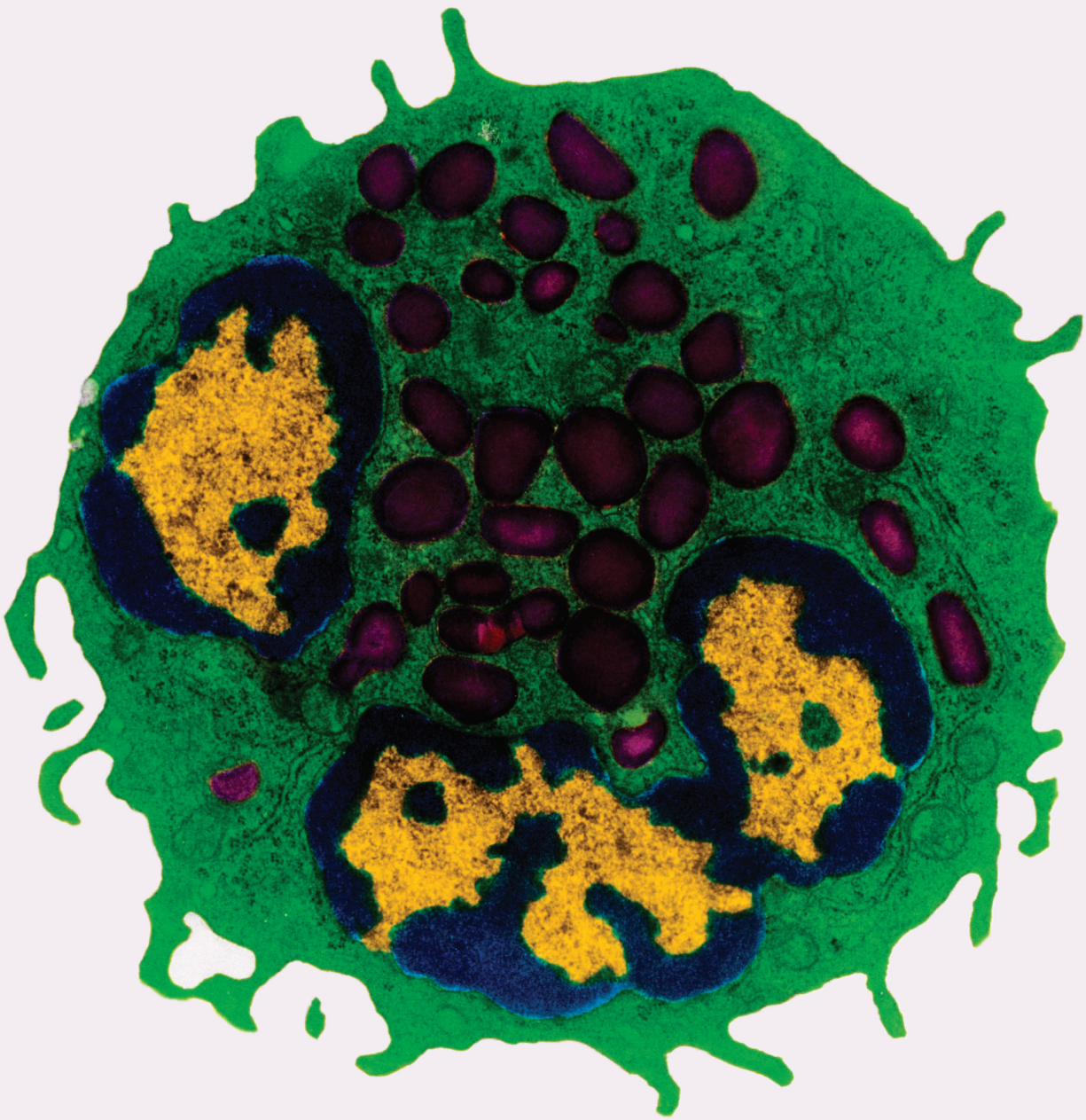
Mediators of Inflammation

Inflammatory Mediators in Obesity

Lead Guest Editor: Divya P. Kumar
Guest Editors: Sai Sudha Koka, Chao Li, and Senthilkumar Rajagopal



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Editorial Board

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Editorial

Inflammatory Mediators in Obesity

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The epidemic of obesity is increasing exponentially, and nearly a third of the world's population is now either overweight or obese [1]. The prevalence of obesity is higher in women than men and increases with age. There is also an alarming increase in the incidence of obesity in children and adolescent [2]. Although there is genetic influence on body weight, obesity results from complex interactions of many factors, including life style, metabolic, socioeconomic, and environmental factors. Thus, obesity, the accumulation of excessive fat, adversely affects nearly all the physiological functions of the body and has emerged as a serious public health challenge [3].

Obesity-induced inflammation is marked by an increased number and activation of immune cells, including macrophages, neutrophils, and T helper cells, leading to the production of proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukins, and C-reactive proteins (CRP) while simultaneously suppressing anti-inflammatory cells and reducing production of adiponectin, predisposing to various cellular stresses like endoplasmic reticulum (ER) stress, mitochondrial dysfunction, and oxidative stress [4]. Obesity-induced inflammation has been implicated as a risk factor in the pathogenesis of insulin resistance, type 2 diabetes mellitus (T2DM), cardiovascular diseases, and metabolic syndrome. It is also associated with the development of other diseases such as psoriasis, renal diseases, polycystic ovary syndrome (PCOS), and cancer [5].

This special issue features a clinical study, original research articles, and review articles that provide insights on various inflammatory markers in obesity-associated diseases. This topic had 21 manuscripts submitted, among which only 9 were accepted for publication.

R. Gomez-Huelgas and colleagues have investigated the impact of intensive life style modification on the levels of adipokine and inflammatory biomarkers in metabolically healthy obese women. The study was a 2-year personalized intervention related to life style modification including a calorie-restricted Mediterranean diet and physical exercise. S. J. Sidles et al. determined how high-fat diet (HFD) alters immunogenic properties of circulating and myeloid-derived $CD45^+$ $DDR2^+$ cells in adipose tissue. They have analyzed myeloid-derived $CD45^+$ $DDR2^+$ cells and $CD4^+$ T cells from peripheral blood (PB), mammary gland-associated adipose tissue (MGAT), and visceral adipose tissue (VAT). They found that myeloid-derived $CD45^+$ $DDR2^+$ cells were more activated in the adipose tissue of HFD-fed preobese mice promoting Th1-type skewing and the production of inflammatory cytokines. The immune system is known to play a key role in the development and progression of T2DM and is characterized by the alterations in the profile of circulating immune cells. M. Šiklová and colleagues have examined the circulating monocyte and lymphocyte populations in association with genetic predisposition to T2DM and the response of these cells to short-term hyperinsulinemia in healthy first-degree relatives of T2D when compared to control subjects. The authors, in addition to providing evidence that there exists an interplay between immune system homeostasis and insulin levels, also showed that there are alterations of

the CD4/CD8 lymphocyte ratio, relative content of Th17 cells, and intermediate monocytes in FDR signifying the role of the immune system in the pathogenesis of T2DM. Furthermore, studies by E. Dozio et al. elucidated the role of the receptor for advanced glycation end products (RAGE) in lipid accumulation in the heart of obese Zucker rats. The authors showed that increased levels of sRAGE (soluble), especially esRAGE (endogenous secretory form), might protect against obesity-induced intromyocardial lipid accumulation by preventing RAGE hyperexpression, therefore allowing lipids to be metabolized. L. Elizondo-Montemayor and colleagues have investigated the concentration of Irisin, a myokine, and its association with high-sensitivity C-reactive proteins (hs-CRP) as well as with metabolic and anthropometric parameters in children and adolescents with T2DM compared to healthy controls. They have explained the possible Irisin-inflammatory crosstalk in overt T2DM and exacerbation of metabolic derangements due to hypoirisinemia emphasizing the need of a detailed study to better understand the mechanisms involved.

In the study by A. R. Kolodziej et al., the authors have performed a systematic review and meta-analysis to explore the prognostic role of elevated myeloperoxidase (MPO) in patients with acute coronary syndrome (ACS). They observed that high MPO levels were associated with the risk of mortality in ACS patients and hence recommended incorporating MPO in risk stratification models that guide therapy of high-risk ACS patients. J. Zhao et al. have elicited the mechanisms of how podocyte injury is caused in obesity-related glomerulopathy (ORG). The authors have showed that in the pathogenesis of ORG, increased expression of CD36 promotes lipid accumulation and activation of NLRP3 inflammasome leading to the secretion of inflammatory cytokines, which causes the injury of podocytes. C. Rodríguez-Cerdeira and colleagues have reviewed the literature about the biomarkers of inflammation in obesity-psoriatic patients. The data available so far strongly suggested that the inflammatory state associated with obesity is a predisposing factor for the development of psoriasis and that obesity aggravates the existing psoriasis. The review summarizes the diagnostic, prognostic, and treatment response biomarkers of inflammation in obesity-psoriatic patients. In the clinical study, D. Pu et al. revealed that the levels of serum ANGPTL8 were elevated in PCOS patients with metabolic syndrome relative to those without metabolic syndrome and this was associated with insulin resistance and adiponectin levels.

Taken together, this special issue aims to emphasize the critical need for the development of effective therapeutic interventions targeting inflammatory pathways in obesity.

Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of this special issue.

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Divya P. Kumar
Saisudha Koka
Chao Li
Senthilkumar Rajagopal

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Clinical Study

Circulating ANGPTL8 Is Associated with the Presence of Metabolic Syndrome and Insulin Resistance in Polycystic Ovary Syndrome Young Women

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Background. ANGPTL8 has been reported to be a regulator of lipid metabolism, and it is associated with insulin resistance (IR) and metabolic syndrome (MetS). We investigated whether ANGPTL8 plays a role in MetS. *Methods.* ANGPTL8 and adiponectin concentrations were measured in PCOS patients with or without MetS and in their corresponding healthy controls. The association of circulating ANGPTL8 with adiponectin and other parameters was also examined. *Results.* Circulating ANGPTL8 concentrations were higher in PCOS women with MetS than in those without MetS and in the controls ($P < 0.01$). ANGPTL8 was positively correlated with age, BMI, FAT%, WHR, SBP, TG, FBG, HbA1c, Fins, and HOMA-IR (all $P < 0.01$) in the study populations and negatively associated with adiponectin and M-values ($P < 0.001$). In addition, ANGPTL8 was positively correlated with PRL, LH, TEST, and FAI and negatively correlated with SHBG (all $P < 0.01$). ROC curve analyses showed that the AUC_{MetS} was 0.87 ($P < 0.001$), with a sensitivity of 92.4% and specificity of 75.4%, and the AUC_{IR} was 0.82 ($P < 0.01$), with a sensitivity of 76.4% and specificity of 75.6%. *Conclusion.* ANGPTL8 levels progressively decrease from PCOS patients with MetS to those without MetS and may be a serum marker associated with the degree of metabolic disorders.

1. Introduction

Metabolic syndrome (MetS) is a cluster of dysmetabolic diseases that increase the risk of cardiovascular disease (CVD), hypertension, and type 2 diabetes mellitus (T2DM) [1, 2]. The working definitions of MetS include abdominal obesity, hyperlipidaemia, hyperglycaemia, and hypertension [3]. MetS subjects have a 5-fold increased risk of T2DM and a 2-fold increased risk of CVD [4, 5]. Atherosclerosis in humans is induced by various components of MetS, and when these components occur together, they are more significant in promoting atherosclerosis [6, 7]. Therefore, in both developed and developing countries, MetS is a fairly serious public health problem [8–10]. It is thus important to improve the preventive and therapeutic strategies of MetS.

Recent studies have reported that some cytokines, such as bone morphogenetic protein-9 (BMP-9) [11], angiopoietin-like protein 8 (ANGPTL8) [12], and irisin [13], are associated with MetS in humans. Therefore, the relationship between these cytokines and the occurrence of MetS has been widely studied. ANGPTL8 is a liver-produced protein that has been found to be related to lipid metabolism, MetS, and insulin resistance (IR) [12, 14–16]. ANGPTL8 expression in adipose tissues and the liver was found to be higher relative to that in

other tissues [15, 16]. As a typical member of the ANGTPL family, ANGPTL8 regulates triacylglycerol (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels by interacting with ANGTPL3 [14, 16–18]. In type 1 diabetes mellitus (T1DM) and insulin-deficient animals, serum ANGPTL8 levels were increased [14, 19]. In T2DM animals, hepatic ANGPTL8 expression was upregulated, suggesting that ANGPTL8 is regulated by IR [19]. Recently, some contrary reports have found that ANGPTL8 is not associated with IR and the proliferation of islet β cells [20]. Thus, with regard to the association between ANGPTL8 and IR, the present results are contradictory, and further study is needed.

Polycystic ovary syndrome (PCOS) is a complex endocrine and metabolic disorder, characterized by chronic anovulation and hyperandrogenism. It is well known that insulin resistance play an important role in the pathogenesis of PCOS and Mets. Whether there is any difference between PCOS subjects with Mets and those without Mets in the level of insulin resistance and the ANPTL8 are not known.

More recently, Abu-Farha et al. reported that circulating ANGPTL8 is elevated in MetS individuals and is significantly related to high-sensitivity C-reactive protein (CRP), highlighting its role in dysmetabolism and chronic inflammation [12]. To exclude the effects of age and sex, in the current study, teenage women were employed as study individuals. Our results showed that serum ANGPTL8 levels in MetS women are significantly elevated relative to healthy controls and associated with adiponectin (ADI) levels and IR.

2. Materials and Methods

In the current study, 241 teenage women (98 healthy controls and 143 polycystic ovary syndrome (PCOS) subjects) were recruited from the community through advertisement or routine medical check-up in the Department of Endocrinology at the Second Affiliated Hospital of Chongqing Medical University between 2016 and 2017. The diagnosis of PCOS was based on the 2003 Rotterdam consensus (the Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group) [21]. MetS was diagnosed by three or more of the following metabolic risk factors which were clearly defined by the International Diabetes Federation and the American Heart Association in 2009: (1) central obesity (waist circumference (WC) ≥80 cm in females and ≥90 cm in males), (2) hypertriglyceridaemia (triglyceride (TG) ≥1.69 mmol/L), (3) HDL-C <1.29 mmol/L in females and <1.04 mmol/L in males, (4) hyperglycaemia (FBG ≥5.6 mmol/L or T2DM), and (5) hypertension (sitting blood pressure (BP) ≥130/85 mmHg, taken as a mean of two readings obtained after resting for 10-15 minutes or taking oral antihypertensive medication). The exclusion criteria include cancer, cirrhosis, positive infection, heart failure, long-term treatment with steroids, or other medical problems. All patients with MetS were newly diagnosed without any drug treatment. Healthy controls without clinical evidence of major diseases were screened from the community through advertisement or routine medical check-up. These individuals did not take any medicine. This study was conducted according to the Declaration of Helsinki and was supported by the ethics committee of our hospital. Informed consent was obtained from participants, which were given a full explanation of the study. This study was registered with the Chinese Clinical Trial Registry at https://www.chictr.org (CHICTR-OCC-13003185).

2.1. Anthropometric and Biochemical Analyses. All participants underwent a physical examination. Anthropometry was performed under standardized conditions by trained staff before breakfast. Body weight and height were examined by a trained nurse, with participants wearing light indoor clothing and nothing on their feet (barefooted), using calibrated portable electronic weighing scales. The body mass index (BMI) was calculated as weight (kg) divided by squared height (metres). WC and hip circumference (HC) were measured by the same nurse and recorded to the nearest 0.1 cm. The waist-to-hip ratio (WHR) was calculated by WC/HC. The BP was measured on the nondominant arm using a mercury sphygmomanometer in all individuals after resting for at least 10 minutes. We used bioelectrical impedance (BIA-101; RJL Systems) to examine the percentage of body fat (FAT%). The homeostasis model assessment of IR (HOMA-IR) was calculated by the following equation: HOMA − IR = fasting insulin (FIns, mU/L) × fasting blood glucose (FBG, mmol/L)/22.5 [22]. Blood samples were collected after fasting for 10-14 h and centrifuged to separate the serum. HbA1c, glucose, insulin, and lipids were measured routine chemistry laboratory at the hospital.

2.2. Hormone Measurement. Blood samples were collected in the early follicular phase (days 3 to 5 of the menstrual cycle) in the control group. Blood samples were collected after a spontaneous bleeding episode or upon first examination in PCOS women. Serum hormone concentrations, including luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, and progesterone (Prog), were measured with a well-established electrochemiluminescence immunoassay using COBAS E immunoassay analysers (Roche Diagnostics GmbH). Total testosterone levels were measured with a coated tube radioimmunoassay (RIA; DiaSorin, S. p. A, Saluggia, Italy, and Diagnostic Products Corporation).

Dehydroepiandrosterone sulfate (DHEA-S) and sex hormone-binding globulin (SHBG) were detected using an automated analyser (Abbott Architect; Abbott Laboratories). The free androgen index (FAI) was calculated by the following equation: FAI = testosterone (nmol/L) × 100/SHBG (nmol/L) × 100 [23].

