BMJ Open

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Journal:	*BMJ Open*
Manuscript ID	bmjopen-2017-015910
Article Type:	Research
Date Submitted by the Author:	09-Jan-2017
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Primary Subject Heading:	Nutrition and metabolism
Secondary Subject Heading:	Nutrition and metabolism, Public health
Keywords:	free fatty acids, lipopolysaccharide binding protein, metabolic health, abdominal obesity

Association of endotoxemia with serum free fatty acids in metabolically healthy and unhealthy abdominally obese individuals: a case-control study

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Running Title: Association of endotoxemia with serum free fatty acids

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Abstract

Objectives: We compared serum FFAs and lipopolysaccharide binding protein (LBP) between metabolically healthy abdominally obese (MHAO) and metabolically unhealthy abdominally obese (MUAO) individuals. We also examined the association between serum FFAs and LBP in the participants.

Methods: In this age- and gender-matched case-control study, 164 abdominally obese subjects were recruited from June to November, 2015 in the Northwest of Iran. Demographic data, dietary intake, body composition, anthropometric indices, and physical activity were assessed. Basal blood samples were collected to determine serum metabolic parameters, FFAs, and LBP. Abdominal obesity was defined as having waist circumference (WC)≥ 95cm. Those with 3 or more metabolic alterations were defined as MUAO and those having 2 or less were classified as MHAO.

Results: There were no significant differences in dietary intake, anthropometric indices, body composition, and physical activity between the two groups. The odds of MUAO significantly increased by increments in serum FBS (OR= 3.79, 95 % CI: 2.25-6.40), TG (OR= 1.10, 95 % CI: 1.05-1.15), SBP (OR= 1.02, 95 % CI: 1.00-1.04), and DBP (OR= 1.03, 95 % CI: 1.01-1.06) and decreased by increase in serum HDL-C (OR= 0.32, 95 % CI: 0.20-0.52). The levels of LBP and FFAs showed no significant differences between the two groups. However, significant correlations were found between LBP and FFAs in pooled population (r=0.712; *P*<0.001) as well as in cases (r=0.717; *P*<0.001) and controls (r=0.704; *P*<0.001). Neither FFAs nor LBP was significantly correlated with dietary intake or metabolic parameters.

Conclusion: The results indicated that serum LBP and FFAs are highly correlated both in MHAO and MUAO states. In addition, the levels of LBP and FFAs seem to be more related to abdominal obesity than to the presence or absence of metabolic health.

Key words: free fatty acids; lipopolysaccharide binding protein; metabolic health; abdominal obesity

Strengths and limitations of this study

1) The association of LBP and FFAs levels in MHAO and MUAO individuals for the first time and found significant differences between the two parameters.

2) This was a case-control study in which causality could not be assessed.

3) Insulin levels were not measured in our study population; therefore, insulin resistance was not studied.

Introduction

Obesity, a major public health concern, is increasingly prevalent worldwide. This condition is estimated to influence more than one billion people by 2030 [1]. There are well-established health consequences of obesity. In particular, the increased risk of type 2 diabetes, metabolic syndrome (MetS), and cardiovascular disease is thought to be highly ascribed to obesity [2-5]. Therefore, obesity can lead to the increased risk of premature death and higher all-cause mortality [6]. However, not all obese people are at higher risk of metabolic diseases. For these subjects, described as "metabolically healthy obese (MHO)", the obese phenotype may exist in the absence of metabolic dysfunction such as insulin resistance, dyslipidemia, and unfavorable inflammatory profile [7-9]. Despite there is still no uniform definition for MHO, it is thought to account for approximately one fifth of the obese population [10, 11].

Evidence increasingly identifies inflammation as a potential mechanism linking adiposity especially abdominal fat and metabolic dysfunction [12]. However, it is unclear whether inflammation accounts for the metabolic differences observed between metabolically healthy and unhealthy individuals. Published results are rare and conflicting [13-16]. Studies on postmenopausal obese women suggest that the MHO may have more favorable inflammatory profiles [17], less visceral fat, and possibly less hepatic fat [18] than their counterparts with insulin resistance and other metabolic abnormalities [19]. In contrast, Wildman et al. [20] reported that, despite not finding increased 10-year risk of cardiovascular disease among metabolically healthy overweight/ obese women, these subjects still displayed abnormal levels of inflammatory profile.

Basic mechanism accounted for inflammation in adipose tissue is still unknown, but some factors including free fatty acids (FFAs) concentrations in blood is suggested [21-23]. It was clarified that plasma FFAs are increased among the obese as it is released from inflamed adipose tissue [24] and through the lipolysis of adipocytes [25]. However, little is known about the contribution of FFAs to the development of inflammation in obesity. Therefore, examining the association of FFAs with inflammatory markers seems to be warranted.

Lipopolysaccharide (LPS) molecules, also known as bacterial endotoxins, may trigger inflammation, leading to immunity activation and cytokine release. LPS infusion and consequent subclinical endotoxemia results in elevated levels of proinflammatory markers, dyslipidemia, fasting hyperglycemia, insulin resistance, and obesity - factors known to be associated with MetS [26-28]. LPS has a short half-life [29] and there is no agreement on the measurement of its plasma level [30]. Hence, lipopolysaccharide-binding protein (LBP) is introduced with longer half-life and more reliable measurement [31, 32]. Also, serum LBP level is a proxy of serum LPS level [33]. A population-based study [34] on Chinese people found that LBP was significantly associated with MetS in normal-weight participants. Another study [35] on adult population reported that among MetS components, LBP concentration was independently associated with abdominal obesity.

In prior studies, inflammatory parameters including LPS/ LBP and FFAs were assessed and compared between obese and non-obese or lean subjects [31, 36, 37]. Therefore, it remains unclear whether the observed alterations in serum FFAs and/or inflammatory parameters in metabolically unhealthy obese patients are due to excess adipose tissue mass and/or directly

associated with their metabolic state. To distinguish between the influence of obesity and metabolic disorder, we hypothesized that altered serum inflammatory marker including LBP and/or FFAs levels are merely related to the abdominally obese state and are not highly concerned with metabolic aberrations. Therefore, regarding the significant confounding effect of abdominal obesity, we used waist circumference (WC), which is a reflection of visceral adipose tissue [38], to define abdominal obesity, and examined differences in characteristics and inflammatory markers (serum LBP and FFAs) between "metabolically healthy" and "unhealthy" abdominally obese individuals. We also examined the association between serum FFAs and LBP in pooled population as well as in each group.

Methods

Study design and participants

A total of 81 metabolically healthy abdominally obese (MHAO) with 83 age- and gender-matched metabolically unhealthy abdominally obese (MUHAO) were recruited in this case-control study, carried out from June 15th to November 6th, 2015 in the Northwest of Iran. Individuals aged 18-60 years with abdominal obesity were included in the study. We excluded pregnant or lactating women, those with diarrhea for 3 consecutive days within the previous 3 months, diagnosed diabetes, coronary heart disease, stroke, myocardial infarction, cardiovascular and kidney, liver or infectious diseases including tuberculosis, AIDS, and hepatitis; thyroid problems, severe mental disorders or physical disabilities and malignancies; taking oral antidiabetic agents or insulin or other drugs for the last 2 months, or antibiotics used for 3 consecutive days within the previous 3 months; smokers or alcohol consumers; misreported dietary intakes (less than 800 kcal/d or more than 4200 kcal/d), or being on specific diets in the past 6 months; and having gastrointestinal surgery within past 1 year.

Abdominal obesity was defined as having waist circumference (WC)≥95cm according to the Iranian new cut-point [39]. According to Meigs *et al.*, metabolic health was defined as the presence of <3 of the following metabolic abnormalities including WC≥95cm for both genders; high serum triglyceride (TG) concentration (≥150 mg/dL); low serum high density lipoprotein cholesterol (HDL-C) (<40 mg/dL for men and <50 mg/dL for women); elevated blood pressure (BP) (≥130/85 mmHg); and fasting blood sugar (FBS) (≥100 mg/dL). Eighty-one individuals had

≥3 criteria for MUAO and formed the case group (MUAO), whereas 83 had ≤2 criteria and entered the control group (MHAO).

Sampling procedures

After public announcement for the study, 500 volunteers were recruited from general population. Of these, 178 people could enter the study based on the defined eligibility criteria for the present study. Informed consent was taken from each participant before the study. After taking blood samples and anthropometric measurements, 14 of them were excluded due to FBS≥126 mg/d, leaving 164 people (82 men, 82 women) to conduct the research.

Biochemical assays

After a 12-h overnight fast, 5cc blood was obtained for serum analyses. After centrifugation at 3000 rpm for 5 min, metabolic parameters were analyzed immediately and serum FFAs and LBP which were analyzed after supplying in -80°C.

FBS was measured by the enzymatic colorimetric method using glucose oxidase. Serum TG concentration was measured by commercially available enzymatic reagents with glycerol phosphate oxidase. Serum HDL-C was measured after precipitation of the apolipoprotein B containing lipoproteins with phosphotungistic acid. Assays were performed using Pars Azmoon kits (Pars Azmoon Inc., Tehran, Iran) and a Selectra 2 auto-analyzer (Vital Scientific, Spankeren, Netherlands). Inter- and intra- assay coefficient of variation (CV) was < 5% for all assays. Serum

samples for both LBP and FFAs assays were stored at -80°C until analysis. Both serum LBP and FFAs levels were determined by a sandwich ELISA (Bioassay Technology Laboratory, Shanghai Korean Biotech Co., LTD; Shanghai City, China) according to the manufacturer's instructions. The intra-assay and inter-assay coefficients of variation were <8 and <10%, respectively.

Measurements

All anthropometric indices were measured by a trained researcher. Height (without shoes in standard situation with precision of 0.1 cm and with an inelastic measuring tape) and weight (with Seca scale, light clothes and precision of 0.1 kg) were measured and BMI was calculated as weight in kgs divided by the square of height in meters [40]. WC was measured using a non-stretchable fiber measuring tape. The subjects were asked to stand erect in relaxed position with both feet together on flat surface. WC was measured as the smallest horizontal girth between the costal and iliac crests at minimal respiration. Hip circumference was taken as the greatest circumference at the level of greater trochanters (the widest portion of the hip) on both sides. Waist to hip ratio (WHR) was calculated by dividing WC (cm) by hip circumference (cm) [41]. BP was recorded in a comfortable sitting position in the left arm after at least a 5-min rest, using the mercury sphygmomanometer. Two measurements were taken and the mean of the two measurements was considered as the BP [42]. Bioelectrical Impedance Analysis (BIA: BC-418MA, Tanita, Japan) was used to describe fat percent, fat Mass (FM), and fat free mass (FFM). Dietary intake was assessed using a 3-day food record (one weekend day and two workdays).

Nutritionist IV software (Axxya Systems, Stafford, TX), modified for Iranian foods, were used for dietary data analysis. Physical activity (PA) was measured via IPAQ-long form questionnaire [43].

Statistical analysis and sample size

To examine the normal distribution of variables, Kolmogrov-Smirnov tests and histograms were applied. The independent samples t-test was used to compare the means (SD) of normally distributed variables between the two groups. The Mann–Whitney U test was used for values with skewed distribution and in such conditions, median (25^{th}, 75^{th}) was reported. In order to assess the association of two categorical variables, Chi-square test was applied. The correlation between serum FFAs and LBP was assessed using Spearman correlation coefficient analysis. Odds ratios and their 95% confidence intervals (CI) were reported using Logistic Regression test.

The sample size estimation was based upon 80% power with α-error of 5% and a case to control ratio of 1:1. It was predicted that 79 people in each group would detect changes in serum FFAs (31) as well as serum LBP level (32), using the two-means formula. Data were analyzed using SPSS ver. 17.0 for Windows (PASW Statistics; SPSS Inc., Chicago, IL, USA). P-value less than 0.05 was considered significant.

Results

Males comprised about 50% of the study participants in the two groups (p=0.87). The age range of the subjects was 20-59 years. Participants of the two study groups similarly had WC≥95 cm i.e. the cut-off point of WC for Iranian population. Overall, there were no significant differences in age, gender, anthropometric indices, and body composition between the two groups. Dietary parameters especially total fat, SFA, MUFA, PUFA intakes were more or less the same between the two study groups (Table 1). Marital status, education level, and job of the cases and controls were also similar. However, mean number of metabolic aberrations were significantly higher in cases than controls (3.25±0.72 vs. 1.67±0.50; P<0.001) (data not shown).

Except for WC which was matched between the two groups, metabolic aberrations including low HDL-C (82% vs. 34%), high TG (78% vs. 24%), high FBS (33% vs. 0%), and Htn (34% vs. 10%) were significantly higher in case than controls, respectively (Figure 1). The current study indicated that each 10 unit increment in serum FBS level increased the risk of MUAO about 3.8 times (OR= 3.79, 95 % CI: 2.25-6.40). Additionally, the odds of MUAO was significantly increased per one increment in serum TG level (OR= 1.10, 95 % CI: 1.05-1.15), the systolic BP (OR= 1.02, 95 % CI: 1.00-1.04), and diastolic BP (OR= 1.03, 95 % CI: 1.01-1.06). However, the odds of having MUAO was significantly decreased by 68% per 10 unit increment in serum HDL-C level (OR= 0.32, 95 % CI: 0.20-0.52) (Table 2).

The median of LBP (12.32 µg/mL in cases vs. 12.76 µg/mL in controls, P=0.483) and FFAs (1294 nmol/L in cases vs. 1333 nmol/L in controls; P=0.686) showed no significant difference between the two groups (Figure 2). However, a significant correlation was found between LBP and FFAs

in pooled population (r=0.712; *P*<0.001) as well as in cases (r=0.717; *P*<0.001) and controls (r=0.704; *P*<0.001) (Figure 3). The results of partial correlation indicated much stronger correlation between LBP and FFAs, when controlling for WC (r=0.961; *P*<0.001). Moreover, number of metabolic aberrations were significantly correlated with HDL-C (r=-0.537; *P*<0.001), TG (r=0.468; *P*<0.001), FBS (r=0.534; *P*<0.001), Systolic BP (r=0.247; *P*=0.001), and Diastolic BP (r=0.315; *P*<0.001). Neither FFAs nor LBP was significantly correlated with dietary intake of total fat, SFA, MUFA, and PUFA (data not shown). There were also no significant correlations of LBP and FFAs with metabolic parameters (Table 3).

Discussion

The present study, to the best of our knowledge, examined the association of LBP and FFAs levels in MHAO and MUAO individuals for the first time and found significant differences between the two parameters. Anthropometric indices as well as body composition profile were similar between the two groups. Moreover, there were no significant differences in LBP and FFAs between MHAO and MUAO.

