Original Communication

A calorie-restricted diet enriched with tree nuts and peanuts reduces the expression of CX3CR1 in peripheral blood mononuclear cells in patients with coronary artery disease

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Abstract: *Background:* The modification of the gut microbiome has been proposed to alter immune response which is a key driver in low-grade inflammation as well as metabolic markers. This study was conducted to determine the effects of a low-calorie diet with and without nuts on some gut bacterial abundance, metabolic markers, and gene expression in peripheral blood mononuclear cells (PBMCs) in stable coronary artery disease patients with overweight or obesity. *Methods:* Overweight or obese patients with stable coronary artery disease of both genders were randomly allocated to a nut-free calorie-restricted diet as 25% of energy deficit (CRD) or a CRD enriched with 39–60 g/d of mixed nuts (CRDEN) for 8 weeks (32 patients in CRD and 35 patients in CRDEN). Mixed nuts consisted of equal amounts of unsalted pistachios, almonds, and peanuts. Microbiota analysis was performed by quantitative real-time polymerase chain reaction method on feces collected before and after the intervention, using primers targeting 16S ribosomal DNA of 4 different bacterial genera, including *Bacteroides*, *Prevotella, Bifidobacterium*, and *Lactobacillus*. We examined the plasma concentrations of glucose, insulin, adiponectin as well as expression of toll-like receptor-4 (TLR4) and fractalkine receptor (CX3CR1) in PBMCs. *Results:* A significant reduction in expression of CX3CR1 (p=0.04) and a tendency to lower expression of TLR4 in PBMCs (p=0.06) was observed in the CRDEN group at the end of the study compared to the CRD group. The abundance of fecal *Prevotella* also tended to increase in CRDEN compared to the CRD group (p=0.06). Plasma insulin and adiponectin had no significant changes. There was a positive correlation between fecal *Prevotella* abundance and plasma adiponectin at baseline (r=0.315, p=0.015) and the end of the study (r=0.380, p=0.003). *Conclusion:* Our results suggest that the inclusion of mixed tree nuts and peanuts in a low-calorie diet for 8 weeks led to a lower CX3CR expression in PBMCs in a cohort of overweight or obese patients with stable CAD. This finding provides another beneficial effect of diet supplemented with nuts on factors associated with inflammation. Trial registration: this clinical study has been registered at the clinical trial registration center (clinicaltrial.gov): NCT04078919 on September 6, 2019.

Keywords: Gut microbiome, low-grade inflammation, mixed nuts, toll-like receptor-4, CX3CR1

The gut microbiota has been mechanistically linked to physiological processes that affect a number of health conditions and diseases, including obesity and cardiovascular disease [1, 2]. The gut microbiota can contribute to systemic immune responses associated with the outside of the intestine diseases [3]. The composition of the intestinal microbiota can have important effects on the production of metabolites, and consequently on host inflammatory activation and metabolic markers [4].

Evidence has suggested that disturbances in the composition of the gut microbiota may be a cause of low-grade systemic inflammation [5]. The gut microbiota in obese participants may alter intestinal permeability with a consequent increase in the release of some pathogen-associated molecular patterns which communicate with the host immune system by eliciting responses involving receptors, such as Toll-like receptors (TLRs) that may lead to chronic low-grade inflammation [6, 7]. Altering gut microbiota-derived molecules by diet and fermentable foods may suppress TLRs expression and reduce levels of inflammation in overweight and obese individuals [8, 9]. Chronic low-grade inflammation facilitates the overexpression of various cell

adhesion molecules, such as chemokines, and the subsequent migration of immune cells from the circulating blood to the arterial wall [10]. CX3CL1 (fractalkine) is a chemokine with a documented role in atherosclerosis, and a reduction in the CX3CL1 and its receptor (CX3CR1) pathway can improve the severity of atherosclerosis. [11, 12]. In patients with coronary artery disease (CAD), increased CX3CL1/CX3CR1 expression in atherosclerotic coronary plaques compared with normal arteries has been observed [13]. Microbiota-derived molecules, could travel through systemic blood circulation and reach the bone marrow, be captured by CX3CR1+ mononuclear cells via toll-like receptors to produce inflammatory cytokines [14]. Furthermore, gut microbiota and the metabolites produced by them may drive the development of insulin resistance in obesity, possibly by triggering an inflammatory response [15].

