAF, fasting-induced adipose factor; GLP-1, glucagon-like peptide 1; HDL, high density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high sensitive C reactive protein; LDL, low density lipoprotein cholesterol; LPL, lipoprotein lipase; TC, total cholesterol; TG, triglycerides; WHR, waist to hip ratio; WHtR, waist to height ratio.

Effect of intervention on microbial diversity, overall microbiota composition and enterotypes

Comparable microbial diversity (Richness and Simpson indices) was observed in both groups before and after treatment ($P>0.05$, Fig. 2). Before metformin treatment, diversity indices correlated positively with LPL (Richness, $r=0.47$, $P=0.036$, Simpson, $r=0.58$, $P=0.007$). After the intervention, Simpson index was negatively correlated with BMI, waist, insulin and HOMA ($r=-0.48$, -0.51, -0.57 and -0.53, respectively, $P<0.05$). We visualized Bray–Curtis distances between samples using principle coordinate analysis (PCoA, Fig. 3). There was no separation based on the interventions, indicating that the overall gut microbiota was more dependent on interindividual variation rather than interventions.

Distribution of enterotypes, community clusters characterized by differences in the abundance of signature taxa, is shown in Fig. 4. Although Bacteroides 2 showed an increasing trend after metformin intake and the Bacteroides 1 enterotype increased after placebo intake in exchange for Bacteroides 2, these changes were not statistically significant ($P=0.85$), and the distribution of enterotypes was not statistically different between before and after metformin intake ($P=0.77$).

Effect of intervention on specific fecal bacterial genus abundances

Despite the absence of global signals, we did observe changes in the relative abundance of specific fecal bacterial genera after metformin intake (Fig. 5). For this analysis, we focused on the alteration of taxa whose concentration was changed after metformin treatment in previous studies (12, 15, 31). Using this targeted analysis, we observed a significant increase in *Escherichia/Shigella* abundance (FDR-adjusted P value=0.012). Moreover,

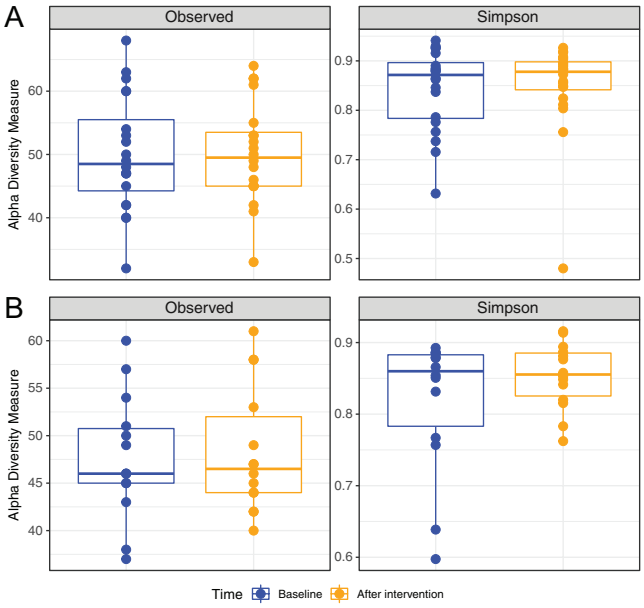

Figure 2
Gut microbial diversity indices before and after (A) metformin (n = 20) or (B) placebo (n = 16) treatment in obese women. Color is indicative of time. A full colour version of this figure is available at https://doi.org/10.1530/EJE-18-0826.