In the present study, we matched metabolically healthy with metabolically unhealthy individuals on abdominal fat which might explain why we did not find any differences in levels of FFAs and LBP, as inflammatory markers, and body composition between the two groups. Therefore, the levels of LBP and FFAs seem to be more related to abdominal obesity than to the presence or absence of metabolic health. This is further supported by the observation that serum FFAs or LBP levels were not correlated with metabolic parameters. Therefore, our findings suggest that increased levels of these two markers are not necessarily related to the presence of metabolic aberrations.

A few smaller studies have examined differences in body composition and/or inflammatory profile between metabolically healthy and unhealthy obese postmenopausal women [44-46]. In line with our result, Engström *et al.* [45] in a research on 58 obese postmenopausal women found no significant differences in levels of inflammatory markers between those with metabolic syndrome (MetS) compared to those without MetS. Additionally, in the population-based study of Philips *et al.* [47], no significant difference was noted in CRP level between MHAO and MUAO,

based on metabolic health criteria of Meigs *et al.* [48] It is noteworthy that in the present study we used Meigs's metabolic health definition in which WC has also been considered [48].

A recent study revealed that the association between inflammatory biomarkers and metabolically healthy obesity depends on the criteria used. Since in that research, a significant difference was noted in the levels of CRP and IL-6 with some but not all MHAO definitions, which disappeared after adjustment for abdominal obesity or percent body fat [49]. This study confirms our results. However, Phillips *et al.* [6] showed that obese women and men with MetS had significantly higher levels of inflammatory cytokines than obese persons without MetS. Beasley *et al.* [50] showed that visceral adiposity, and not abdominal subcutaneous fat, was most consistently associated with significantly higher levels of IL-6 and CRP levels in black and white men and women in the Health ABC study. We could not measure visceral fat in our study, though, abdominal obesity measured through WC, can reflect visceral adiposity [38]. On the other hand, a recent work observed no significant differences in visceral fat between the obese-insulin resistant and obese-insulin sensitive persons [51].

In the present work, dietary intake was compared between the two obese groups; therefore, no significant difference was found in terms of energy or macronutrients especially fat intake. Moreover, habitual PA was controlled between the study groups. Since different levels of habitual PA might affect levels of serum inflammatory markers [52].

Obesity, as a well-known metabolic risk factor, is usually associated with mild chronic inflammation [53]. The relationship between obesity and increased inflammation may be justified, in part, by FFAs [54] which are released from adipocytes through lipolysis and are elevated in

obesity due to increased adipose tissue [55]. Inflammatory cytokines such as interleukin-6 (IL-6) can stimulate lipolysis and increase levels of FFAs [56]. On the other hand, in healthy persons an acute increase in FFAs can induce inflammatory changes [57]. Therefore, FFAs are not only increased by inflammation, but also promote inflammation. The results of the present research showed that FFAs are positively correlated with LBP levels either in the pooled population or in each group. It shows that any increase in the level of FFAs, observed in the abdominally obese, regardless of their metabolic aberrations can lead to a significant elevation in the level of LBP.

LBP has been considered a key inflammatory marker which mediates LPS-triggered innate immunity [58]. Although LBP concentration was previously reported to be associated with various anthropometric and metabolic factors such as BMI, WC, and so on [35-37], in our study the relationship only existed between the two biomarkers, FFAs and LBP, but not with the metabolic or anthropometric parameters. It is notable that the positive relationship between LBP and BMI was not observed in either normal weight or obese groups in Yang *et al.* [59] study after multivariate analyses. In their research, the level of LBP significantly reduced after bariatric surgery and consequent reduction in WC (from 121.6 cm to 90.6 cm; $P<0.001$) which indicates the strong association of LBP with WC. Liu *et al.* [34] in a population-based follow-up study on 2529 Chinese also found that the association of LBP with MetS was only significant in normal-weight participants, but not in their overweight/obese counterparts after multivariate adjustments including BMI, which supports our study findings. It is assumed that the association between serum LBP level and MetS observed in previous studies [31, 36, 37] is mediated

by BMI or WC, and finding no association between serum LBP level and incidence of MetS in our study, in which the WC-matched controls were included, is not unexpected.

Overall, what makes our research different from most of previous ones is that in our study we matched the two groups based on WC, rarely observed in prior reports. Most of the previous studies have examined either MetS patients *vs.* those without the syndrome or metabolically healthy *vs*. metabolically unhealthy, regardless of their BMI or WC status and based on different metabolic health criteria [7, 9, 17, 48]. A few have examined inflammatory markers between metabolically healthy and unhealthy persons, considering WC or abdominal obesity [45, 47, 49].

Conclusion

Our study indicated that WC could be a strong mediator of the association between serum LBP, FFAs, and metabolic alterations. In fact, the levels of LBP and FFAs seem to be more related to abdominal obesity than to the presence or absence of metabolic health. The results also suggested a significant correlation between serum FFAs and LBP in abdominally obese population, which seems to be independent of metabolic aberrations.

Competing of interests:

The authors declare that they have no competing interests

Authors' contributions

MS and NK conceived the study design and wrote the study protocol. MS and NK analyzed and interpreted the data. MS, PA, MN, SMG and NK have been involved in drafting the manuscript or revising it critically for content. All authors have given final approval of the version to be published.

Acknowledgments

We would like to thank those who participated in the present study.

Ethical considerations

All protocols were approved by regional and organizational ethics committee of TBZMED and MRGUMS, respectively. Written informed consent was taken from each participant. Research was carried out in compliance with the Helsinki Declaration.

Consent for publication

Not applicable.

Funding

The present study was funded by the TBZMED and MRGUMS.

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Table 1. Demographic, anthropometric, and dietary intake parameters between MUAO and MHAO patients

Variables	MUAO (n=81)	MHAO (n=83)	OR (95% CI)	*p*
Age (y)†	38.23 (8.52)	37.13 (8.64)	1.01 (0.97-1.05)	0.412 ξ
Men (%)	50.6	49.4	1.05 (0.57-1.93)	0.876 £
Physical activity score ¥	3144 (1416, 5166)	2412 (1260, 5211)	1.00 (0.99, 1.00)	0.451 ш
Weight (Kg)†	87.21 (13.90)	84.78 (13.98)	1.01 (0.99, 1.03)	0.266 ξ
Height (cm)†	165.09 (11.56)	164.56 (10.60)	1.00 (0.97, 1.03)	0.762 ξ
Waist circumference (cm) †	106.02 (8.30)	105.06 (8.63)	1.01 (0.97, 1.05)	0.470 ξ
Hip circumference (cm)†	110.90 (6.92)	111.31 (8.26)	0.99 (0.95, 1.03)	0.730 ξ
Waist to hip ratio†	0.95 (0.05)	0.94 (0.06)	2.73 (0.15, 48.00)	0.209 ξ
BMI (Kg/m^2)†	32.16 (4.25)	31.35 (4.12)	1.04 (0.97, 1.12)	0.214 ξ
Body Fat Percentage (%)†				
Males	26.86 (5.15)	25.07 (4.86)	1.07 (0.98, 1.17)	0.093 ξ
Females	38.59 (4.38)	39.88 (4.79)	0.93 (0.85, 1.03)	0.227 ξ
Body Fat Mass (kg)†				
Males	25.91 (7.27)	22.79 (6.76)	1.06 (1.00, 1.13)	0.062 ξ
Females	31.11 (7.33)	32.4 (8.63)	0.98 (0.92, 1.03)	0.441 ξ
Body Fat Free Mass (kg)†				
Males	69.09 (6.69)	66.98 (7.85)	1.04 (0.98, 1.10)	0.130 ξ
Females	48.72 (5.08)	47.61 (4.94)	1.04 (0.95, 1.14)	0.425 ξ
Total energy intake (Kcal/day)†	2152.9 (765.1)	2206.8 (862.9)	1.00 (0.97-1.04)	0.700 ξ
Carbohydrate intake (% energy)†	60.20 (10.09)	59.29 (9.11)	1.01 (0.97-1.04)	0.499 ξ
Protein intake (% energy)†	14.22 (2.95)	14.17 (4.10)	1.00 (0.91-1.09)	0.937 ξ

Total fat intake (% energy)†	25.58 (10.54)	26.54 (12.14)	0.98 (0.95-1.02)	0.380 ξ
Total SFA intake (% energy)¥	14.06 (10.6, 21.87)	14.21 (10.18, 21.49)	0.99 (0.96-1.02)	0.780 ψ
Total MUFA intake (% energy)¥	16.39 (11.01, 24.68)	18.26 (11.76-26.27)	0.99 (0.97-1.01)	0.183 ψ
Total PUFA intake (% energy)¥	12.7 (9.59-22.19)	14.41 (8.94, 19.01)	1.01 (0.98-1.03)	0.943 ψ

†Variables with normal numeric scales are reported as Mean (standard deviation).

¥ Variables with non-normal numeric scales are reported as Median (25^{th}, 75^{th}).

ξ Independent Samples t-test

ψ Mann- Whitney U test

£ Chi Square test

MHAO, Metabolically Healthy Abdominally Obese; MUAO, Metabolically Unhealthy Abdominally Obese; OR, Odds Ratio; CI, Confidence Interval; BMI, Body Mass Index; SFA, Saturated Fatty Acids; MUFA, Mono Unsaturated fatty Acids; PUFA, Poly Unsaturated Fatty Acids.

Table 2. Biochemical characteristics between MUAO and MHAO patients

Variables	MUAO (n=81)	MHAO (n=83)	OR (95% CI)	*p*
FBS (mg/dL) ¥	87.72 (5.82)	95.50 (9.76)	3.79 (2.25-6.40)*	<0.001ξ
TG (mg/dL) ¥	193 (151, 241)	112 (88, 146)	1.10 (1.05-1.15)*	<0.001Ш
HDL-C (mg/dL) †	39.53 (6.65)	46.44 (9.20)	0.32 (0.20-0.52)*	<0.001ξ
SBP (mg/dL) ¥	115 (16.45)	108.13 (16.60)	1.02 (1.00-1.04)*	0.009 ξ
DBP (mg/dL)¥	77.31 (13.86)	70.84 (12.94)	1.03 (1.01-1.06)*	0.002 ξ
Cholesterol (mg/dL)†	193.60 (41.37)	187.37 (32.91)	1.00 (0.99, 1.01)	0.286 ξ

† Variables with normal numeric scales are reported as Mean (standard deviation).

¥ Variables with non-normal numeric scales are reported as Median (25^{th}, 75^{th}).

ξ Independent Samples t- test

Ш Mann- Whitney U test

MHAO, Metabolically Healthy Abdominally Obese; MUAO, Metabolically Unhealthy Abdominally Obese; OR, Odds Ratio; CI, Confidence Interval; FBS, Fasting Blood Sugar; TG, Triglycerides; HDL-C, High-Density Lipoprotein Cholesterol; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure

Table 3. correlation of FFAs and LBP with metabolic parameters in MUAO and MHAO individuals

	MUAO		MHAO	
Variables	FFAs	LBP	FFAs	LBP
WC	0.07 (0.51)	0.02 (0.85)	0.06 (0.58)	0.03 (0.74)
TG	-0.02 (0.79)	0.008 (0.94)	-0.07 (0.48)	0.07 (0.49)
FBs	-0.005 (0.96)	-0.18 (0.09)	0.07 (0.50)	0.09 (0.40)
HDL-C	0.01 (0.91)	0.08 (0.45)	0.10 (0.33)	0.41 (0.09)
SBP	-0.03 (0.78)	-0.08 (0.46)	-0.05 (0.64)	-0.11 (0.29)
DBP	-0.08 (0.43)	-0.18 (1.00)	0.06 (0.58)	-0.09 (0.39)

P=NS, using Spearman correlation coefficient test.

MHAO, Metabolically Healthy Abdominally Obese; MUAO, Metabolically Unhealthy Abdominally Obese; FBS, Fasting Blood Sugar; TG, Triglycerides; HDL-C, High-Density Lipoprotein Cholesterol; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; LBP, Lipopolysaccharide Binding Protein; FFAs, Free Fatty Acids

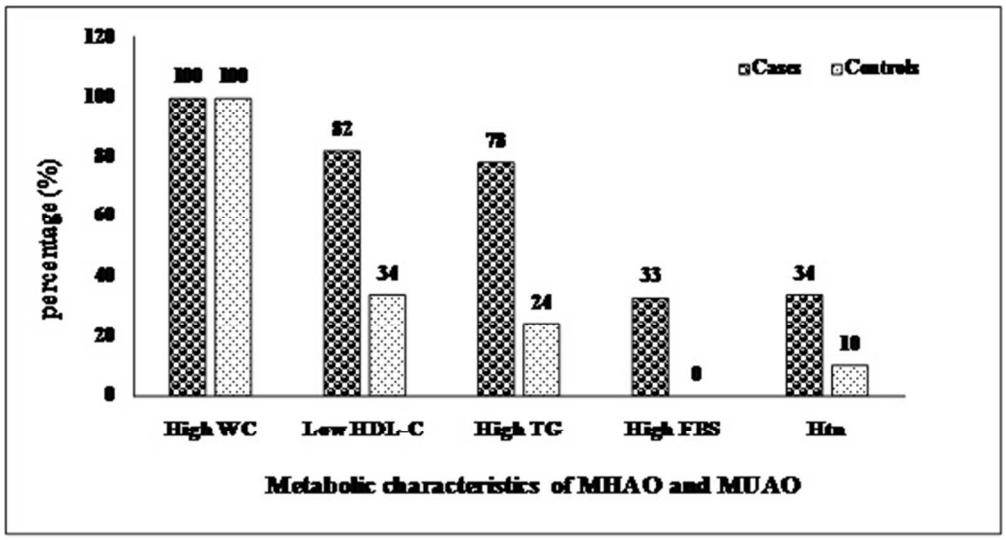

Figure 1. Metabolic characteristics of MHAO and MUAO subjects.
P<0.001 for all except WC, using X2.

MHAO, Metabolically Healthy Abdominally Obese; MUAO, Metabolically Unhealthy Abdominally Obese; FBS, Fasting Blood Sugar; TG, Triglycerides; HDL-C, High-Density Lipoprotein Cholesterol; Htn, Hypertension.

