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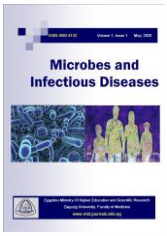

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Original article

The relationship between the gut microbiota shifts and the inflammatory biomarkers in obese and normal weight adults

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ABSTRACT

Background: Healthy obesity means obese individuals remain free of metabolic health complications. Several researches link microbiota changes to obesity, focus on metabolic role of IL-6 and its ability to alter fat metabolism and correlate circulating calprotectin concentration to morbid obesity. This study evaluated the relative abundance of gut microbiota (*Prevotella* and *γ-Proteobacteria*), estimated the serum levels of inflammatory mediators (IL-6 and calprotectin) and studied the correlation between them in healthy obese and normal weight adults. **Methods:** 33 healthy obese (HO) and 14 normal weight (NW) controls were included. Serum levels of IL-6 and calprotectin were estimated by ELISA. Abundances of *Prevotella* and *γ-Proteobacteria* were determined in stool using real time PCR. **Results:** IL-6 and calprotectin levels were significantly lower in HO than in NW (p=0.001, p=0.001 respectively) with significant negative correlation between IL-6 and body mass index (BMI) in HO (r=-0.438, p=0.011) and negative but statistically insignificant correlation with BMI in NW groups (r=-0.024, p=0.935). There was negative but statistically insignificant correlation between calprotectin and BMI in HO (r=-0.075, p=0.677) and NW (r=-0.381, p=0.179). *Prevotella* and γ-*Proteobacteria* abundances were higher in HO than in NW and insignificant (p=0.31, p=0.55 respectively) with significant positive correlation between *Prevotella* and γ-*Proteobacteria* abundance in HO (r=0.436, p=0.011), positive and insignificant correlations between abundance of Prevotella and levels of IL-6 in HO and NW (r=0.303, P=0.086 vs r=0.316, p=0.272 respectively). **Conclusion:** IL-6 and calprotectin have role in regulation of energy homeostasis. Elevated abundances of *Prevotella* and *γ-Proteobacteria* may be the primary shifts in gut microbiota of HO.

Introduction

The 'microbiota' collectively describes all microorganisms on and in the human body (skin, gut, and other tissues). The majority of up to 100 trillion microbes reside in the colon [1]. Gut microbiota exert numerous functions such as nutrient metabolism, maintenance of gut barrier function, development of the gastrointestinal immune system, and protection against pathogens [1,2]. Imbalance in the gut microbiota could result in various disease, such as obesity, diabetes mellitus and inflammatory diseases. Modulation of the gut microbiota represents a novel target for disease therapies through dietary interventions, probiotics, exercise and/or fecal microbiota transplantation [3,4]. Exercise was recognized to stabilize the progression of obesity and modify the gut microbiota composition by increasing the microbial

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diversity, improving the Firmicutes/Bacteroidetes ratio that could potentially contribute to decrease body weight, obesity-associated pathologies and gastrointestinal disorders. Overall, promoting exercise could help to restore the balance toward an improvement of dysbiosis in obesity [5].

Obesity is a growing health problem and global challenge for health care [6,7]. Healthy obesity or metabolically healthy obese is a subtype of obesity which means obese individuals remain free of metabolic health complications such as type 2 diabetes mellitus and cardiovascular disease. It is more important to reach to new strategy to prevent healthy obese transition to metabolic unhealthy obese [8,9]. IL-6 has traditionally been known for its immunomodulatory effects, but some studies postulated that IL-6 administration could alter fat metabolism and lipolysis [10].

In obese humans, circulating IL-6 levels are sometimes slightly elevated and IL-6 resistance has been suggested as one possible underlying cause for the metabolic disturbances associated with obesity. In fact, lacking the IL-6 signal is associated with expansion of adipose tissue mass in mice and humans. Individuals with abdominal obesity treated with tocilizumab (IL-6 receptor antibody) failed to reduce visceral and epicardial adipose tissue mass in response to exercise training. However, how exactly endogenous IL-6 regulates adipose tissue mass in humans is largely unknown [11].