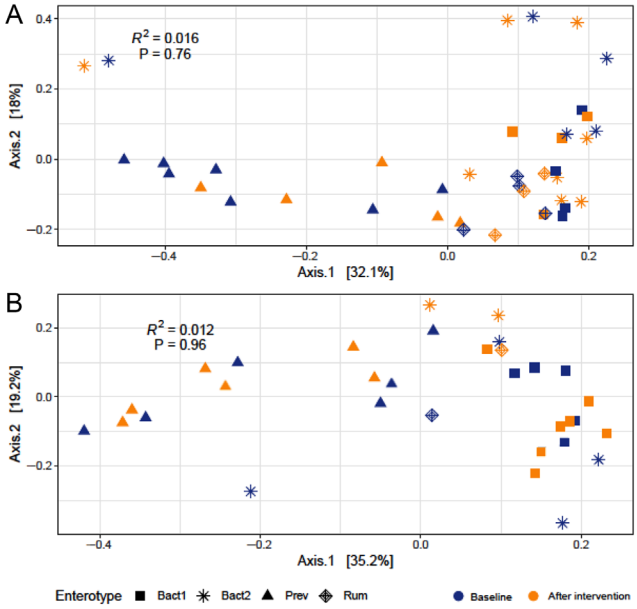

Figure 3
PCoA ordination of Bray–Curtis distances between samples before and after (A) metformin (n = 20) or (B) placebo (n = 16) treatment in obese women. Each data point represents an individual sample. Symbol is indicative of enterotypes and color is indicative of time. A full colour version of this figure is available at https://doi.org/10.1530/EJE-18-0826.

a decrease in *Intestinibacter* abundance was observed, which did not remain significant following correction for multiple testing (FDR-adjusted P value=0.19). The abundances of some of the SCFA-producing bacteria including *Prevotella*, *Faecalibacterium*, *Lactobacillus* and *Akkermansia* had mild non-significant decrease and some other ones including *Roseburia*, *Blautia*, *Bacteroides* and *Butyrivibrio* had mild non-significant increase in the metformin group. The abundance of *Streptococcus* had non-significant increasing trend in the metformin group. There was no significant change in relative abundances of bacterial genus in the placebo group. *Escherichia/Shigella* was also more abundant in the post-metformin group compared to the post-placebo group when adjusted for age and baseline BMI (P=0.026).

The *Prevotella*-to-*Bacteroides* ratio remained unchanged in eight subjects and decreased in most of people (11 subjects) after metformin treatment. This ratio increased in five subjects after placebo+LCD and decreased or stayed unchanged in other participants of this group.

Effect of intervention on fecal SCFAs

Fecal SCFA concentrations were decreased in the metformin group during the study; however, they showed an increasing trend in placebo (Fig. 6). Fecal concentration of acetate was significantly higher in the placebo group after intervention adjusted for baseline value and BMI (P=0.002). Differences between the two groups in fecal propionate and butyrate levels were not statistically significant.

Discussion

In this 8-week randomized double-blind controlled clinical trial on obese women, we demonstrated that metformin+LCD, compared with placebo+LCD, results in a reduction of anthropometric indices, insulin concentration and fecal SCFA concentrations as well as an increased HDL-C without significantly changing the overall fecal microbial composition and diversity. We observed a significant specific increase in the *Escherichia/Shigella* abundance after metformin intervention. We also found no significant changes in distribution of enterotypes, lipid metabolism, liver markers and satiety-regulating hormone.

We found a significant reduction in weight, waist and hip circumferences in obese women who