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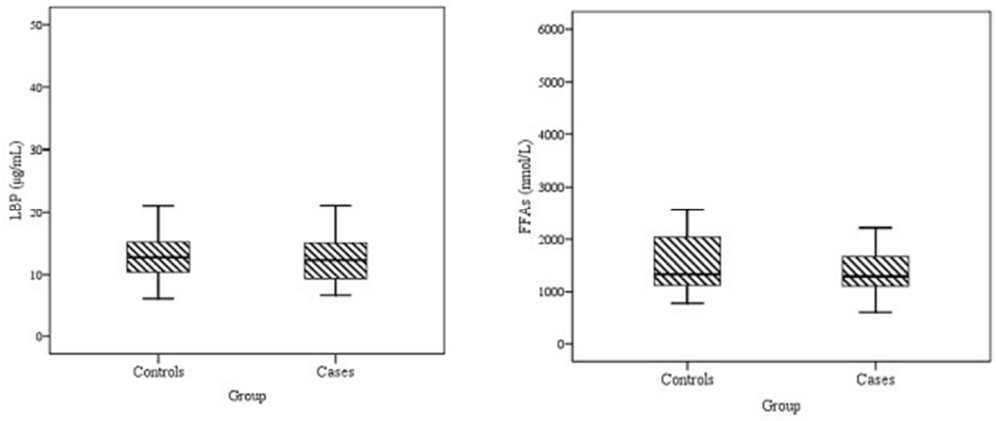

Figure 2. Lipopolysaccharide binding protein (LBP) (µg/mL) (a) and free fatty acids (FFAs) concentrations (nmol/L) (b) in subjects with MHAO (Controls) and MUAO (Cases).
P=NS
Data are presented as box plot where boxes represent the interquartile range [IQR], the line within boxes represent the median, and the lines outside the boxes represent the lower quartile minus 1.5 times the IQR or the upper quartile plus 1.5 times the IQR.

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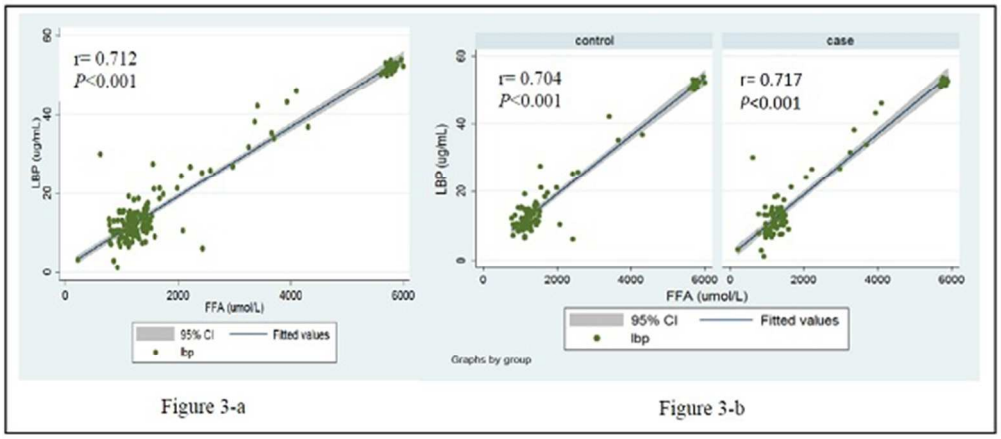

Figure 3. Spearman correlation between LBP and FFAs in pooled population (Fig. 3-a) as well as in each study group (Fig. 3-b).

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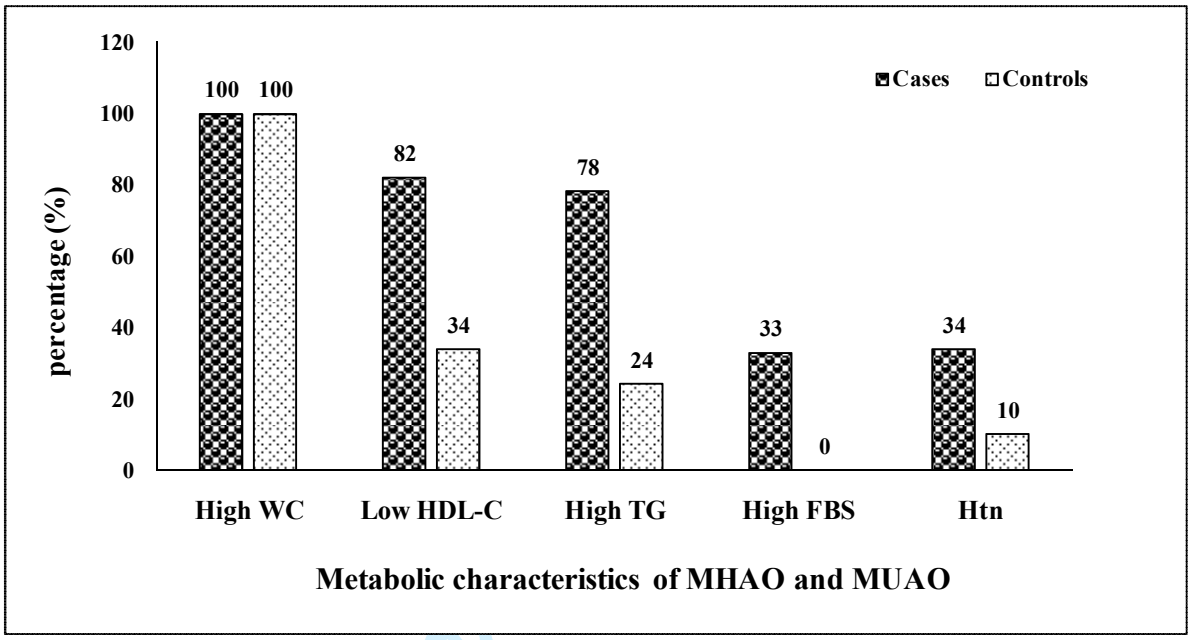
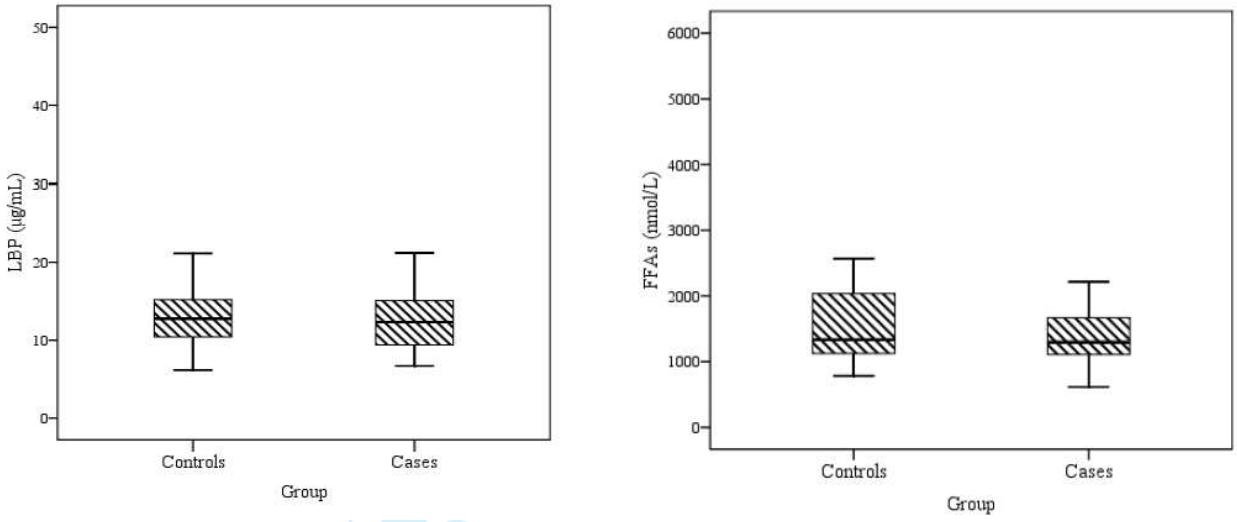


Figure 1. Metabolic characteristics of MHAO and MUAO subjects.
$P<0.001$ for all except WC, using X^2.

MHAO, Metabolically Healthy Abdominally Obese; MUAO, Metabolically Unhealthy Abdominally Obese; FBS, Fasting Blood Sugar; TG, Triglycerides; HDL-C, High-Density Lipoprotein Cholesterol; Htn, Hypertension.



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P=NS
Data are presented as box plot where boxes represent the interquartile range [IQR], the line within boxes represent the median, and the lines outside the boxes represent the lower quartile minus 1.5 times the IQR or the upper quartile plus 1.5 times the IQR.

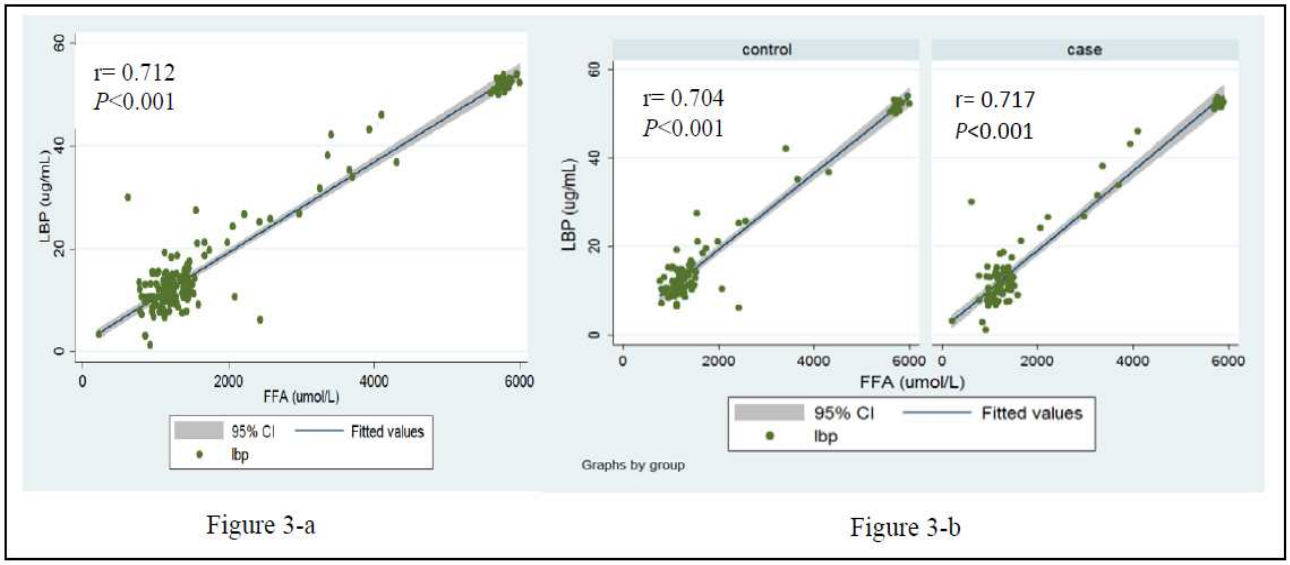

Figure 3-a Figure 3-b

Figure 3. Spearman correlation between LBP and FFAs in pooled population (Fig. 3-a) as well as in each study group (Fig. 3-b).

BMJ Open

Association of endotoxemia with serum free fatty acids in metabolically healthy and unhealthy abdominally obese individuals: a case-control study in northwest of Iran

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2017-015910.R1
Article Type:	Research
Date Submitted by the Author:	24-Feb-2017
Complete List of Authors:	Saghafi-Asl, Maryam; Tabriz University of Medical Sciences, Nutrition Research Center, Department of Biochemistry and Diet Therapy, School of Nutrition and Food Sciences, Tabriz University of Medical Sciences, Tabriz, Iran Amiri, Parichehr; Tabriz University of Medical Sciences, Student Research Committee, School of Nutrition and Food Sciences, Tabriz University of Medical Sciences, Tabriz, Iran Naghizadeh, Mahsa; Tabriz University of Medical Sciences, Student Research Committee, School of Nutrition and Food Sciences, Tabriz University of Medical Sciences, Tabriz, Iran Ghavami, Seyed Mostafa; Tabriz University of Medical Sciences, Department of Radiology, School of Paramedicine, Tabriz University of Medical Sciences, Tabriz, Iran Karamzad, Nahid; Maragheh University of Medical Sciences, Department of Public Health, School of Nursing and Midwifery, Maragheh University of Medical Sciences, Maragheh, Iran
Primary Subject Heading:	Nutrition and metabolism
Secondary Subject Heading:	Nutrition and metabolism, Public health
Keywords:	PUBLIC HEALTH, NUTRITION & DIETETICS, Diabetes & endocrinology < INTERNAL MEDICINE

Association of endotoxemia with serum free fatty acids in metabolically healthy and unhealthy abdominally obese individuals: a case-control study in northwest of Iran

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Running Title: Association of endotoxemia with serum free fatty acids

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Association of endotoxemia with serum free fatty acids in metabolically healthy and unhealthy abdominally obese individuals: a case-control study in northwest of Iran

Abstract

Objectives: This study was aimed to compare serum free fatty acids (FFAs) and lipopolysaccharide binding protein (LBP) between metabolically healthy abdominally obese (MHAO) and metabolically unhealthy abdominally obese (MUAO) individuals. We also examined the association between serum FFAs and LBP in the participants.

Methods: In this age- and gender-matched case-control study, 164 abdominally obese subjects were recruited from June to November, 2015 in the Northwest of Iran. Demographic data, dietary intake, body composition, anthropometric indices, and physical activity were assessed. Basal blood samples were collected to determine serum metabolic parameters, FFAs, and LBP. Abdominal obesity was defined as having waist circumference (WC)≥ 95cm. Those with 3 or more metabolic alterations were defined as MUAO and those having 2 or less were classified as MHAO. Data were analyzed using SPSS ver. 17.0.

Results: There were no significant differences in dietary intake, anthropometric indices, body composition, and physical activity between the two groups. The odds of MUAO significantly increased by increments in serum fasting blood sugar (OR= 3.79, 95 % CI: 2.25-6.40), TG (OR= 1.10, 95 % CI: 1.05-1.15), systolic blood pressure (OR= 1.02, 95 % CI: 1.00-1.04), and diastolic blood pressure (OR= 1.03, 95 % CI: 1.01-1.06) and decreased by increase in serum high-density lipoprotein- cholesterol (OR= 0.32, 95 % CI: 0.20-0.52). The levels of LBP and FFAs showed no significant differences between the two groups. However, significant correlations were found between LBP and FFAs in pooled population (r=0.712; *P*<0.001) as well as in cases (r=0.717; *P*<0.001) and controls (r=0.704; *P*<0.001). Neither FFAs nor LBP were significantly correlated with dietary intake or metabolic parameters (p>0.05).

Conclusion: The results indicated that serum LBP and FFAs are highly correlated both in MHAO and MUAO states. In addition, the levels of LBP and FFAs seem to be more related to abdominal obesity than to the presence or absence of metabolic health.

Key words: free fatty acids; lipopolysaccharide binding protein; metabolic health; abdominal obesity

Strengths and limitations of this study

1) The association of LBP and FFAs levels in MHAO and MUAO individuals for the first time and found significant differences between the two parameters.

2) This was a case-control study in which causality could not be assessed.

3) Insulin levels were not measured in our study population; therefore, insulin resistance was not studied.

4) The present work was carried out on volunteer participants. Though all volunteers were randomly recruited from general population after public announcement and based on the eligible criteria.