Calprotectin is a cytoplasmic protein complex, derived from neutrophils, monocytes/macrophages and dendritic cells and released from contracting skeletal muscle. Circulating levels of calprotectin are elevated in various inflammatory diseases, including inflammatory bowel disease, atherosclerosis and type 2 diabetes mellitus. It has been proven that calprotectin may be more useful for both detecting and monitoring inflammation in adults, related to metabolic complications of obesity, as compared with conventional inflammatory markers, such as white blood cell count or C-reactive protein [12].

This study was designed to evaluate the relative abundance of gut microbiota (*Prevotella* and *γ-Proteobacteria*), estimate the serum levels of inflammatory mediators (IL-6 and calprotectin) and study the correlation between them in healthy obese and normal weight adults in order to suggest gut microbiota and/ or inflammatory profile manipulation as management lines of obesity.

Methods

Ethics approval

This study was approved by the Human Ethics Committee of Faculty of Medicine in Assiut University IRB no: 17100872. Informed consent was obtained before collecting samples from all patients and control subjects.

Study design: case control study.

Subjects: A total of 47 adults attending the outpatient obesity clinic at Assiut University Hospital from January to March 2020 including 33 were diagnosed as healthy obese (after clinical examination and routine laboratory investigation in obesity clinic at Assiut University Hospital) and 14 were normal weight participants as control group. The exclusion criteria included individuals with any chronic disease, febrile conditions or had febrile condition within the previous 2 months, chronic diarrhea, Crohn's disease or ulcerative colitis. Also subjects had recived non-steroidal anti-inflammatory, corticosteroids, laxatives or antibiotic at least two months before sample collection or vaccinated in the last 30 days were exculded.

Anthropometric measurements

Height, weight, and waist circumference were measured and body mass index (BMI) was calculated (kg/m^2).The participants were classified into normal weight and healthy obese groups according to the criteria of the WHO for adults. Body mass index of normal weight was from 18.5 to 24.99 kg/m^2, and for the obese was ≥ 30 kg/m^2 [7,13].

Diet quality scores

According to manual of KomPAN questionnaire based on the usual food frequency consumption of 24 food items two diet quality scores were calculated; the pro-Healthy Diet Index (pHDI) and the non-Healthy Diet Index (nHDI) [14,15].

Life style

According to KomPAN questionnaire, the physical activity of participants was described at work or school time and during the leisure time [15].

Samples

Blood and fresh stool samples were collected from all the subjects included in the study. Stool samples were collected using sterile containers and stored at −80°C. Serum was separated from blood samples by centrifugation (at 2000 x g for 15 minutes at room temperature) and stored at −80°C.

Measurement of calprotectin and IL-6 concentrations

Serum concentrations of calprotectin and IL-6 were measured by ELISA technique using Human Calprotectin ELISA Kit (Bioassay Technology Laboratory Shanghai China Cat. No. E4010 Hu, Lot. No.1811011) and Human IL6 ELISA Kit (Bioassay Technology Laboratory Shanghai. China Cat. No. E0090 Hu, Lot. No.201902007) respectively according to the manufacturer's protocol.

Determination of the abundances of *Prevotella* and *γ-Proteobacteria* in stool samples using real time PCR:

Genomic DNA extraction from stool samples:

DNA extraction was performed using DNA stool Mini Kit (Cat.No.:FASTI 001, 50 Preps, Lot.No.:BJ318119408) according to the manufacturer's instructions. The concentration of extracted DNA was determined using a spectrophotometer (GeneQuart 1300, Germany) and the DNA was standardized to a final concentration of 4 ng/µl [7].

Real time PCR technique for determination of Prevotella and γ-Proteobacteria abundance in stool samples

Abundance of *Prevotella* and γ-*Proteobacteria* in stool samples was determined by real time PCR using 16S rRNA gene specific primers **(Table 1)** [7].

The optimal annealing temperatures of the PCR primer pairs and the correct product sizes were determined with end-point gradient PCR (7500 Fast Thermal Cycler; AMRC.EGYPT).

The reaction was done at a final volume of 10µl (3 µl 5xHot FIREPOl EvaGreen qPCR Mix plus (ROX) reagent mix, 1 µl of each forward and reverse primer, 2 µl of genomic DNA template (8 ng) and 3 µl of distilled water).