2.3. Euglycaemic-Hyperinsulinaemic Clamping (EHC). EHC was used to as the gold standard to the diagnosis of IR and performed in all participants as previously reported [24]. Briefly, after fasting for 10-12 h, a catheter was placed in the antecubital vein to infuse insulin and glucose. Another catheter was placed retrograde in the dorsal vein of the contralateral hand for blood withdrawal. Regular human insulin (1 mU/kg minute) was infused for 2 h, and a variable infusion of 20% glucose was administered to maintain plasma glucose at the fasting level. During clamping, blood glucose levels

were measured every 10-15 minutes to guide the glucose infusion. The rate of glucose disposal (GRd) was defined as the glucose infusion rate (GIR) during the stable period of the clamp and was related to body weight (M-value). Blood samples for ANGPTL8 measurements were obtained at 0, 80, 100, and 120 minutes. The samples were immediately cooled, and serum/plasma was prepared within 1 h and stored at -80°C until further use.

2.4. Cytokine Measurements. Serum ANGPTL8 concentration was determined with an ELISA obtained from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA) by using the manufacturer's protocol. The intra-assay and interassay variations were <10% and <15%, respectively. The linear range of the assay was 0-100 μg/L. The assay has high sensitivity and excellent specificity for the detection of ANGPTL8 with no significant cross-reactivity or interference. Serum ADI levels were also measured with an ELISA from Adipobiotech as previously described [25].

2.5. Statistical Analysis. All analyses were performed with Statistical Package for the Social Sciences version 19.0 (SPSS Inc., Chicago, IL). Normally distributed data were expressed as the mean ± SD. The data for nonnormal distribution were skewed and logarithmically transformed to obtain a normal distribution, which was expressed as the median with interquartile range (IQR). An unpaired t test or one-way ANOVA was performed to analyse the differences between two or more groups. Spearman's correlation analysis was used to examine the association of circulating ANGPTL8 with other parameters. Relationships between the ANGPTL8 and the other variables were investigated by using a multiple stepwise regression analysis, with ANGPTL8 as a dependent variable. A multivariate logistic regression analysis was used to investigate the association of ANGPTL8 with MetS. Receiver operating characteristic (ROC) curves were used to analyse the predictive values of serum ANGPTL8 for MetS and IR. All data were based on two-sided tests. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Main Clinical Features, Hormone, and Serum ANGPTL8 Levels in Study Populations. The anthropometric and biochemical parameters in the study populations are shown in Table 1. PCOS women with MetS have higher BMI, Fat%, WHR, BP, TG, total cholesterol (TC), LDL-C, FBG, FIns, HbA1c, and HOMA-IR and lower HDL-C and M-values than PCOS women without MetS and/or healthy controls ($P < 0.05$ or $P < 0.01$; Table 1 and Figure 1(c)). Furthermore, in PCOS women with MetS, serum TEST, DHEAS levels, and the FAI were markedly higher, while PRL and SHBG was lower than those in PCOS women without MetS and/or healthy controls ($P < 0.05$ or $P < 0.01$; Table 1). Importantly, serum ANGPTL8 levels in PCOS women with MetS were significantly higher than those in PCOS women without MetS and healthy controls ($P < 0.05$ or $P < 0.01$; Table 1 and Figure 1(a)). Serum ANGPTL8 levels remained significantly different after adjustment for age and BMI (Table 1). Serum ADI concentrations, an adipocytokine-related insulin sensitivity, were markedly lower in MetS individuals than in non-MetS individuals and healthy controls ($P < 0.05$ or $P < 0.01$; Table 1 and Figure 1(b)).

3.2. The Association between Serum ANGPTL8 and Other Parameters in Study Populations. Spearman's correlation analysis showed that serum ANGPTL8 at baseline was correlated positively with age ($r = 0.16$, $P < 0.01$), BMI ($r = 0.48$, $P < 0.001$), FAT% ($r = 0.43$, $P < 0.001$), WHR ($r = 0.43$, $P < 0.001$), SBP ($r = 0.20$, $P < 0.001$), TG ($r = 0.42$, $P < 0.001$), FBG ($r = 0.28$, $P < 0.001$), HbA1c ($r = 0.15$, $P < 0.05$), FIns ($r = 0.53$, $P < 0.001$), and HOMA-IR ($r = 0.53$, $P < 0.001$) and negatively correlated with ADI ($r = -0.44$, $P < 0.001$) and M-values ($r = -0.51$, $P < 0.001$) in the study populations (Table 2). In addition, circulating ANGPTL8 was positively correlated with PRL ($r = 0.17$, $P < 0.01$), LH ($r = 0.22$, $P < 0.001$), TEST ($r = 0.27$, $P < 0.001$), and FAI ($r = 0.34$, $P < 0.001$) and negatively correlated with SHBG ($r = -0.35$, $P < 0.001$) in the study populations (Table 3). Furthermore, ADI was negatively correlated with BMI, FAT%, WHR, TG, FBG, FIns, HbA1c, HOMA-IR, LH, TEST, DHEAS, and FAI but positively correlated with M-value and SHBG (Tables 2 and 3). In all study populations, regression analyses of all-factor and stepwise models indicated that the main predictors of circulating ANGPTL8 were LDL-C and BMI (Figure 1(d)). The multiple regression equation was $Y_{\text{ANGPTL8}} = 0.130 + 0.013X_{\text{BMI}} + 0.003X_{\text{FIns}} + 0.053\,X_{\text{LDL-C}} - 0.012X_{M\text{-value}} - 0.050X_{\text{HDL-C}} + 0.005X_{\text{LH}}$ ($R^2 = 0.406$, $P < 0.01$).

In fully adjusted logistic regression models controlling for anthropometric variables, BP, lipid profile, and hormone, higher serum ANGPTL8 concentrations were markedly related to the high onset of MetS in individuals with PCOS (Table 4).

3.3. Effects of EHC on Circulating ANGPTL8 in the Study Populations. To investigate whether serum ANGPTL8 levels are affected by hyperinsulinaemia, EHC was performed in PCOS women with and without MetS and in healthy women. Insulin levels during EHC were elevated from 8.0 ± 3.3 to 110.1 ± 15.2 mU/L in healthy women and from 25.0 ± 6.0 to 105.2 ± 20.3 mU/L in PCOS women. Blood glucose was clamped at euglycaemic levels (~5.5 mmol/L) by an infusion of 25% glucose without significant hypoglycaemic events in these individuals. During EHC, M-values were markedly lower in MetS individuals than those in non-MetS and healthy women (Table 1), indicating more obvious IR in PCOS women with MetS. In response to hyperinsulinaemia, serum ANGPTL8 concentrations decreased significantly in all study individuals (Figure 1(e)). During the stable clamping state, circulating ANGPTL8 was maintained at a lower level in all three groups due to hyperinsulinaemia (from 0.38 ± 0.17 to 0.15 ± 0.09 μg/L for the controls, from 0.49 ± 0.15 to 0.20 ± 0.08 μg/L for non-MetS, and from 0.68 ± 0.14 to 0.20 ± 0.08 μg/L for MetS). However, in MetS individuals, circulating ANGPTL8 levels in the stable clamping state were still significantly higher than those in healthy individuals (0.20 ± 0.08 vs. 0.15 ±

Table 1: Main clinical features and circulating betatrophin levels in study populations.

Group	PCOS		Controls	P value
	MetS	No-MetS		
N	65	78	98	—
Age (yr)[b]	26.2 ± 3.4	25.4 ± 4.9	25.7 ± 2.3	NS
BMI (kg/m^2)[a]	25.9(23.2-30.4)$^{**\blacktriangle\blacktriangle}$	23.5(19.7-26.4)**	20.0(18.6-21.3)	<0.001
FAT (%)	39.27±9.23$^{**\blacktriangle\blacktriangle}$	31.8±9.0**	26.6 ± 5.5	<0.001
WHR[b]	0.89(0.83-0.93)$^{**\blacktriangle\blacktriangle}$	0.84(0.80-0.87)**	0.78(0.75-0.84)	<0.001
SBP (mmHg)[b]	119(109-125)**	114(107-120)**	108(102-116)	<0.001
DBP (mmHg)	78 ± 9$^{*\blacktriangle}$	75 ± 6	75 ± 8	<0.05
TG (mmol/L)[a]	2.30(1.66-3.08)$^{**\blacktriangle\blacktriangle}$	1.06(0.64-1.77)*	0.80(0.59-1.29)	<0.001
TC (mmol/L)	4.64±0.95$^{**\blacktriangle}$	4.24 ± 1.11*	3.86 ± 1.00	<0.001
HDL-C (mmol/L)[b]	1.20(1.04-1.25)$^{**\blacktriangle\blacktriangle}$	1.41(1.19-1.58)**	1.17(0.99-1.43)	<0.001
LDL-C (mmol/L)	2.69±0.86$^{**\blacktriangle}$	2.34 ± 0.83	2.16 ± 0.87	<0.01
FFA (μmol/L)	0.63 ± 0.18	0.55 ± 0.21	0.56 ± 0.28	NS
FBG (mmol/L)[a]	5.15(4.74-5.76)$^{**\blacktriangle\blacktriangle}$	4.81(4.43-5.05)**	4.42(4.03-4.74)	<0.001
FIns (pmol/L)[b]	19.24 (13.3-28.3)$^{**\blacktriangle\blacktriangle}$	9.59(6.80-17.75)**	7.02(6.10-8.45)	<0.001
HbA1c (%)[b]	5.30(5.10-5.65)**	5.30(5.10-5.50)**	5.20(5.00-5.30)	<0.001
HOMA-IR[b]	4.55(2.68-6.34)$^{**\blacktriangle\blacktriangle}$	2.05(1.52-3.55)**	1.35(1.16-1.73)	<0.001
M-value (mg/kg/min)[a]	4.12(30.48-5.71)$^{**\blacktriangle\blacktriangle}$	5.91(4.95-8.77)**	10.29(8.07-11.94)	<0.001
ADI (μg/mL)	26.4±12.6$^{**\blacktriangle\blacktriangle}$	36.5±16.9**	47.06 ± 14.04	<0.001
ANGPTL8 (μg/L)	0.67±0.14$^{**\blacktriangle\blacktriangle}$	0.52±0.16**	0.33 ± 0.16	<0.001
PRL (mIU/L)[b]	337.1(230.8-561.6)**	381.6(216.8-490.0)	374.6(235.0-404.6)	NS
PROG (nmol/L)[b]	2.81(1.87-3.12)	2.81(2.18-3.12)	2.50(1.87-3.20)	NS
LH (IU/L)[a]	7.32(4.56-11.90)**	10.80(6.21-15.09)**	4.31(3.04-6.16)	<0.001
FSH (IU/L)[a]	7.60(6.10-8.63)	7.42(6.18-9.10)	7.80(6.73-9.26)	NS
TEST (nmol/L)[b]	2.99(1.95-3.86)**	2.79(2.20-3.43)**	1.65(1.21-2.29)	NS
E2 (pmol/L)[b]	212.9(126.6-280.8)	194.5(98.4-244.1)	183.5(119.6-255.1)	NS
DHEAS (μg/dL)[b]	202.9(163.3-149.5)*	183.4.0(150.4-214.3)	182.1(141.9-217.0)	<0.05
SHBG (nmol/L)[a]	30.6(17.8-42.2)$^{**\blacktriangle\blacktriangle}$	40.6(24.2-75.2)**	57.4(42.0-75.6)	<0.001
FAI[a]	9.45(4.83-16.96)$^{**\blacktriangle}$	6.78(3.47-10.21)**	2.59(1.80-4.97)	<0.001

BMI: body mass index; FAT%: body fat %; WHR: waist-to-hip ratio; SBP: systolic blood pressure; DBP: diastolic blood pressure; TG: triglyceride; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; FFA: free fatty acid; FBG: fasting blood glucose; FIns: fasting insulin; HOMA-IR: HOMA-insulin resistance index; M-value: whole body glucose uptake rate; ADI: adiponectin; PRL: prolactin; PROG: progestogen; LH: luteinizing hormone; FSH: follicle-stimulating hormone; TEST: total testosterone; E2: estradiol; DHEAS: dehydroepiandrosterone sulfate; SHBG: sex hormone-binding globulin. FAI: free androgen index. Data are median (interquartile range) or frequency (percent). [a]Log transformed before analysis; [b]nonparametric test was used in comparisons between those groups. $^{*}P < 0.05$, compared with controls; $^{\blacktriangle}P < 0.05$, compared with no MetS; $^{**}P < 0.01$, compared with the controls; $^{\blacktriangle\blacktriangle}P < 0.01$, compared with no MetS.

0.09 μg/L, $P < 0.05$; Figure 1(e)). Therefore, in response to hyperinsulinaemia during EHC, circulating ANGPTL8 levels were significantly decreased. These results indicate that circulating ANGPTL8 is associated with dysmetabolism and IR.

3.4. The Predictive Value of Circulating ANGPTL8 in Detecting MetS and IR. Finally, we performed the ROC curve of circulating ANGPTL8 for predicting MetS and IR. The results showed that the area under the ROC curves was 0.87 ($P < 0.001$) with a sensitivity of 92.4% and specificity of 75.4% for MetS (AUC_{MetS}) (Figure 2(a)) and 0.82 ($P < 0.01$) with a sensitivity of 76.4% and specificity of 75.6% for IR (AUC_{IR}) (Figure 2(b)). The best cutoff values for serum ANGPTL8 levels to predict MetS and IR were 0.53 and 0.51 μg/L, respectively.

4. Discussion

MetS and PCOS have some similar clinical manifestations such as obesity and lipid metabolism disorders. The mechanism lies in the MetS, and PCOS is still unclear. Researchers hold there may have some correlation between MetS and PCOS that include: (1) insulin resistance is the milestone between MetS and PCOS; (2) some adipokines are associated with MetS and PCOS, such as leptin, insulin-like growth

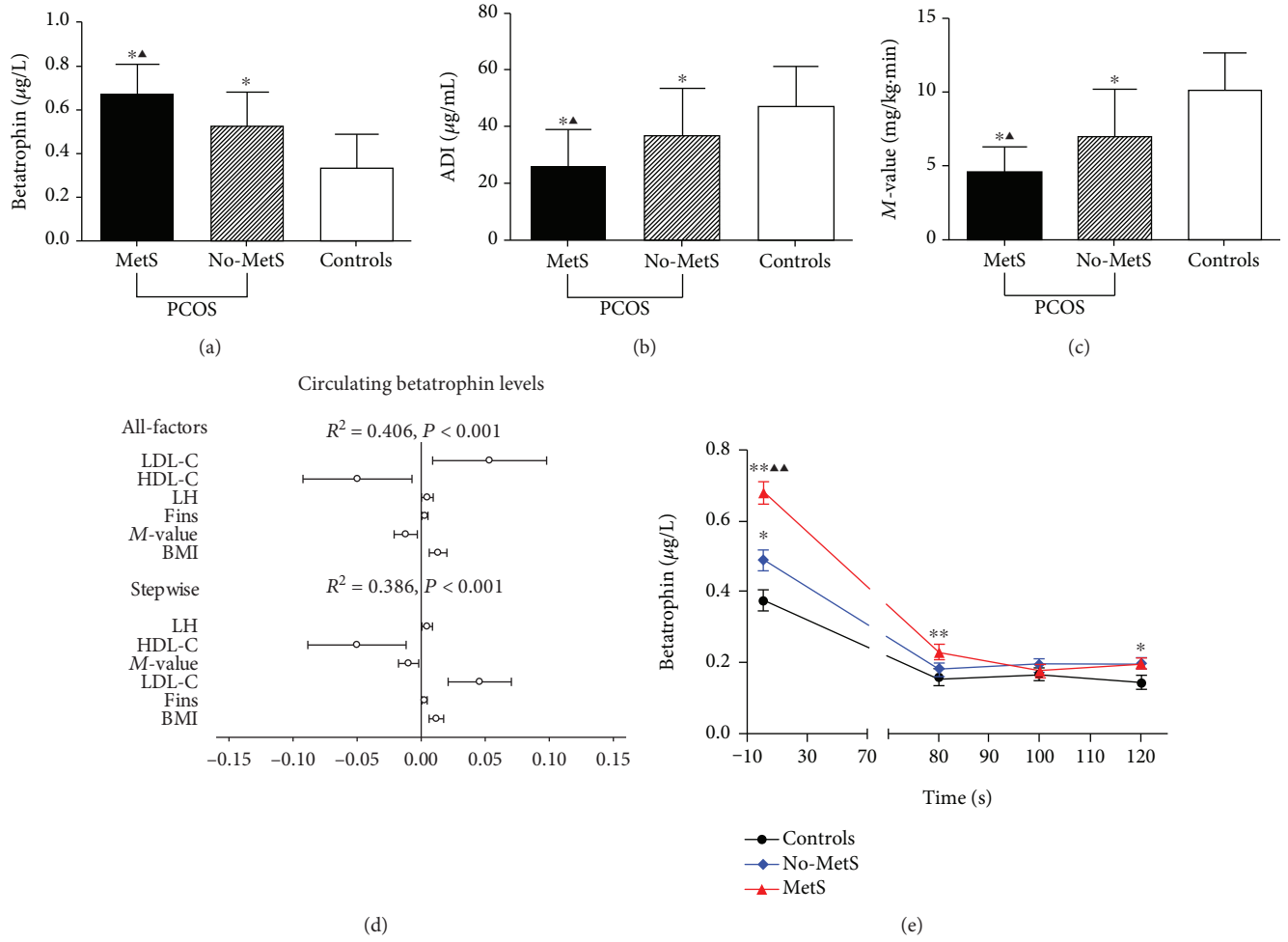

Figure 1: Parameters in PCOS women with MetS or without MetS and healthy controls. (a) Serum betatrophin levels in PCOS women with MetS were significantly higher than that of PCOS women without MetS and healthy controls ($P < 0.05$ or $P < 0.01$); (b) serum ADI concentrations, an adipocytokine-related insulin sensitivity, were markedly lower in MetS individuals than no-MetS individuals and healthy controls ($P < 0.05$ or $P < 0.01$); (c) PCOS women with MetS have lower M-values compare with PCOS women without MetS and/or healthy controls ($P < 0.05$ or $P < 0.01$); (d) in all study populations, regression analyses of all-factor and stepwise models indicated that the main predictors of circulating ANGPTL8 were LDL-C and BMI; (e) serum ANGPTL8 concentrations decreased significantly in all study individuals and in MetS individuals; circulating ANGPTL8 levels at the stable state of clamp were still significantly higher than that of healthy individuals.

factor-1, and adiponectin. Insulin resistance is the common soil between MetS and PCOS, which has been generally recognized. Therefore, it is a promising work to find a specific target for regulating insulin resistance [3, 9, 13].