Introduction

Obesity is increasingly prevalent worldwide[1]. There are well-established health consequences of obesity such as type 2 diabetes, metabolic syndrome (MetS), and cardiovascular disease [2]. However, not all obese people are at higher risk of metabolic diseases. In a subtype of obese persons, described as "metabolically healthy obese (MHO)", the obese phenotype may exist devoid of metabolic dysfunction [3]. Despite there is still no uniform definition for MHO, it is thought to account for approximately one fifth of the obese population [4].

Evidence increasingly identifies inflammation as a potential mechanism linking adiposity especially abdominal fat and metabolic dysfunction [5]. However, published results are rare and conflicting regarding the role of inflammation in the metabolic differences observed between metabolically healthy and unhealthy individuals [6-7].. Studies on postmenopausal obese women suggest that the MHO may have more favorable inflammatory profiles [8] and less visceral fat [9] than their counterparts with insulin resistance (IR) and other metabolic abnormalities [10]. In contrast, another stduy [11] reported that MHO women displayed abnormal levels of inflammatory profile, despite not having increased 10-year risk of cardiovascular disease.

Basic mechanism accounted for inflammation in adipose tissue is still unknown, but some factors including plasma free fatty acids (FFAs) are suggested [12]. It was clarified that plasma FFAs are increased among the obese as they are released from inflamed adipose tissue [13] and through the lipolysis of adipocytes [14]. However, little is known about the contribution of FFAs to the development of inflammation in obesity. Therefore, examining the association of FFAs with inflammatory markers seems to be warranted.

Lipopolysaccharide (LPS) molecules, also known as bacterial endotoxins, may trigger inflammation, leading to activation of immunity and cytokine release. LPS infusion and consequent subclinical endotoxemia results in elevated levels of proinflammatory markers and metabolic aberrations [15-16]. LPS has a short half-life [17] and there is no agreement on the measurement of its plasma level [18]. Hence, lipopolysaccharide-

binding protein (LBP) is introduced with longer half-life and more reliable measurement [19-20]. Also, serum LBP level is a proxy of serum LPS level [21]. A population-based study [22] found that LBP was significantly associated with MetS in normal-weight participants. Another study [23] reported that among MetS components, LBP concentration was independently associated with abdominal obesity.

In prior studies, inflammatory parameters were compared between obese and lean subjects [19, 24-25]. Therefore, it remains unclear whether the observed alterations in serum FFAs and/or inflammatory parameters in metabolically unhealthy obese patients are due to adiposity and/or metabolic state. Therefore, regarding the significant confounding effect of abdominal obesity, we used waist circumference (WC), as a reflection of visceral adipose tissue [26], to define abdominal obesity, and examined differences in characteristics and inflammatory markers (serum LBP and FFAs) between "metabolically healthy" and "unhealthy" abdominally obese individuals. We also examined the association between serum FFAs and LBP in pooled population as well as in each group.

Methods

Study design and participants

A total of 81 metabolically healthy abdominally obese (MHAO) with 83 age- and gender-matched metabolically unhealthy abdominally obese (MUHAO) were recruited in this case-control study, carried out from June 15th to November 6th, 2015 in the Northwest of Iran. Frequency matching was carried out for the present study. Apparently healthy individuals aged 18-60 years with abdominal obesity were included in the study. We excluded pregnant or lactating women, those with diarrhea for 3 consecutive days within the previous 3 months, diagnosed diabetes, coronary heart disease, stroke, myocardial infarction, cardiovascular and kidney, liver or infectious diseases including tuberculosis, AIDS, and hepatitis; thyroid problems, severe mental disorders or physical disabilities and malignancies; taking oral antidiabetic agents or insulin or other drugs for the last 2 months, or antibiotics used for 3 consecutive days within the

previous 3 months; smokers or alcohol consumers; misreported dietary intakes (less than 800 kcal/d or more than 4200 kcal/d), or being on specific diets in the past 6 months; and having gastrointestinal surgery within past 1 year.

Abdominal obesity was defined as having waist circumference (WC)≥95cm according to the Iranian National Committee of Obesity [27]. According to Meigs *et al.*[28], metabolic health was defined as the presence of <3 of the following metabolic abnormalities including abdominal obesity (WC≥95cm for both genders)[27]; high serum triglyceride (TG) concentration (≥150 mg/dL); low serum high density lipoprotein cholesterol (HDL-C) (<40 mg/dL for men and <50 mg/dL for women); elevated blood pressure (BP) (≥130/85 mmHg); and fasting blood sugar (FBS) (≥100 mg/dL). Eighty-one individuals with ≥3 criteria entered the case group (MUAO) and 83 with 2 or less criteria formed the control group (MHAO).

Sampling procedures

After public announcement for the study, 500 volunteers were recruited from general population. Of these, 178 people could enter the study based on the defined eligibility criteria for the present study. Informed consent was taken from each participant before the study. After taking blood samples and anthropometric measurements, 14 of them were excluded due to diabetes (FBS≥126 mg/dl [29] in two occasions), leaving 164 people (82 men, 82 women) to conduct the research.

Biochemical assays

After a 12-h overnight fast, 5cc blood were obtained for serum analyses. After centrifugation at 3000 rpm for 5 min, metabolic parameters were analyzed immediately, butserum FFAs and LBP were analyzed after supplying in -80°C.

FBS was measured by the enzymatic colorimetric method using glucose oxidase. Serum TG concentration was measured by commercially available enzymatic reagents with glycerol phosphate oxidase. Serum HDL-C was measured after precipitation of the apolipoprotein B containing lipoproteins with phosphotungistic

acid. Assays were performed using Pars Azmoon kits (Pars Azmoon Inc., Tehran, Iran) and a Selectra 2 auto-analyzer (Vital Scientific, Spankeren, Netherlands). Inter- and intra- assay coefficient of variation (CV) was < 5% for all assays. Serum samples for both LBP and FFAs assays were stored at -80°C until analysis. Both serum LBP and FFAs levels were determined by a sandwich ELISA (Bioassay Technology Laboratory, Shanghai Korean Biotech Co., LTD; Shanghai City, China) according to the manufacturer's instructions. The intra-assay and inter-assay coefficients of variation were <8 and <10%, respectively.

Measurements

All anthropometric indices were measured by a trained researcher. Height (without shoes in standard situation with precision of 0.1 cm and with an inelastic measuring tape) and weight (with Seca scale, light clothes and precision of 0.1 kg) were measured and BMI was calculated as weight in kg divided by the square of height in meters [30]. WC was measured using a non-stretchable fiber measuring tape. The subjects were asked to stand erect in relaxed position with both feet together on flat surface. WC was measured as the smallest horizontal girth between the costal and iliac crests at minimal respiration. Hip circumference was taken as the greatest circumference at the level of greater trochanters (the widest portion of the hip) on both sides. Waist to hip ratio (WHR) was calculated by dividing WC (cm) by hip circumference (cm) [31]. BP was recorded in a comfortable sitting position in the left arm after at least a 5-min rest, using the mercury sphygmomanometer. Two measurements were taken and the mean of the two measurements was considered as the BP [32]. Bioelectrical Impedance Analysis (BIA: BC-418MA, Tanita, Japan) was used to describe fat percent, fat Mass (FM), and fat free mass (FFM). Dietary intake was assessed using a 3-day food record (one weekend day and two workdays). *Nutritionist IV* software (Axxya Systems, Stafford, TX), modified for Iranian foods, were used for dietary data analysis. Physical activity (PA) was measured via IPAQ-long form questionnaire [33].

Statistical analysis and sample size

To examine the normal distribution of variables, Kolmogrov-Smirnov tests and histograms were applied. The independent samples t-test was used to compare the means (SD) of normally distributed variables between the two groups. The Mann–Whitney U test was used for values with skewed distribution and in such conditions, median (25^{th}, 75^{th}) was reported. In order to assess the association of two categorical variables, Chi-square test was applied. The correlation between serum FFAs and LBP was assessed using Spearman correlation coefficient analysis. Odds ratios and their 95% confidence intervals (CI) were reported using Logistic Regression test.

The larger sample size was calculated for serum FFAs compared to LBP, using literature-derived data [34] for nonalcoholic fatty liver disease (NAFLD) patients; the effect size for serum FFAs was 0.20 nmol/l (SD1=0.34 nmol/l and SD2=0.53 nmol/l). Therefore, sample size estimation was based upon this parameter with 80% power and α-error of 5% and a case to control ratio of 1:1. It was predicted that 79 persons in each group would detect changes in serum FFAs as well as serum LBP level, using the two-means formula. Data were analyzed using SPSS ver. 17.0 for Windows (PASW Statistics; SPSS Inc., Chicago, IL, USA). P-value less than 0.05 was considered significant.

Results

Males comprised about 50% of the study participants in the two groups (p=0.87). The age range of the subjects was 20-59 years. Participants of the two study groups similarly had WC≥95 cm i.e. the cut-off point of WC for Iranian population. Overall, there were no significant differences in age, gender, anthropometric indices, and body composition between the two groups. Dietary parameters especially total fat, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) intakes were more or less the same between the two study groups (Table 1). Marital status, education level, and job of the cases and controls were also similar. However, mean number of metabolic aberrations were significantly higher in cases than controls (3.25±0.72 vs. 1.67±0.50; P<0.001) (data not shown). The flow chart of the study is shown in Figure 1.

Except for WC which was matched between the two groups, metabolic aberrations including low HDL-C (82% vs. 34%), high TG (78% vs. 24%), high FBS (33% vs. 0%), and hypertension (34% vs. 10%) were significantly higher in the cases than controls, respectively (Figure 2). The current study indicated that each 10 unit increment in serum FBS level increased the risk of MUAO about 3.8 times (OR= 3.79, 95 % CI: 2.25-6.40). Additionally, the odds of MUAO was significantly increased per one increment in serum TG level (OR= 1.10, 95 % CI: 1.05-1.15), the systolic BP (OR= 1.02, 95 % CI: 1.00-1.04), and diastolic BP (OR= 1.03, 95 % CI: 1.01-1.06). However, the odds of having MUAO was significantly decreased by 68% per 10 unit increment in serum HDL-C level (OR= 0.32, 95 % CI: 0.20-0.52) (Table 2).

The median of LBP (12.32 µg/mL in cases vs. 12.76 µg/mL in controls, *P*=0.483) and FFAs (1294 nmol/L in cases vs. 1333 nmol/L in controls; *P*=0.686) showed no significant difference between the two groups (Figure 3). However, a significant correlation was found between LBP and FFAs in pooled population (r=0.712; *P*<0.001) as well as in cases (r=0.717; *P*<0.001) and controls (r=0.704; *P*<0.001) (Figure 4). The results of partial correlation indicated much stronger correlation between LBP and FFAs, when controlling for WC (r=0.961; *P*<0.001). Moreover, number of metabolic aberrations were significantly correlated with HDL-C (r=-0.537; *P*<0.001), TG (r=0.468; *P*<0.001), FBS (r=0.534; *P*<0.001), Systolic BP (r=0.247; *P*=0.001), and Diastolic BP (r=0.315; *P*<0.001). Neither FFAs nor LBP were significantly correlated with dietary intake of total fat, SFA, MUFA, and PUFA (data not shown). There were also no significant correlations of LBP and FFAs with metabolic parameters (Table 3).

Discussion

The present study, to the best of our knowledge, examined the association of LBP and FFAs levels in MHAO and MUAO individuals for the first time and found significant differences between the two parameters. Anthropometric indices as well as body composition profile were similar between the two groups. Moreover, there were no significant differences in LBP and FFAs between MHAO and MUAO.

In the present study, we matched metabolically healthy with metabolically unhealthy individuals on abdominal fat which might explain why we did not find any differences in levels of FFAs and LBP, as inflammatory markers, and body composition between the two groups. Therefore, the levels of LBP and FFAs seem to be more related to abdominal obesity than to the presence or absence of metabolic health. This is further supported by the observation that serum FFAs or LBP levels were not correlated with metabolic parameters. Therefore, our findings suggest that increased levels of these two markers are not necessarily related to the presence of metabolic aberrations.

A few smaller studies have examined differences in body composition and/or inflammatory profile between metabolically healthy and unhealthy obese postmenopausal women [35-37]. In line with our result, Engström *et al.* [36] in a research on 58 obese postmenopausal women found no significant differences in levels of inflammatory markers between those with metabolic syndrome (MetS) compared to those without MetS. Additionally, in the population-based study of Philips *et al.* [38], no significant difference was noted in CRP level between MHAO and MUAO, based on metabolic health criteria of Meigs *et al.* [28] study. It is noteworthy that in the present study we used Meigs's metabolic health definition in which WC has also been considered.

A recent study revealed that the association between inflammatory biomarkers and metabolically healthy obesity depends on the criteria used. Since in that research, a significant difference was noted in the levels of CRP and IL-6 with some but not all MHAO definitions, which disappeared after adjustment for abdominal obesity or percent body fat [39]. This study confirms our results. However, Phillips *et al.* [40] showed that obese women and men with MetS had significantly higher levels of inflammatory cytokines than obese persons without MetS. Beasley *et al.* [41] showed that visceral adiposity, and not abdominal subcutaneous fat, was most consistently associated with significantly higher levels of IL-6 and CRP levels in black and white men and women in the Health ABC study. We could not measure visceral fat in our study, though, abdominal obesity measured through WC, can reflect visceral adiposity [26]. On the other hand, a recent

work observed no significant differences in visceral fat between the obese-insulin resistant and obese-insulin sensitive persons [42].

Several studies have demonstrated a strong association of IR with obesity, low HDL-C, hypertriglyceridemia, and hypertension [10, 43] as well as inflammatory factors [44]. However, in our study, we could not assess IR, due to some financial deficits.

In the present work, dietary intake was compared between the two obese groups; therefore, no significant difference was found in terms of energy or macronutrients especially fat intake. Moreover, habitual physical activity (PA) was controlled between the study groups. And, in our previous report, there were no significant differences between the two groups in terms of PA (unpublished data). Since different levels of habitual PA might affect levels of serum inflammatory markers [45].

Obesity, as a well-known metabolic risk factor, is usually associated with mild chronic inflammation [46]. The relationship between obesity and increased inflammation may be justified, in part, by FFAs [47] which are released from adipocytes through lipolysis and are elevated in obesity due to increased adipose tissue [48]. Inflammatory cytokines such as interleukin-6 (IL-6) can stimulate lipolysis and increase levels of FFAs [49]. On the other hand, in healthy persons an acute increase in FFAs can induce inflammatory changes [50]. Therefore, FFAs are not only increased by inflammation, but also promote inflammation. The results of the present research showed that FFAs are positively correlated with LBP levels either in the pooled population or in each group. It shows that any increase in the level of FFAs, observed in the abdominally obese, regardless of their metabolic aberrations can lead to a significant elevation in the level of LBP.