The amplification program was performed as follow*:*

- Polymerase activation cycle at 95.0 ˚C for 2.0 min, 40 cycles.
- Denaturation was at 95.0 ˚C for 5 sec.
- Annealing was at the indicated Ta (Table 1) for 10 sec .
- Extension at 72.0 ˚C for 20 sec.

The product specificity was assessed by melting-curve analysis after denaturation at 95.0 ˚C for 10 sec and slowly heating the mixture from 65.0 ˚C to 95.0 ˚C with plate readings every 0.5 ˚C for 5 sec. Negative control was used for every run in which 2.0 µl of distilled H_2O were used instead of DNA template.

Table 1. List of primers used for quantification of extracted DNA by real time PCR.

Targeted prokaryotic taxa	Primer set	Primer sequence (5'→3')	Amplicon size (bp)	Ta (°C)
Total bacteria	Uni926F Uni1062R	F:AAACTCAAAKGAATTGACGG R:CTCACRRCACGAGCTGAC	180	60
Prevotella	Prov_F1 Prov_R1	F:GCCGCGGTAATACGGAAGG R:CTAATCCTGTTYGATACCCGCAC	271	56
γ-*Proteobacteria*	1080γF 1202γ R	F:TCGTCAGCTCGTGTYGTGA R: CGTAAGGGCCATGATG	170	53

Statistical analysis

The Statistical Package for Social Sciences version 16.0 (SPSS, Chicago, IL, USA) was used to analyze the data. Data were presented as mean± standard error of mean (SEM). Shapiro–Wilk test was used to test the normal distribution of all the variables. For non-normal distributions, values are log transformed before statistical tests. The statistical significance of differences between groups was evaluated by independent T tests and Mann–Whitney U test. The correlations between parameters were evaluated with Spearman's correlation test, p value <0.05 was regarded as statistically significant.

To evaluate differences in the composition of microbiota in feces samples obtained from study groups, we compared the relative abundances in both groups. The registered Ct values of interested bacteria normalized to the Ct of total bacteria as internal control with the formula:

$\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\text{internal control}}$ Then the data is presented as fold change in the expression of gene of interest between groups [16,17].

Results

For all the 47 participants in the study; demographic data, anthropometric measurements, physical activity and diet quality scores are presented in **table (2)**.

The serum levels of IL-6 pg/ml (Mean ± SEM) in healthy obese group were significantly lower than that in normal weight control group (15.3±3.9 vs.57.8±14.7) respectively, p = 0.0001 with t-test and p = 0.001 with Mann-Whitney test) **(Figure 1A)**. In addition, the serum levels of calprotectin ng/ml (Mean ± SEM) were significantly low in healthy obese group in comparison to normal weight group (270.21±87.5 vs. 631.03.± 122) respectively, p = 0.0001 with t-test and p = 0.001 with Mann-Whitney test) **(Figure 1B)**. The relative abundances of *Prevotella* in healthy obese group were higher than that in normal weight control group (12±0.038 vs. 9±0.04) respectively, but were not statistically significant (p=0.35 with t-test and p=0.313 with Mann-Whitney test) **(Figure 1 C)**. The relative abundances of γ-*Proteobacteria* in healthy obese group were higher than that in normal weight control group (22.8 ± 0.05 vs. 9 ± 0.06) respectively, but were not statistically significant (p=0.39 with t-test and p=0.557 with Mann-Whitney test) **(Figure 1 D)**.

The correlations between serum levels of the IL-6, BMI and calprotectin are listed in **table (3)** and **figure (2)**.

The correlations of the relative abundance of *Prevotella* and γ-*Proteobacteria* in healthy obese and normal weight groups with different variables in the study are listed in **table (4)** and **figure (3)**.

Table 2. Demographic data, anthropometric measurements, physical activity and diet quality scores of the study participants.