Although circulating ANGPTL8 levels have been reported to be related to IR [26], T2DM [27–30], obesity [27, 30], PCOS [31–33], nonalcoholic fatty liver disease (NAFLD) [34], and MetS [12, 35], ANGPTL8 is mainly secreted and expressed by hepatocytes. It reduces the cleavage of triglycerides by inhibiting the activity of lipoprotein esterase and increases the level of triglycerides. ANGPTL8 also promotes the proliferation of islet beta cells. Some researchers suggested that the serum ANGPTL8 level was significantly increased in type 2 diabetes patients; the ANGPTL8 level was positively correlated with insulin resistance and negatively correlated with insulin sensitivity [29]. High insulin level increases the ANGPTL8 expression by activating the PI3K/Akt pathway, while insulin resistance inhibits the ANGPTL8 expression [36]. Other researches declared that overexpression of ANGPTL8 may inhibit the PI3K/Akt pathway, reduce insulin sensitivity, and enhance insulin resistance in hepatocytes [31]. The results are inconsistent, and the regulation factors of ANGPTL8 are not clear. Therefore, as with most new discoveries, the association of circulating ANGPTL8 with these diseases needs to be studied repeatedly.

In the current study of PCOS and ANGPTL8, regardless of BMI, the circulatory ANGPTL8 levels are elevated in PCOS patients compared to controls. PCOS patients with higher insulin resistance had substantially higher circulating ANGPTL8 concentrations [37]. Other research suggested that ANGPTL8 levels were increased in women with PCOS and were associated with insulin resistance, hs-CRP, and free testosterone in these patients (Table 5). Elevated ANGPTL8

Table 2: Spearman's correlation coefficients between circulating ANGPTL8 and other parameters.

Group	ANGPTL8	ADI	*M*-value	Age	BMI	FAT (%)	WHR	SBP	DBP	TG	HDL-C	FBG	FIns	HbA1c	HOMA-IR
ANGPTL8	1	-0.440 [a]	-0.507[a]	0.159[b]	0.475[a]	0.433[a]	0.425[a]	0.202[a]	0.065	0.424[a]	-0.003	0.280[a]	0.527[a]	0.147[c]	0.533[a]
ADI		1	0.605[a]	-0.127	-0.479[a]	-0.453[a]	-0.387[a]	-0.138	0.065	-0.299[a]	0.056	-0.272[a]	-0.475[a]	-0.244[a]	-0.483[a]
M-value			1	-0.110	-0.599[a]	-0.708[a]	-0.495[a]	-0.270[a]	-0.100	-0.460[a]	-0.028	-0.360[a]	-0.701[a]	-0.368[a]	-0.0709
Age				1	0.160[b]	0.168[a]	0.154[c]	0.50	0.106	0.074	0.064	0.167[a]	0.063	-0.013	0.098
BMI					1	0.771[a]	0.517[a]	0.288[a]	0.130[c]	0.368[a]	0.041	0.247[a]	0.547[a]	0.094	0.555[a]
FAT (%)						1	0.551[a]	0.358[a]	0.167[a]	0.409[a]	-0.009	0.383[a]	0.645[a]	0.148[c]	0.655[a]
WHR							1	0.267[a]	0.101	0.292[a]	-0.043	0.167[a]	0.456[a]	0.204[a]	0.448[a]
SBP								1	0.417[a]	0.071	-0.066	0.111	0.223[a]	0.120	0.224[a]
DBP									1	-0.069	-0.022	-0.008	0.025	0.039	0.020
TG										1	-0.038	0.289[a]	0.392[a]	0.182[a]	0.415[a]
HDL-C											1	0.039	-0.015	0.008	-0.003
FBG												1	0.410[a]	0.179[a]	0.555[a]
FIns													1	0.249[a]	0.981[a]
HbA1c (%)														1	0.255[a]
HOMA-$_{IR}$															1

a: $P < 0.001$; b: $P < 0.01$; c: $P < 0.05$.

Table 3: Spearman's correlation coefficients between ANGPTL8 and sex hormone.

Group	ANGPTL8	PRL	PROG	LH	FSH	TEST	E2	DHEAS	SHBG	FAI
ANGPTL8	1	0.167[b]	-0.078	0.219[a]	-0.055	0.271[a]	0.070	0.117	-0.349[a]	0.342[a]
M-value		-0.172 [a]	0.024	-0.199[a]	0.099	-0.314[a]	-0.024	-0.205[a]	0.494[a]	-0.466[a]
ADI		-0.101	0.048	-0.204[a]	0.096	-0.291[a]	0.022	-0.207[a]	0.392[a]	-0.416[a]
PRL		1	0.007	0.024	0.020	0.201[a]	0.145[c]	0.027	-0.274[a]	0.249[a]
PROG			1	-0.039	-0.119	-0.003	0.055	0.055	0.055	-0.081
LH				1	0.115	0.457[a]	0.018	-0.060	-0.299[a]	0.418[a]
FSH					1	0.008	-0.050	-0.051	-0.008	0.028
TEST						1	0.191[a]	0.284[a]	-0.361[a]	0.814[a]
E2							1	0.152[c]	-0.191[a]	0.199[a]
DHEAS								1	-0.329[a]	0.347[a]
SHBG									1	-0.798[a]
FAI										1

a: $P < 0.001$; b: $P < 0.01$.

Table 4: Association of circulating ANGPTL8 with MetS in fully adjusted models.

Model adjust	MetS			Insulin resistance		
	OR	95% CI	*P*	OR	95% CI	*P*
Age	6.69	3.90-11.47	<0.001	4.82	3.17-7.32	<0.001
Age, BMI	4.79	2.74-8.36	<0.001	3.19	2.07-4.93	<0.001
Age, BMI, WHR	4.47	2.49-8.03	<0.001	3.27	2.11-5.08	<0.001
Age, BMI, WHR, HbA1c	4.51	2.49-8.17	<0.001	3.24	2.06-5.12	<0.001
Age, BMI, WHR, HbA1c, FIns	3.95	2.15-7.26	<0.001	2.19	1.29-3.71	<0.01
Age, BMI, WHR, HbA1c, FIns, lipid profile	4.12	2.16-7.87	<0.001	2.49	1.42-4.36	<0.01
Age, BMI, WHR, HbA1c, FIns, lipid profile, hormone	5.71	2.51-13.0	<0.001	2.43	1.29-4.61	<0.01

Results of binary logistic regression analysis are presented as the odds ratio (OR) of being in MetS status decrease in circulating. BMI: body mass index; WHR: waist-to-hip ratio; FAT (%): the percentage of fat *in vivo*; SBP: systolic blood pressure; DBP: diastolic blood pressure; lipid profile: including total cholesterol, FFA, triglyceride, and LDL- and HDL-cholesterol. Hormone: including SHBG, DHEAS, E2, TEST, LH, FSH, PRL, and PROG.

levels were found to increase the odds of having PCOS [38]. And one research declared that ANGPTL8 levels are reduced in full-blown PCOS patients and positively associated with low-density lipoprotein cholesterol [39]; therefore, further research is needed to elucidate the role of ANGPTL8 in PCOS and insulin resistance.

We investigated ANGPTL8 circulation levels in PCOS patients with or without MetS and in healthy women. The significance of this design is that we can exclude the effects of gender and age on the results. Our data showed that serum ANGPTL8 levels in healthy young women were lower than those in normal individuals, as reported by Abu-Farha et al. (0.33 ± 0.16 vs. 0.71 (0.59–1.15) μg/L). In our study, MetS individuals had lower circulating ANGPTL8 levels than the MetS individuals reported by Abu-Farha et al. (0.67 ± 0.14 vs. 1.14 (0.17–1.17) μg/L) [12]. In addition, circulating ANGPTL8 levels in our study were lower than those reported by Liu et al. (0.12 ± 0.08 μg/L for MetS subjects and 0.13 ± 0.01 μg/L for the controls) [35]. We consider that the difference between the present and previous studies may be due to the effects of age and sex on study populations. In the previous two studies, the study subjects were selected from individuals aged 18-70 years and with different sexes, while in our study, the age of the subjects was limited to 18-35 years, and only women were included in the study.

In the current study, we found that circulating ANGPTL8 levels were higher in PCOS women than in healthy women, and PCOS women with MetS also had higher circulating ANGPTL8 levels than PCOS women without MetS. These results suggest that with the aggravation of this metabolic disorder, the circulating levels of ANGPTL8 are progressively increased from normal young women to PCOS patients and then to PCOS patients with MetS. Therefore, ANGPTL8 may be a serum marker related to the degree of dysmetabolism *in vivo*.

It is noteworthy that our results were consistent with two published studies performed by Crujeiras et al. and Abu-Farha et al., in which circulating ANGPTL8 was increased in MetS individuals [12, 40]. However, in another study, there was no significant difference in circulating ANGPTL8 between the MetS and normal subjects [41]. This disparity may be due to confounding factors, such as anthropometric characteristics, age, and gender. All MetS individuals enrolled in this study were newly diagnosed without any drug treatment and were PCOS women aged 18-35 years and without T2DM.

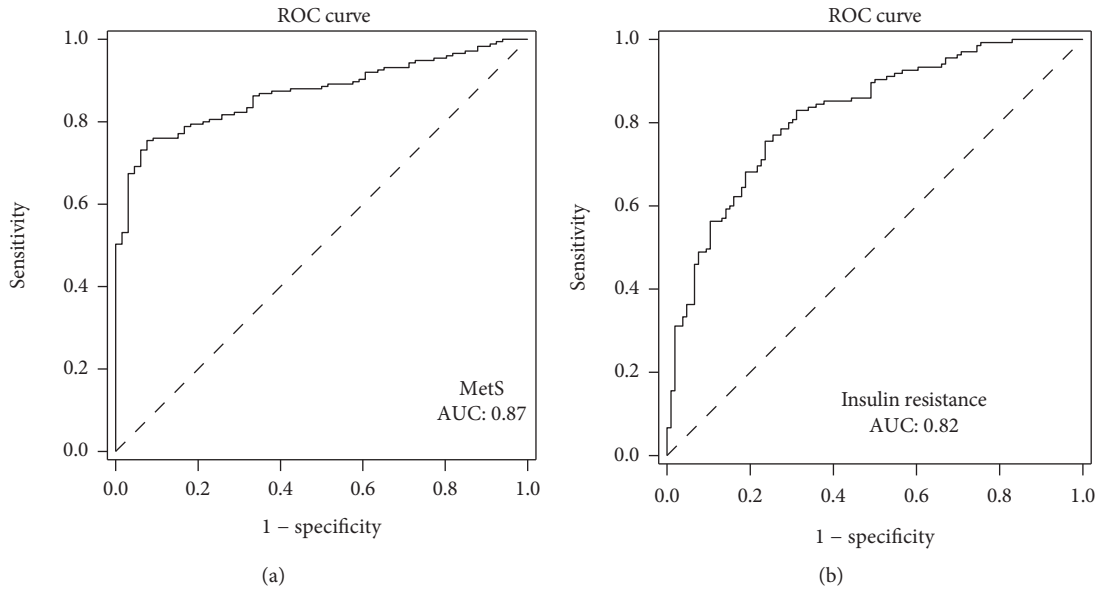

Figure 2: The ROC curve of circulating ANGPTL8 for predicting MetS and IR. (a) The area under the ROC curves was 0.87 ($P < 0.001$) with a sensitivity of 92.4% and specificity of 75.4% for MetS (AUC_{MetS}); (b) the area under the ROC curves was 0.82 ($P < 0.01$) with a sensitivity of 76.4% and specificity of 75.6% for IR (AUC_{IR}).

Table 5: Row mean scores and Cochran-Armitage trend test of the impact of circulating ANGPTL8 levels on MetS.

	MetS	
	x^2	P value
Row mean scores test	76.8259	<0.001
Cochran-Armitage trend test	5.5862	<0.001

The circulating ANGPTL8 levels of all subjects were cut-off and adjusted for age, sex, BMI, WHR, BP, and lipid profile.

In this study, we also found that fasting serum full-length ANGPTL8 levels were positively correlated with markers of adiposity (BMI, FAT%, and WHR), the glycometabolism index (FBF, 2 h-BG, and HbA1c), and IR markers (FIns and HOMA-IR) but negatively correlated with ADI and M-values. Consistent with the current results, previous reports from different groups also showed these correlations between ANGPTL8 and other parameters [28, 29]. In addition, our ROC curve analysis also indicated that circulating ANGPTL8 could predict MetS with a relatively high sensitivity and specificity. Therefore, the association between ANGPTL8 and glucose, adiposity, ADI, and IR parameters confirmed the potential role of ANGPTL8 in metabolic disorders and IR and thus contributed to the occurrence and development of MetS.

Our study had some strengths. First, ANGPTL8 levels were examined in young women; thus, miscellaneous factors in sex and age were excluded. Second, we used age- and gender-matched controls, making between group comparisons more feasible. Third, all individuals in this study were drug-naïve and untreated with diet control or exercise. Fourth, EHC, a gold standard for IR, was performed in all participants. Insulin sensitivity is accurately evaluated.

Our study has some limitations: (1) the design of this cross-sectional study cannot suggest causality, (2) our data could be affected by some outliers due to the related small sample size, and (3) circulating ANGPTL8 levels are based on single measurements, which may not reflect the alternations in ANGPTL8 levels over time. Serial alternations in circulating CTRP-5 concentrations should be measured at different stages of T2DM and IR to investigate the role of CTRP-5 in the development of T2DM. In addition, the study population consisted entirely of Chinese people. Thus, the application of these data to other ethnic populations should be undertaken with caution. Nevertheless, the use of newly diagnosed PCOS patients with and without MetS and their age- and gender-matched controls prevents pharmacotherapy complications or other confounding variables.

In conclusion, the current study shows that circulating ANGPTL8 concentrations are progressively increased from healthy controls to PCOS patients and then to PCOS patients with MetS. The high concentrations of ANGPTL8 in PCOS populations were related to the incidence of MetS. Our results highlight a possible role for ANGPTL8 in IR and MetS. In future studies, this cytokine might be used as a biomarker for MetS and IR.

Abbreviations

PCOS: Polycystic ovary syndrome
BMI: Body mass index
FAT%: Body fat %
WHR: Waist-to-hip ratio
SBP: Systolic blood pressure
TG: Triglyceride
FBG: Fasting blood glucose
FIns: Fasting insulin
HOMA-IR: HOMA-insulin resistance index
PRL: Prolactin

LH: Luteinizing hormone
TEST: Total testosterone
FAI: Free androgen index
SHBG: Sex hormone-binding globulin
AUC: The area under the receiver operating characteristic (ROC) curve.

Data Availability

The individual unidentified participant data (including data dictionaries), study protocol, and statistical analysis plan will be available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

D.P. and J.Y. researched and analysed the data. L.L. and R.L. performed the ELISA assay and handled data analysis. Y.G. and Y.L. wrote the manuscript. Q.W. was the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors approved the final version of the manuscript. Danlan Pu, Ling Li, and Jingxia Yin contributed equally to this project.

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Review Article

Prognostic Role of Elevated Myeloperoxidase in Patients with Acute Coronary Syndrome: A Systemic Review and Meta-Analysis

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Background. Myocardial inflammation following acute ischemic injury has been linked to poor cardiac remodeling and heart failure. Many studies have linked myeloperoxidase (MPO), a neutrophil and inflammatory marker, to cardiac inflammation in the setting of acute coronary syndrome (ACS). However, the prognostic role of MPO for adverse clinical outcomes in ACS patients has not been well established. *Methods*. MEDLINE and Cochrane databases were searched for studies from 1975 to March 2018 that investigated the prognostic value of serum MPO in ACS patients. Studies which have dichotomized patients into a high MPO group and a low MPO group reported clinical outcomes accordingly and followed up patients for at least 30 days to be eligible for enrollment. Data were analyzed using random-effects model. Sensitivity analyses were conducted for quality control. *Results*. Our meta-analysis included 13 studies with 9090 subjects and a median follow-up of 11.4 months. High MPO level significantly predicted mortality (odds ratio (OR) 2.03; 95% confidence interval (CI): 1.40-2.94; $P < 0.001$), whereas it was not significantly predictive of major adverse cardiac events and recurrent myocardial infarction (MI) (OR 1.28; CI: 0.92-1.77, $P = 0.14$ and OR 1.23; CI: 0.96-1.58, $P = 0.101$, respectively). Hypertension, diabetes mellitus, and age did not affect the prognostic value of MPO for clinical outcomes, whereas female gender and smoking status have a strong influence on the prognostic value of MPO in terms of mortality and recurrent MI (metaregression coefficient -8.616: 95% CI -14.59 to -2.633, $P = 0.0048$ and 4.88: 95% CI 0.756 to 9.0133, $P = 0.0204$, respectively). *Conclusions*. Our meta-analysis suggests that high MPO levels are associated with the risk of mortality and that MPO can be incorporated in risk stratification models that guide therapy of high-risk ACS patients.