Dietary changes are considered to be the main shaper of the intestinal microbiota composition [16]. The inclusion of tree nuts or peanuts in the diet can be considered as a valuable dietary intervention for improving cardiovascular risk factors, including markers of glycemic control [17] or inflammation [18]. Furthermore, improvement in adipokines such as adiponectin levels has been observed with nuts consumption [19, 20]. Although nuts are energy-dense foods, clinical interventions have reported that a nut-enriched diet will not cause weight gain [21] and higher nuts intake has been associated with a lower risk of becoming obese [22]. Interestingly, the measured metabolizable energy of whole natural forms of nuts is less than the predicted amount due to incomplete absorption of macronutrients in the gastrointestinal tract [23, 24]. Since unabsorbed nutrients in the proximal gastrointestinal tract can be exposed to microbial fermentation in the colon [25], dietary consumption of nuts can potentially affect the composition of the gut microbiota. Thus, the health effects associated with nuts consumption may not only be related to their absorbable nutrients but also their impact on the gastrointestinal microbiota. Based on these assumptions, a prebiotic effect on the colonic microbiota has been suggested [26].

The current evidence indicates that tree nuts and peanuts consumption may affect gut microbiota composition at the genus level [27, 28] but in general, a few studies have evaluated the impact of nuts consumption on gut microbiota composition [29, 30]. In the present study, the effect of two low-calorie diets, with and without nuts, on some gut bacterial abundance, metabolic, as well as toll-like receptor-4 (TLR4) and CX3CR1 expression in peripheral blood mononuclear cells (PBMCs) were investigated in overweight or obese patients with stable CAD. We specifically were interested in the gut abundance of some bacterial genera, including *Prevotella* and *Bifidobacterium* because they have been inversely related to markers of low-grade inflammation [31] and also others such as *Lactobacillus* since it was reported to have health benefits and widely being used as a probiotic. Furthermore, the *Prevotella*-to-*Bacteroides* ratio in the fecal communities has been suggested for better assessment of possible effects of dietary intervention on the gut microbiota and physiological biomarkers [32]. The *Prevotella-to-Bacteroides* ratio has been suggested as a biomarker for optimal weight management, as overweight adults with high *Prevotella* abundances may lose more weight [33] and have better weight loss maintenance [34] than subjects with low *Prevotella* abundances.

Method

Study design, participants, and treatments

The current report is a follow-up to a previously conducted trial (NCT04078919). The details for the study design were previously described [35]. Herein, outcomes related to the effects of the two diets on the gut bacteria and PBMCs gene expression were assessed. Briefly, this was a randomized, controlled dietary intervention study in which 70 overweight or obese stable CAD patients were randomized to study groups for 8 weeks. The inclusion criteria were stable CAD, body mass index (BMI) $\geq$25 kg/m^2, age between 35 and 75 years, having at least one of the cardiovascular risk factors (diabetes, elevated blood pressure, hyperlipidemia), and the readiness to follow a calorie-restricted diet. The exclusion criteria were allergy to nuts, advanced chronic kidney disease, regular consumption of nuts more than 50 grams in a week, constant consumption (more than once in a week) of vitamin and minerals supplements during the past month, and change in type or dose of medications during the study. Study groups included the nuts-free calorie-restricted diet (CRD) or a calorie-restricted diet enriched with nuts (CRDEN). In both groups, a healthy low-calorie diet was prescribed. For this purpose, calorie requirements for weight maintenance were estimated by Mifflin St Jeor's equation [36] multiplied by the physical activity level and then 25% of the estimated calories were subtracted to prescribe a low-calorie diet. In the CRD group, calories were distributed as ~55% from carbohydrates, ~18% from protein and ~27% from fat, and participants were asked not to consume tree nuts and peanuts during the research period. In the CRDEN group, 20% of the calorie of the low-calorie diet was considered to be obtained from tree nuts and peanuts (equal amounts of unsalted pistachio, almond, and peanut) and the remaining calories were distributed as ~55% from carbohydrates, ~18% protein, and ~27% fat. Participants in the nuts diet group received 60 packets of free mixed tree nuts and peanuts for daily consumption during the study. Both diets were