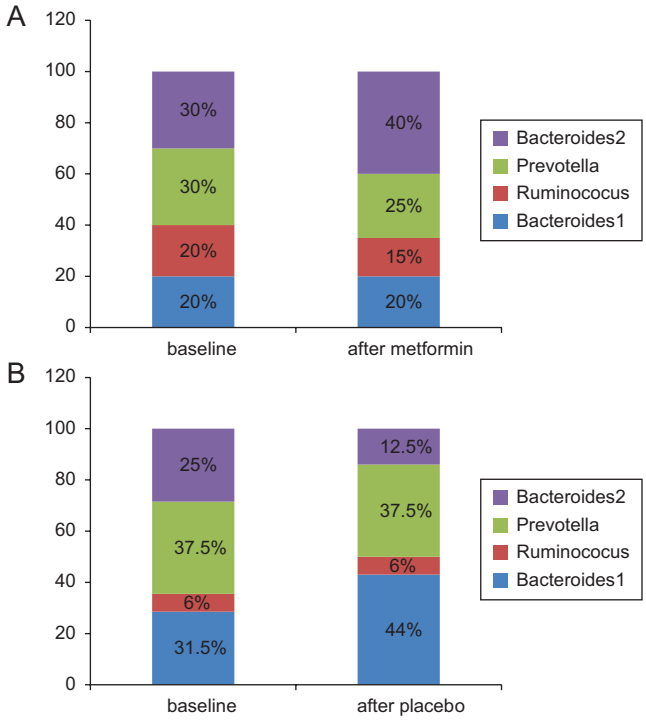

Figure 4

Distribution of the gut microbiome enterotypes before and after (A) metformin ($n = 20$) or (B) placebo ($n = 16$) treatment in obese women. Color is indicative of enterotypes. A full colour version of this figure is available at https://doi.org/10.1530/EJE-18-0826.

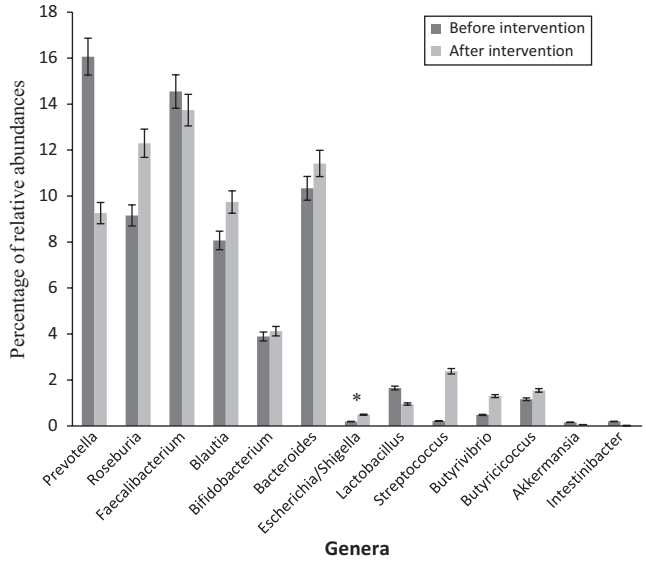

Figure 5

Changes in the relative abundance of fecal bacterial genus after metformin ($n = 20$) treatment in obese women. Color is indicative of time. *$P < 0.05$.

were treated with metformin. A German multicenter analysis on 9108 diabetic patients showed that women had a significantly higher reduction of body weight compared to men after metformin treatment (32). There is growing evidence suggesting the weight-reducing potential of metformin in nondiabetic obese subjects (6, 33). A systematic review indicated that orlistat and metformin had similar effects in reducing BMI of overweight/obese women with polycystic ovary syndrome (PCOS) (34).

In the present study, global gut microbial diversity indices were unaffected in the metformin group. However, in the metformin group, we identified negative correlations between Simpson Index and BMI, and waist and insulin resistance. A large study of American adults also showed that gut bacterial richness was lower in obese subjects compared to healthy-weight participants (35).

As our prescribed diet was mild, no notable effects on gut microbiota had been seen in the placebo group of this study, although previous restricted dietary interventions showed alterations in gut microbiota of obese individuals (36, 37). Since the dietary recommendations were the same in both groups and no significant change in gut microbiota was observed in the placebo group, gut microbial alteration in the metformin group could be attributed to the metformin intervention.