1. Introduction

Cardiovascular disease is the leading cause of death worldwide [1]. Acute coronary syndrome (ACS) has the worst prognosis among cardiovascular diseases with significant impact on morbidity and mortality. However, ACS patients are a heterogonous population with variable pathologies and clinical outcomes. Methods for risk stratification that incorporate biological variables such as heightened inflammation after cardiac injury are lacking. While troponin and other cardiac markers have been shown to estimate the degree of initial ischemic insult and long-term clinical events [2], the prognostic value of markers of inflammation is not well established.

Cardiomyocyte damage has been shown to initiate a systemic and local inflammatory response that results in worsening cardiac remodeling and long-term cardiac and clinical adverse events [3, 4]. This response initiates the mobilization, recruitment, and activation of neutrophils and other inflammatory cells. Upon activation, neutrophils degranulate and release inflammatory cytokines such as myeloperoxidase (MPO) which aids in the clearance of dead cells and tissues but has been shown to exert atherogenic and adverse vascular effects [5, 6]. A robust body of well-designed, well-controlled

foundational studies conducted in humans and animal models collectively supports the premise that inflammation and circulating inflammatory cells after myocardial infarction are detrimental for cardiac recovery [7, 8]. All these properties make MPO a potential prognostic tool for predicting future adverse clinical outcomes in ACS patients.

Studies that have been conducted to evaluate the prognostic value of MPO in ACS patients showed discrepant results [9, 10] and included a heterogeneous patient population, and their sample size was insufficient to provide solid conclusions. Therefore, we conducted this protocol-driven systematic review and meta-analysis to explore the prognostic value of MPO in ACS patients. We focused on studies that included ACS patients and stratified patients' outcomes based on the plasma MPO levels.

2. Materials and Methods

We conducted this protocol-driven systematic review and meta-analysis according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [11]. We sought to compare the 30-day prognosis of ACS patients with high vs. low MPO levels. MEDLINE, Scopus, and Cochrane databases were searched from inception of myeloperoxidase until March 2018. Further details about the search strategy and terms are shown in the Supplemental Table 1. The references of relevant papers were also screened for potential eligible studies. The abstracts of the American Heart Association, American College of Cardiology, and European Society of Cardiology were screened over the last 2 years for eligible studies.

To be eligible for inclusion in our analysis, studies had to meet the following criteria: (1) patients are divided according to a cutoff value of serum MPO into "high" and "low," (2) patients were followed up for at least 30 days, and (3) absolute numbers of clinical outcome events were reported. Exclusion criteria were (1) irretrievable data, (2) review articles and editorials, and (3) studies including less than 50% subjects with an index diagnosis of ACS. ACS was defined as either ST segment elevation myocardial infraction (STEMI), non-STEMI, or unstable angina. STEMI is defined according to previously published criteria [12, 13] or the WHO criteria [14]. Non-STEMI is defined as at least 10-15 minutes of chest pain at rest and elevated biomarkers of myonecrosis, ST-segment deviation, or T-wave abnormalities. Unstable angina was defined as a typical chest pain at rest with new ST segment changes and peak cardiac troponin I levels within the normal range. Prespecified outcomes of our analyses were mortality, major adverse cardiac events (MACE), and recurrent myocardial infarction. Because of the variability of the definition of the composite of MACE, we included only studies that specifically reported MACE or used a traditional definition of its components.

2.1. Data Extraction and Critical Appraisal. Two reviewers (A.A-L and M.A) independently screened the full text of the retrieved studies and used a standardized form to extract the data from each study. For each outcome, absolute event numbers were included and results are expressed as a ratio of total participants with complete follow-up. Patients were divided into 2 groups, above and below the median level of MPO. Regarding reports that investigated the same subjects at different follow-up time points, we extracted data pertaining to outcomes from the longest follow-up report. We used the Newcastle-Ottawa quality assessment scale (NOS) to assess the quality of included studies [15].

2.2. Statistical Analyses. The prespecified outcomes of our analyses were mortality, major adverse cardiac events (MACE), and recurrent myocardial infarction. Summary estimates were calculated as odds ratios (OR) with 95% confidence intervals (CI) using the random-effects model based on DerSimonian and Laird's meta-analytic statistical method [16]. Considering that the heterogeneity of the included studies might influence the prognostic effects, we prespecified the use of the random-effects model to assess effect sizes. The I^2 index was used to summarize the proportion of the total variability in the estimate. The I^2 statistic is derived from the Q statistic and describes the percentage of total variation across studies that is due to heterogeneity; values of 25%, 50%, and 75% correspond to low, moderate, and high heterogeneity, respectively [17] [18]. Sensitivity analyses were performed using the one-study-removed and the cumulative analysis methods in order to assess the influence of each study on the overall pooled results of the meta-analysis. We used Egger's test and visual inspection of Funnel plots to assess for publication bias [19].

2.3. Metaregression Analysis. Using log-transformed OR as dependent variable, metaregression analyses were performed to determine whether the prognostic value of MPO was modulated by prespecified study-level factors including age and percentage of female gender, patients with index diagnosis ACS, smoker, diabetes mellitus, and hypertension among study populations. Metaregression was performed with unrestricted maximum-likelihood method (inverse variance-weighted regression) on the event rate log-transformed before being used as independent variables in linear metaregression analyses [20]. The statistical level of significance was 2-tailed $P < 0.05$. All statistical analyses were performed using Comprehensive Meta-Analysis version 3.0 software (Biostat Inc., New Jersey, USA).

3. Results

The final analysis included 13 studies that enrolled 9090 subjects with a median follow-up of 11.4 months. The selection process is summarized in Figure 1. Interreviewer agreement on study eligibility was 100%. The baseline characteristics of the included patients are shown in Table 1. Overall, patients with high MPO had similar baseline characteristics compared to those with low MPO. The different definitions of MACE in the included studies in the meta-analysis are shown in Supplemental Table 2. The quality assessment of each included study is shown in Supplemental Table 3.

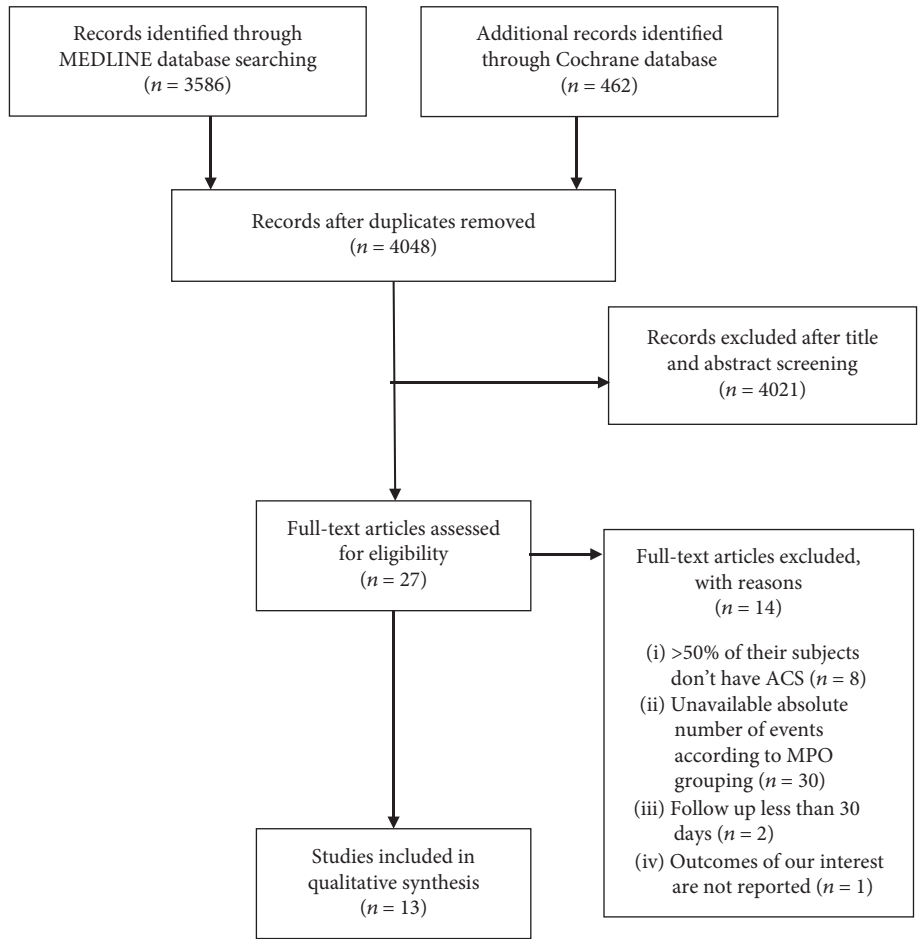

Figure 1: Flow chart of search strategy.

The primary endpoint, all-cause mortality, was significantly higher in patients with high MPO compared to those with low MPO (OR 2.03; CI: 1.40-2.94, $P < 0.001$) (Figure 2). The incidence of MACE and recurrent myocardial infraction trended higher among patients with high MPO (OR 1.28; CI: 0.92-1.77, $P = 0.14$ and OR 1.23; CI: 0.96-1.58, $P = 0.101$, respectively) (Figures 3 and 4). The heterogeneity in our analyses was moderate based on the I^2 statistic of 17%, 48%, and 77% for recurrent MI, mortality, and MACE, respectively.

Metaregression analysis of the primary endpoints stratified by baseline characteristics, such as age, prevalence of hypertension, percentage of ACS in the study population, and diabetes mellitus, showed no significant interactions (Supplemental Figures 1-3). However, there was a significant inverse correlation between female gender and the prognostic value of MPO for both mortality (correlation coefficient -4.23, 95% CI: -7.88 to -0.59, $P = 0.02$) and recurrent MI (correlation coefficient -2.37, 95% CI: -4.69 to -0.03, $P = 0.047$) (Supplemental Figures 1 and 3). On the other hand, smoking showed a significant direct correlation with the OR of recurrent MI; hence, the prognostic value of high MPO on recurrent MI was greater among smokers than nonsmokers (correlation coefficient 5.21, 95% CI: 1.08 to 9.34, $P = 0.01$) (Supplemental Figure 3).

3.1. Sensitivity Analyses. Sensitivity analyses using the "one-study-removed" method did not show significant changes in the summary odds ratio estimates for any outcome assessed (Supplemental Figure 4). Cumulative meta-analysis showed a relatively stable accumulation of evidence for primary endpoints assessed (Supplemental Figure 5). We also stratified the studies based on sample collection method. The results were inconclusive for the sample collection tube because there was a significant imbalance with higher number of studies that utilized EDTA collection tube compared to those using heparin or citrate collection tubes, thus precluding a definitive conclusion regarding the impact of sample collection method on the prognostic value of MPO in our analysis.

We also stratified the studies based on sample collection timing. There was heterogeneity in the sample collection time in relation to the onset of chest pain as detailed in Table 2. There was no correlation between the timing of blood collection and the prognostic value of MPO in mortality (-0.00, 95% CI: -0.02 to 0.02, $P = 0.99$), MACE (-0.02, 95% CI: -0.15 to 0.11, $P = 0.78$), or recurrent MI (-0.00, 95% CI: -0.03 to 0.03, $P = 0.99$).

3.2. Publication Bias. No clear evidence of publication bias was observed on visual inspection of the Funnel plots

Table 1: Patients' characteristics of the studies included in the meta-analysis.

	STEMI (%)	NSTEMI (%)	UAP (%)	MPO cutoff value	Sample size	Age	Female (%)	Smoking (%)	Diabetes (%)	Hypertension (%)
Apple et al.* [31]	≥50% of patients have cTnI ≥ 0.09		NA	≤125.6 mcg/L	172	57 ± 16	43	NR	24.9	57.9
				>125.6 mcg/L	285					
Baldus et al. [34]	0	0	100	<350 μg/L	376	61.4 ± 10.5	28.7	42.5	8.2	35.4
				≥350 μg/L	171	62.5 ± 10.4	31	40	12.5	36.9
Brugger-Andersen et al. [30]	AMI 100		0	≤26.8 mcg/L	142	64 ± 13	20.8	38.9	10.4	24.4
				>26.8 mcg/L	141					
Cavusoglu et al. [38]	12	43	45	≤20.34 ng/mL	91	65 ± 9.3	0.0	32	59	84
				>20.34 ng/mL	91	64.7 ± 10.8	0.0	43	34	83
Chang et al. [28]	AMI 53.9		NA	<1150 ng/mL	95	59.9 ± 12.8	10.55	34.7	33.7	60
				≥1150 ng/mL	33	64.3 ± 12.1	15.1	39.4	51.5	57.6
Eggers et al. [9]	AMI 36.6		21.8	≤208.1 pmol/L	235	66 (55, 76)	33.9	17.2	16.2	37.3
				>208.1 pmol/L	61					
Kaya et al. [10]	100	0	0	≤68 ng/mL	37	56 ± 11	26	61	20	37
				>68 ng/mL	36	57 ± 13	21	66	32	55
Koch et al.§ [26]	43	NA	NA	≤306.3 pmol/L	396	63.7 ± 13.0	30.3	32.9	19.2	70.9
				>306.3 pmol/L	267	65 ± 12	31.1	33	23.6	67.8
Morrow et al. [39]	0	35	65	≤884 pg/mL	762	61 (52, 69)	32.1	28.7	25.1	67.2
				>884 pg/mL	762	61 (53, 70)	34	29.5	28.9	63.9
Mocatta et al. [27]	81.1	18.9	0	≤55 ng/mL	242	61.7 ± 11.0	19.9	NA	12.7	NR
				>55 ng/mL	243					
Oemrawsingh† et al. [40]	0	0	100	<350 μg/L	376	62 (54, 69)	20	40	14	42
				≥350 μg/L	171					
Rahman et al. [32]	65	30	5	<285.5 pmol/L	30	NR	20	NR	NR	NR
				≥285.5 pmol/L	70					
Scirica et al. [29]	0	48.3	49.2	≤670 pg/mL	2507	64	35.1	25	32.3	74.6
				>670 pg/mL	1845					

STEMI: ST-elevation myocardial infarction; NSTEMI: non-ST-elevation myocardial infarction; UAP: unstable angina; MPO: myeloperoxidase; CTn1: cardiac troponin I; AMI: acute myocardial infraction; NA: not available. Continuous variables are presented in either median or mean ± SD. Categorical variables are presented in percentages. *Apple et al. reported that the median cardiac troponin of the whole cohort is 0.09 μg/L. †Oemrawsingh et al. is a longer follow-up report of Baldus et al.'s study subjects. §Reported that ACS-negative patients are 10.8% of the study population.

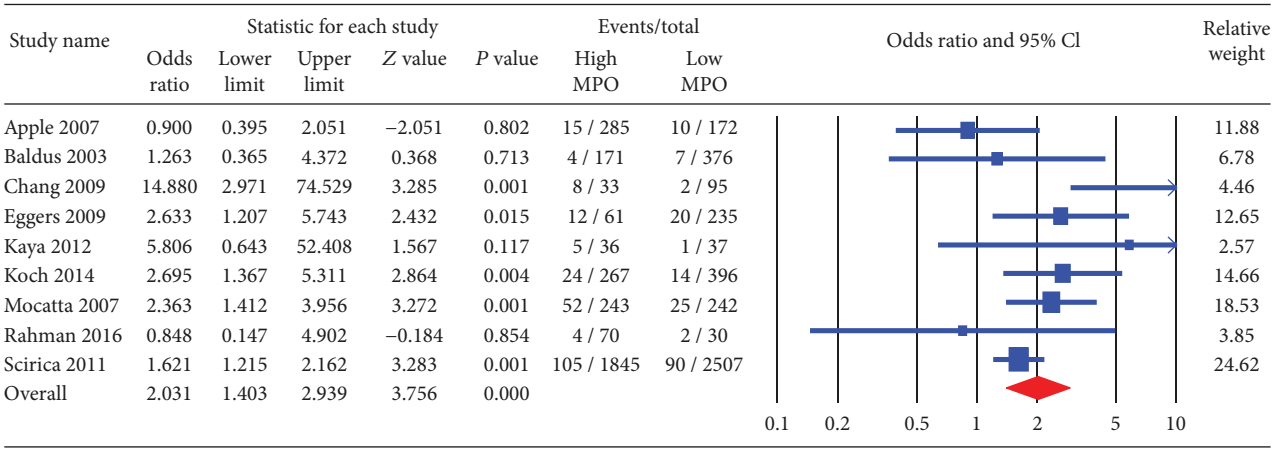

Figure 2: Forest plot for all-cause mortality. High myeloperoxidase level was associated with significantly higher risk of mortality (odds ratio 2.03; 95% confidence interval (CI): 1.403-2.939; $P < 0.001$).