aimed to modify the frequency and type of foods, such as substituting lower saturated fat foods instead of foods with higher content, daily consumption of at least 5 servings of fruits and vegetables, and lower consumption of salt and simple sugars. Patients were asked about their regular nut consumption prior to the study, and if they ate nuts more than once a week, they were given a two-week washout period before being admitted to the study. All participants were individually counseled by the study dietitian. Compliance with the consumption of nuts was monitored by contacting participants once a week. In addition, at the end of the study, individuals were asked to return any bags of unused nuts. The study was in adherence to the Declaration of Helsinki. The study was approved by National Nutrition and Food Technology Research Institute Ethics Committee (code: IR.SBMU.NNFTRI.REC.1398.014) and informed consent was obtained from all patients before the study.

In the original study, the sample size was determined based upon C-reactive protein as the main outcome variable, considering an effect size of 0.8 generated in Zhao et al. [37], with type I error (α) of 0.05, and power of 0.85. By considering an attrition rate of 15%, a total of 70 participants (35 in each group) were regarded as the study sample size.

Fecal sample collection and analysis

Fecal samples were collected at baseline and the end of 8 weeks of dietary treatment periods and stored at −20 °C until extraction. For DNA extraction, by using a small blade grinder, each frozen stool sample was pulverized and homogenized, and a 200 mg pulverized sample was used for DNA extraction. The grinder was cleaned using water and ethanol after each fecal sample was processed. Total microbial DNA was extracted from all stool specimens using Stool DNA Isolation Mini Kit (Favorgen Biotech Corp, Taiwan) according to the manufacturers protocol. DNA quality and concentrations were determined by a Nanodrop spectrophotometer (Nanodrop Technologies, USA). After extraction, extracted DNAs were immediately frozen at −20 °C until analysis.

For each sample, the levels of total bacteria and *Bacteroides, Prevotella, Bifidobacterium*, and *Lactobacillus* were estimated from fecal DNA by quantitative real-time PCR (qPCR) by using primers targeting 16S ribosomal DNA of bacteria. The primers used in the present study have been tested previously (Table 1). The amplification reactions were carried out with 1 µl of extracted DNA, the corresponding forward and reverse primer (0.5 µL each), and 10 µl qPCRBIO SYBR Green MIX Hi-ROX (PCR Biosystems Ltd., London, UK) in a total volume of 20 µl. Parallel analysis of non-template controls (water) was also included. All reactions were performed on a Step-one plus system (Applied Biosystems, Inc., Foster City, CA) by using the following program: 2 min at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C, followed by melt curve generation for assessing amplicon specificity.

The relative abundances of the 4 different bacterial genera (*Bacteroides, Prevotella, Bifidobacterium*, and *Lactobacillus*) were obtained by normalizing the Ct values of each bacteria for the Ct values of total bacterial (ΔCt values=Ct target bacteria−Ct total bacteria). Due to inter-individual variation, fold changes for specific bacteria were calculated as the differences between ΔCts values at baseline and ΔCts values at 8-week for all participants before further analysis.

Blood sample collection and analysis

At both baseline and end of the study visits, venous blood samples were drawn after a 10–12 h overnight fasting for plasma and PBMCs isolation. PBMC were isolated using Lymphoprep (Nyegaard & Co., Oslo, Norway) by gradient centrifugation methods within 45 to 60 min after sampling and stored at −80 °C until RNA isolation was performed. Commercial enzyme-linked immunosorbent assays kits were used to measure plasma concentrations of adiponectin (Biolegend, San Diego, CA) and insulin (Momobind Inc, CA, USA).

The RNA from PBMC samples was extracted using an RNA extraction kit (Sinaclon Co., Tehran, Iran) in accordance with the manufacturer's protocol and stored at −80 °C. RNA concentration and purity were determined using a NanoDrop-2000 (Nanodrop Technologies, USA). The extracted RNA was DNase treated to eliminate the contamination of genomic DNA and was then converted to complementary DNA (cDNA) using the cDNA synthesis kit (Sinaclon Co., Tehran, Iran) according to the manufacturer's protocol. Quantitative real-time PCR (qPCR) was performed using gene-specific primers and qPCRBIO SYBR Green MIX Hi-ROX (PCR Biosystems Ltd., London, UK) on a Step-one plus system (Applied Biosystems, Inc., Foster City, CA). Sequences of primers were as follow: TLR4 (NM138556) forward: 5′-GCTCACAACCATCCTGGT CATT-3′, reverse: 5′-TTTGAAGCACGTCTTAAACAAC CTTA-3′; CX3CR1 (NM001337) forward: 5′-AATGCTAAA GAAAAAGTCATCCAATCTAAC-3′, CX3CR1 reverse: 5′-AAAGCGAGCACTATTTGTGGTCTAA-3′.