LBP has been considered a key inflammatory marker which mediates LPS-triggered innate immunity [51]. Although LBP concentration was previously reported to be associated with various anthropometric and metabolic factors such as BMI, WC, and so on [23-25], in our study the relationship only existed between the two biomarkers, FFAs and LBP, but not with the metabolic or anthropometric parameters. It is notable that the positive relationship between LBP and BMI was not observed in either normal weight or obese groups

in Yang *et al.* [52] study after multivariate analyses. In their research, the level of LBP significantly reduced after bariatric surgery and consequent reduction in WC (from 121.6 cm to 90.6 cm; P<0.001) which indicates the strong association of LBP with WC. Liu *et al.* [22] in a population-based follow-up study on 2529 Chinese also found that the association of LBP with MetS was significant only in normal-weight participants, but not in their overweight/obese counterparts after multivariate adjustments including BMI, which supports our study findings. It is assumed that the association between serum LBP level and MetS observed in previous studies [19, 24-25] is mediated by BMI or WC, and finding no association between serum LBP level and incidence of MetS in our study, in which the WC-matched controls were included, is not unexpected.

Overall, what makes our research different from most of previous ones is that in our study we matched the two groups based on WC, rarely observed in prior reports. Most of the previous studies have examined either MetS patients *vs.* those without the syndrome or metabolically healthy *vs*. metabolically unhealthy, regardless of their BMI or WC status and based on different metabolic health criteria[3, 8, 28]. A few have examined inflammatory markers between metabolically healthy and unhealthy persons, considering WC or abdominal obesity [36, 38, 39].

Conclusion

Our study indicated that WC could be a strong mediator of the association between serum LBP, FFAs, and metabolic alterations. In fact, the levels of LBP and FFAs seem to be more related to abdominal obesity than to the presence or absence of metabolic health. The results also suggested a significant correlation between serum FFAs and LBP in abdominally obese population, which seems to be independent of metabolic aberrations.

Competing interests:

The authors have none to declare.

Authors' contributions

MSA and NK conceived the study design and wrote the study protocol. MSA and NK analyzed and interpreted the data. MSA, PA, MN, SMG and NK were involved in drafting the manuscript or revising it critically for content. All authors have given final approval of the version to be published.

AcknowledgmentsWe would like to thank those who participated in the present study.

Ethical approval

All protocols were approved by regional and organizational ethics committee of TBZMED and MRGUMS, respectively. Written informed consent was taken from each participant. Research was carried out in compliance with the Helsinki Declaration.

Consent for publication

Not applicable.

Funding

This work was financially supported by Research Vice-Chancellor, Tabriz University of Medical Sciences and Maragheh University of Medical Sciences, Iran. The results of this paper were extracted from MSc. Thesis of Nahid Karamzad, registered at Tabriz University of Medical Sciences.

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Table 1. Demographic, anthropometric, and dietary intake parameters in MUAO and MHAO patients

Variables	MUAO (n=81)	MHAO (n=83)	OR (95% CI)	*p*
Age (y)†	38.23 (8.52)	37.13 (8.64)	1.01 (0.97-1.05)	0.412 ξ
Men (%)	50.6	49.4	1.05 (0.57-1.93)	0.876 £
Physical activity score ¥	3144 (1416, 5166)	2412 (1260, 5211)	1.00 (0.99, 1.00)	0.451 ш
Weight (Kg)†	87.21 (13.90)	84.78 (13.98)	1.01 (0.99, 1.03)	0.266 ξ
Height (cm)†	165.09 (11.56)	164.56 (10.60)	1.00 (0.97, 1.03)	0.762 ξ
Waist circumference (cm) †	106.02 (8.30)	105.06 (8.63)	1.01 (0.97, 1.05)	0.470 ξ
Hip circumference (cm)†	110.90 (6.92)	111.31 (8.26)	0.99 (0.95, 1.03)	0.730 ξ
Waist to hip ratio†	0.95 (0.05)	0.94 (0.06)	2.73 (0.15, 48.00)	0.209 ξ
BMI (Kg/m^2)†	32.16 (4.25)	31.35 (4.12)	1.04 (0.97, 1.12)	0.214 ξ
Body Fat Percentage (%)†				
Males	26.86 (5.15)	25.07 (4.86)	1.07 (0.98, 1.17)	0.093 ξ
Females	38.59 (4.38)	39.88 (4.79)	0.93 (0.85, 1.03)	0.227 ξ
Body Fat Mass (kg)†				
Males	25.91 (7.27)	22.79 (6.76)	1.06 (1.00, 1.13)	0.062 ξ
Females	31.11 (7.33)	32.4 (8.63)	0.98 (0.92, 1.03)	0.441 ξ
Body Fat Free Mass (kg)†				
Males	69.09 (6.69)	66.98 (7.85)	1.04 (0.98, 1.10)	0.130 ξ
Females	48.72 (5.08)	47.61 (4.94)	1.04 (0.95, 1.14)	0.425 ξ
Total energy intake (Kcal/day)†	2152.9 (765.1)	2206.8 (862.9)	1.00 (0.97-1.04)	0.700 ξ
Carbohydrate intake (% energy)†	60.20 (10.09)	59.29 (9.11)	1.01 (0.97-1.04)	0.499 ξ
Protein intake (% energy)†	14.22 (2.95)	14.17 (4.10)	1.00 (0.91-1.09)	0.937 ξ
Total fat intake (% energy)†	25.58 (10.54)	26.54 (12.14)	0.98 (0.95-1.02)	0.380 ξ

Total SFA intake (% energy)[¥]	14.06 (10.6, 21.87)	14.21 (10.18, 21.49)	0.99 (0.96-1.02)	0.780 [Ш]
Total MUFA intake (% energy)[¥]	16.39 (11.01, 24.68)	18.26 (11.76-26.27)	0.99 (0.97-1.01)	0.183 [Ш]
Total PUFA intake (% energy)[¥]	12.7 (9.59-22.19)	14.41 (8.94, 19.01)	1.01 (0.98-1.03)	0.943 [Ш]

[†]Variables with normal numeric scales are reported as Mean (standard deviation).

[¥] Variables with non-normal numeric scales are reported as Median (25^{th}, 75^{th}).

[ξ] Independent Samples t-test

[Ш] Mann- Whitney U test

[£] Chi Square test

MHAO, Metabolically Healthy Abdominally Obese; MUAO, Metabolically Unhealthy Abdominally Obese; OR, Odds Ratio; CI, Confidence Interval; BMI, Body Mass Index; SFA, Saturated Fatty Acids; MUFA, Mono Unsaturated fatty Acids; PUFA, Poly Unsaturated Fatty Acids.

Table 2. Biochemical characteristics in MUAO and MHAO patients

Variables	MUAO (n=81)	MHAO (n=83)	OR (95% CI)	*p*
FBS (mg/dL) ¥	87.72 (5.82)	95.50 (9.76)	3.79 (2.25-6.40)*	<0.001[ξ]
TG (mg/dL) ¥	193 (151, 241)	112 (88, 146)	1.10 (1.05-1.15)*	<0.001[Ψ]
HDL-C (mg/dL) †	39.53 (6.65)	46.44 (9.20)	0.32 (0.20-0.52)*	<0.001[ξ]
SBP (mg/dL) ¥	115 (16.45)	108.13 (16.60)	1.02 (1.00-1.04)*	0.009[ξ]
DBP (mg/dL)¥	77.31 (13.86)	70.84 (12.94)	1.03 (1.01-1.06)*	0.002[ξ]
Cholesterol (mg/dL)†	193.60 (41.37)	187.37 (32.91)	1.00 (0.99, 1.01)	0.286[ξ]

† Variables with normal numeric scales are reported as Mean (standard deviation).

¥ Variables with non-normal numeric scales are reported as Median (25^{th}, 75^{th}).

[ξ] Independent Samples t- test

[Ψ] Mann- Whitney U test

MHAO, Metabolically Healthy Abdominally Obese; MUAO, Metabolically Unhealthy Abdominally Obese; OR, Odds Ratio; CI, Confidence Interval; FBS, Fasting Blood Sugar; TG, Triglycerides; HDL-C, High-Density Lipoprotein Cholesterol; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure

Table 3. correlation of FFAs and LBP with metabolic parameters in MUAO and MHAO individuals

	MUAO		MHAO	
Variables	FFAs	LBP	FFAs	LBP
WC	0.07 (0.51)	0.02 (0.85)	0.06 (0.58)	0.03 (0.74)
TG	-0.02 (0.79)	0.008 (0.94)	-0.07 (0.48)	0.07 (0.49)
FBs	-0.005 (0.96)	-0.18 (0.09)	0.07 (0.50)	0.09 (0.40)
HDL-C	0.01 (0.91)	0.08 (0.45)	0.10 (0.33)	0.41 (0.09)
SBP	-0.03 (0.78)	-0.08 (0.46)	-0.05 (0.64)	-0.11 (0.29)
DBP	-0.08 (0.43)	-0.18 (1.00)	0.06 (0.58)	-0.09 (0.39)

P=NS, using Spearman correlation coefficient test.

MHAO, Metabolically Healthy Abdominally Obese; MUAO, Metabolically Unhealthy Abdominally Obese; FBS, Fasting Blood Sugar; TG, Triglycerides; HDL-C, High-Density Lipoprotein Cholesterol; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; LBP, Lipopolysaccharide Binding Protein; FFAs, Free Fatty Acids

Figure 1. Flowchart of the study

Figure 2. Metabolic characteristics of MHAO and MUAO subjects.

$p<0.001$ for all except WC, using X^2.

MHAO, Metabolically Healthy Abdominally Obese; MUAO, Metabolically Unhealthy Abdominally Obese; FBS, Fasting Blood Sugar; TG, Triglycerides; HDL-C, High-Density Lipoprotein Cholesterol; Htn, Hypertension.

Figure 3. Lipopolysaccharide binding protein (LBP) (µg/mL) (a) and free fatty acids (FFAs) concentrations (nmol/L) (b) in subjects with MHAO (Controls) and MUAO (Cases).
p=NS
Data are presented as box plot where boxes represent the interquartile range [IQR], the line within boxes represent the median, and the lines outside the boxes represent the lower quartile minus 1.5 times the IQR or the upper quartile plus 1.5 times the IQR.

Figure 4. Spearman correlation between LBP and FFAs in pooled population (Fig. 4-a) as well as in each study group (Fig. 4-b).

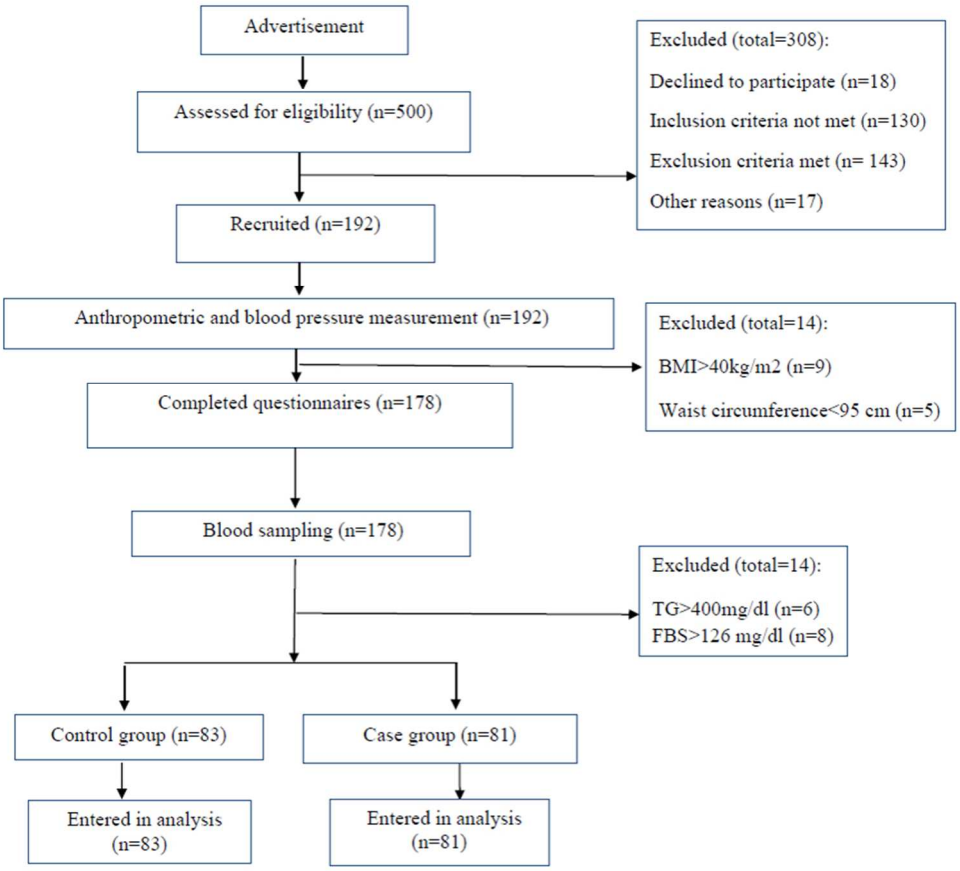

Figure 1. Flowchart of the study

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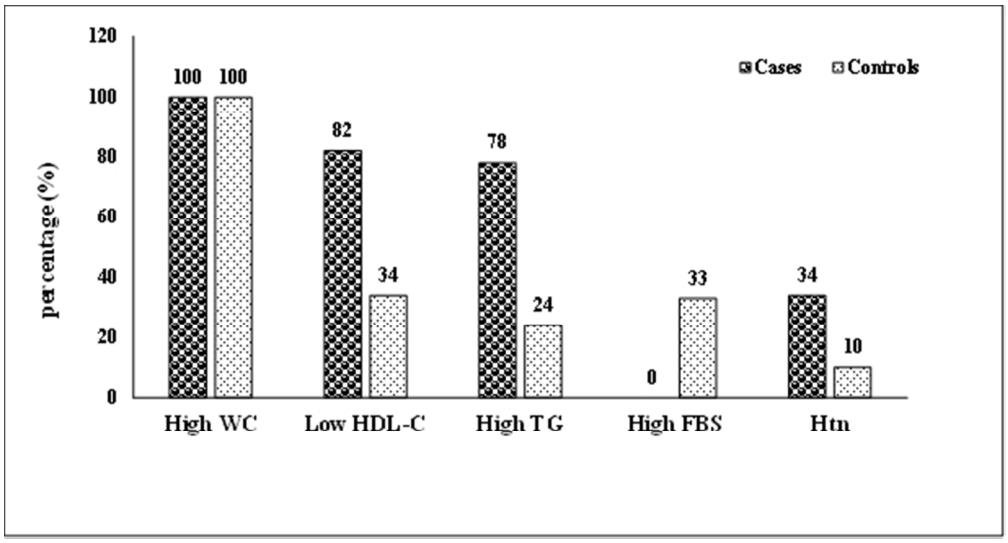