Study name	Statistic for each study					Events/total		Odds ratio and 95% Cl	Relative weight
	Odds ratio	Lower limit	Upper limit	Z value	P value	High MPO	Low MPO		
Apple 2007	1.895	0.869	4.132	1.608	0.108	27 / 285	9 / 172		11.28
Brügger-Andersen 2008	0.674	0.361	1.257	−1.240	0.215	16 / 75	64 / 223		14.65
Chang 2009	1.003	1.001	1.005	2.944	0.003				31.32
Eggers 2009	1.497	0.752	2.981	1.148	0.251	14 / 61	39 / 235		13.12
Kaya 2012	4.125	1.184	14.366	2.226	0.026	12 / 36	4 / 37		5.64
Oemrawsingh 2011	1.500	1.086	2.073	2.458	0.014				23.99
Overall	1.278	0.921	1.773	1.469	0.142				

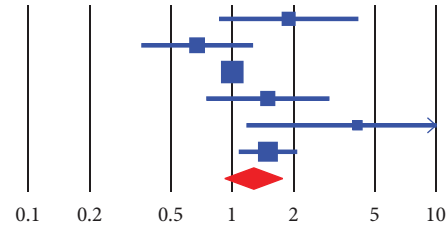

Figure 3: Forest plot for major adverse cardiac events (MACE). High myeloperoxidase showed a trend towards higher risk of MACE (odds ratio 1.27; CI: 0.92-1.77, $P = 0.14$).

Study name	Statistic for each study					Events/total		Odds ratio and 95% Cl	Relative weight
	Odds ratio	Lower limit	Upper limit	Z value	P value	High MPO	Low MPO		
Cavusoglu 2007	2.319	1.050	5.120	2.081	0.037	22 / 91	11 / 91		8.62
Eggers 2009	0.806	0.318	2.045	−0.453	0.651	6 / 61	28/ 235		6.47
Kaya 2012	5.435	0.252	117.236	1.080	0.280	2 / 36	0 / 37		0.65
Koch 2014	2.500	0.592	10.550	1.247	0.212	5 / 267	3 / 396		2.85
Marrow 2008	1.280	0.965	1.696	1.715	0.086	127 / 762	103 / 762		36.71
Scirica 2011	1.050	0.841	1.311	0.430	0.667				44.70
Overall	1.231	0.960	1.578	1.641	0.101				

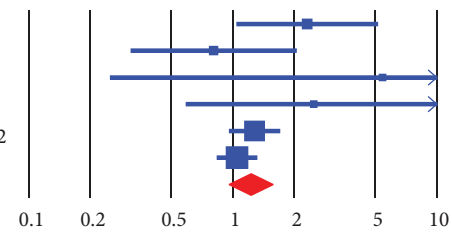

Figure 4: Forest plot for recurrent myocardial infraction (MI). High myeloperoxidase showed a trend towards higher risk of recurrent MI (odds ratio 1.23; CI: 0.96-1.57, $P = 0.101$).

(Supplemental Figures 6-8). Our Egger's regression test did not show significant risk of publication bias ($P = 0.39$ for all-cause mortality, 0.06 for MACE, and 0.2 for recurrent MI).

4. Discussion

Risk stratification for patients with ACS is an evolving field, and the prognostic role of inflammatory markers such as MPO has not been fully investigated. In this comprehensive systematic review and meta-analysis, we confirm the strong correlation between elevated plasma MPO levels and cardiac outcomes, including mortality, among patients with acute coronary syndrome. More importantly, our results were consistent across multiple study designs and patient characteristics. These results support a potential role for MPO as part of multimarker risk stratification model to guide future individualized therapies to the highest risk population. Future prospective studies examining the prognostic value of MPO in comparison of other biomarkers of inflammation such as high-sensitivity C-reactive protein (hs-CRP) are warranted.

Myocardial injury triggers a series of signaling events to communicate with the bone marrow and peripheral blood cells (PBCs) through processes that are just now being elucidated. After myocardial infarction, circulating inflammatory cells such as neutrophils are a poor prognostic indicator, in part because of their contribution to infarct expansion and impaired cardiac remodeling, thereby promoting the progression to adverse remodeling and heart failure [21, 22]. Indeed, this initial injury response may actually confer long-term harm because reduction in the initial recruitment of inflammatory cells can reduce infarct size and prevent cardiac remodeling following cardiac injury [23]. In addition to effects on the myocardium, circulating inflammatory cells following ACS accelerate experimental atherosclerosis in animal models thus initiating a vicious cycle; thus, this type of cycle may contribute to recurrent coronary events in humans [24]. Therefore, identifying markers of inflammation and inflammatory cell activity can help risk stratify ACS patients and guide future therapies. MPO is a product of inflammatory neutrophils during their degranulation and can aid in the process of clearing dead cells. However, MPO has been linked to atherosclerosis and recurrent coronary events. MPO enhances LDL cholesterol oxidation, hence destabilizes coronary atherosclerotic plaque [25]. Additionally, MPO limits endothelial-derived nitric oxide bioavailability which impairs coronary vessel dilatation and worsens cardiac ischemia [6].

Myeloperoxidase as a prognostic marker in ACS patients has generated conflicting results in clinical studies. The majority of clinical data has confirmed the prognostic value of MPO in predicting mortality [9, 26–29], and our analysis confirmed this correlation to be highly significant. However, although there was strong correlation between MPO levels

Table 2: Sample collection methods and time of sample collection in relation to onset of chest pain/hospital admission of the included studies.

	Apple et al. [31]	Baldus et al.* [34]	Brugger-Andersen et al. [30]	Cavusoglu et al. [38]	Chang et al. [28]	Eggers et al. [9]	Kaya et al. [10]	Koch et al. [26]	Mocatta et al. [27]	Morrow et al. [39]	Rahman et al. [32]	Scirica et al. [29]
Sample collection method	Heparin-containing tubes	NR	Citrate-anticoagulated tubes	NR	NR	EDTA-anticoagulated tubes	NR	EDTA-anticoagulated tubes	EDTA-anticoagulated tubes	Citrate-anticoagulated tubes	NR	EDTA-anticoagulated tubes
Time of sample collection	Within 3.1 hours from the onset of symptoms	Within 8.7 hours from the onset of symptoms	Within 4-6 days from the onset of symptoms	≥12 hours after hospital admission	At hospital admission	After 0.8 hour from hospital admission	Within 6 hours of the onset of symptoms	After 26.1 hours from hospital admission	From 24 to 96 hours after hospital admission	NR	NR	NR

*Oemrawsingh et al. is a longer follow-up report of Baldus et al.'s study; the study's subjects received heparin before blood samples were withdrawn.

and other clinical events such as MACE and recurrent myocardial infarction, this association did not reach statistical significance in individual trials or our analysis [9, 30, 31]. There are multiple factors that can explain the lack of this correlation. Some of the studies were underpowered to reach a valid conclusion especially in individual endpoints [10, 30, 32]. Other studies enrolled a heterogeneous population of patients with chest pain (mixture of ACS and non-ACS). Indeed, it has been shown that MPO levels correlate with the severity of ACS pathology [33]. In accordance with these findings, we found that the prognostic value of MPO was the highest among studies with high proportion of AMI patients [10, 27] compared to those with higher percentage of unstable angina subjects [29, 34]. Additionally, timing of sample collection could have played a role in the variable results since MPO level was significantly higher immediately after STEMI [26, 35]. Although studies adopted different MPO cutoff values, our analysis was primarily focused on the prognostic value of MPO rather than its absolute value since the included studies used different MPO assays. Therefore, despite the fact that MPO cutoff value was not the same, stratifying patients based on a certain MPO cutoff provided valuable prognostic information in patients with acute coronary syndrome.

We performed additional sensitivity analyses attempting to unify the included studies based on methodology and sample collection. When we focused our analyses on studies that reported using similar methodology, we observed consistent prognostic value of MPO for all endpoints examined. Similarly, we did not see significant interaction between most of the baseline characteristics or time of sample collection and the prognostic value of plasma MPO. Additionally, the predictive value of MPO for all-cause mortality and MACE was consistent in the "one-study-removed" and cumulative analyses suggesting the generalizability of our findings.

There are limitations to our analysis inherent in conducting a meta-analysis using published patient data and the methodological differences among the included studies. Included studies enrolled heterogeneous patient populations and adopted different definition of clinical outcomes which could have influenced the results of the pooled analyses. While we attempted to address this limitation by using comprehensive sensitivity and metaregression analyses, we could have failed to include other clinical parameters that were not reported in the published manuscripts. Furthermore, the sample withdrawal timing was different across the included studies which could have influenced the results; however, there was no significant correlation between the time of sample withdrawal and the prognostic value of MPO for any of the outcomes. Finally, statin therapy, which is known to downregulate MPO expression [36], was not reported in most of the included studies, and therefore, we could not conduct sensitivity analysis based on the proportion of patients receiving statin.

This meta-analysis attempted to focus on a homogeneous population of studies with high percentage of ACS patients, thus addressing some of the variability in the literature. Our results have significant implications in clinical practice. Integrating MPO in risk stratification models could have an additional value in identifying patients at higher risk of developing heart failure, recurrent ischemia, and clinical events specially mortality. The predictive value of MPO is more specific in patients with STEMI and high-risk NSTEMI where the damage is higher and more inflammatory cells are more activated [28, 37].

5. Conclusions

MPO is a powerful prognostic marker for clinical outcomes in patients with acute coronary syndrome. Our results advocate for more comprehensive risk assessment tools that incorporate MPO to more personalized medical and invasive management for patients with ACS. Further studies examining management strategies based on peak MPO level are needed to assess the clinical utility of this novel biomarker.

Disclosure

Part of this work was presented at the Basic Cardiovascular Sciences annual meeting in 2015.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

Authors' Contributions

Andrew R. Kolodziej and Mohamed Abo-Aly contributed equally to this manuscript.

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Supplementary Materials

Supplemental Table 1: (A) MEDLINE search strategy. (B) Scopus search strategy. Supplemental Table 2: the definition of major adverse cardiac events of the included studies in the meta-analysis. Supplemental Table 3: assessment of the quality of the included studies using the Newcastle-Ottawa quality assessment scale. Supplemental Figure 1: metaregression for risk factors of mortality. X axis represents the observed effect size of studies. Y is the metaregression coefficient. Age ($Y = -0.12$; $P = 0.32$). Female (-8.61; $P = 0.0048$). ACS ($Y = -0.071$; $P = 0.94$). DM ($Y = 3.522$; $P = 0.32$). Hypertension ($Y = -0.20$; $P = 0.911$). Smoking ($Y = 1.44$; $P = 0.55$). Supplemental Figure 2: metaregression for risk factors of major adverse cardiac events. X axis represents the observed effect size of studies. Y is the metaregression coefficient. Age ($Y = -0.112$; $P = 0.28$). Female (-3.15; $P = 0.301$). ACS ($Y = -0.22$; $P = 0.897$). DM ($Y = -0.353$; $P = 0.89$). Hypertension ($Y = -0.74$; $P = 0.785$). Smoking ($Y = 2.04$; $P = 0.28$). Supplemental Figure 3: metaregression for risk factors of recurrent myocardial infraction. X axis represents the observed effect size of studies. Y is the metaregression

coefficient. Age ($Y = -0.03$; $P = 0.68$). Female (-2.23; $P = 0.06$). ACS ($Y = -0.11$; $P = 0.89$). DM ($Y = 2.0$; $P = 0.286$). Hypertension ($Y = 1.13$; $P = 0.37$). Smoking ($Y = 4.8$; $P = 0.204$). Supplemental Figure 4: forest plot displays sensitivity analysis using the one-study-removed method. High myeloperoxidase is significantly associated with mortality (odds ratio 2.040; 95% confidence interval (CI): 1.405-2.960, $P = 0.000$). High MPO showed a trend for developing major adverse cardiac events (odds ratio 1.421; 95% confidence interval (CI): 1.010-1.999, $P = 0.044$) and recurrent MI (odds ratio 1.241; 95% confidence interval (CI): 0.996-1.545, $P = 0.054$). Supplemental Figure 5: forest plot displays cumulative meta-analysis. High myeloperoxidase is significantly associated with mortality (odds ratio 2.040; 95% confidence interval (CI): 1.405-2.960, $P = 0.000$). High MPO showed a trend for developing major adverse cardiac events (odds ratio 1.421; 95% confidence interval (CI): 1.010-1.999, $P = 0.044$) and recurrent MI (odds ratio 1.241; 95% confidence interval (CI): 0.996-1.545, $P = 0.054$). Supplemental Figure 6: funnel plot of all studies included in the meta-analysis. The standard error (SE) of the log odds ratio of each study was plotted against the odds ratio for mortality. No skewed distribution was observed, suggesting no publication bias. Supplemental Figure 7: funnel plot of all studies included in the meta-analysis. The standard error (SE) of the log odds ratio of each study was plotted against the odds ratio for major adverse cardiac events. No skewed distribution was observed, suggesting no publication bias. Supplemental Figure 8: funnel plot of all studies included in the meta-analysis. The standard error (SE) of the log odds ratio of each study was plotted against the odds ratio for recurrent myocardial infraction. No skewed distribution was observed, suggesting no publication bias. *(Supplementary Materials)*

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Review Article

Biomarkers of Inflammation in Obesity-Psoriatic Patients

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Psoriasis is a common chronic inflammatory multisystemic disease with a complex pathogenesis consisting of genetic, immunological, and environmental components. It is associated with a number of comorbidities, including diabetes, metabolic syndrome, obesity, and myocardial infarction. In addition, the severity of psoriasis seems to be related to the severity of obesity. Patients with higher levels of obesity show poorer response to systemic treatments of psoriasis. Several studies have demonstrated that white adipose tissue is a crucial site of the formation of proinflammatory adipokines such as leptin, adiponectin, and resistin and classical cytokines such as interleukin- (IL-) 6 and tumour necrosis factor-α. In psoriasis, due to the proliferation of Th1, Th17, and Th22 cells, IL-22, among others, is produced in addition to the abovementioned cytokines. With respect to leptin and resistin, both of these adipokines are present in high levels in obese persons with psoriasis. Further, the plasma levels of leptin and resistin are related to the severity of psoriasis. These results strongly suggest that obesity, through proinflammatory pathways, is a predisposing factor to the development of psoriasis and that obesity aggravates existing psoriasis. Different inflammatory biomarkers link psoriasis and obesity. In this paper, the most important ones are described.

1. Introduction

Currently, obesity is considered a chronic, multifactorial disease and a result of interactions between genetic load and the environment that affect a large percentage of the population across all ages, sexes, and social conditions. It is defined as an abnormal or excessive accumulation of adipose tissue that can be harmful to health, and it affects a greater proportion of women than men [1].

It is characterized by an elevation of plasma levels of proinflammatory cytokines, including tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and acute-phase proteins such as C-reactive protein (CRP). This condition associated with obesity is explained by the inflammatory

activity of adipocytes. Adipose tissue, classically considered as an energy reservoir, is able to communicate with the rest of the body by secreting adipokines, which are molecules with proinflammatory, thrombotic, and vasoactive activity [2]. Adipokines include TNF-α, plasminogen activator inhibitor 1, IL-6, and leptin [3].

A decrease in adiponectin, a cytokine with anti-inflammatory activity, has also been observed. In response to these signals, macrophages are attracted to adipose tissue. Once infiltrated trapped between adipocytes, mature macrophages stimulate cytokine secretion, leading to local primary inflammation. Subsequently, cytokines trigger the production of inflammatory proteins in the liver and thus lead to the low-grade systemic inflammation observed in obesity [4]. In addition, cytokines increase lipolysis, which is the constant release of free fatty acids by adipose tissue into peripheral circulation.

Thus, glycoprotein 130 cytokines have presented different effects on adipogenesis, lipolysis, insulin sensitivity, and food intake. In this review, we have summarized the current knowledge about gp130 cytokines, including IL-6, LIF, CNTF, CT-1, and OSM, in adipocyte biology and metabolic activities in conditions such as obesity and type 2 diabetes [5].

Free fatty acids have been considered an important link between chronic inflammation and adipose tissue activity, as they are capable of increasing oxidative stress and, therefore, the inflammatory environment and vascular activity. It should be noted that adipose tissue of central predominance is associated with a greater amount of visceral fat, compared with its peripheral distribution. Adipocytes of visceral fat are metabolically more active, releasing more cytokines and fatty acids. As such, we would expect to find a more pronounced inflammatory environment in patients with abdominal obesity [6].

Psoriasis is a chronic autoimmune disease of genetic predisposition and multifactorial triggers that affects the skin, semi-mucosa, mucosa, annexes, and joints [7]. Globally, it affects 2%-5% of the population [8].

The interactive relationship between hyperproliferative keratinocytes (KCs), inflammatory dendritic cells (DCs), neutrophils, mast cells, and T cells leads to the apparition of psoriatic lesions. These lesions, from the clinical point of view, are characterized by sharply demarked, erythematous, and scaly plaques. They are found predominantly in the scalp, knees, elbows, lumbosacral area, and body folds, with symmetrical distribution, and can develop in sites of injury (Koebner phenomenon). At the moment, there are no differential diagnostic criteria for psoriasis, and its diagnosis is based on examination of the morpho- and histological characteristics of the lesions, clinical history of patients, and the psoriasis area and severity index (PASI). It is usually accompanied by other morbidities that can further affect the quality of life and survival of patients [9].

Therefore, epidemiological studies have identified a greater risk of development of metabolic alterations in these patients, among which obesity is highlighted [10, 11].

Obesity and psoriasis are linked by a common pathophysiological mechanism, which is explained by low-grade chronic inflammation [12]. Obesity is not only associated with a higher incidence and severity of psoriasis, but it also affects the response to treatment [13]. The inflammatory state associated with obesity [14, 15] has been proposed as a link between various pathological conditions that usually coexist, a condition known as "metabolic syndrome," another comorbidity of psoriasis [16, 17].