Variable	**Healthy obese N=33 (70.2%)**	**Normal weight N= 14 (29.7%)**	***p* value**
Age (years, Mean ± SEM†)	36.6±1.4	31±2.03	
Gender Female/Male (F/M)	33 F (100%)	14 F (100%)	
Waist circumferences (cm, Mean ± SEM†)	117.3±2.88	84±1.4	0.0001*
BMI (kg/m^2, Mean ± SEM†)	37.5±5.3	23.5±1.5	0.0001*
Physical activity (2 weeks before) Low activities Moderate activities High activities	 17 (51.51%) 8 (24.24%) 8 (24.24%)	 1 (7%) 2 (14.28%) 11 (78.5%)	0.282
Diet quality scores (2 weeks before) pHDI nHDI	 3.8 11.3	 10.3 6.8	 0.0001* 0.0001*

**p* value < 0.05 was considered statistically significant.
SEM: Standard Error of Mean
pHDI pro-Healthy Diet Index
nHDI non-Healthy Diet Index

Table 3. Correlations between serum levels of IL-6, BMI and calprotectin.

The serum levels of IL-6	**The serum levels of calprotectin**
Negative significant correlation with BMI in healthy obese group (r = - 0.438, p = 0.011). Negative but statistically insignificant correlation with BMI in normal weight group (r = - 0.024, p = 0.935).	Negative but statistically insignificant correlation with BMI in healthy obese group (r = - 0.075, p =0.677). Negative but statistically insignificant correlation with BMI in normal weight group (r = -0.381, p = 0.179).
Positive significant correlation between serum levels of IL-6 and serum levels of calprotectin in healthy obese group and normal weight group (r = 0.491, p = 0.004 vs. r =0.560, p = 0.037 respectively).	

Table 4. The correlations of the relative abundance of *Prevotella* and γ-*Proteobacteria* in healthy obese and normal weight groups with different variables in the study.

	The mean relative abundance *Prevotella*	**The mean relative abundance of γ-*Proteobacteria***
BMI	Negative and significant correlation in healthy obese group (r = -0.362, p = 0.039). Positive and insignificant correlation in normal weight group (r = 0.081, p = 0.782).	Positive but insignificant correlation in healthy obese group (r = 0.206, p = 0.251). Negative but insignificant correlation in normal weight group (r = -0.183, p = 0.53)
Serum levels of calprotectin	Positive but insignificant correlation in healthy obese group (r = 0.136, p = 0.451). Negative but insignificant correlation in normal weight group (r = -0.114, p = 0.697)).	Negative but insignificant correlation in healthy obese group (r = -0.202, p = 0.260). Positive but insignificant correlation in normal weight group (r = 0.289, p= 0.316).
Serum levels IL-6	Positive but insignificant correlation in healthy obese group (r = 0.303, p = 0.086). Positive but insignificant correlation in normal weight group (r = 0.316, p = 0.272).	Negative but insignificant correlation in healthy obese group (r = -0.170, p = 0.345). Positive but insignificant correlation in normal weight group (r = 0.408, p = 0.147).
	Positive significant correlation between the relative abundances of *Prevotella* and γ-*Proteobacteria* in healthy obese group (r = 0.436, p = 0.011). Positive but statistically insignificant correlation between the relative abundances of *Prevotella* and γ-*Proteobacteria* in normal weight group (r = 0.37, p = 0.193).	

Figure 1. Serum levels of IL-6 (A) and calprotectin (B) in normal weight and healthy obese groups. The mean relative abundance of *Prevotella* (C) and γ-*Proteobacteria* (D) in normal weight and obese groups.

