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ORIGINAL ARTICLE

Insulin resistance in relation to inflammatory gene expression and metabolic features in apparently healthy obese individuals

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Abstract

The present study aimed to investigate the association of insulin resistance (IR) with inflammatory gene expression levels, metabolic health, lipid profile, and body composition in the apparently healthy obese. In this cross-sectional study, 88 apparently healthy obese subjects were recruited and divided into insulin-resistant and non-insulin-resistant (NIR) groups. Fasting blood samples were taken to determine serum metabolic features. mRNA expression of inflammatory genes were assessed in freshly isolated peripheral blood mononuclear cells (PBMCs), using quantitative real-time PCR (qPCR). Bioelectrical impedance analysis (BIA) was used to describe body composition. Among inflammatory genes, toll-like receptor 4 (TLR4) mRNA revealed significant upregulation in PBMCs of IR group compared with NIR individuals ($p = 0.035$). High-density lipoprotein cholesterol (HDL-C, $p = 0.04$), low-density lipoprotein cholesterol (LDL-C, $p < 0.001$), waist circumference ($p = 0.025$), and waist to hip ratio ($p = 0.013$) were significantly different between the two groups. A significant but weak correlation of HDL-C was observed with TLR4 ($r = -0.305$; $p = 0.011$) and myeloid differentiation factor 88 (MyD88, $r = -0.27$; $p = 0.024$) expression level. Also, LDL-C was found to be correlated with TLR4 ($r = 0.302$; $p = 0.012$) and MyD88 ($r = 0.267$; $p = 0.027$) expression levels. There was also a significant correlation between HOMA-IR and HDL-C ($r = -0.25$; $p = 0.019$). The results of this study indicated the possible link between IR and TLR4. Also, there was a significant correlation between HDL-C and LDL-C as well as between TLR4 and MyD88. Some inflammatory genes and metabolic parameters were also significantly correlated.

Keywords Insulin resistance · Inflammatory genes · Metabolic health · Lipid profile · Healthy obese

Abbreviations

BP	Blood pressure
BMI	Body mass index
FBS	Fasting blood sugar
HDL-C	High-density lipoprotein cholesterol
HOMA-IR	Homeostasis model of insulin resistance
IR	Insulin resistance
LDL-C	Low-density lipoprotein cholesterol
MetS	Metabolic syndrome
MHO	Metabolically healthy obese
MUO	Metabolically unhealthy obese
MyD88	Myeloid differentiation factor 88
NFκB	Nuclear factor kappa B
NIR	Non-insulin resistance
PBMCs	Peripheral blood mononuclear cells
QUICKI	Quantitative insulin sensitivity check index
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
TRIF	TIR-domain containing adaptor-inducing interferon-β
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
WC	Waist circumference
WHR	Waist/hip ratio

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Introduction

Obesity as a serious concern to public health is reaching epidemic rates worldwide. The major health complications associated with the growing prevalence of obesity are type 2 diabetes mellitus (T2DM), fatty liver disease, cardiovascular disease, and obesity-related cancers [1]. Obesity is also associated with abnormal inflammatory responses that are a risk for obesity-related insulin resistance (IR) [2].

IR is linked to various chronic diseases such as hypertension, hyperlipidemia, and atherosclerosis, the hallmarks of metabolic syndrome (MetS) [3]. The dyslipidemia of IR seems to be triggered by features of IR than obesity. However, not all subjects with diminished insulin sensitivity develop dyslipidemia [4]. In fact, adipocyte size is proposed as a key factor for determining the contributing degree of adipose tissue to dyslipidemia. Enlargement of adipocytes, enhanced in lipolysis, can lead to more circulating free fatty acids and their delivery to the liver to increase triglyceride synthesis, exacerbate IR, and promote dyslipidemia [5]. The study of Veilleux et al. [6] reported the association between enlargement of visceral adipocytes and dyslipidemia independent of body composition in obese subjects (181). In another study [7], serum triglyceride and high-density lipoprotein cholesterol (HDL-C) levels were directly correlated with IR and visceral fat. A research [8] on two groups with normal BMI showed that individuals were severely IR. Previous studies propose that the distribution of fat, particularly visceral obesity, may be a more important determinant of IR than overall obesity [9–11]. The key role of adipose tissue in the development of IR by releasing a wide range of proinflammatory cytokines and chemokines is clarified, but molecular basis for the link between obesity, IR, and metabolic state is not thoroughly identified.