The effect of cytokines on insulin sensitivity in the liver and muscle has been widely studied [18]. Unlike psoriasis, its association with obesity during the last decades has been demonstrated at a clinical level, without an extensive study of all the molecular mechanisms involved in this association. However, both diseases are inflammatory pathologies, with a common pathophysiological substrate, such as inflammatory pathways and cytokine accumulation [18].

It is known that tumour necrosis factor alpha (TNF-α)—a cytokine that is elevated in patients with psoriasis, rheumatic diseases, and obesity—induces insulin resistance through various mechanisms. Exposure of cells to TNF-α causes inhibitory phosphorylation by receptor 1 of TNF-α (TNF-R1) to the serine residues of substrate 1 of the insulin receptor (IRS-1), favouring development of insulin resistance [19].

Insulin resistance contributes to the pathogenesis of the metabolic syndrome by generating hyperglycaemia and compensatory hyperinsulinaemia. This favours development of obesity, hepatic steatosis, dyslipidemia, atherosclerotic disease, and, eventually, diabetes mellitus type 2 [20, 21].

Obesity and psoriasis share common pathogenic mechanisms, including increased proinflammatory cytokines (IL-1, IL-6, TNF-α, and adiponectin). Several studies have shown that control of these pathologies favours good evolution of psoriasis and concurrently that treatment with methotrexate and anti-TNF would reduce the risk of these comorbidities [22] (Figure 1).

Therefore, TNF-α is probably one of the cytokines responsible for the increased risk of cardiovascular disease experienced by patients with psoriasis. TNF-α and IL-1β inflammatory cytokines are central mediators of immunity and are involved in cytokines, monoclonal antibodies that target cell surface proteins and receptors. Examples of anti-TNFs are infliximab, etanercept, adalimumab, certolizumab, and golimumab. Examples of monoclonal Abs include ustekinumab, secukinumab, and ixekizumab. Most of them are subcutaneous treatments. They are expensive treatments, and systemic immunosuppression can lead to infections and disease recurrence if there is a discontinuous pattern of treatment. There is also a drug delivery treatment, apart from phototherapy and biological treatment [23], based on two pathways: either to normalize the keratinocyte differentiation or to modulate immune responses.

In psoriasis, the main expressed component of biomarkers is related to hyperproliferation of keratinocytes. This is why the level of certain proteins allows distinguishing between psoriatic and normal skin. Certain biomarkers are still unknown, and other predictions are made based on similarities with other diseases.

In this manuscript, the main diagnostic, prognostic, and treatment response biomarkers were collected in obesity-psoriatic patients.

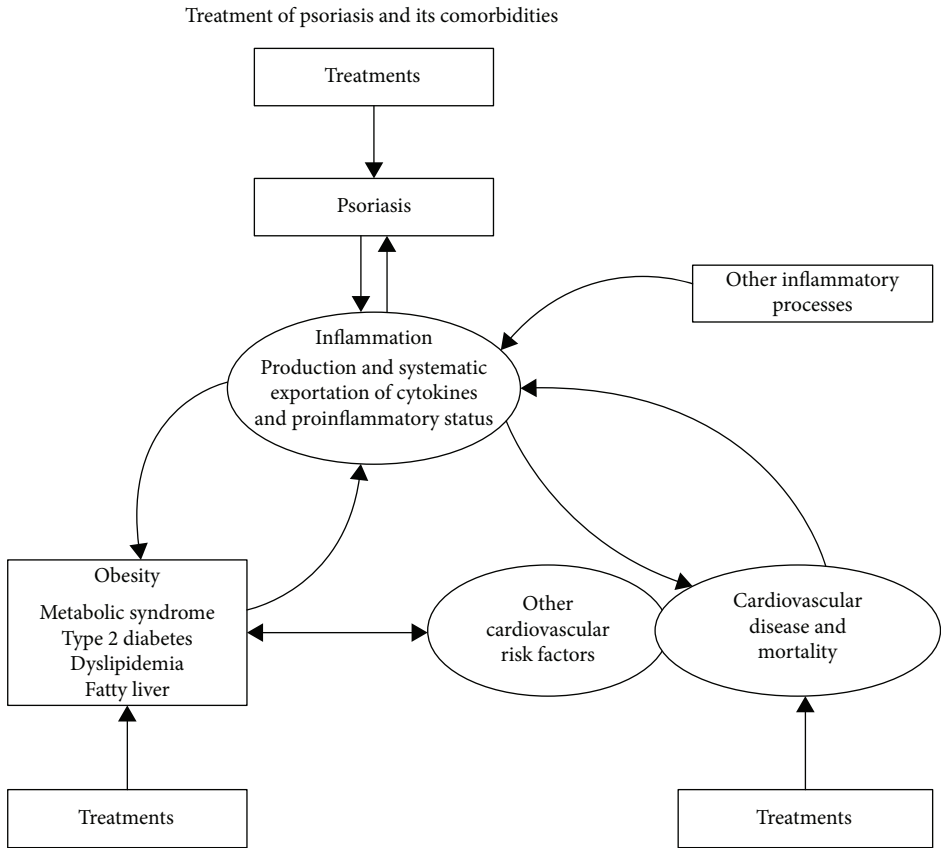

Figure 1: Metabolic pathway of lipoinflammation. The interactive association among psoriasis, obesity, type 2 diabetes, cardiovascular disease, and mortality would be based on the inflammation observed in each of these diseases and transported systemically, especially to other inflammatory processes.

2. Correlation between Obesity—Body Weight and Body Mass Index (BMI)—and Severity of Psoriasis and Response to Treatment

In this review, Alotaibi aimed to study the effects of weight loss on the symptoms of psoriasis in obese patients. Using Ovid, the search since 1990 to December 2017 yielded 14 results [24]. Debbaneh at al. [25] conducted a literature review of observational and clinical literature on the effects of weight loss on the severity of psoriasis. Naldi et al. [26]conducted a randomized controlled trial on 303 patients, which incorporated 20 weeks of dietary and exercise interventions as an adjunct treatment for obese and overweight patients with psoriasis. All of these studies showed significantly improved PASI scores in the group with intervention compared to those in the control group. Al-Mutairi and Nour [27] observed in 2014 that body weight reduction could improve PASI scores in a trial of 262 obese patients on anti-TNF-α biologic therapy.

Both psoriasis and obesity are related to an underlying common cause of inflammation. A review of published literature clearly shows that diet and exercise will be considered as adjunct treatments for psoriasis, as they are easily accessible and inexpensive. Weight loss improved the overall health of a patient and was effective in combating oxidative stressors, with secondary positive impacts on the PASI scores. Therefore, the authors recommend that physicians encourage their patients to follow a healthier lifestyle aimed at following an exercise regimen and reducing weight as a method to improve psoriasis symptoms [24].

A meta-analysis was performed with results by Budu-Aggrey et al. [28], using both adults' and children's data separately and in combination. Investigating causal relationships, the analysis included 753,421 individuals from the UK Biobank and Nord-Trøndelag Health Study (HUNT), Norway. A two-sample MR was performed with 356,926 individuals from the published body mass index (BMI) and psoriasis genome-wide association studies (GWASs). For the observational analysis, logistic regression models were used to estimate the observational association between BMI and psoriasis.

Briefly, 56 studies reporting data about the relationship between psoriasis and BMI, obesity, or being overweight were identified. Among them, 35 compared BMI between psoriasis cases and controls, which were considered to be meta-analysed. It was then found a significant difference in BMI between cases and controls of 1.26 kg/m^2 (95% CI 1.02–1.51) in adults (69,844 psoriasis cases and 617,844 controls) and 1.55 kg/m^2 (95% CI 1.13–1.98) in children (5–18 years old). In the additional 21 studies, the researchers sought to find an association between BMI and obesity, which reported a positive association.

The Genetic Risk Score for Body Mass Index (BMI GRS) was strongly associated with BMI in the UK Biobank and HUNT [29]. In both UK Biobank and HUNT, a higher BMI was associated with increased risk of psoriasis [30].

Higher BMI increases the risk of psoriases, both diseases, presenting a rising prevalence [31]. Type 2 diabetes and obesity were associated with a significant increased risk of liver fibrosis. The association between psoriasis and obesity should be properly considered when choosing a systemic treatment, because it could exert negative effects on metabolic parameters, including liver enzymes, serum lipids, and renal function. Obesity may increase the risk of liver and renal toxicity from methotrexate and cyclosporine [28].

As reported by Carrascosa et al. [32], obese patients with psoriasis have a higher risk of adverse effects with conventional systemic drugs. Biological drugs in which the dose is not adjusted to weight, such as etanercept and adalimumab, are usually less effective, whereas other biological drugs, such as infliximab and ustekinumab, can be adjusted to weight.

Rui et al. [33] showed that psoriatic patients with MS showed much less reduction of IL-17 and IL-6 before and after 10 sections of NB-UVB treatment, respectively, than patients without MS did ($P < 0.05$). Psoriatic patients with MS had poorer improvement in comparison with those without MS using NB-UVB treatment. MS was an independent factor affecting NB-UVB treatment. In addition, psoriatic patients with MS showed much less reduction of systemic biomarkers (interleukin- (IL) 17, TNF-α, and IL-6) than patients without MS did. That is, they may need a longer treatment course to achieve improved skin lesions.

Since 2004, it is possible to treat psoriasis with molecules generated by molecular biology using recombinant DNA technology. Biological treatments in psoriasis are directed against cytokines or surface proteins of lymphocytes blocking specific steps in the pathogenesis of psoriasis [34, 35].

The most commonly active principles used in the treatment of psoriasis are described in Table 1 [33–42].

Baerdazzi et al. [43] have studied 33 patients (27 men and 6 women), 25–45 years old, treated with biological therapies, and "nonresponders" to at least two traditional systemic therapies. The results and follow-up were as follows: mean BMI = 30.59 ($\pm$6.94) and average PASI = 25.03 ($\pm$12.43), obese patients average PASI = 32.36 ($\pm$12.79) and patients with grade III obesity average PASI = 44 ($\pm$3.37). Differences were not statistically significant in most cases due to the low number of patients. Overweight and obese patients (BMI > 25) were invited to lose weight. Reevaluation of the PASI after 4 months showed that the reductions were attributable, for the most part, to the treatment. However, if weight reduction was obtained, there was a remarkable improvement in the PASI. At the end of the study, BMI did not change in 22 patients, increased in 4, and decreased in 7. Among the 7 patients who lost weight, they achieved a PASI of 75 or higher (statistically significant difference). Finally, weight loss could help improve psoriasis and make the treatments used more effective [43].

As in Puig et al. [44], in this study, the correlation between PASI and WhtR was analysed in a population of 289 patients with psoriasis for which anthropometric measurements were available. It was seen that PASI in 243 patients with a WhtR greater than or equal to 0.5 had a higher median than in the 48 patients with a WhtR less than 0.5. The correlation between WhtR and BMI was 0.86, while that between WhtR and PASI was 0.14. WhtR is an accessible method, with an equal cutoff point for both sexes and good correlation in obesity, cardiovascular disease, and diabetes mellitus. In this study, we have seen in obese patients with psoriasis a better correlation of WhtR with PASI than BMI. For each unit of increase in BMI, the risk of suffering psoriasis increased by 9% and PASI 7%. One factor to consider is that therapy with TNF-α contributed to weight gain in patients with chronic plaque psoriasis. Thus, the clearance of adalimumab or ustekinumab was greater in obese patients, and the PASI75 or PASI90 response rate was higher in patients with low weight. In the case of etanercept, patients with lower weight had better response rates to the drug than those with obesity. However, PASI75 response rates at 10 weeks of treatment with infliximab were independent of BMI. Thus, in those patients with psoriasis and obesity, a treatment with infliximab could be more advisable in comparison with other biologics that are administered in fixed doses.

In addition, the response of obese patients with moderate or severe psoriatic plaques to treatment with low-dose cyclosporine showed improvements after loss of passage through diet or bariatric surgery, although there are studies that show a worsening of the disease after surgery or with rapid drops in weight [44].

Petridis et al. [45], in a multicentre, prospective, observational study, examined the impact of risk factors, such as BMI and waist circumference, on quality of life improvement and clinical response in moderate-to-severe plaque-type psoriasis patients treated with infliximab in routine care settings in Greece.

Fleming et al. [46] reviewed 254 articles, of which they included only 9. The sample size was 134,823 psoriasis patients. They included data on age, sex, body mass index (BMI), obesity proportion, and psoriasis area severity index (PASI) score. They found a statistically significant association between the BMI and PASI.

According to Klingberg et al. [47], psoriasis affects 2-3% of the population in Sweden, and 20-30% of these patients develop psoriatic arthritis (PsA), both of which are strongly associated with obesity and the metabolic syndrome (MetS). Obesity increases the risk of developing both psoriasis and PsA, and this is associated with higher disease activity and a poorer treatment response. The aim of this study was to determine the effects of weight loss treatment with very low energy diet (VLED) on the disease activity in joints, entheses, and skin in patients with PsA and obesity.

Briefly, 41 patients with psoriatic arthritis (CASPAR criterion) completed the study, showing a BMI $\geq 33\,kg/m^2$ with weight loss treatment with very low energy diet (VLED) and daily intake of 640 kcal, including recommended doses of vitamins, minerals, and other essentials, following an initial period of 12 or 16 weeks. The association between BMI and disease activity at baseline BMI was positively correlated with

Table 1: Treatment of psoriasis.

	Mechanisms of action	Administration via
Conventional therapies		
Methotrexate [36]	Inhibits replication of T and B lymphocytes and suppresses secretion of various cytokines, including IL-1 (interleukin-1), interferon-gamma, and TNF-alpha.	Oral and subcutaneous
Cyclosposrin A [34, 36]	Inhibits T cell activation by inhibiting interleukin-2 (IL-2) and interferon-gamma production through inhibition of calcineurin.	Oral
Acitretin [36]	Is a second-generation monoaromatic retinoid. It acts by modulating proliferation of epidermal keratinocytes, joining the nuclear receptor RAR or RXR.	Oral
Phototherapy [33, 36]	Causes alteration of the antigen-presenting cell population (Langerhans cells) and modifies intra- and intercellular signalling mechanisms, leading to development of Th2 preferentially to Th1 responses. It also causes apoptosis of activated T lymphocytes.	Ultraviolet A and B radiation
Biological treatments		
Infliximab [35, 37]	Mouse antibody to TNF-alpha	Intravenous
Etanercept [35, 37]	Competitive inhibitor of tumour necrosis factor-alpha. It binds to TNF-alpha to inactivate it.	Subcutaneous
Adalimumab [38]	Anti-TNF IgG1 antibody of an entirely human nature, produced in genetically modified CHO cells.	Subcutaneous
Ustekinumab [39]	Is a fully human IgG1κ monoclonal antibody that binds with high affinity and specificity to the p40 protein subunit of the human cytokines IL-12 and IL-23.	Subcutaneous
Ixekizumab [40]	Is a humanized monoclonal antibody. The substance acts by blocking interleukin-17, reducing inflammation. The antibody has affinity to the homodimer IL-17A and heterodimer IL-17A/F.	Subcutaneous
Secukinumab [41]	Is a recombinant monoclonal antibody, entirely human, selective to interleukin-17A.	Subcutaneous
Others		
Apremilast [42]	Is a novel phosphodiesterase 4 inhibitor.	Oral

several measures of disease activity and function. BMI decreased from median 35.2 kg/m^2 to 29.7 kg/m^2, and a significant reduction was seen in a majority of the disease activity measures. The improvement of the skin occurred later than for the other disease activity parameters (3 months). The treatment was generally well tolerated without serious adverse events occurring. Significant improvement in disease activity in joints, entheses, and skin and reduction in CRP, PLT, and parameters assessing function were found at the 6-month follow-up.

Data from Giunta et al. [48] indicated that obesity (BMI $\geq$ 30 kg/m^2) was a negative cause for psoriasis treatment with etanercept. Thus, an increase in BMI may be a predictor to suspending use of the anti-TNF-α drug. Therefore, there is a consensus that a BMI of 25 kg/m^2 is for a good response to treatment.

In this study by Hansel et al. [49], 30 patients received complete treatment with complete clearance; the treatment was optimally applied with dose spacing up to 21 or 28 days. Patients were divided into two groups in the body mass index. Group A of the body mass index remained at a level of 60%, and group B of 40% had to return to the standard dose. In addition, the time to achieve PASI-100 with the standard dose was also less for group A.

In another study conducted by Prussick et al. [50] with psoriatic patients and controls treated with adalimumab vs. methotrexate, PASI responses through 16 weeks of treatment were related to the BMI represented in three categories (<25 kg/m^2, 25 to <30 kg/m^2, and $\geq$30 kg/m^2). In normal weight, overweight, and patients at week 16, the respective PASI-75 response rates were 85.0%, 85.7%, and 61.3% with adalimumab; 43.3%, 29.3%, and 26.1% with methotrexate; and 28.6%, 16.7%, and 0% with placebo. Adalimumab was superior in all cases, although the result was also influenced by BMI.

In this study, Takamura et al. [51] aimed to investigate the effects of infliximab, ustekinumab, and secukinumab on body weight (BW) and body mass index (BMI) in patients with psoriasis. This retrospective study examined changes in BW and BMI between patients treated with these biologics. Patients presented similar values of BMI and BW at the beginning of the study. The number of patients was as follows: infliximab ($n = 18$), ustekinumab ($n = 30$), and secukinumab ($n = 20$). The treatment results appeared better in patients treated with ustekinumab and secukinumab.