In this clinical trial, we investigated the effects of metformin on the gut microbiota composition of non-diabetic obese adults, while prior studies were done on diabetic patients. Ma *et al.* (2018) showed that the microbiota profile of healthy mice treated with metformin had similarity with microbiota of prediabetes and irritable bowel syndrome situations (17). Intestinal discomforts which were prevalent side effects of metformin could be a consequence of relative increase in *Escherichia* abundance (38). We observed an increase of *Escherichia* abundance in the metformin-treated obese women. *Escherichia* is a gram-negative hydrogen producer bacterial genus contributing to hydrogen sulfide production (39). Despite the difference in the physiological conditions of target groups, we observed an increase in *Escherichia* abundance in the gut microbiota of metformin-treated obese women in agreement with previous observational studies on metformin-treated type 2 diabetic patients (12, 15, 40, 41); however, *in vitro* analysis suggested that the effect of metformin on *Escherichia* was indirect and might be results of other changes within the gut environment including reduced intestinal lipid absorption and LPS caused

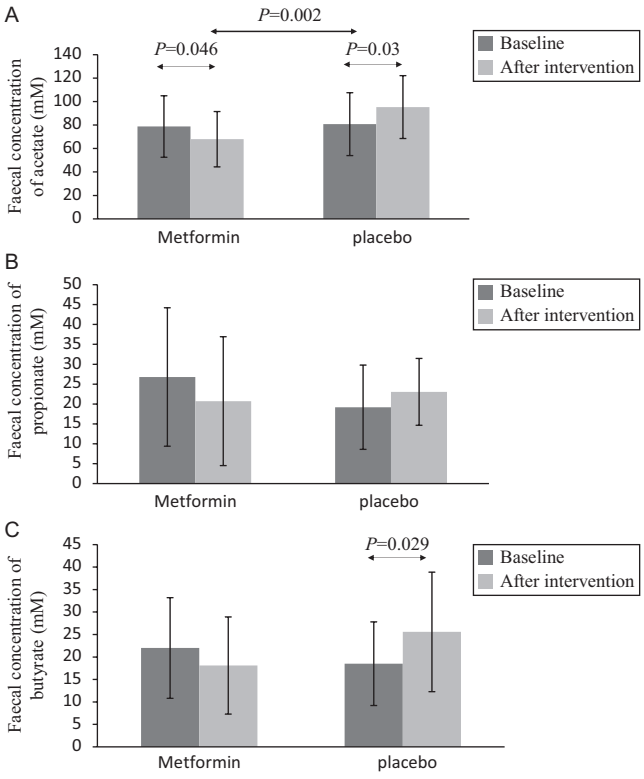

Figure 6

Concentration of fecal short chain fatty acids (SCFAs) before and after metformin (n = 20) or placebo (n = 16) treatment in obese women. Color is indicative of time. (A) Acetate, (B) Propionate, (C) Butyrate.

inflammation (31, 42). Besides, metagenomic analysis reported that LPS biosynthesis by gram-negative bacteria was increased by metformin intervention (15, 42).

Million *et al*. (2013) in a study on 263 obese, overweight and lean individuals found for the first time that *E. coli* was negatively correlated with BMI (43). In alignment with this finding, we showed that BMI reduction associated with *Escherichia* increase in gut microbiota after metformin intake. As a probable underlying mechanism, Breton *et al*. (2016) in an animal study showed that meal-induced release of commensal *Escherichia coli* proteins into gut lumen could induce satiety and affect food intake. This α-MSH-like protein, caseinolytic protease B (ClpB), activates gut enteroendocrine cells and stimulates secretion of the satietogenic hormones including glucagon-like peptide-1 (GLP-1) and peptide YY (PYY). ClpB also activates anorexigenic neurons in the brain, influencing short-term and long-term dietary intakes (44). Confirming this hypothesis, an increasing trend observed in GLP-1 concentration after metformin intervention in this study could be a result of increased in *Escherichia* abundance and its satietogenic protein, although this increasing trend did not reach significance.