There is considerable evidence that activation of membrane receptors such as toll-like receptors (TLRs) plays a significant role in the initiation and development of IR [2, 12, 13]. Glucose and saturated fatty acids can contribute to TLR expression and activation in human mononuclear cells and induce inflammatory cytokine production [14]. TLR4 recognizes free fatty acids which contribute to the pathogenesis of IR [15, 16]. Animal studies remarkably show that lack of TLR4 can protect mice from diet-induced obesity [13, 17, 18]. Furthermore, inhibition of TLR2 improved insulin sensitivity and signaling in muscles and white adipose tissue of mice [19]. Hardy et al. [20] reported increased TLR2 and TLR4 expression in adolescents with MetS compared to BMI-matched controls. However, studies between metabolically healthy and unhealthy obese individuals showed no significant differences in TLR2 and TLR4 gene expression levels [21, 22].

TLRs act via two downstream molecules, myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor-inducing interferon-β (TRIF), which have connections with insulin homeostasis [2, 14]. An animal survey indicated that mice lacking MyD88 have decreased β cell mass compared to wild-type controls and they have normal glucose tolerance [23]. Also, another study revealed that TRIF deficiency induces decreased glucose tolerance and β-cell dysfunction [24].

In the study of Jialal et al. [25], there was a positive correlation between HOMA-IR and increased levels of TLR2 and TLR4 in patients with MetS. However, they did not report any downstream signaling proteins. While these important findings imply that TLR2 and TLR4 activation is important in the pathogenesis of IR, the association of IR and inflammatory genes is rarely studied. Therefore, the purpose of this study was to test the association of IR with TLR2 and TLR4 and downstream signaling in peripheral blood mononuclear cells (PBMCs) isolated from apparently healthy obese persons. Also, the association of IR with lipid profile, metabolic health, and body composition was studied.

Materials and methods

Study participants

In this cross-sectional study, 88 apparently healthy obese persons aged 29–43 years were recruited from numerous clinics in the northwest of Iran from June 15th to November 6th, 2015. All of the participants were classified as abdominally obese (waist circumference (WC) ≥ 95 cm) based on the Iranian National Committee of Obesity [26]. Informed consent was obtained from each participant and the study was approved by regional ethics committee of Tabriz University of Medical Sciences, Tabriz, Iran. The whole investigation was conducted according to the principles of the Declaration of Helsinki (ethical code: TBZMED.REC.1394.1191). Metabolic health status was defined using Meigs et al.'s [27] criteria in which the presence of less than three of the following components was considered as metabolically healthy state: high WC (≥ 95 cm), 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).

The exclusion criteria were pregnancy and lactation, postmenopausal, recent change or misreport in energy intake (i.e., < 800 or ≥ 4200 kcal/d), chronic high-intensity exercise (> 100 min/week), smoking, alcohol consumption, individuals with serum TG (> 400 mg/dl), malabsorption, irritable bowel

syndrome, recent gastrointestinal surgery in the past 1 year, and diarrhea for 3 consecutive days in the past 3 months. Patients with diabetes mellitus, acute or chronic infectious or inflammatory disease, thyroid disease, cardiovascular disease, abnormal complete blood count, malignant disease, kidney disease, and mental disorders were excluded from the study. Individuals receiving medications/therapies including anticoagulant therapy, anti-obesity drugs, steroid therapy, anti-inflammatory drugs, antibiotics, beta blockers, corticosteroids, oral contraceptives, and dietary supplements in the past 2 months were also excluded. Demographic data, medical history, and physical history questionnaires were obtained. Anthropometric indices were measured according to standard measurement protocols as described in our previous study [28]. Bioelectrical impedance analysis (BIA: BC-418MA, Tanita, Japan) was used to define the fat percentage, fat mass (FM), and fat-free mass (FFM).