There is still not much known in patients with a high BMI and high body weight with ixekizumab; however, the results were similar so that a high rate of BMI seemed to decrease the effectiveness of treatment in patients with moderate-to-severe plaque-type psoriasis [52].

Finally, 48 patients were included in a study by Vujic et al. [53], who received apremilast between 1 April 2015 and 19 January 2017 and were evaluated every 4 weeks. Further, we documented the following: age, weight, height, smoking psoriasis area severity index (PASI) scores, and the onset and duration of adverse events (AE). Three patients (6.3%) reached PASI-90, nine (18.8%) PASI-75, and eight (16.7%) PASI-50. Patient weight was inversely correlated with PASI-50.

3. Association between Some Obesity Biomarkers and Psoriasis Severity

Recent studies have revealed that there are some possible biological markers that can be used to detect psoriasis and evaluate the prognosis and response to treatment.

3.1. Biomarker for Diagnosis. In this work from Yadav et al. [54], a global view of the biomarker for psoriasis protein is made. The signal transducer and activator of transcription (STAT) has a key role in psoriasis because of its involvement in some biological actions related to immune pathways, such as cell division, growth, and apoptosis. It is believed that the JAK/STAT intracellular signalling pathways control inflammatory reactions in psoriasis and other diseases. STAT is a transcriptional protein family (consisting of STAT1, STAT2, STAT3, STAT4, STAT5A/5B, and STAT6) involved in the expression of main cytokines and nuclear transmission of extracellular signals. Various studies have suggested the importance of STAT1 in psoriasis, as its elevated activity (upregulated by hyperphosphorylation of TAT1) appears in psoriatic skin. STAT3 plays another important role because its activation in T cells and keratinocytes is involved in the pathogenesis of psoriasis. The high expression of STAT2 is significant in psoriatic lesions. Psoriasis has also a characteristic raised level of IFNγ-producing Th1 cells and IL-17A-producing Th17 cells.

Following with S100 proteins, there are 18 S100 family proteins. Among them, 13 are responsible for cellular differentiation of the epidermis and located on chromosome 1q21. In psoriasis, S100A7, S100A8, and S100A9 are expressed. S100A7 (Psoriasin) is involved in abnormal keratinocyte differentiation. Its overexpression is noticed in the nucleus and cytoplasm in keratinocytes and secretion from epithelial cells in psoriatic skin. S100A8 and S100A9 are very significant in psoriatic arthritis and rheumatoid arthritis as they are strongly expressed in inflamed tissue fluid. This is the reason why S100A8 and S100A9 are strong protein candidates for therapeutic targeting of psoriasis and PsA. Another important biomarker is Wnt5a, which is a transmission protein that has a key role in carcinogenesis and embryogenesis and regulates tissue regeneration in the intestine and skin. Wnt5a and its receptors fzd3 and fzd5 have a central role in adult skin cell differentiation and are located in hair follicles of normal epidermal skin, signifying their crucial role in skin cell differentiation in adults. In psoriasis, Wnt5a and fzd5 are overexpressed and relocated, which leads to anomalous differentiation of keratinocytes.

p53/TP53 or tumour protein, one key factor of psoriasis, is a phosphoprotein that regulates cell cycle. Its presence in the layers of psoriatic skin suggests its involvement in the disease.

As for enzyme biomarkers, exfoliations of epidermal cells followed by hyperproliferation are a distinct indication in psoriasis. The development, repair, and proliferation of keratinocytes are controlled by several proteins, enzymes, ions, cytokines, and growth factors. Targeting psoriasis with enzymes could be a unique treatment strategy as they offer insight into the disease progression by prognosis, diagnosis, and assessment of responses. Phospholipase C (PLC) has a key role in the formation of the stratum corneum barrier and keratinocyte differentiation.

Although psoriasin (S100A7) and koebnerisin (S100A15) are distinct in tissue distribution, regulation, and function, both have functional roles in innate immunity, epidermal cell maturation, and epithelial tumourigenesis [55].

Ekman et al. [56] suggest that IL-22 links the inflammatory response to differentiation of immature cells and epithelial regeneration by acting directly on keratinocytes to promote cell stemness. Additionally, IL-22 may have a very important role in the triggering of psoriasis. IL-22 is found in the dermal infiltrate of psoriasis plaques, as well as in the blood of patients with psoriasis.

Importantly, banal infections or small lesions persisting over a long period of time may stimulate IL-22 production. According to Sabat et al. [57], endogenous production of IL-22 initiates the immune system-mediated limitation of adiposity development and metabolic alteration. Further, the authors believe that IL-22 could be used for treatment of the abovementioned disorders.

Upon activation, keratinocytes synthesize thymic stromal lymphopoietin (TSLP), which is considered an initiator of Th2-mediated immune responses in the skin [58] (Figure 2).

Adipocytes and cells residing within the adipose tissue secrete various soluble mediators involved in regulating organ function, metabolism, immunity, and inflammation. The plasma levels of adiponectin increase with weight loss and decrease in obesity. Adiponectin is an important regulator of metabolism and energy homeostasis, enhancing insulin sensitivity and decreasing hepatic glycogenesis.

In this study from Batycka-Baran et al. [59], it included 30 patients diagnosed as chronic plaque psoriasis and 30 healthy controls. Psoriasin, koebnerisin, human IL-23, and IL-12 were measured, and statistical analysis using SPSS version 16 was performed.

Psoriasin, koebnerisin, IL-12, and IL-23 were significantly increased in all cases, and the risk of psoriasis development was directly related to BMI greater than 30. IL-12 was a good predictor of the psoriasis response to treatment, and IL-23 was decreased in psoriatic arthritis. The risk of developing psoriasis was directly related to increases in BMI greater than 30; thus, obesity will play as another factor along with genetic factors in developing psoriasis [60, 61]. Chronic inflammation and hyperhomocysteinaemia may explain the association with atheroma plaque and metabolic syndrome. The pathophysiology of both psoriasis and obesity showed many shared cytokines that are known to contribute to hypertension, dyslipidaemia, and insulin resistance in the metabolic syndrome [59]. Psoriasin and IL-23 will be important new therapeutic targets for patients with skin psoriasis [62].

In this study from Vachatova et al. [63], the authors selected inflammatory markers. Specifically, they extracted blood samples and determined the following: C-reactive protein (CRP) level was assessed by immunonephelometry on an IMMAGE 800 (Beckman, USA), and the results were expressed in milligrams (mg) per litre of serum.

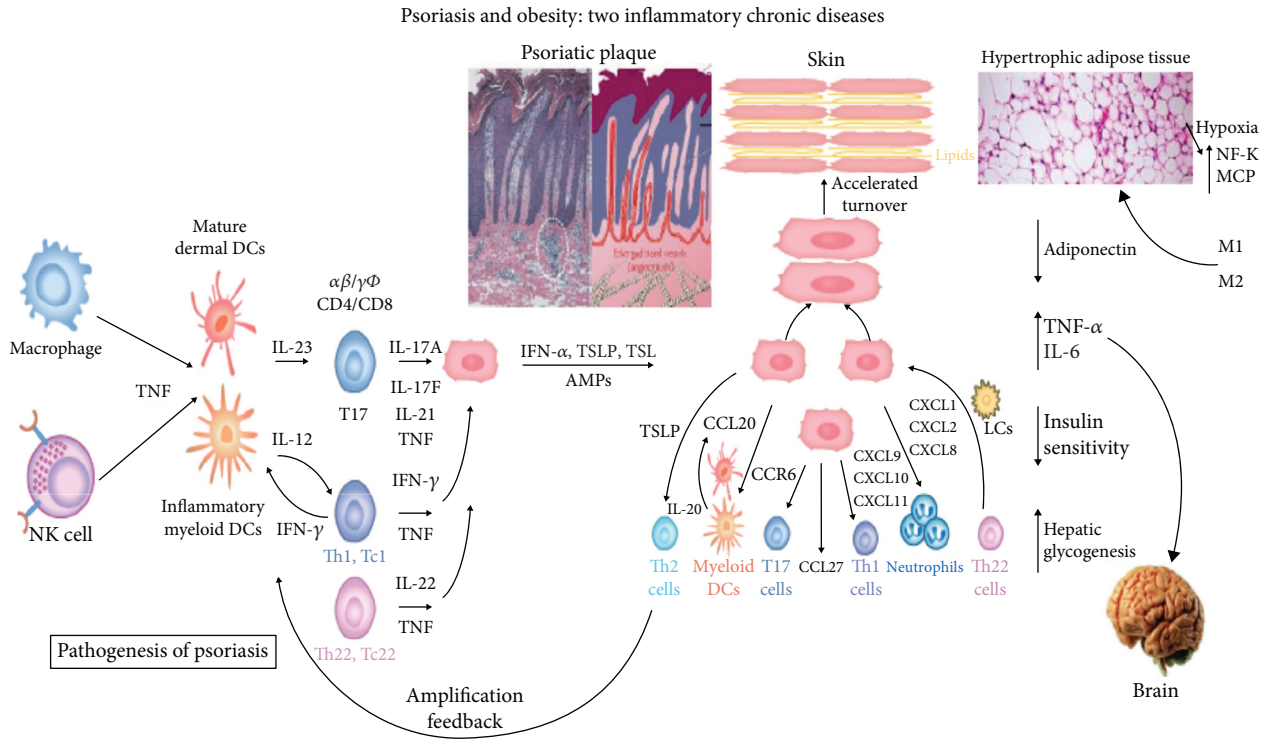

Figure 2: IL-22 stimulation leads to lymphocyte proliferation, which in turn accelerates the synthesis of molecules, elimination of germs, and desquamation. Thus, the epidermis can participate in innate and adaptive immunity in response to infection or stimulation with cytokines. This is due to an increase in the levels of molecules, such as AMP; in the production of chemokines, such as IFN-α, TSL, and TSLP; and in the number of Th17 and IL-17-producing CD4+ and CD8+ T cells and T- helper cells (Th). This mechanism responds to the pathogenesis of psoriasis.

Adiponectin, leptin, resistin, and lipoprotein-associated phospholipase A2 levels were determined using commercial ELISA kits following the manufacturer's instructions. All of them were considered selective inflammatory markers in psoriatic patients.

Another group of researchers led by Kyriakou et al. [64] collected and analysed 38 papers that discussed the levels of these adipokines in psoriasis (26 of these papers about leptin, 15 about resistin, and 25 about adiponectin). All concluded that leptin and resistin had higher levels in patients with psoriasis, compared to the healthy control population, and that adiponectin levels were lower in psoriasis patients than in healthy patients. However, the heterogeneity of these studies was high, since factors such as sex, BMI, or PASI, among others, can affect these levels.

There is significant evidence that systemic inflammation increases cardiovascular risk and leads to metabolic dysregulation in psoriasis.

In this study of cases and controls by Baran et al. [65], they evaluated the serum irisin levels in patients with psoriasis and associated them with disease, inflammatory, and metabolic parameters and topical treatment in 37 patients with flare of plaque psoriasis (35–64) and 15 sex-, age-, and BMI-matched healthy controls. Initial blood samples were taken and another one after 2 weeks of topical treatment (5% salicylic acid ointment and 0,3% anthralin). Irisin serum level was measured using ELISA. There was a significant statistical correlation between serum irisin levels and age or disease duration. The median irisin serum levels in psoriatic patients did not differ compared to the controls; however, it was 2.5 times higher. In patients with psoriasis, serum irisin levels did not correlate with PASI score or BMI. Assessing irisin levels depending on the severity of psoriasis in each group of patients, no statistical correlations in comparison to the controls were noted. There were no significant relations between the study and control groups in terms of liver enzyme activity, glucose, or lipid levels. Further, there were no significant differences between the groups depending on BMI as compared to the healthy subjects.

Myśliwiec et al. [66] conducted a study on the possible correlation that exists between sphingolipids, such as ceramides and sphingosine-1-phosphate, with psoriasis and possible psoriatic comorbidities such as inflammatory (arthritis) and metabolic diseases (overweight and obesity). To carry out this study, 85 patients with plaque-type psoriasis and 32 healthy controls were used, and the sphingosine-1-phosphate analyses were carried out using high-performance liquid chromatography (HPLC) and silica thin-layer chromatography for ceramides. According to the results obtained in these tests and the correlation with patients in the study, it was determined that in those who presented only plaque psoriasis, ceramides were observed to diminish. However, in those who presented with psoriatic arthritis, the values were observed to increase. Likewise, when they evaluated the concentration of ceramide FA-C22, it was observed to be much higher in patients who presented psoriasis with obesity.

In agreement with the previous results, it was determined that changes in the concentrations of certain sphingolipid species aided diagnosis of psoriasis and some of its comorbidities.

Hyperuricaemia is usually associated with skin psoriasis or PsA, and variations in genetic factors, diet habits, and living areas might contribute to the wide range in its prevalence, as a manifestation of metabolic disorders [67].

Lai et al. [68] carried out a multicentre, cross-sectional observational study to assess the prevalence of asymptomatic hyperuricaemia in Hong Kong Chinese patients with PsA and to investigate the associated factors for hyperuricaemia among them. A total of 160 eligible participants were recruited, from May 2016 to August 2017. The frequency of hyperuricaemia was determined by calculating the percentage of patients with SUA level ≥ 360 mol/L in females and ≥420 mol/L in males. Briefly, 46.9% of the patients were overweight and 9.4% were obese. The overweight patients were found to have the strongest association with hyperuricaemia in PsA, and the SUA level was found to have a statistically significant positive relationship with BMI. No associations were found between lipid profile, renal function, enthesitis index, dactylitis count, CRP, tender and swollen joint counts, duration of the psoriatic conditions, or SUA levels.

Hong Kong Chinese PsA patients had 30.6% of hyperuricaemia (considered as high prevalence), and overweight PsA patients were associated with hyperuricaemia, independent of psoriasis, arthritis severity, renal function, and disease duration. Further, BMI was significantly associated with SUA level, with a positive linear relationship [68].

Baran et al. [69] reported that serum lipocalin-2 levels were much higher in psoriatic patients than in controls, although they were not significantly related to the inflammation markers BMI or PASI. They concluded by saying that lipocalin-2 may be a good predictor of psoriasis and cardiovascular risk, but not a reliable indicator of inflammation, severity of psoriasis, or antipsoriatic treatment outcome.

Based on their study, Watarai et al. [70] suggested that nestin- and FABP5-expressing keratinocytes might be an important diagnostic marker of psoriasis.

He et al. [71] believe that IL-21 is associated with disease severity. In addition, they advocate that it plays an important role in its pathogenesis.

3.2. Biomarkers for Prognosis. Gerdes et al. [72] conducted a comprehensive review of the relationship between adipokines and psoriasis, highlighting that these bioactive products (adipokines) are directly related between psoriasis and its comorbidities (insulin resistance, obesity, diabetes mellitus type 2, and cardiovascular diseases). In the case of adiponectin (Acrp30), it has been found that their levels are low in psoriasis in correlation with obesity, but not in the case of leptin and resistin, which correlate with their exacerbation.

With respect to visfatin, there is a correlation between increased level of this with severity of disease. Regarding the retinol-binding protein (RBP4) and omentin, a direct relationship with psoriasis has not been found; however, they have been associated with metabolic processes such as diabetes mellitus type 2 [73].

Finally, altered serum values of tumour necrosis factor alpha (TNF-α) and interleukin- (IL-) 6 have been found as important markers in obese patients with psoriasis diagnosis. According to the above, they highlighted that these adipokines can serve as biomarkers to determine the degree of disease advancement, the level of risk posed by comorbidities, and the success that can be obtained by different treatment options [74, 75].

In this research with cases and controls, El-Boghdady et al. [76] evaluated psoriatic patients from an Egyptian hospital (2015–2016) and matching apparently healthy volunteers. The objective was to establish if psoriasin, nestin, Krt16, and IL-21 were possible biomarkers of psoriasis and to correlate them with Body Mass Index (BMI), leptin, and resistin (biomarkers of obesity). Additionally, the researchers sought to identify the bidirectional relationship between psoriasis and obesity. Blood collections (5 mL) from the participants were processed using the ELISA test, and BMI and PASI (when psoriasis was present) were calculated.

They observed that leptin and resistin were significantly increased in obese psoriatic patients as well as in obese and psoriatic groups. Among those groups, they were significantly elevated in obese psoriatic subjects, and all psoriatic patients demonstrated a significant raise by 20.3% (leptin) and 12% (resistin) when compared with the obese group. They were significantly correlated with PASI, plasma psoriasin, nestin, Krt16, and IL-21. IL-21 (inflammatory biomarker) was significantly increased in obese, psoriatic, and obese psoriatic patients. This interleukin has a key role in keratinocyte proliferation. Psoriasin and nestin were significantly raised in psoriatic and obese psoriatic patients. Psoriasin causes inhibition of differentiation in the epidermis because it reduces Krt1 and Krt10 expression. BMI has significant correlations with Krt16 and IL-21. Krt16 produces alterations in Krt filament organization, cell adhesion, differentiation, and migration, and PASI remained significantly associated with psoriasin. Finally, resistin showed a significant correlation with psoriasin, IL-21, and leptin [76].

This study shows the possibility of using psoriasin, nestin, Krt16, and IL-21 as biochemical markers of psoriasis and highlights the correlation of these with biomarkers of obesity (BMI, leptin, and resistin). Furthermore, it reveals the bidirectional association between psoriasis and obesity.