Figure 2. Metabolic characteristics of MHAO and MUAO subjects.┼ P<0.001 for all except WC, using X2.┼ ┼ MHAO, Metabolically Healthy Abdominally Obese; MUAO, Metabolically Unhealthy Abdominally Obese; FBS, Fasting Blood Sugar; TG, Triglycerides; HDL-C, High-Density Lipoprotein Cholesterol; Htn, Hypertension.┼ ┼

51x27mm (300 x 300 DPI)

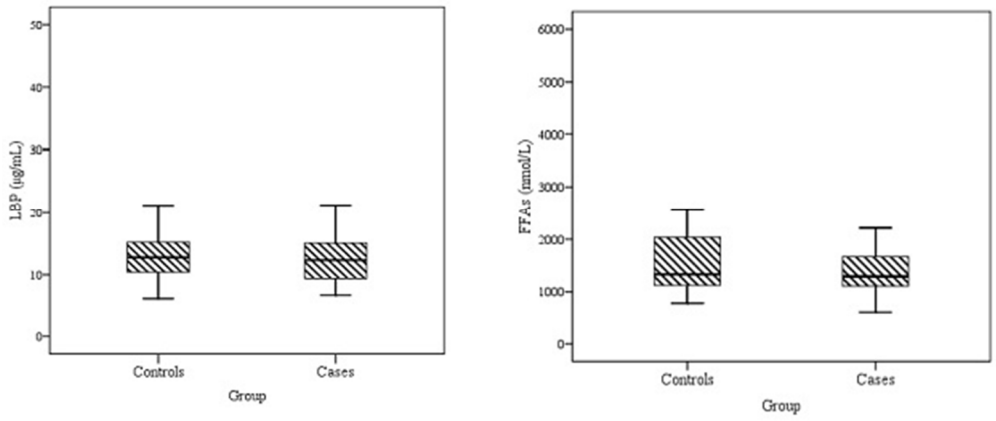

Figure 3. Lipopolysaccharide binding protein (LBP) (µg/mL) (a) and free fatty acids (FFAs) concentrations (nmol/L) (b) in subjects with MHAO (Controls) and MUAO (Cases). ┼ P=NS┼ Data are presented as box plot where boxes represent the interquartile range [IQR], the line within boxes represent the median, and the lines outside the boxes represent the lower quartile minus 1.5 times the IQR or the upper quartile plus 1.5 times the IQR.┼ ┼

52x22mm (300 x 300 DPI)

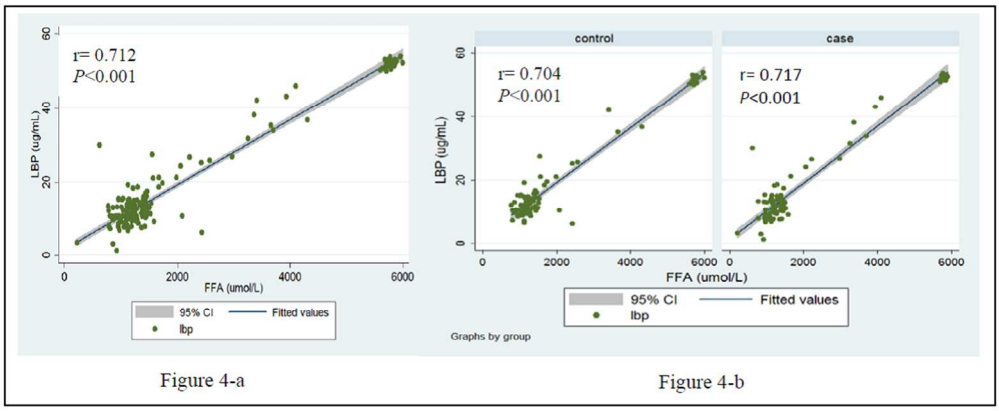

Figure 4. Spearman correlation between LBP and FFAs in pooled population (Fig. 4-a) as well as in each study group (Fig. 4-b).

96x39mm (300 x 300 DPI)

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *case-control studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	2
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2, 3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4, 5
Objectives	3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Participants	6	(a) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	5, 6
		(*b*) For matched studies, give matching criteria and the number of controls per case	5
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	6,7
Bias	9	Describe any efforts to address potential sources of bias	3
Study size	10	Explain how the study size was arrived at	8
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	8
		(*b*) Describe any methods used to examine subgroups and interactions	Not applicable
		(*c*) Explain how missing data were addressed	Not applicable
		(*d*) If applicable, explain how matching of cases and controls was addressed	5
		(*e*) Describe any sensitivity analyses	Not applicable
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	6
		(b) Give reasons for non-participation at each stage	6
		(c) Consider use of a flow diagram	Figure 1
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	Table 1
		(b) Indicate number of participants with missing data for each variable of interest	Table 1
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure	Table 1,2
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	Table 1, 2
		(*b*) Report category boundaries when continuous variables were categorized	6
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	Not applicable
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	Not applicable
Discussion			
Key results	18	Summarise key results with reference to study objectives	9
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	3
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	10-12
Generalisability	21	Discuss the generalisability (external validity) of the study results	3, 12
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	13

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Association of endotoxemia with serum free fatty acids in metabolically healthy and unhealthy abdominally obese individuals: a case-control study in northwest of Iran

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2017-015910.R2
Article Type:	Research
Date Submitted by the Author:	24-Mar-2017
Complete List of Authors:	Saghafi-Asl, Maryam; Tabriz University of Medical Sciences, Nutrition Research Center, Department of Biochemistry and Diet Therapy, School of Nutrition and Food Sciences, Tabriz University of Medical Sciences, Tabriz, Iran Amiri, Parichehr; Tabriz University of Medical Sciences, Student Research Committee, School of Nutrition and Food Sciences, Tabriz University of Medical Sciences, Tabriz, Iran Naghizadeh, Mahsa; Tabriz University of Medical Sciences, Student Research Committee, School of Nutrition and Food Sciences, Tabriz University of Medical Sciences, Tabriz, Iran Ghavami, Seyed Mostafa; Tabriz University of Medical Sciences, Department of Radiology, School of Paramedicine, Tabriz University of Medical Sciences, Tabriz, Iran Karamzad, Nahid; Maragheh University of Medical Sciences, Department of Public Health, School of Nursing and Midwifery, Maragheh University of Medical Sciences, Maragheh, Iran
Primary Subject Heading:	Nutrition and metabolism
Secondary Subject Heading:	Nutrition and metabolism, Public health
Keywords:	PUBLIC HEALTH, NUTRITION & DIETETICS, Diabetes & endocrinology < INTERNAL MEDICINE

Association of endotoxemia with serum free fatty acids in metabolically healthy and unhealthy abdominally obese individuals: a case-control study in northwest of Iran

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Running Title: Association of endotoxemia with serum free fatty acids

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Association of endotoxemia with serum free fatty acids in metabolically healthy and unhealthy abdominally obese individuals: a case-control study in northwest of Iran

Abstract

Objectives: This study was aimed to compare serum free fatty acids (FFAs) and lipopolysaccharide binding protein (LBP) between metabolically healthy abdominally obese (MHAO) and metabolically unhealthy abdominally obese (MUAO) individuals. We also examined the association between serum FFAs and LBP in the participants.

Methods: In this age- and gender-matched case-control study, 164 abdominally obese subjects were recruited from June to November, 2015 in the Northwest of Iran. Demographic data, dietary intake, body composition, anthropometric indices, and physical activity were assessed. Basal blood samples were collected to determine serum metabolic parameters, FFAs, and LBP. Abdominal obesity was defined as having waist circumference (WC)≥ 95cm. Those with 3 or more metabolic alterations were defined as MUAO and those having 2 or less were classified as MHAO. Data were analyzed using SPSS ver. 17.0.

Results: There were no significant differences in dietary intake, anthropometric indices, body composition, and physical activity between the two groups. The odds of MUAO significantly increased by increments in serum fasting blood sugar (OR= 3.79, 95 % CI: 2.25-6.40), TG (OR= 1.10, 95 % CI: 1.05-1.15), systolic blood pressure (OR= 1.02, 95 % CI: 1.00-1.04), and diastolic blood pressure (OR= 1.03, 95 % CI: 1.01-1.06) and decreased by increase in serum high-density lipoprotein- cholesterol (OR= 0.32, 95 % CI: 0.20-0.52). The levels of LBP and FFAs showed no significant differences between the two groups. However, significant correlations were found between LBP and FFAs in pooled population (r=0.712; *P*<0.001) as well as in cases (r=0.717; *P*<0.001) and controls (r=0.704; *P*<0.001). Neither FFAs nor LBP were significantly correlated with dietary intake or metabolic parameters (p>0.05).

Conclusion: The results indicated that serum LBP and FFAs are highly correlated both in MHAO and MUAO states. In addition, the levels of LBP and FFAs seem to be more related to abdominal obesity than to the presence or absence of metabolic health.

Key words: free fatty acids; lipopolysaccharide binding protein; metabolic health; abdominal obesity

Strengths and limitations of this study

1) The association of LBP and FFAs levels in MHAO and MUAO individuals for the first time and found significant differences between the two parameters.

2) This was a case-control study in which causality could not be assessed.

3) Insulin levels were not measured in our study population; therefore, insulin resistance was not studied.

4) The present work was carried out on volunteer participants. Though all volunteers were randomly recruited from general population after public announcement and based on the eligible criteria.

Introduction

Obesity is increasingly prevalent worldwide[1]. There are well-established health consequences of obesity such as type 2 diabetes, metabolic syndrome (MetS), and cardiovascular disease [2]. However, not all obese people are at higher risk of metabolic diseases. In a subtype of obese persons, described as "metabolically healthy obese (MHO)", the obese phenotype may exist devoid of metabolic dysfunction [3]. Despite there is still no uniform definition for MHO, it is thought to account for approximately one fifth of the obese population [4].

Evidence increasingly identifies inflammation as a potential mechanism linking adiposity especially abdominal fat and metabolic dysfunction [5]. However, published results are rare and conflicting regarding the role of inflammation in the metabolic differences observed between metabolically healthy and unhealthy individuals [6-7].. Studies on postmenopausal obese women suggest that the MHO may have more favorable inflammatory profiles [8] and less visceral fat [9] than their counterparts with insulin resistance (IR) and other metabolic abnormalities [10]. In contrast, another stduy [11] reported that MHO women displayed abnormal levels of inflammatory profile, despite not having increased 10-year risk of cardiovascular disease.

Basic mechanism accounted for inflammation in adipose tissue is still unknown, but some factors including plasma free fatty acids (FFAs) are suggested [12]. It was clarified that plasma FFAs are increased among the obese as they are released from inflamed adipose tissue [13] and through the lipolysis of adipocytes [14]. However, little is known about the contribution of FFAs to the development of inflammation in obesity. Therefore, examining the association of FFAs with inflammatory markers seems to be warranted.

Lipopolysaccharide (LPS) molecules, also known as bacterial endotoxins, may trigger inflammation, leading to activation of immunity and cytokine release. LPS infusion and consequent subclinical endotoxemia results in elevated levels of proinflammatory markers and metabolic aberrations [15-16]. LPS has a short half-life [17] and there is no agreement on the measurement of its plasma level [18]. Hence, lipopolysaccharide-

binding protein (LBP) is introduced with longer half-life and more reliable measurement [19-20]. Also, serum LBP level is a proxy of serum LPS level [21]. A population-based study [22] found that LBP was significantly associated with MetS in normal-weight participants. Another study [23] reported that among MetS components, LBP concentration was independently associated with abdominal obesity.

In prior studies, inflammatory parameters were compared between obese and lean subjects [19, 24-25]. Therefore, it remains unclear whether the observed alterations in serum FFAs and/or inflammatory parameters in metabolically unhealthy obese patients are due to adiposity and/or metabolic state. Therefore, regarding the significant confounding effect of abdominal obesity, we used waist circumference (WC), as a reflection of visceral adipose tissue [26], to define abdominal obesity, and examined differences in characteristics and inflammatory markers (serum LBP and FFAs) between "metabolically healthy" and "unhealthy" abdominally obese individuals. We also examined the association between serum FFAs and LBP in pooled population as well as in each group.

Methods

Study design and participants

A total of 81 metabolically healthy abdominally obese (MHAO) with 83 age- and gender-matched metabolically unhealthy abdominally obese (MUHAO) were recruited in this case-control study, carried out from June 15th to November 6th, 2015 in the Northwest of Iran. Frequency matching was carried out for the present study. Apparently healthy individuals aged 18-60 years with abdominal obesity were included in the study. We excluded pregnant or lactating women, those with diarrhea for 3 consecutive days within the previous 3 months, diagnosed diabetes, coronary heart disease, stroke, myocardial infarction, cardiovascular and kidney, liver or infectious diseases including tuberculosis, AIDS, and hepatitis; thyroid problems, severe mental disorders or physical disabilities and malignancies; taking oral antidiabetic agents or insulin or other drugs for the last 2 months, or antibiotics used for 3 consecutive days within the

previous 3 months; smokers or alcohol consumers; misreported dietary intakes (less than 800 kcal/d or more than 4200 kcal/d), or being on specific diets in the past 6 months; and having gastrointestinal surgery within past 1 year.

Abdominal obesity was defined as having waist circumference (WC)≥95cm according to the Iranian National Committee of Obesity [27]. According to Meigs *et al.*[28], metabolic health was defined as the presence of <3 of the following metabolic abnormalities including abdominal obesity (WC≥95cm for both genders)[27]; high serum triglyceride (TG) concentration (≥150 mg/dL); low serum high density lipoprotein cholesterol (HDL-C) (<40 mg/dL for men and <50 mg/dL for women); elevated blood pressure (BP) (≥130/85 mmHg); and fasting blood sugar (FBS) (≥100 mg/dL). Eighty-one individuals with ≥3 criteria entered the case group (MUAO) and 83 with 2 or less criteria formed the control group (MHAO).

Sampling procedures

After public announcement for the study, 500 volunteers were recruited from general population. Of these, 178 people could enter the study based on the defined eligibility criteria for the present study. Informed consent was taken from each participant before the study. After taking blood samples and anthropometric measurements, 14 of them were excluded due to diabetes (FBS≥126 mg/dl [29] in two occasions), leaving 164 people (82 men, 82 women) to conduct the research.

Biochemical assays

After a 12-h overnight fast, 5cc blood were obtained for serum analyses. After centrifugation at 3000 rpm for 5 min, metabolic parameters were analyzed immediately, butserum FFAs and LBP were analyzed after supplying in -80°C.