Laboratory assays

After a 12-h overnight fast, 5 cm^3 blood was obtained. Instantly, after centrifugation at 3000 rpm for 5 min, metabolic parameters were examined. FBS, total cholesterol (TC), TG, and HDL-C were assayed, using the standard enzymatic methods via 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 analyses. Low-density lipoprotein cholesterol (LDL-C) was calculated with the Friedewald formula: LDL-C (mg/dl) = [TC] − [HDL-C] − [TG] 5.0. Insulin was assayed using ELISA Monobind kit.

The homeostasis model of insulin resistance (HOMA-IR) was calculated as

$$[\text{fasting insulin}\ (\mu\text{IU/ml}) \times \text{fasting glucose}\ (\text{mg/dl})/405] \quad [29]$$

Quantitative insulin sensitivity check index (QUICKI), an indicator of insulin sensitivity, was calculated as

$$[1/(\log \text{fasting insulin} + \log \text{fasting glucose in mg/dl})] \quad [30]$$

The patients were divided into insulin-resistant (IR) and non-insulin-resistant (NIR) groups.

Peripheral blood mononuclear cell isolation

PBMCs were isolated using Ficoll-Hypaque gradient density centrifugation (Baharafshan, Tehran, Iran).

Real-time PCR

RNA was extracted from PBMCs, using Accusol (Bioneer Pacific, USA). cDNA from total RNA was synthesized, using the Revert Aid First Strand cDNA Synthesis kit (Fermentas, Thermo fisher Scientific, USA). Real-Time PCR was performed using primers specific for TLR2, TLR4, MyD88, TRIF, and NFκB (Invitro Gen), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as normalizer (Invitro Gen). Reactions were performed in triplicate, using a light cycler 96 real-time PCR instrument (Roche, Switzerland). Data were calculated via the $2^{-\Delta\Delta CT}$ method [31, 32].

Statistical analysis

All analyses were performed using SPSS software version 17.0 for Windows (PASW Statistics; SPSS Inc., Chicago, IL, USA). Normality of the data was checked, using Kolmogorov-Smirnov test. Mean ± standard deviation (SD) was used for parametric data and median (25th, 75th) for

Table 1 General characteristics and anthropometric measurements between IR and NIR groups of obese individuals

Variable	NIR group ($n = 27$)	IR group ($n = 61$)	$p^{\dagger}$
Age (years)	36.11 ± 7.95	36.83 ± 7.21	0.675
Weight (kg)	83.75 ± 11.80	85.68 ± 15.03	0.557
Height (cm)	165.66 ± 9.21	164.15 ± 12.89	0.584
Body mass index (kg/m^2)	30.66 ± 3.36	31.77 ± 4.22	0.232
Waist circumference (cm)	102.37 ± 6.32	106.022 ± 7.71	0.025
Hip circumference (cm)	110.14 ± 6.34	110.62 ± 8.30	0.792
Waist/hip ratio (cm)	0.92 ± 0.054	0.95 ± 0.056	0.013
Body fat (%)	32.25 ± 8.12	33.20 ± 7.70	0.603
Body fat mass (kg)	26.82 ± 6.33	28.32 ± 7.98	0.390
Fat-free mass (kg)	57.26 ± 11.86	57.40 ± 12.60	0.962
Systolic blood pressure (mmHg)	113.70 ± 19.24	112 ± 13.52	0.680
Diastolic blood pressure (mmHg)	73.33 ± 13.08	76.58 ± 11.36	0.243