Statistical analysis

Statistical analysis of data was performed using the Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL, USA) for Windows version 21.0. Natural log transformations were used for variables with skewed distributions. The normal distribution of variables was assessed using

Table 1. Primer sequences for quantitative real-time PCR assays for bacteria

Target bacteria	Primer	Sequence (5′-3′)
Total bacteria [64]	F	CCTACGGGAGGCAGCAG
	R	ATTACCGCGGTGCTGG
Prevotella spp [65]	F	CACCAAGGCGACGATCA
	R	GGATAACGCCYGGACCT
Bifidobacterium spp [65]	F	GCGTGCTTAACACATGCAAGTC
	R	CACCCGTTTCCAGGAGCTATT
Bacteroides spp [65]	F	CGATGGATAGGGGTTCTGAGAGGA
	R	GCTGGCACGGAGTTAGCCGA
Lactobacillus spp [66]	F	AGCAGTAGGGAATCTTCCA
	R	CACCGCTACACATGGAG

The Kolmogorov–Simonov test. To compare the 8-week values between the groups using baseline values and body weight changes as covariates, a general linear model analysis of covariance (ANCOVA) test was used. The paired t-test was performed to analyze within-group differences between baseline and post-intervention in each group. For qPCR results, differences in relative expression between the two groups were analyzed using an independent t-test. The Pearson correlation was used to evaluate the correlation between the changes in gene expression and plasma metabolic values. The results are expressed as mean (SD), and differences were considered statistically significant at $p \leq 0.05$. For fecal bacteria, normal distributions were evaluated, and comparisons between CRD and CRDEN groups for individual bacterial groups were done by Student's t-test (if normally distributed) or Mann-Whitney U test (if not normally distributed) using fold change values.

Results

Of the 70 CAD patients who were randomly assigned to diet groups, 3 patients in the CRD group were withdrawn because of a lack of cooperation. (CRDEN n=35, CRD n=32). Participant's baseline characteristics were similar between the two groups. Baseline characteristics of the participants in CRDEN and CRD were respectively as follows: age 58±7 and 59±8 y; body weight 80.5±13.9 and 84.8±13.5 kg; BMI 30±3.7 and 31.8±4.2 kg/m^2; male/female distribution 19/16 and 18/14 and history of diabetes (yes, no) 8/27 and 10/22. The diabetic patients in the study were treated with oral hypoglycemic drugs and none received exogenous insulin. Almost all patients in the study were treated with statins. The dietary intakes did not show any significant differences between the two groups at baseline (Table 2). Calorie intake at the end of the study (1355.49±536.55 and 1414.93±473.56 kcal/day for CRD and CRDEN respectively) was lower than the intake at baseline in both groups (1640.14±649.23 and 1742.13±935.99 kcal/day for CRD and CRDEN respectively), but there was no difference between the two groups. The amount of total fiber at baseline (16.33±9.76 and 15.36±7.55 g/day for CRD and CRDEN respectively) and week 8 (17.93±9.04 and 16.47±6.96 g/day for CRD and CRDEN respectively) were not different among the groups. Also, the intake of carbohydrates and protein did not differ between the two groups. However, the mean dietary intake of total fat, polyunsaturated, and monounsaturated fatty acids in the mixed nuts diet group was significantly higher compared to the control diet group after 8 weeks of the study.

Gut microbiota

No statistically significant differences were found in the fecal abundance of the *Bifidobacter, Lactobacillus*, or *Bacteroides* between groups. The abundance of *Prevotella* tended to increase in CRDEN compared to the CRD group (p=0.06). However, no significant difference was found in the fecal *Prevotella/Bacteroides* ratio between study groups (Figure 1).