FBS was measured by the enzymatic colorimetric method using glucose oxidase. Serum TG concentration was measured by commercially available enzymatic reagents with glycerol phosphate oxidase. Serum HDL-C was measured after precipitation of the apolipoprotein B containing lipoproteins with phosphotungistic

acid. Assays were performed using Pars Azmoon kits (Pars Azmoon Inc., Tehran, Iran) and a Selectra 2 auto-analyzer (Vital Scientific, Spankeren, Netherlands). Inter- and intra- assay coefficient of variation (CV) was < 5% for all assays. Serum samples for both LBP and FFAs assays were stored at -80°C until analysis. Both serum LBP and FFAs levels were determined by a sandwich ELISA (Bioassay Technology Laboratory, Shanghai Korean Biotech Co., LTD; Shanghai City, China) according to the manufacturer's instructions. The intra-assay and inter-assay coefficients of variation were <8 and <10%, respectively.

Measurements

All anthropometric indices were measured by a trained researcher. Height (without shoes in standard situation with precision of 0.1 cm and with an inelastic measuring tape) and weight (with Seca scale, light clothes and precision of 0.1 kg) were measured and BMI was calculated as weight in kg divided by the square of height in meters [30]. WC was measured using a non-stretchable fiber measuring tape. The subjects were asked to stand erect in relaxed position with both feet together on flat surface. WC was measured as the smallest horizontal girth between the costal and iliac crests at minimal respiration. Hip circumference was taken as the greatest circumference at the level of greater trochanters (the widest portion of the hip) on both sides. Waist to hip ratio (WHR) was calculated by dividing WC (cm) by hip circumference (cm) [31]. BP was recorded in a comfortable sitting position in the left arm after at least a 5-min rest, using the mercury sphygmomanometer. Two measurements were taken and the mean of the two measurements was considered as the BP [32]. Bioelectrical Impedance Analysis (BIA: BC-418MA, Tanita, Japan) was used to describe fat percent, fat Mass (FM), and fat free mass (FFM). Dietary intake was assessed using a 3-day food record (one weekend day and two workdays). *Nutritionist IV* software (Axxya Systems, Stafford, TX), modified for Iranian foods, were used for dietary data analysis. Physical activity (PA) was measured via IPAQ-long form questionnaire [33].

Statistical analysis and sample size

To examine the normal distribution of variables, Kolmogrov-Smirnov tests and histograms were applied. The independent samples t-test was used to compare the means (standard deviation: SD) of normally distributed variables between the two groups. The Mann–Whitney U test was used for values with skewed distribution and in such conditions, median (25^{th}, 75^{th}) was reported. In order to assess the association of two categorical variables, Chi-square test was applied. The correlation between serum FFAs and LBP was assessed using Spearman correlation coefficient analysis. Odds ratios and their 95% confidence intervals (CI) were reported using Logistic Regression test.

The larger sample size was calculated for serum FFAs compared to LBP, using literature-derived data [34] for nonalcoholic fatty liver disease (NAFLD) patients; the effect size for serum FFAs was 0.20 nmol/l (SD_1=0.34 nmol/l for controls and SD_2=0.53 nmol/l for NAFLD patients). Therefore, sample size estimation was based upon this parameter with 80% power and α-error of 5% and a case to control ratio of 1:1. It was predicted that 79 persons in each group would detect changes in serum FFAs as well as serum LBP level, using the two-means formula. Data were analyzed using SPSS ver. 17.0 for Windows (PASW Statistics; SPSS Inc., Chicago, IL, USA). P-value less than 0.05 was considered significant.

Results

Males comprised about 50% of the study participants in the two groups (p=0.87). The age range of the subjects was 20-59 years. Participants of the two study groups similarly had WC≥95 cm i.e. the cut-off point of WC for Iranian population. Overall, there were no significant differences in age, gender, anthropometric indices, and body composition between the two groups. Dietary parameters especially total fat, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) intakes were more or less the same between the two study groups (Table 1). Marital status, education level, and job of the cases and controls were also similar. However, mean number of metabolic aberrations were significantly higher in cases than controls (3.25±0.72 vs. 1.67±0.50; P<0.001) (data not shown). The flow chart of the study is shown in Figure 1.

Except for WC which was matched between the two groups, metabolic aberrations including low HDL-C (82% vs. 34%), high TG (78% vs. 24%), high FBS (33% vs. 0%), and hypertension (34% vs. 10%) were significantly higher in the cases than controls, respectively (Figure 2). The current study indicated that each 10 unit increment in serum FBS level increased the risk of MUAO about 3.8 times (OR= 3.79, 95 % CI: 2.25-6.40). Additionally, the odds of MUAO was significantly increased per one increment in serum TG level (OR= 1.10, 95 % CI: 1.05-1.15), the systolic BP (OR= 1.02, 95 % CI: 1.00-1.04), and diastolic BP (OR= 1.03, 95 % CI: 1.01-1.06). However, the odds of having MUAO was significantly decreased by 68% per 10 unit increment in serum HDL-C level (OR= 0.32, 95 % CI: 0.20-0.52) (Table 2).

The median of LBP (12.32 µg/mL in cases vs. 12.76 µg/mL in controls, *P*=0.483) and FFAs (1294 nmol/L in cases vs. 1333 nmol/L in controls; *P*=0.686) showed no significant difference between the two groups (Figure 3). However, a significant correlation was found between LBP and FFAs in pooled population (r=0.712; *P*<0.001) as well as in cases (r=0.717; *P*<0.001) and controls (r=0.704; *P*<0.001) (Figure 4). The results of partial correlation indicated much stronger correlation between LBP and FFAs, when controlling for WC (r=0.961; *P*<0.001). Moreover, number of metabolic aberrations were significantly correlated with HDL-C (r=-0.537; *P*<0.001), TG (r=0.468; *P*<0.001), FBS (r=0.534; *P*<0.001), Systolic BP (r=0.247; *P*=0.001), and Diastolic BP (r=0.315; *P*<0.001). Neither FFAs nor LBP were significantly correlated with dietary intake of total fat, SFA, MUFA, and PUFA (data not shown). There were also no significant correlations of LBP and FFAs with metabolic parameters (Table 3).

Discussion

The present study, to the best of our knowledge, examined the association of LBP and FFAs levels in MHAO and MUAO individuals for the first time and found significant differences between the two parameters. Anthropometric indices as well as body composition profile were similar between the two groups. Moreover, there were no significant differences in LBP and FFAs between MHAO and MUAO.

In the present study, we matched metabolically healthy with metabolically unhealthy individuals on abdominal fat which might explain why we did not find any differences in levels of FFAs and LBP, as inflammatory markers, and body composition between the two groups. Therefore, the levels of LBP and FFAs seem to be more related to abdominal obesity than to the presence or absence of metabolic health. This is further supported by the observation that serum FFAs or LBP levels were not correlated with metabolic parameters. Therefore, our findings suggest that increased levels of these two markers are not necessarily related to the presence of metabolic aberrations.

A few smaller studies have examined differences in body composition and/or inflammatory profile between metabolically healthy and unhealthy obese postmenopausal women [35-37]. In line with our result, Engström *et al.* [36] in a research on 58 obese postmenopausal women found no significant differences in levels of inflammatory markers between those with metabolic syndrome (MetS) compared to those without MetS. Additionally, in the population-based study of Philips *et al.* [38], no significant difference was noted in CRP level between MHAO and MUAO, based on metabolic health criteria of Meigs *et al.* [28] study. It is noteworthy that in the present study we used Meigs's metabolic health definition in which WC has also been considered.

A recent study revealed that the association between inflammatory biomarkers and metabolically healthy obesity depends on the criteria used. Since in that research, a significant difference was noted in the levels of CRP and IL-6 with some but not all MHAO definitions, which disappeared after adjustment for abdominal obesity or percent body fat [39]. This study confirms our results. However, Phillips *et al.* [40] showed that obese women and men with MetS had significantly higher levels of inflammatory cytokines than obese persons without MetS. Beasley *et al.* [41] showed that visceral adiposity, and not abdominal subcutaneous fat, was most consistently associated with significantly higher levels of IL-6 and CRP levels in black and white men and women in the Health ABC study. We could not measure visceral fat in our study, though, abdominal obesity measured through WC, can reflect visceral adiposity [26]. On the other hand, a recent

work observed no significant differences in visceral fat between the obese-insulin resistant and obese-insulin sensitive persons [42].

Several studies have demonstrated a strong association of IR with obesity, low HDL-C, hypertriglyceridemia, and hypertension [10, 43] as well as inflammatory factors [44]. However, in our study, we could not assess IR, due to some financial deficits.

In the present work, dietary intake was compared between the two obese groups; therefore, no significant difference was found in terms of energy or macronutrients especially fat intake. Moreover, habitual physical activity (PA) was controlled between the study groups. And, in our previous report, there were no significant differences between the two groups in terms of PA (unpublished data). Since different levels of habitual PA might affect levels of serum inflammatory markers [45].

Obesity, as a well-known metabolic risk factor, is usually associated with mild chronic inflammation [46]. The relationship between obesity and increased inflammation may be justified, in part, by FFAs [47] which are released from adipocytes through lipolysis and are elevated in obesity due to increased adipose tissue [48]. Inflammatory cytokines such as interleukin-6 (IL-6) can stimulate lipolysis and increase levels of FFAs [49]. On the other hand, in healthy persons an acute increase in FFAs can induce inflammatory changes [50]. Therefore, FFAs are not only increased by inflammation, but also promote inflammation. The results of the present research showed that FFAs are positively correlated with LBP levels either in the pooled population or in each group. It shows that any increase in the level of FFAs, observed in the abdominally obese, regardless of their metabolic aberrations can lead to a significant elevation in the level of LBP.

LBP has been considered a key inflammatory marker which mediates LPS-triggered innate immunity [51]. Although LBP concentration was previously reported to be associated with various anthropometric and metabolic factors such as BMI, WC, and so on [23-25], in our study the relationship only existed between the two biomarkers, FFAs and LBP, but not with the metabolic or anthropometric parameters. It is notable that the positive relationship between LBP and BMI was not observed in either normal weight or obese groups

in Yang *et al.* [52] study after multivariate analyses. In their research, the level of LBP significantly reduced after bariatric surgery and consequent reduction in WC (from 121.6 cm to 90.6 cm; P<0.001) which indicates the strong association of LBP with WC. Liu *et al.* [22] in a population-based follow-up study on 2529 Chinese also found that the association of LBP with MetS was significant only in normal-weight participants, but not in their overweight/obese counterparts after multivariate adjustments including BMI, which supports our study findings. It is assumed that the association between serum LBP level and MetS observed in previous studies [19, 24-25] is mediated by BMI or WC, and finding no association between serum LBP level and incidence of MetS in our study, in which the WC-matched controls were included, is not unexpected.

Overall, what makes our research different from most of previous ones is that in our study we matched the two groups based on WC, rarely observed in prior reports. Most of the previous studies have examined either MetS patients *vs.* those without the syndrome or metabolically healthy *vs*. metabolically unhealthy, regardless of their BMI or WC status and based on different metabolic health criteria[3, 8, 28]. A few have examined inflammatory markers between metabolically healthy and unhealthy persons, considering WC or abdominal obesity [36, 38, 39].

Conclusion

Our study indicated that WC could be a strong mediator of the association between serum LBP, FFAs, and metabolic alterations. In fact, the levels of LBP and FFAs seem to be more related to abdominal obesity than to the presence or absence of metabolic health. The results also suggested a significant correlation between serum FFAs and LBP in abdominally obese population, which seems to be independent of metabolic aberrations.

Competing interests

None declared.

Authors' contributions

MSA and NK conceived the study design and wrote the study protocol. MSA and NK analyzed and interpreted the data. MSA, PA, MN, SMG and NK were involved in drafting the manuscript or revising it critically for content. All authors have given final approval of the version to be published.

Acknowledgments

We would like to thank those who participated in the present study.

Ethical approval

All protocols were approved by regional and organizational ethics committee of TBZMED and MRGUMS, respectively. Written informed consent was taken from each participant. Research was carried out in compliance with the Helsinki Declaration.

Consent for publication

Not applicable.

Funding

This work was financially supported by Research Vice-Chancellor, Tabriz University of Medical Sciences and Maragheh University of Medical Sciences, Iran. The results of this paper were extracted from MSc. thesis of Nahid Karamzad, registered at Tabriz University of Medical Sciences, Tabriz, Iran.

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Table 1. Demographic, anthropometric, and dietary intake parameters in MUAO and MHAO patients

Variables	MUAO (n=81)	MHAO (n=83)	OR (95% CI)	*p*
Age (y)†	38.23 (8.52)	37.13 (8.64)	1.01 (0.97-1.05)	0.412 ξ
Men (%)	50.6	49.4	1.05 (0.57-1.93)	0.876 £
Physical activity score ¥	3144 (1416, 5166)	2412 (1260, 5211)	1.00 (0.99, 1.00)	0.451 ш
Weight (Kg)†	87.21 (13.90)	84.78 (13.98)	1.01 (0.99, 1.03)	0.266 ξ
Height (cm)†	165.09 (11.56)	164.56 (10.60)	1.00 (0.97, 1.03)	0.762 ξ
Waist circumference (cm) †	106.02 (8.30)	105.06 (8.63)	1.01 (0.97, 1.05)	0.470 ξ
Hip circumference (cm)†	110.90 (6.92)	111.31 (8.26)	0.99 (0.95, 1.03)	0.730 ξ
Waist to hip ratio†	0.95 (0.05)	0.94 (0.06)	2.73 (0.15, 48.00)	0.209 ξ
BMI (Kg/m^2)†	32.16 (4.25)	31.35 (4.12)	1.04 (0.97, 1.12)	0.214 ξ
Body Fat Percentage (%)†				
Males	26.86 (5.15)	25.07 (4.86)	1.07 (0.98, 1.17)	0.093 ξ
Females	38.59 (4.38)	39.88 (4.79)	0.93 (0.85, 1.03)	0.227 ξ
Body Fat Mass (kg)†				
Males	25.91 (7.27)	22.79 (6.76)	1.06 (1.00, 1.13)	0.062 ξ
Females	31.11 (7.33)	32.4 (8.63)	0.98 (0.92, 1.03)	0.441 ξ
Body Fat Free Mass (kg)†				
Males	69.09 (6.69)	66.98 (7.85)	1.04 (0.98, 1.10)	0.130 ξ
Females	48.72 (5.08)	47.61 (4.94)	1.04 (0.95, 1.14)	0.425 ξ
Total energy intake (Kcal/day)†	2152.9 (765.1)	2206.8 (862.9)	1.00 (0.97-1.04)	0.700 ξ
Carbohydrate intake (% energy)†	60.20 (10.09)	59.29 (9.11)	1.01 (0.97-1.04)	0.499 ξ
Protein intake (% energy)†	14.22 (2.95)	14.17 (4.10)	1.00 (0.91-1.09)	0.937 ξ
Total fat intake (% energy)†	25.58 (10.54)	26.54 (12.14)	0.98 (0.95-1.02)	0.380 ξ

Total SFA intake (% energy)[¥]	14.06 (10.6, 21.87)	14.21 (10.18, 21.49)	0.99 (0.96-1.02)	0.780 [Ш]
Total MUFA intake (% energy)[¥]	16.39 (11.01, 24.68)	18.26 (11.76-26.27)	0.99 (0.97-1.01)	0.183 [Ш]
Total PUFA intake (% energy)[¥]	12.7 (9.59-22.19)	14.41 (8.94, 19.01)	1.01 (0.98-1.03)	0.943 [Ш]

[†]Variables with normal numeric scales are reported as Mean (standard deviation).