Data are presented as mean ± SD

† Based on independent sample *t* test

Table 2 Metabolic parameters between IR and NIR groups of obese individuals

Variable	NIR group ($n = 27$)	IR group ($n = 61$)	$p^{\dagger}$
TG (mg/dl)	198.40 ± 92.54	170.37 ± 80.30	0.250
HDL-C (mg/dl)	46.00 ± 7.44	40.60 ± 8.14	0.040
LDL-C (mg/dl)	102.40 ± 29.17	121.36 ± 30.00	0.302
Cholesterol (mg/dl)	183.88 ± 34.87	196.54 ± 34.63	0.137
FBS (mg/dl)	90.88 ± 8.67	93.32 ± 9.08	0.242
Insulin (μU/ml)	6.14 ± 1.72	22.26 ± 8.28	< 0.001
HOMA-IR	1.38 ± 0.41	5.13 ± 1.99	< 0.001
QUICKI	0.57 ± 0.03	0.43 ± 0.03	< 0.001

Data are presented as mean ± SD

TG triglycerides, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *FBS* fasting blood sugar, *HOMA-IR* homeostasis model of insulin resistance, *QUICKI* quantitative insulin sensitivity check index

† Based on independent sample *t* test

nonparametric variables. Standard statistical tests for comparison of the means of the two groups were independent sample *t* test for normal values and Mann-Whitney *U* test for abnormal data. Correlation between gene expression levels and other quantitative variables was assessed, using the Spearman correlation test. A *p* value less than 0.05 was considered significant. The sample size was estimated to be 67 persons, according to a previous study [33] based on serum HDL-C level, with 80% power and an α-error of 5%. Considering a drop-out rate of 30%, the total sample size required was 88.

Results

The general characteristics and anthropometric measurements of the participants are given in Table 1. Despite similar age (36.11 vs. 36.83 years) and BMI (30.66 vs. 31.77 kg/m^2), the waist

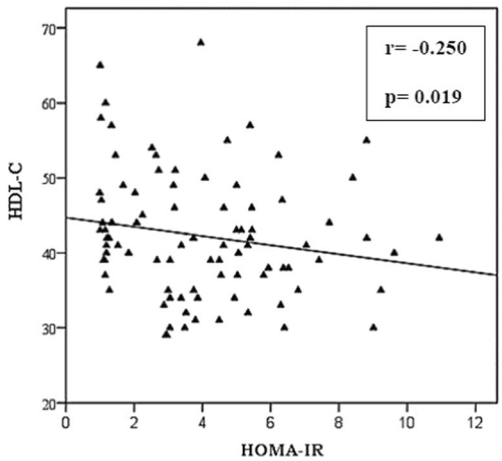

Fig. 1 Pearson correlation between HDL-C and HOMA-IR. HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model of insulin resistance

Table 3 Gene expression results between IR and NIR groups of obese individuals

Variable	NIR group ($n = 19$)	IR group ($n = 51$)	p^{+}
FC TLR2	1.84 (1.01–9.41)	0.611 (0.26–5.09)	0.074
FC Myd88	1.03 (0.27–3.78)	4.02 (0.48–15.56)	0.063
FC NFκB	1.21 (0.21–5.85)	2.05 (0.43–4.11)	0.383
FC TLR4	0.52 (0.01–12.80)	4.89 (0.46–16)	0.035
FC TRIF	0.702 (0.17–3.73)	0.92 (0.27–4.9)	0.409

Data are presented as median (25th, 75th)

FC fold change, *TLR2* toll-like receptor 2, *MyD88* myeloid differentiation factor 88, *NFκB* nuclear factor κB, *TLR4* toll-like receptor 4, *TRIF* TIR domain-containing adaptor-inducing interferon-β

+ Based on the Mann-Whitney *U* test

circumference (WC) ($p = 0.025$) and the waist/hip ratio (WHR) ($p = 0.013$) were higher in the IR group than the NIR group. There was no significant difference in body fat mass (FM) and fat-free mass (FFM) measured by bioelectric impedance between the two groups. The IR and NIR groups had no significant difference in terms of systolic and diastolic blood pressure.