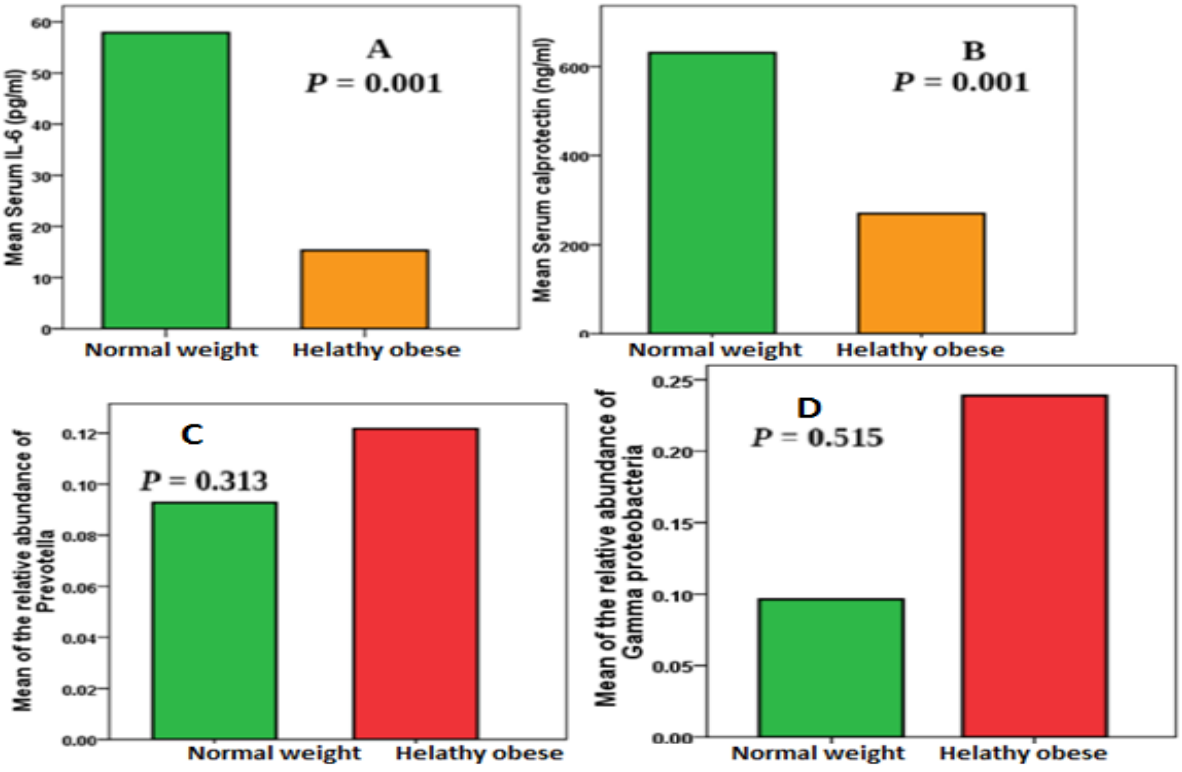


Figure 2. Correlation between BMI (kg/m^2) and log serum levels of IL-6 (pg/ml) in healthy obese and normal weight groups (A). Correlation between BMI (kg/m^2) and log serum levels of calprotectin (ng/ml) in healthy obese and normal weight groups (B) Correlation between log serum levels of calprotectin (ng/ml) and log serum levels of IL-6 (pg/ml) in healthy obese and normal weight groups (C).