PBMC mRNA expression and plasma metabolic markers

Gene expression of TLR4 in PBMC increased in the CRD group but did not change in the CRDEN group. The difference between the two groups tended to be statistically significant (p=0.06). A significant reduction in expression of CX3CR1 in PBMC was observed in the CRDEN group at the end of the study compared to the CRD group (p=0.04) (Table 3).

The changes in fasting glucose and insulin were similar and non-statistically different between the two intervention diets (Table 4). HOMA-IR values decreased in both groups, but the change was not statistically different between the two groups. Adiponectin concentrations increased in both groups, with no significant differences between the two groups (Table 4). Correlation analysis was performed to evaluate the relevance of relative abundance fecal bacteria and metabolic markers. We found that fecal *Prevottela* was

Table 2. Dietary intakes of participants during study[a]

	CRD	CRDEN	p*
Energy (kcal/d)			
Baseline	1640.14 (649.23)	1742.13 (935.99)	0.20
Change	−284.65 (250.6)	−327.2 (854.8)	0.92
Carbohydrate (g/d)			
Baseline	270.4 (113.27)	273.52 (147.79)	0.38
Change	−46.93 (28.0)	−64.38 (0.49)	
Protein (g/d)			
Baseline	66.04 (20.74)	60.09 (26.02)	0.99
Change	−5.04 (28.40)	−8.38 (29.78)	0.95
Total fat (g/d)			
Baseline	41.28 (15.80)	48.80 (39.13)	0.69
Change	−8.24 (25.13)	−0.46 (34.32)	0.07
Saturated fat (g/d)			
Baseline	14.00 (7.28)	12.93 (5.86)	0.89
Change	−2.38 (9.12)	−0.98 (7.00)	0.15
Monounsaturated fat (g/d)			
Baseline	10.36 (6.16)	13.61 (15.78)	0.60
Change	−1.81 (6.36)	4.97 (15.82)	0.005
Polyunsaturated fat (g/d)			
Baseline	9.61 (8.01)	8.57 (9.55)	0.56
Change	−3.56 (9.72)	2.39 (13.36)	0.01
Total fiber (g/d)			
Baseline	16.33 (9.76)	15.36 (7.55)	0.80
Change	1.60 (12.76)	1.11 (6.77)	0.58

[a]Some of the results in this table were presented previously [35]. Values are mean (SD). CRD: calorie-restricted diet; CRDEN: calorie-restricted diet enriched with nuts. *Statistical analysis using independent t-test or Mann-Whitney test between two groups.

positively correlated with plasma adiponectin at baseline (r=0.315, p=0.015) and at the end of the study (r=0.380, p=0.003).

Discussion

Mixed tree nuts and peanuts consumption in a low-calorie diet for 8 weeks reduced relative expression of CX3CR1 in PBMCs compared with a nut-free low-calorie diet. CX3CR1 is expressed in mononuclear and vascular endothelial cells which have a crucial role in the early development of atherosclerosis. CX3CR1 expressing cells contribute to vascular remodeling and plaque formation by producing cytokines involved in angiogenesis [38]. CX3CR1 and its ligand CX3CL1 are critical for the recruitment of circulating monocytes to the injured atherosclerotic vascular wall [39]. In addition, they may be involved in a range of kidney diseases, and circulating CX3CR1+ monocytes have been found to increase reversibly in patients with renal insufficiency and correlate with their cardiovascular risk [40]. Lower expression of CX3CR1 may reduce some of the CX3CL1/CX3CR1 mediated responses in the endothelium. Damas et al. demonstrated that 6-month statin therapy reduced the excessive expression of CX3CL1 and CX3CR1 in CAD patients [41]. Several nutrients in nuts may contribute to the reduction of inflammatory molecules, including CX3CR1. Tree nuts and peanuts are a rich source of oleic acid, which has been shown to have anti-inflammatory effects [32]. Furthermore, the observed effects may partly be related to the polyphenols in nuts [15, 39].