The IR group had significantly lower HDL-C ($p = 0.04$) and higher low-density lipoprotein cholesterol (LDL-C) ($p = 0.02$) compared with the NIR group; however, there were no significant differences in other components of lipid profile (Table 2). Fasting insulin concentration and HOMA-IR were significantly higher ($p < 0.001$) and QUICKI was significantly lower ($p < 0.001$) in the IR compared to the NIR group (Table 2). There was a significant correlation between HOMA-IR and HDL-C ($r = -0.25$; $p = 0.019$) (Fig. 1).

TLR4 mRNA revealed significant upregulation in PBMCs of the IR group compared with the NIR individuals ($p = 0.035$). However, TLR2 mRNA expression revealed no significant difference between the two groups. There was also no significant difference in mRNA expression of downstream signaling proteins of TLRs including MyD88, TRIF, and NFκB between the two groups (Table 3). But there was a significant correlation between HDL-C and TLR4 ($r = -0.305$; $p = 0.011$) and MyD88 ($r = -0.27$; $p = 0.024$) expression level in all the persons (Fig. 2). A significant correlation of LDL-C was also observed with TLR4 ($r = 0.302$; $p = 0.012$) as well as with MyD88 ($r = 0.267$; $p = 0.027$) expression level (Fig. 3). Gene mRNA expression revealed no significant difference between the IR groups compared with the NIR individuals when analyzed based on gender (Table 4).

Discussion

The present study investigated the relationship of IR with inflammatory gene expression, lipid profile, metabolic health, and body composition in apparently healthy obese subjects. We found that TLR4 mRNA expression was significantly

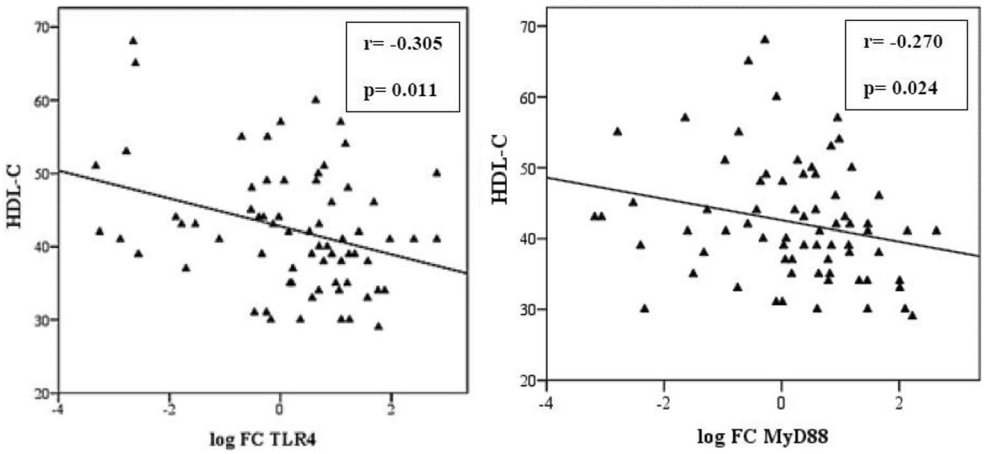

Fig. 2 Spearman correlations of HDL-C with log FC TLR4 as well as log FC MyD88. HDL-C: high-density lipoprotein cholesterol; FC: fold change, TLR4: toll-like receptor 4, Myd88: myeloid differentiation factor 88

different in PBMCs of the IR group compared with the NIR individuals. Though Myd88 and NFκB gene expression were increased in the IR group compared with the NIR group, it did not reach the significant level. However, there was a significant link of HDL-C and LDL-C with TLR4 and Myd88 expression. Approximately 70% of the participants were insulin-resistant. According to prior studies, high-risk abdominally obese patients are characterized by the cluster of metabolic abnormalities particularly high fasting insulin concentration [3]. Furthermore, it is proved that IR in population is related more to abdominal obesity than to general adiposity [8, 34]. Hence, there is a cause and effect relationship between them.