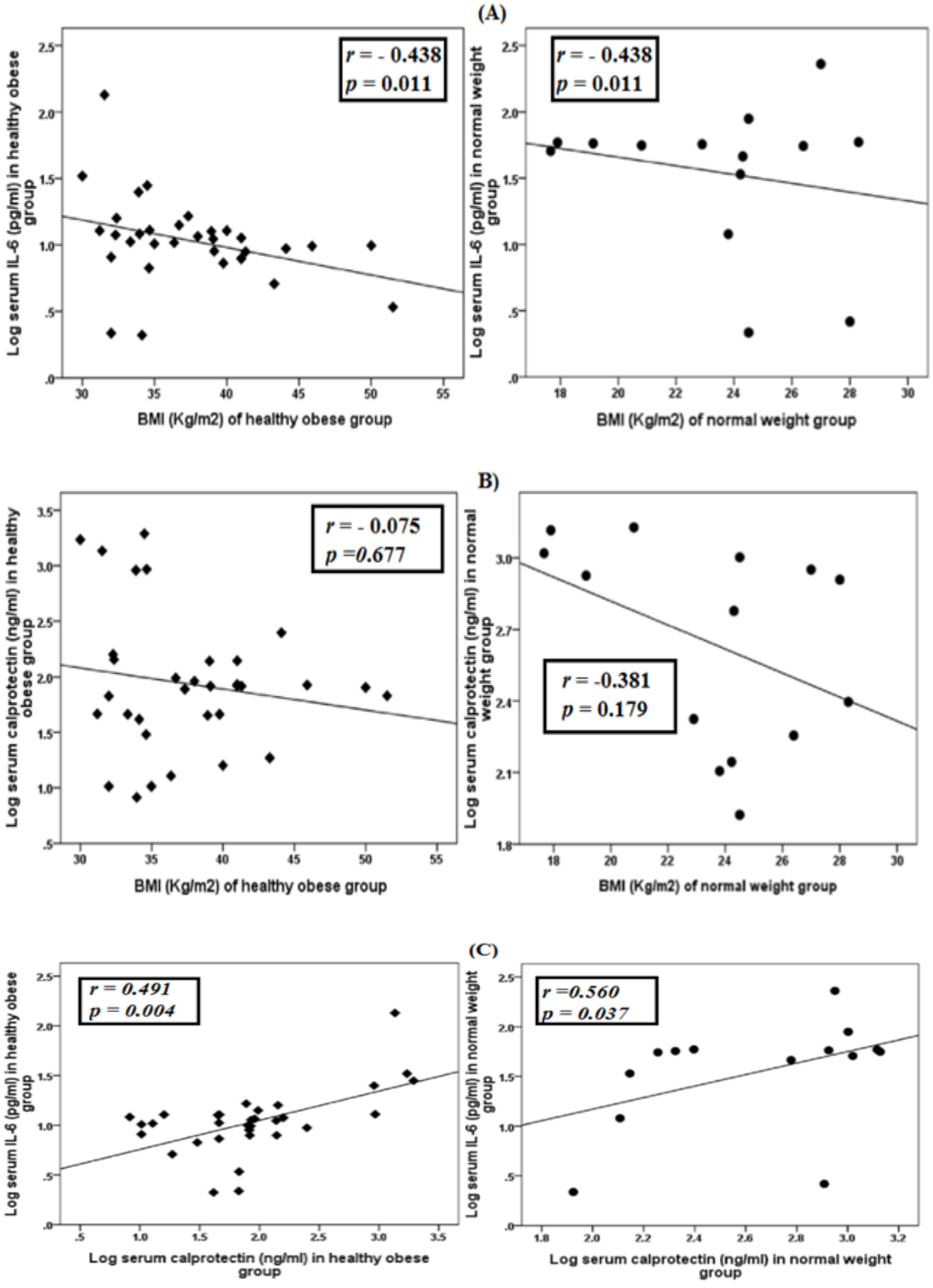
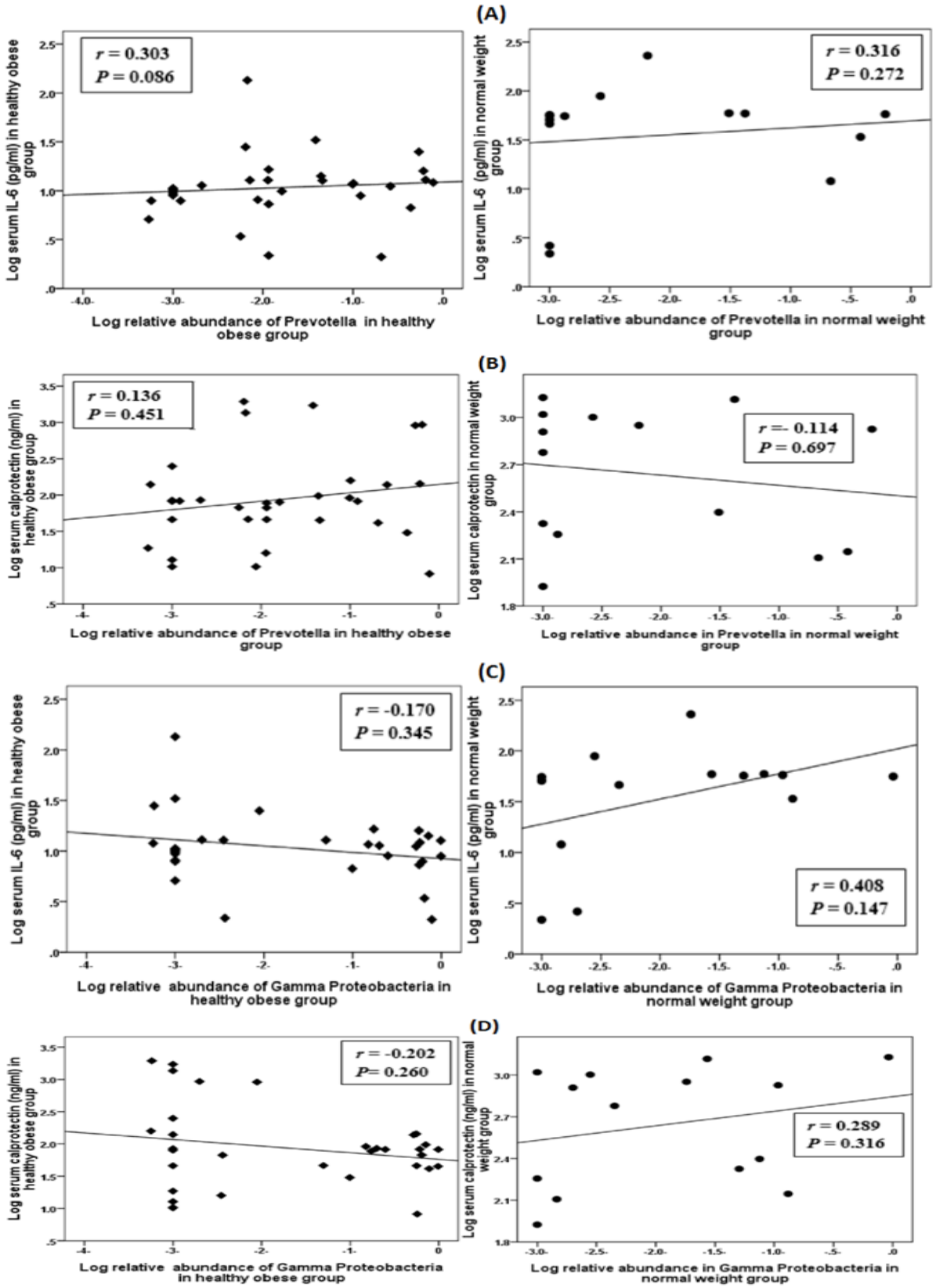