[¥] Variables with non-normal numeric scales are reported as Median (25^{th}, 75^{th}).

[ξ] Independent Samples t-test

[Ш] Mann- Whitney U test

[£] Chi Square test

MHAO, Metabolically Healthy Abdominally Obese; MUAO, Metabolically Unhealthy Abdominally Obese; OR, Odds Ratio; CI, Confidence Interval; BMI, Body Mass Index; SFA, Saturated Fatty Acids; MUFA, Mono Unsaturated fatty Acids; PUFA, Poly Unsaturated Fatty Acids.

Table 2. Biochemical characteristics in MUAO and MHAO patients

Variables	MUAO (n=81)	MHAO (n=83)	OR (95% CI)	*p*
FBS (mg/dL) ¥	87.72 (5.82)	95.50 (9.76)	3.79 (2.25-6.40)*	<0.001[ξ]
TG (mg/dL) ¥	193 (151, 241)	112 (88, 146)	1.10 (1.05-1.15)*	<0.001[Ψ]
HDL-C (mg/dL) †	39.53 (6.65)	46.44 (9.20)	0.32 (0.20-0.52)*	<0.001[ξ]
SBP (mg/dL) ¥	115 (16.45)	108.13 (16.60)	1.02 (1.00-1.04)*	0.009[ξ]
DBP (mg/dL)¥	77.31 (13.86)	70.84 (12.94)	1.03 (1.01-1.06)*	0.002[ξ]
Cholesterol (mg/dL)†	193.60 (41.37)	187.37 (32.91)	1.00 (0.99, 1.01)	0.286[ξ]

† Variables with normal numeric scales are reported as Mean (standard deviation).

¥ Variables with non-normal numeric scales are reported as Median (25^{th}, 75^{th}).

[ξ] Independent Samples t- test

[Ψ] Mann- Whitney U test

MHAO, Metabolically Healthy Abdominally Obese; MUAO, Metabolically Unhealthy Abdominally Obese; OR, Odds Ratio; CI, Confidence Interval; FBS, Fasting Blood Sugar; TG, Triglycerides; HDL-C, High-Density Lipoprotein Cholesterol; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure

Table 3. correlation of FFAs and LBP with metabolic parameters in MUAO and MHAO individuals

	MUAO		MHAO	
Variables	FFAs	LBP	FFAs	LBP
WC	0.07 (0.51)	0.02 (0.85)	0.06 (0.58)	0.03 (0.74)
TG	-0.02 (0.79)	0.008 (0.94)	-0.07 (0.48)	0.07 (0.49)
FBs	-0.005 (0.96)	-0.18 (0.09)	0.07 (0.50)	0.09 (0.40)
HDL-C	0.01 (0.91)	0.08 (0.45)	0.10 (0.33)	0.41 (0.09)
SBP	-0.03 (0.78)	-0.08 (0.46)	-0.05 (0.64)	-0.11 (0.29)
DBP	-0.08 (0.43)	-0.18 (1.00)	0.06 (0.58)	-0.09 (0.39)

P=NS, using Spearman correlation coefficient test.

MHAO, Metabolically Healthy Abdominally Obese; MUAO, Metabolically Unhealthy Abdominally Obese; FBS, Fasting Blood Sugar; TG, Triglycerides; HDL-C, High-Density Lipoprotein Cholesterol; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; LBP, Lipopolysaccharide Binding Protein; FFAs, Free Fatty Acids

Figure 1. Flowchart of the study

Figure 2. Metabolic characteristics of MHAO and MUAO subjects.

$p<0.001$ for all except WC, using X^2.

MHAO, Metabolically Healthy Abdominally Obese; MUAO, Metabolically Unhealthy Abdominally Obese; FBS, Fasting Blood Sugar; TG, Triglycerides; HDL-C, High-Density Lipoprotein Cholesterol; Htn, Hypertension.

Figure 3. Lipopolysaccharide binding protein (LBP) (µg/mL) (a) and free fatty acids (FFAs) concentrations (nmol/L) (b) in subjects with MHAO (Controls) and MUAO (Cases).
p=NS
Data are presented as box plot where boxes represent the interquartile range [IQR], the line within boxes represent the median, and the lines outside the boxes represent the lower quartile minus 1.5 times the IQR or the upper quartile plus 1.5 times the IQR.

Figure 4. Spearman correlation between LBP and FFAs in pooled population (Fig. 4-a) as well as in each study group (Fig. 4-b).

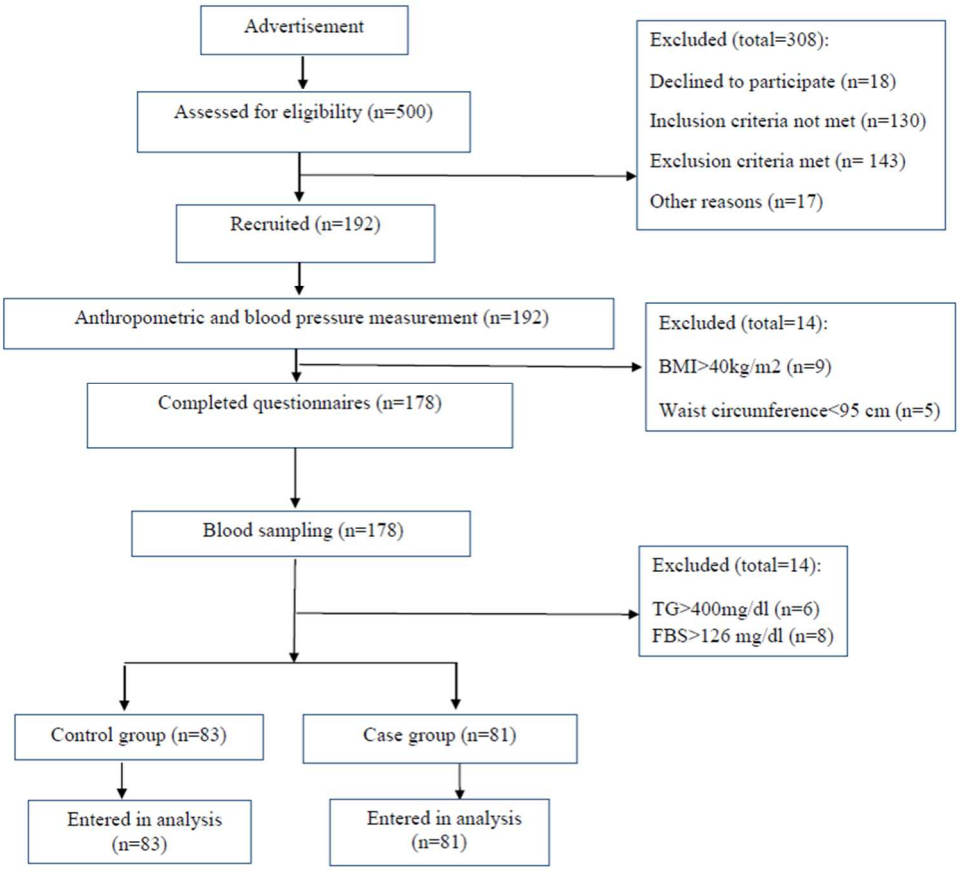

Figure 1. Flowchart of the study

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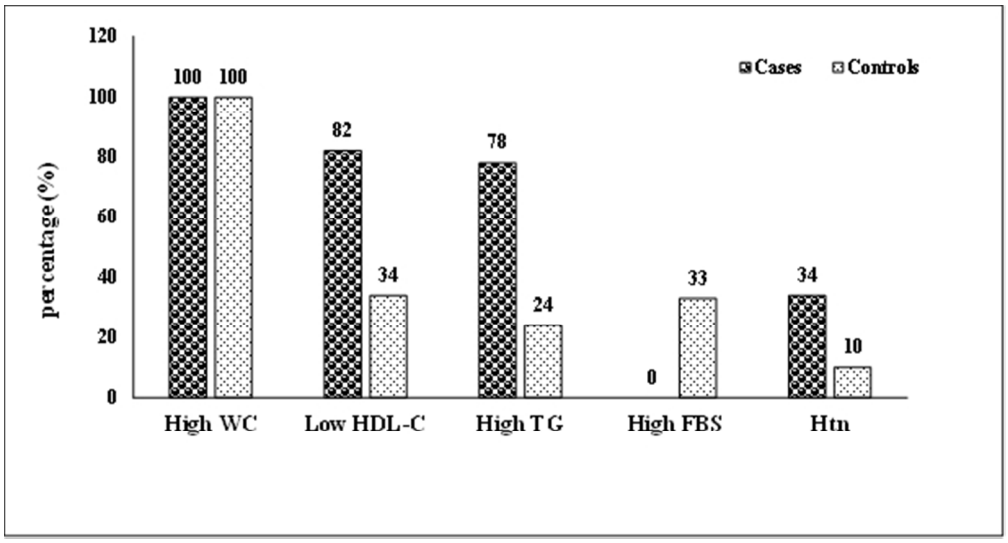

Figure 2. Metabolic characteristics of MHAO and MUAO subjects.┼ P<0.001 for all except WC, using X2.┼ ┼ MHAO, Metabolically Healthy Abdominally Obese; MUAO, Metabolically Unhealthy Abdominally Obese; FBS, Fasting Blood Sugar; TG, Triglycerides; HDL-C, High-Density Lipoprotein Cholesterol; Htn, Hypertension.┼ ┼

51x27mm (300 x 300 DPI)

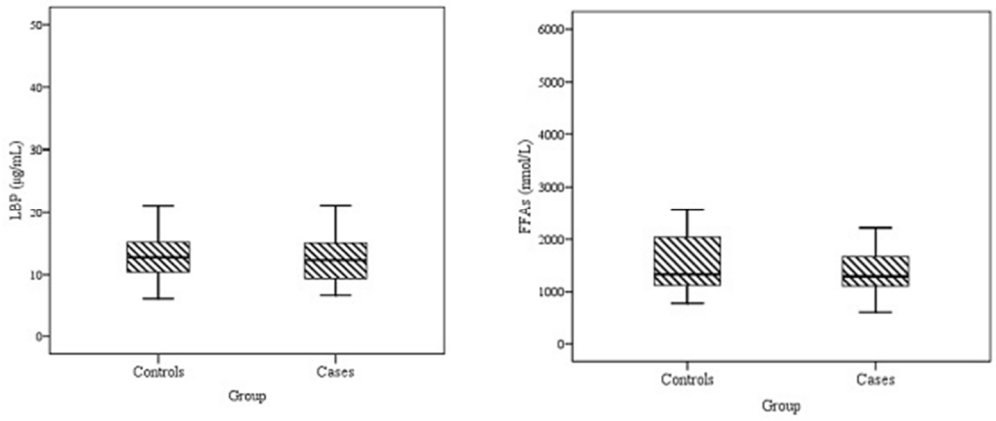

Figure 3. Lipopolysaccharide binding protein (LBP) (µg/mL) (a) and free fatty acids (FFAs) concentrations (nmol/L) (b) in subjects with MHAO (Controls) and MUAO (Cases). ┼ P=NS┼ Data are presented as box plot where boxes represent the interquartile range [IQR], the line within boxes represent the median, and the lines outside the boxes represent the lower quartile minus 1.5 times the IQR or the upper quartile plus 1.5 times the IQR.┼ ┼

52x22mm (300 x 300 DPI)

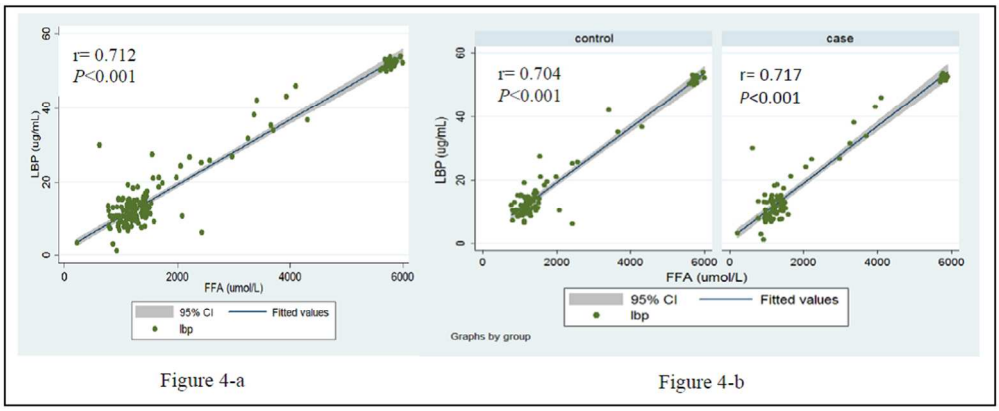

Figure 4. Spearman correlation between LBP and FFAs in pooled population (Fig. 4-a) as well as in each study group (Fig. 4-b).

96x39mm (300 x 300 DPI)

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *case-control studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	2
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2, 3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4, 5
Objectives	3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Participants	6	(a) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	5, 6
		(*b*) For matched studies, give matching criteria and the number of controls per case	5
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	6,7
Bias	9	Describe any efforts to address potential sources of bias	3
Study size	10	Explain how the study size was arrived at	8
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	8
		(*b*) Describe any methods used to examine subgroups and interactions	Not applicable
		(*c*) Explain how missing data were addressed	Not applicable
		(*d*) If applicable, explain how matching of cases and controls was addressed	5
		(*e*) Describe any sensitivity analyses	Not applicable
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	6
		(b) Give reasons for non-participation at each stage	6
		(c) Consider use of a flow diagram	Figure 1
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	Table 1
		(b) Indicate number of participants with missing data for each variable of interest	Table 1
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure	Table 1,2
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	Table 1, 2
		(*b*) Report category boundaries when continuous variables were categorized	6
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	Not applicable
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	Not applicable
Discussion			
Key results	18	Summarise key results with reference to study objectives	9
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	3
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	10-12
Generalisability	21	Discuss the generalisability (external validity) of the study results	3, 12
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	13

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.