In the present study, we hypothesized that TLR4 signaling might be activated in obese condition which may mediate IR. We observed that mRNA expression of TLR4 was significantly increased in PBMCs of the IR group compared with the NIR group. In line with our study, the research conducted by Kim et al. [35] showed that TLR4 is a mediating key factor in the development of vascular inflammation and IR in diet-induced obesity via upregulation of transcriptional factors such as NFκB. Also, it was recently proved that free fatty acids (FFAs) can activate inflammatory pathways by activating TLR4 signaling in different cells like adipocytes and macrophages. In fact, in the absence of TLR4, initiation of inflammatory signaling pathways cannot occur [13].

The research conducted by Dasu et al. [36] on subjects with T2DM indicated that TLR4 expression was significantly increased compared to control subjects. It also had a positive correlation with HOMA-IR. Moreover, Reyna et al. [37] in a study on skeletal muscle of insulin-resistant subjects ($n = 22$) reported abnormal TLR4 expression with little information on TLR4-MyD88 signaling pathway and its correlation to IR. In addition, Creely et al. [38] showed increased TLR2 expression in the adipose tissue of T2DM patients with strong correlates to endotoxin levels and with no change in TLR4 expression.

We failed to find a significant difference in the mRNA expression of TLR2 and downstream signaling proteins of TLRs including MyD88, TRIF, and NFκB between the two groups, though significant correlations were observed between expression levels of inflammatory genes. Likewise, Telle-Hansen et al. [22] reported no differences in the expression level of TLR2 and TLR4 in the PBMCs of metabolically unhealthy obese (MUO) subjects compared with metabolically healthy obese (MHO) persons, although in MUO group, HOMA-IR was significantly higher than MHO group. In a study conducted by Gomez-Ambrosi et al. [21], no significant

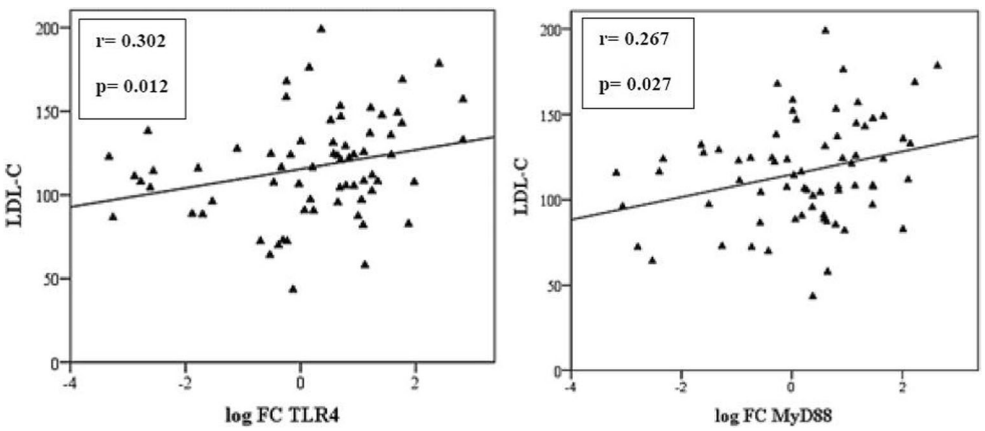

Fig. 3 Spearman correlations of LDL-C with log FC TLR4 as well as log FC MyD88. LDL-C: low-density lipoprotein cholesterol; FC: fold change, TLR4: toll-like receptor 4, Myd88: myeloid differentiation factor 88

Table 4 Gender differences in gene expression levels between IR and NIR groups of obese individuals