Figure 3. Correlation between the relative abundance of *Prevotlla* and log serum levels of IL-6 (A) and log serum levels of calprotectin (B) in healthy obese group and normal weight groups. Correlation between the relative abundance of γ- *Proteobacteria* and log serum levels of IL-6 (C) and log serum levels of calprotectin (D) in healthy obese group and normal weight groups.



Discussion

Our results showed the serum levels of IL-6 were significantly higher in normal weight group compared with healthy obese group. These results were in agreement with **Pederse et al.** [18] and **Mortensen et al.** [19] who related this elevation to the increased physical activity of normal weight group compared to obese group due to muscle-derived IL-6 released into the circulation in large amounts. The systemic effects of IL-6 include enhancement of lipolysis and oxidation of fatty acids, without any changes in plasma levels of catecholamines, glucagon or insulin.

Another explanation was declared by the study of **Punder and Pruimboom** [20]; the diet quality scores of normal weight group showed elevation in (pHDI) that contains more fibers, whole grains and proteins than health obese group that led to changes in the cytokine pattern towards a more pro-inflammatory cytokine profile.

The results of our study demonstrated that there were negative significant correlation between serum levels of IL-6 and BMI in healthy obese group. Our findings were supported by the study of **Wallenius et al.** [21] who concluded that IL-6 suppress body fat at the level of the CNS, presumably the hypothalamus, mainly by increasing energy expenditure rather than by suppression of feeding. Therefore, the low endogenous IL-6 production contributes to development of obesity.

Our results showed significant elevation in serum levels of calprotectin in normal weight group in comparison to healthy obese group due to more physical activity and diet quality that depends on low carbohydrate, high fiber and protein and whole grains in normal weight group. The study of **Ohlsson et al.** [22] supports this assumption as it showed that the serum calprotectin levels were increased after weight reduction using dietary intervention using lower carbohydrate and fat diet with higher fiber, whole-grain and protein diet. **Mortensen et al.** [18] and **Maharaj et al.** [23] were reported that exercises activate of neutrophils or macrophages embedded in muscle tissue lead to the calprotectin release following exercise.