In the present study, mixed tree nuts and peanuts consumption in a low-calorie diet also tended to decrease TLR4 expression in PBMCs as well as a tendency to increase the abundance of fecal *Prevotella*. Diet and obesity can both affect intestinal integrity and subsequently lead to the penetration of intestinal microbiota or their bacterial products into the bloodstream, activating immune cells' TLRs and consequently low-grade systemic inflammation in the host [42]. The immune system via the measurement of microbiome-derived short-chain fatty acid may detect whether the gut microbiota is properly functioning. A diet low in plant products may confuse the immune system into thinking that the microbiome is not functioning properly, leading

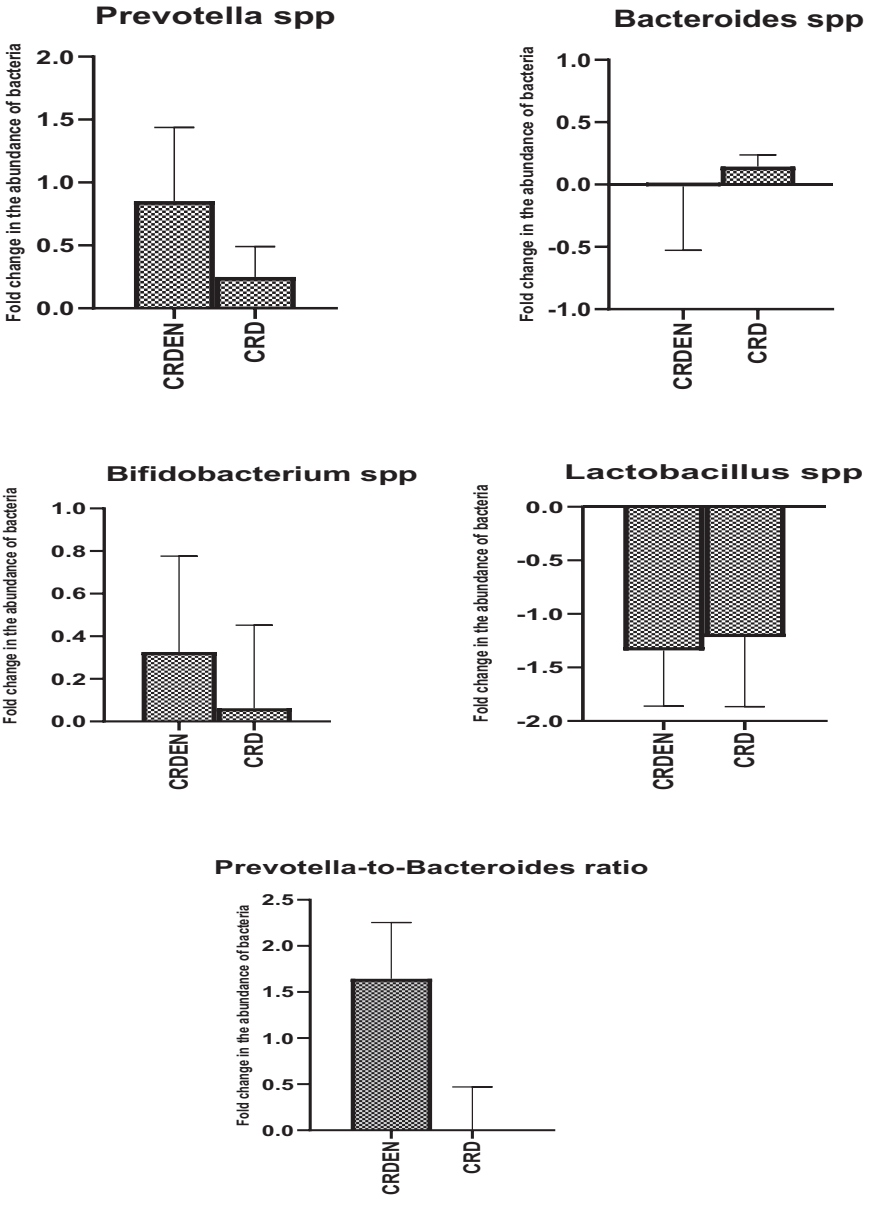

Figure 1. Changes in relative abundances of *Prevotella, Bacteroides, Bifidobacterium*, and *Lactobacillus* in stool isolated from study participants. The relative abundances were calculated normalizing the Ct values of each bacteria for the Ct values of total bacterial (ΔCt values=Ct target bacteria−Ct total bacteria). Fold changes in the abundance of bacteria were calculated as the differences between ΔCts values at baseline and ΔCts values at 8-week. Higher values indicate a higher abundance. Bacteria Data are mean±standard error. CRD: calorie-restricted diet; CRDEN: calorie-restricted diet enriched with nuts.