Variable	NIR group ($n = 19$)			IR group ($n = 51$)		
	Females ($n = 10$)	Males ($n = 9$)	p	Females ($n = 26$)	Males ($n = 25$)	p^+
FC TLR2	3.13 (1.01–8.56)	1.34 (0.65–11.08)	0.71	0.64 (0.30–8.23)	0.53 (0.23–4.99)	0.48
FC Myd88	1.67 (0.54–3.08)	0.48 (0.21–6.84)	0.46	4.25 (0.5–11.48)	3.98 (0.14–28.7)	0.79
FC NFκB	2.60 (0.23–5.83)	0.84 (0.05–6)	0.46	1.61 (0.42–3.20)	2.15 (0.05–6)	0.64
FC TLR4	0.93 (0.007–15.63)	0.4 (0.14–6.34)	0.65	3.31 (0.26–14.54)	4.92 (1.62–18.5)	0.32
FC TRIF	0.77 (0.3–5.2)	0.61 (0.062–4.81)	0.56	0.81 (0.25–4.45)	1.0 (0.26–6.68)	0.94

Data are presented as median (25th, 75th)

FC fold change, *TLR2* toll-like receptor 2, *MyD88* myeloid differentiation factor 88, *NFκB* nuclear factor κB, *TLR4* toll-like receptor 4, *TRIF* TIR domain-containing adaptor-inducing interferon-β

[+] Based on the Mann-Whitney *U* test

difference was noticed in the expression level of TLR4 and TNF-α between MHO and MUO groups with similar IR status. Although some studies [21, 22] showed that metabolic state of obese persons can be an important factor in determining inflammatory gene expression, our smaller sample size might have contributed to such results.

Our study also showed that among lipid profile, the levels of HDL-C and LDL-C were significantly different between the two groups, a finding which is consistent with prior reports [39–43]. Additionally, HDL-C levels inversely and LDL-C levels positively correlated with TLR4 and Myd88 expression levels. Although these correlations were significant, they were not strong, possibly due to lower sample size of our study. In line with our results, in a microarray study on obese subjects, TLR4 and Myd88 were inversely associated with plasma HDL-C levels [44]. Also, another study revealed that HDL-C causes MyD88-specific downregulation of TLR4 expression and signaling [45]. Moreover, several researchers [12, 46, 47] have shown that accumulation of LDL-C can trigger TLR2 and TLR4 signaling. Therefore, there is a strong link between HDL-C and LDL-C with TLR4 signaling and its relation with metabolic disorders like IR.

As the present work showed, there was a significant correlation of HOMA-IR with HDL-C and FBS. Similar to our finding, in a study [48] on 2283 patients with coronary heart disease (CHD), HDL-C was correlated with HOMA-IR levels. Another research on people with T2DM or MetS indicated significant positive correlations between the HOMA-IR and TC/HDL and between HOMA-IR and TG/HDL in MetS and T2DM patients [49]. Thus, low HDL-C may contribute to the high prevalence of IR; also, the combination of HOMA-IR and HDL-C as available and economic markers would help in identifying high-risk patients in clinical practice.

In this research, the mean WC and WHR were higher in the IR than those in the NIR patients. In line with this result, a study [50] from Quebec Heart Institute revealed that high WC is powerfully related to plasma insulin levels. Besides, Reaven et al. [51] showed that WC is a better predictor than BMI of insulin-mediated glucose uptake. Therefore, WC not only contributes to IR, but also can be used as a powerful predictor of clinical outcomes linked to IR.

Overall, to the best of our knowledge, this study is the first to be conducted on Tabriz population and to investigate the association of IR in relation to inflammatory gene expression levels, metabolic health, lipid profile, and body composition in apparently healthy obese individuals. However, some limitations of the study need to be considered, first, the small sample size and second, the case control nature of the study the cause of which could not be assessed through it.

Conclusion

The results of this study indicated the possible link between IR with TLR4. Also, there was significant correlation between HDL-C and LDL-C with TLR4 and MyD88. Some inflammatory genes and metabolic parameters were also significantly correlated.

Acknowledgments Thanks are due to all who participated in the study.

Authors' contribution MSA and PA wrote the study protocol and study design. BB and DS helped with qPCR. MSA and PA analyzed and interpreted the data. PA, MN, and SM helped with the sampling. PA and MSA were involved in drafting the manuscript or revising it critically for content. All authors have given final approval of the version to be published.

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Compliance with ethical standards

Competing interests The authors declare that they have no conflict of interest.

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