The results of current study showed a strong positive significant correlation between serum levels of IL-6 and calprotectin in both groups. This is explained by previous studies of **Mortensen et al.** [19], **Dhas et al.** [24] and **Maharaj et al.** [23] who highlighted the role of IL-6 in the induction of calprotectin, and **Grand et al.** [25] who denoted that calprotectin is an endogenous ligand to TLR4 that causes up-regulation of pro-inflammatory cytokines such as TNF-α IL-6.

In agreement with previous studies by **Chakraborti,** [26] and **Rizzatti et al.** [27], our results showed increased relative abundance of *Prevotella* and γ-*Proteobacteria* in healthy obese group relative to normal weight group but were not statistically significant.

This elevation could be explained according to **Amabebe et al.** [28] as there is no bacteria has the capacity to hydrolyze all nutrients and produce all metabolites observed in the gut lumen, and there is metabolic synergy among the bacterial community so that the entire community collaborate to produce the physiological relationship with the host cells. Dysregulation of the physiological and biochemical interaction between the host and gut microbiota is characteristic of the obese state. A fact that was explained by the study of **Shin et al.** [29] that demonstrated the presence of differences in diet habit and physical activity between healthy obese and normal weight groups so γ-*Proteobacteria* increased with calorie-dense, high-fat and low-fiber diet.

Also **Durban et al.** [30], **Haro et al.** [31] and **Hjorth et al.** [32] concluded that *Prevotella* was increased with intake of high carbohydrate and fiber diet. **Quiroga et al.** [33] concluded that exercise reduces the γ-*Proteobacteria, Prevotella, Bacteroides* and increases the *Lactobacillus*.

In our study, we found positive correlations between the relative abundances of *Prevotella* and γ-*Proteobacteria* in both groups of the study. These positive correlations can be explained by the study of **Shin et al.** [29] who reported that the facultative anaerobes make the habitat suitable for colonization by strict anaerobes (e.g. *Prevotella*) via consuming oxygen, altering the pH, and producing carbon dioxide and nutrients.

The positive correlation between *Prevotella* and serum levels of IL-6 in both healthy obese and normal weight groups could elucidated by the studies of **Larsen,** [34] and **Cani and Jordan,** [35] that demonstrated *Prevotella spp* stimulate release of IL-8 and IL-6 by epithelial cells, favoring Th17 responses and neutrophil recruitment.

The positive correlations between the *γ Proteobacreia* and inflammatory mediators in normal weight group and the negative correlation in

healthy obese group may be explained by the difference in intestinal permeability between the two groups caused by differences in diet habit. **Hiippala et al.** [36] declared that leaky gut allows the intestinal content to be in contact with the host periphery potentially inducing inflammatory responses while **Lin et al.** [37] explained that Hexa-acylated LPS of *γ Proteobacreia* induces pro-inflammatory mediators.

Conclusions

This study conclude that IL-6 and calprotectin have role in the regulation of energy homeostasis and the elevation in relative abundances of *Pevotella* and γ-*Proteobacteria* may be attributed to differences in diet habits and physical activity between the two groups and primary shifts in gut microbiota of healthy obese. These finding represent a target for the development therapeutic strategies to manage obesity. We need to have multipurpose diet intervention that can successes in weight reduction, improved glucose metabolism and lead to reduced degree of inflammation regarding diet-microbiota-inflammation associations and decrease the use of pharmaceutical drugs with harmful effect on the health. However, this study has to be performed on a larger scale to allow more accurate statistical analysis.

Conflict of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Authors' contributions

Shereen M. Mohammed conceived and designed the study and wrote the original draft of the manuscript. Marwa Fayez Hosny administered the project, collected and analyzed the data. Manal Elsayed Ezz Eldeen provided clinical evaluation of study groups and helped in collecting participants' data. While Mohamed Saad Badary and Wegdan A. Mohamed participated in revising the article critically and approved its intellectual content. All authors have reviewed and approved the final version of the manuscript.

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Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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