In obese rats, metformin induced enrichment of SCFA-producing bacteria, including *Bacteriodes*, *Blautia* and *Butyricoccus*, and reduction of microbial diversity (45). However, we identified only subtle and inconsistent changes in different SCFA-producing bacterial genus in the intervention group. We showed a non-significant reduction of *Intestinibacter*, a butyrate-producing bacterium, in the metformin-treated group confirming the findings of previous studies (12, 15). *Akkermansia*, a propionate producer, did not change significantly in the present study. Results of previous studies about *Akkermansia* were not very conclusive too (11, 15). Forslund *et al*. (2015) observed inconsistent trends of *Akkermansia* between different country subsamples after metformin intervention in type 2 diabetes (12). Contrary to our results, some previous studies declared an increase in *Bifidobacterium* of gut microbiome following metformin intake (15). *Roseburia* which has anti-inflammatory and anti-glycemic effects had a lower abundance in obese individuals compared to lean subjects (46). In this study, we showed that the amount of *Roseburia* had a non-significant increase during metformin intervention, making gut microbiota of obese subjects more similar to lean subjects. These differences in observed results about gut bacterial abundance could highlight the complexity of interactions between gut microbiota and interventions and might be explained by the inter-individual variation of microbiota between obesity and diabetic situations and differences of intestinal microbiota composition as a result of confounding factors like immune response, age, dietary intakes, ethnicity and geographical locations (16, 47). The lack of significant results other than *Escherichia*/*Shigella* could be linked to lack of power – the study was powered on clinical endpoints. Recent estimates suggest that several hundreds of individuals in each arm would be necessary toward this aim (29).

Previous studies candidate increased production of SCFAs as a potential mechanism for health benefits of metformin via gut microbiota (11). However, our study failed to show an increase in fecal SCFA concentrations after metformin intake. Wu *et al*. (2017) showed that metformin intervention for 4 months caused an increase in fecal propionate and butyrate concentrations in diabetic men; however, no differences were observed after combining men and women (15). We also observed that SCFA concentrations in obese women were reduced

by metformin, pointing the importance of gender in microbiota manipulation. The gender-dependent effect of dietary interventions on gut microbiota has also been indicated in previous studies (48, 49).

The gut microbiota-regulated FIAF could impact on fat storage via inhibiting LPL activity (20). Previous animal studies have suggested that FIAF is possibly modulated by SCFAs (20); however, in this study, despite the reduced fecal SCFAs, no statistically significant differences existed in FIAF and LPL concentrations after metformin intervention.

Previous studies proposed that *Prevotella*-to-*Bacteroides* ratio in gut microbiota could predict the response of obese subjects to dietary interventions (50, 51). In the present study, we showed that this ratio remained unchanged or decreased after metformin+LCD treatment in obese women.

To the best of our knowledge, the effect of metformin on gut microbiota of healthy obese female has never been reported. By conducting the clinical trial and investigating the effect of metformin on paired samples, the effect of interindividual variations was reduced in this study in comparison with previous case–control studies. Moreover, previous cross-sectional studies could not determine causality, highlighting the need for clinical trials. Furthermore, our healthy participants had not used metformin before, nullifying the potential impact of different duration of therapy on the microbiota. By excluding subjects who had used antibiotics and weight loss drugs a few months prior to study, the effects of those two major confounding factors on gut microbiota were eliminated. Besides, low-calorie diet was the same in the two groups, normalizing the effect of diet in any potential change in the gut microbiota. It should be noted that we cannot conclude if the weight-reducing effect of metformin was through the gut microbiota or other probable mechanisms, including appetite suppression, improvement of insulin sensitivity and regulation of fat oxidation and storage that should be considered. Additional studies combining metagenomics and untargeted metabolomics analyses are needed to clarify the effect of metformin on weight control through gut microbiota modulation. Shotgun sequencing should be applied to allow assessment of gut microbiota response at functional levels. Furthermore, due to the effective role of gender on microbiota, the effect of metformin on obese men should be investigated too. As one of the limitations of this trial is its sample size, large-scale interventional studies with higher power are needed to confirm the results.

Conclusion

In summary, we found that metformin supplementation in addition to low-calorie diet, compared to placebo supplemented low-calorie diet, resulted in BMI reduction and specific increased abundance of gut *Escherichia/Shigella* in non-diabetic obese Iranian women. No statistically significant differences for overall microbiota composition were observed after multiple testing adjustments. To disentangle power issues vs true absence of signal, additional larger microbiome-endpoint powered studies are needed.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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