Table 3. Changes of PBMCs' gene expression across two studied groups

	CRDEN (n=35)			CRD (n=32)			Treatment effects
Gene	Baseline	8-week	Change	Baseline	8-week	Change	p-value[a]
TLR4 (ΔCTs)[+]	2.30 (2.84)	2.69 (2.41)	0.39 (2.99)	3.30 (2.13)	1.93 (3.13)	−1.37 (3.0)	0.061
CX3CR1 (ΔCTs)[+]	3.31 (2.0)	3.97 (1.94)*	0.65 (1.90)	4.17 (1.57)	3.23 (2.2)*	−0.94 (2.7)	0.040

Data are mean (SD). [a]p-values correspond to between-group comparisons by using an analysis of covariance with baseline value and mean change of weight loss as covariates. *p<0.05 for within-group (baseline vs 8-week) comparison by paired t-test. +ΔCt values=Ct target gene−Ct GAPDH. Lower values indicate lower expression. CRD: calorie-restricted diet; CRDEN: calorie-restricted diet enriched with nuts.

Table 4. Changes of plasma metabolic markers across two studied groups

	CRDEN (n=35)			CRD (n=32)			Treatment effects[a]
Parameters	Baseline	8-week	Change	Baseline	8-week	Change	p-value
FBS (mg/dl)	119.4 (50.8)	109.3 (37.4)	−10.0 (32.5)	108.5 (30.6)	103.8 (37.5)	−4.71 (25.0)	0.924
Insulin (μIU/ml)	14.3 (9.6)	13.8 (6.6)	−0.52 (8.5)	15.0 (7.6)	13.2 (6.2)	−1.78 (5.7)	0.524
HOMA-IR	4.0 (2.7)	3.6 (1.8)	−0.44 (2.4)	4.0 (2.1)	3.3 (1.8)	−0.64 (1.7)	0.598
Adiponectin (μg/ml)	7.80 (3.3)	8.36 (2.9)	0.56 (2.6)	7.3 (2.8)	7.7 (2.9)	0.37 (2.5)	0.470

Data are mean (SD). [a]p-values correspond to between-group comparisons by using an analysis of covariance with baseline value and mean change of weight loss as covariates. CRD: calorie-restricted diet; CRDEN: calorie-restricted diet enriched with nuts.

to unnecessary inflammation [43]. Studies involving controlled diets have shown that dietary intake has a significant effect on the human colonic microbial communities [3, 44]. In the human colon, *Bacteroides* and *Prevotella* are generally the two most prevalent genera among the *Bacteroidetes* phylum. Higher dietary fiber intake has been associated with a *Prevotella* dominated microbiota (27). Particular indigestible carbohydrates have great potential for modifying the microbiota of the gut [45]. *Prevotella* may increase the capacity of the gut microbiota to ferment complex polysaccharides from the diet [46]. Most of the microbiota profiling studies in rural, high-fiber consuming populations have reported that the genus *Prevotella* is more prevalent than in urban communities, which typically have low-fiber diets [47, 48, 49, 50]. In the current study, a tendency to higher abundance of fecal *Prevotella* in the mixed nuts group may be due to the chemical composition of the nuts components, including dietary fibers, which reach the large intestine. However, analysis of patients' food intake at the end of the study showed that there was no difference in the amount of total fiber intake between the two groups. It is also possible that due to the consumption of nuts, the type of fiber intake in the CRDEN group may have been different from that of the CRD group. Assessment of the gut microbiota in a cohort of Italian individuals concerning their habitual diets has shown that high-level adherence to a Mediterranean diet characterized by high-level intake of plant-based foods, including nuts is associated with increased levels of fecal *Prevotella* [51]. Nuts are also rich in polyphenols and consumption of several foods rich in polyphenols is known to modulate the composition of the gut microbiota [52, 53]. Furthermore, nuts are rich in fats, in particular monounsaturated fats which as compared with saturated fats, are associated with promoting a positive gut microbiome profile [54]. Lipids in nuts may have low bioaccessibility due to their intact cell walls that may be resistant to digestion [55], and as a result, some of their fat may reach the large intestine, where it is potentially used by microbiota. However, in contrast to the present study, in a randomized controlled crossover study conducted in healthy adults, 42 g/d of almond consumption did not affect the relative abundance of fecal *Prevotella* compared to the control diet period that was devoid of almonds [56]. In the present study, the inclusion of nuts in the low-calorie diet did not change the abundance of other bacteria, including *Bifidobacteria* as well. The lack of an effect on the *Bifidobacteria* genus is consistent with previous studies that have investigated the effect of almond or pistachio consumption on the composition of gut microbiota [56, 57, 58]. In contrast, in a non-randomized clinical trial, Liu et al. reported an increase in fecal *Bifidobacteria* and *Lactobacillus* following 6 weeks of consumption of either almond or almond skin in comparison to commercial fructooligosaccharides [59]. However, it should be noted that the number of almonds consumed (56 g/day) was higher than the amount in the present study.

In this study, we could not found a specific effect of a nuts-enriched diet on insulin resistance. This is in contrast to the findings of a recent meta-analysis in which nuts consumption had a favorable effect on HOMA-IR and fasting insulin [60]. The reason might be attributed to the differences in metabolic state. Most of the participants in the present study were non-diabetic (69% in the CRD group and 77% in the CRDEN group) which could influence the effect of nuts on glycemic control. In studies that did not include patients with type 2 diabetes, the results were similar to those in our study and showed no change in fasting insulin or HOMA-IR [31, 33, 34]. In our study, changes in adiponectin concentration were not statistically significant between groups. In contrast to our finding, some clinical studies have shown the benefits of the low-calorie diet or nuts consumption in adiponectin concentration [19, 61, 62]. Considering that in the present study, the average weight loss in each of the two groups was 3 and 3.5 kg, respectively, one reason may be related to the greater weight loss observed in previous studies, which led to an increase in adiponectin concentration. In addition, since total adiponectin, but not high-molecular-weight adiponectin was measured in the current study, it could influence the lack of significant effect of diets, considering that this isoform is the most active. Although plasma adiponectin concentration did not change significantly, its concentration was significantly associated

with the abundance of fecal *Prevotella*. Interestingly, in an experimental study in mice, the inclusion of one legume (navy bean) in the high-fat diet increased fecal *Prevotella* abundance as well as serum adiponectin levels [63]. This suggests that increased fecal *Prevotella* in overweight/obesity may be linked to some adipose tissue-derived adipokines and that inclusion of nuts or legumes in the diet may not only alter intestinal microenvironment but also adipokines.

The strength of the present study is that to our knowledge, it is the first to investigate the effect of nuts in the context of a low-calorie diet on the fecal abundance of bacteria and expression of PBMCs' TLR4 and CX3CR1 in CAD patients. A major limitation of this study is that the abundance of a limited number of gut microbiota components was examined and the diversity of bacteria was not determined. The duration of the study was also relatively short and a longer duration of study is required to confirm the effects. Furthermore, participants' diet analysis included total fiber intake, and the amount of different types of fiber were not determined. Moreover, the mRNA expression of TLR4 and CX3CR1 were not supplemented by additional experiments such as western blotting.

Conclusion

In conclusion, the inclusion of mixed tree nuts and peanuts in a low-calorie diet for 8 weeks led to a lower CX3CR expression in PBMCs in patients with stable CAD. This finding provides another beneficial effect of diet supplemented with nuts on factors associated with inflammation. Furthermore, consumption of nuts with low calorie diet did not change the abundance of intestinal bacteria or the concentration of some metabolic biomarkers, but the plasma adiponectin concentration showed a positive association with the abundance of fecal *Prevotella*. Further studies may help to better elucidate the association between intestinal bacteria and adipokines.

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Conflict of interest
The authors declare that there are no conflicts of interest.

Statement of ethics
This study was approved (Certificate No. IR.SBMU.NNFTRI.REC.1398.014) by the National Nutrition and Food Technology Research Institute Ethics Committee of Shahid Beheshti University of medical science, Tehran, Iran.

Author contribution
The study was conceptualized by J.N and M.G. The research was conducted by M.G. The authors read and approved the final version of the manuscript.

Data availability
The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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