Enany et al. [77] conducted a study evaluating the carotid intima media (IMT) and leptin in patients with psoriasis, who are known to have atherosclerosis risk. The study included 50 patients diagnosed with psoriasis and 10 healthy patients as controls. The psoriatic patients presented significantly higher levels of leptin and carotid intima than controls did. To determine this, each patient was evaluated for the following parameters: duration of disease, body mass index, severity index of the area of psoriasis (PASI), systolic blood pressure, diastolic blood pressure, leptin levels, LDL (low-density lipoprotein) cholesterol levels, and triglyceride levels. Further, significant or highly significant correlations between each of them were determined, with the exception of HDL (high-density lipoprotein) cholesterol, clarifying that psoriasis is an independent risk factor for subclinical atherosclerosis and that cardiovascular deterioration is mainly influenced

by the severity of disease, serum triglyceride levels, and leptin. The authors proposed that serum levels of leptin and the level of thickness of the carotid middle intima should be routinely evaluated, with the latter involving evaluating hyperlipidaemia, thus reducing the risk of morbidity and cardiovascular mortality and improving the prognosis of psoriasis.

Myśliwiec et al. [78] carried out a study with cases and controls (85 patients with active plaque psoriasis and 32 sex- and aged-matched healthy controls) to evaluate serum concentrations of fatty acids (FAs) and investigate their association with psoriasis activity, markers of inflammation, and possible involvement in psoriatic comorbidity (obesity, type 2 diabetes, and hypertension). The authors found that the total FA concentration was similar in the psoriatic/control group; in particular, FA was different. There was no correlation between total FA concentration and PASI.

Moreover, in the group of psoriatic patients, they observed a significant negative correlation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) with PASI. There was a positive correlation between PASI and the n-6/n-3 ratio.

Nevertheless, in nonobese psoriatic patients, they observed a negative correlation between DHA, n-3 polyunsaturated FA (n-3 PUFA), and PASI and a positive correlation of the percentage of monounsaturated FA (MUFA) and PASI [78].

In obese psoriatic patients, no significant correlations between FA and PASI were found. Further, there were no significant correlations between other metabolic parameters such as cholesterol, triglycerides, and fasting blood glucose and PASI in nonobese and obese psoriatic groups.

Conversely, in the psoriatic patient groups with or without hypertension, hypertensive patients had significantly higher concentrations of total FA, percentage of SFA, n-3 PUFA, and SFA/UFA ratio. They also had a lower percentage of all PUFA and n-6/n-3 ratio in serum. The psoriatic group with type 2 diabetes compared to nondiabetic patients had significantly higher concentrations of total FA and a lower percentage of PUFA. Psoriatic patients with diabetes were older and had higher BMI than psoriatics without diabetes. Similarly, psoriatic patients with hypertension were older and had higher BMI than those without hypertension [78].

Kustán et al. [79], in a study conducted in Hungary in patients with psoriasis, measured the levels of water-phase proteins, such as high-specificity reactive C-protein (hsPCR) and orosomucoid in serum and urine (sepsis; ORM and u-ORM). To perform the study, 87 patients were used with levels of mild, moderate, and severe psoriasis and 41 healthy patients as controls. As a result, the values of hsPCR and u-ORM were significantly higher in patients with severe psoriasis. Further, they found a higher concentration of u-ORM/u-CREAT (creatinine in urine) in patients with psoriatic arthritis in relation to those without joint involvement. Thus, in addition to hsPCR, u-ORM is considered a noninvasive sensitive inflammatory biomarker to determine the severity of disease.

In a revision carried out by Gisondi and Giromoloni [80], they evaluated psoriatic comorbidities and their impact on atherothrombotic risk factors. Similar to comorbidities affecting psoriasis prognosis, they observed that psoriasis patients had increased risk of atherothrombotic diseases independently from the concomitance of traditional cardiovascular risk factors.

Hyperhomocysteinaemia has been reported in psoriasis patients and was directly correlated with psoriasis severity according to the PASI score, whereas it was inversely correlated with plasma folic acid levels. Increased levels of serum C-reactive protein (CRP) have been reported in patients with active psoriasis [81].

Patients with pustular psoriasis have significant elevated hs-CRP compared with that of plaque psoriasis patients. A direct correlation between CRP and psoriasis severity was found. CRP elevation is attributable (at least partially) to psoriasis inflammation.

Platelet activation has been established in psoriasis patients; the hemostatic balance is deranged toward a prothrombotic state in psoriasis patients, which might be mainly sustained by platelet hyperactivity.

Cytokines can also mediate several metabolic effects that, in the short term, result in an appropriate response to injury or infection; however, on a chronic basis, it can prove detrimental by accelerating the development of atherosclerosis and predisposing to thrombosis [82]. All of them are directly involved in poor prognosis of psoriasis.

Psoriasis is associated with obesity, dyslipidaemia, altered levels of HDL cholesterol, and impaired cholesterol efflux capacity.

Oxidized LDL (oxLDL) contributes to the spread of atherosclerosis and is an important target for the treatment of cardiovascular disease (CVD). The oxidized phospholipids (oxPL) of lipoproteins A (LPa) increase the proinflammatory response and favour the progression of CVD. In addition, low levels of oxidized HDL (oxHDL) are also associated with an increased risk of CVD in a young healthy population [82].

Sorokin et al. [83] studied 232 subjects with psoriasis, with an average PASI of 7.9. Almost half had hyperlipidaemia, of which 68% were under treatment. The levels of triglycerides (TG), LPa, ApoB, oxHDL, and oxLPa were higher than in healthy volunteers. The most severe patients (PASI > 10) had a negative correlation between oxLDL and oxLPa levels with a calcified plaque burden (DCB) and positive correlation between oxHDL levels with a noncalcified plaque burden (NCB), while the inverse occurred in patients with a PASI < 10.

The paraoxonase-1 (PON-1) system determines antioxidant properties and contributes to the CVD risk. Subjects with psoriasis have higher paraoxonase activity compared to healthy individuals; however, arylesterase levels are lower. Lactonase activity does not vary between people with psoriasis and the healthy population. In one part of the study, patients with psoriasis of a mean age of 47 years, with low cardiovascular risk, and treatment with anti-TNF and anti-IL7 were included. After 3–5 months of treatment, there was a significant increase in the levels of TG and LPa and a decrease in the levels of oxHDL with respect to the basal levels. After 1 year of follow-up, a reduction in the total

Table 2: Biomarkers of inflammation in obesity-psoriatic patients.

Type	Biomarkers	References
Diagnosis	C-reactive protein (CRP)	[63]
	Adipokines (adiponectin, leptin, resistin)	[63, 64]
	Signal transducer and activator of transcription (STAT)	[54]
	S100 family proteins (S100A7, S100A8, S100A9)	[54]
	Wnt family membrane 5a (Wnt5a) protein	[54]
	p53/TP53 protein	[54]
	Phospholipase C (PLC)	[54]
	Psoriasin (S100A)	[55]
	Interleukin (IL-22)	[56, 57]
	Interleukins (IL-12, IL-23)	[60]
	Koebnerisin	[55]
	Irisin	[65]
	Sphingolipids (ceramide FA-C22, sphingosine-1-phosphate)	[66]
	Hyperuricemia	[67]
	Interleukin (IL-21)	[71]
	Keratin-16 (Krt16)	[76]
	Lipocalin-2	[69]
	Class VI intermediate filament protein (Nestin)	[70]
Prognosis	Hyperhomocysteinaemia	[80]
	C-reactive protein (CRP)	[80, 81]
	Platelet activation	[80, 82]
	Cytokines (TNF-α, IFN-α)	[80, 82]
	Interleukins (IL-1, IL-6, IL-17)	[80]
	Dyslipidemia (↑TG, ↑VLDL, ↑LDL, ↓HDL-C)	[80]
	Body mass index (BMI) > 25	[80]
	Metabolic syndrome	[80]
	Habitual smoking	[80]
	Medications (methotrexate, cyclosporine, retinoids, biologics)	[80]
	Adipokines (adiponectin, leptin, resistin)	[72, 77]
	↑Carotid intima-media thickness	[77]
	Fatty acids (MUFA, SFA UFA, PUFA, EPA, DHA)	[78]
	Urinary orosomucoid (u-ORM). ↑Ratio u-ORM/u-CREAT	[78]
	Oxidation-modified lipoproteins (oxLDL, oxLPa, oxHDL)	[83]
	↑Paraoxonase-1 (PON-1) activity	[83]
Treatment	Serum YKL-40	[83]
	Retinoic acid-related orphan nuclear receptor gamma (RORγ/RORγt)	[85, 86]
	Aquaporin 3 (AQP3)	[87]
	Galectin-3	[88]
	TNF-like weak (TWEAK)	[89]
	C-reactive protein (CRP)	[90]
	Adipokines (chemerin, resistin, visfatin)	[90]
	Psoriasin (S100A)	[60, 61]

coronary plaque burden was observed due to a reduction in NCB with changes in DCB.

In summary, circulating lipid levels modified by oxidation can serve as useful markers of early atherosclerosis in psoriasis. This result could help to redirect the prognosis of psoriasis [83].

3.3. Biomarker for Treatment. Abud El-Hamd et al. [84] evaluated serum levels of YKL-40 in patients with psoriasis vulgaris before and after treatment with narrow-band UVB phototherapy (NB-UVB). Briefly, 30 patients with vulgar-type psoriasis were taken into account for this study, which were evaluated with the severity index of the psoriasis area

(PASI) and 20 control patients. Blood was drawn from both groups before and after the NB-UVB phototherapy. The blood was allowed to clot for a period of 30 minutes, followed by quantification of YKL-40 serum levels by the enzyme-linked immunosorbent assay (ELISA). The results determined that the serum values of YKL-40 were higher in patients with psoriasis vulgaris than the control patients, and the values of YKL-40 and PASI decreased significantly in patients with psoriasis vulgaris after NB-UVB phototherapy. Accordingly, the authors proposed YKL-40 as a biomarker to determine the inflammatory degree of psoriasis.

Gege et al. [85] investigated about the retinoic acid-related orphan nuclear receptor gamma (RORγ/RORγt). RORγt is a nuclear hormone receptor (NHR), which converts CD4+ immune cells into Th17 cells and is involved in cytokine production. GSK2981278 is a powerful inverse agonist of RORγ and appears to hinder Th17 expression, including the topical targeting of molecules of psoriatic lesions [86].

Aquaporin-3 (AQP3) is a protein that acts as a water channel, permitting water transport and glycerol permeability across cell membranes. It is involved in the regulation of differentiation and proliferation of keratinocytes as its expression is reduced in psoriasis. Targeting dry and itchy skin as one of the prime aspects of psoriasis, AQP3 is believed to be very relevant for its treatment [87].

Ritchie et al. [88] found that galectin-3 participates in cell proliferation, differentiation, and apoptosis and has a central role in skin disease (dermatitis and some skin cancers) as its decrease leads to changes in keratinocyte functioning and characterization through the JNK pathway.

Bilgiç et al. [89] studied the tumour necrosis factor-(TNF-) like weak inducer of apoptosis (TWEAK). TWEAK is a protein under the TNF superfamily that participates in cell growth, in apoptosis, and in various immune responses. It is involved in inflammatory and immune disorders, including atopic dermatitis, psoriasis, and multiple sclerosis. A potential target for inflammatory conditions driven by IL-17 could be the obstruction of TWEAK since it plays a crucial role in IL-17 signalling pathways. Clinical trial data of the anti-TWEAK monoclonal antibody in rheumatoid arthritis patients have shown promising results.

Finally, in this study of cases and controls, Gisondi et al. [90] enrolled 40 patients with chronic plaque psoriasis with the absence of systemic antipsoriatic treatment for at least 2 months before inclusion and 40 controls matched for age, sex, and BMI.

The objective was to measure serum adipokines in patients with plaque psoriasis, and serum levels of chemerin, resistin, and CRP were significantly higher in patients with chronic plaque psoriasis compared with controls independent of age, sex, BMI, and metabolic comorbidities. Further, they observed that chemerin levels were higher in patients with psoriatic arthritis than those without psoriatic arthritis. Chemerin was linearly correlated to CRP and resistin, but not with psoriasis severity measured with PASI or body surface area (BSA). After 2 and 12 months of treatment with infliximab, a significant reduction in chemerin, resistin, and CRP levels, as well as the PASI score, was observed. In contrast, visfatin levels significantly increased after 12 months of infliximab.

The main biomarkers of inflammation involved on diagnose, prognosis, and treatment in obesity-psoriatic patients are collated in Table 2 [54–57, 60, 61, 63–66, 68–70, 72, 76–80, 83–85, 90].

4. Conclusions

For some time, the epidemiological association of psoriasis, especially in its severe forms, with various diseases with which it shares a common pathogenic substrate has been known, including the involvement of tumour necrosis factor α (TNF-α) and different target organs (such as arthritis and Crohn's disease) and the increased risk of coronary heart disease and occlusive CVD. The severity of psoriasis measured by PASI, disease progression, and response to new treatments, with the so-called metabolic syndrome, is characterized by abdominal obesity, dyslipidemia aterogénica, hypertension, insulin resistance with or without glucose intolerance, and a proinflammatory and prothrombotic state as a risk factor for cardiovascular disease. Further, there is evidence that, in psoriasis, chronic inflammation has a pathogenic role in the metabolic syndrome and associated comorbidities, and its proper treatment could contribute to reversing it.

It is an obligation of dermatologists to recognize elements of the metabolic syndrome and to propose to psoriatic patients, in addition to optimal treatment of their psoriasis, changes in life habits, and adequate pharmacological treatments to reduce the risk of cardiovascular morbidity and mortality.

Thus, the patient with moderate-severe psoriasis has become the main therapeutic target. However, little has been documented about biomarkers that can objectively discriminate between states of disease activity. A biomarker of activity reflects an underlying inflammatory process, is reproducible, and allows patient monitoring, which is useful in making therapeutic decisions.

In this study, we considered the principal biomarkers that could discriminate subjects with active disease or remission, prognosis, and response to treatment in obese patients with comorbidities derived from overweight and high BMI.

Further research is required to achieve biomarkers that objectively discriminate the phases of reproducibility and can be applied in different clinical scenarios and by physicians with different levels of training.

Abbreviations

IFN-α:	Interferon-α
TNF:	Tumour necrosis factor
IL:	Interleukin
TSLP:	Thymic stromal lymphoprotein
IFN-α:	Interferon-α
LCN:	Lipocalin-2
LCs:	Langerhans cells
CCR:	Chemokine receptors
CXCL:	C-X-C motif chemokine ligand
CLA:	Cutaneous lymphocyte antigen

(CAMP/LL-37): Cathelicidin
CTACK: Cutaneous T cell-attracting chemokine
NF-KB: Nuclear factor kappa B
MCP-1: Monocyte chemoattractant protein 1
CCL: Chemokine (C-C motif) ligand
M: Macrophages (M1>M2).

Conflicts of Interest

The authors declare no potential competing interests with respect to the research, authorship, and/or publication of this article.

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Research Article

CD36-Mediated Lipid Accumulation and Activation of NLRP3 Inflammasome Lead to Podocyte Injury in Obesity-Related Glomerulopathy

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Podocyte injury critically contributes to the pathogenesis of obesity-related glomerulopathy (ORG). Recently, lipid accumulation and inflammatory responses have been found to be involved in podocyte injury. This study is to explore their role and relationship in podocyte injury of ORG. In animal experiments, the ORG mice developed proteinuria, podocyte injury, and hypertriglyceridemia, accompanied with deregulated lipid metabolism, renal ectopic lipid deposition, activation of NOD-like receptor protein 3 (NLRP3) inflammasome, and secretion of IL-1β of the kidney. The expression of adipose differentiation-related protein (ADRP), CD36, sterol regulatory element-binding protein 1 (SREBP-1), and peroxisome proliferator-activated receptor α (PPARα) in renal tissue were increased. In in vitro cell experiments, after cultured podocytes were stimulated with leptin, similar to ORG mice, we found aggravated podocyte injury, formatted lipid droplet, increased expression of ADRP and CD36, activated NLRP3 inflammasome, and released IL-1β. In addition, after blocking CD36 with inhibitor sulfo-N-succinimidyl oleate (SSO) or CD36 siRNA, activation of NLRP3 inflammasome and release of IL-1β are downregulated, and podocyte injury was alleviated. However, after blocking NLRP3 with MCC950, although podocyte injury was alleviated and release of IL-1β was decreased, there was no change in the expression of CD36, ADRP, and intracellular lipid droplets. Taken together, our study suggests that CD36-mediated lipid accumulation and activation of NLRP3 inflammasome may be one of the potential pathogeneses of ORG podocyte injury.

1. Introduction

Obesity is one of the major public health concerns with prevalence rates rapidly rising worldwide. In China, according to the 2010 China chronic disease monitoring program, the prevalence of obesity and central obesity of Chinese adults is 12.0% and 40.7%, respectively [1]. Obesity may directly lead to kidney injury, known as obesity-related glomerulopathy (ORG), and the incidence of ORG has increased concurrently with obesity [2, 3]. A retrospective study by D'Agati et al. showed that among cases that underwent renal biopsy, the percentage of ORG increased 13.5 times from 1986 to 2015 [3]. We also analyzed the cases that underwent renal biopsy in our hospital from 2010 to 2015 and calculated that the incidence of ORG with or without other kidney diseases accounted for 9.85% [4]. Podocyte injury critically contributes to the pathogenesis of ORG. Glomerular hypertrophy accompanied with podocyte hypertrophy and podocyte process fusion are the main pathological features of ORG, secondary focal segmental glomerulosclerosis may have occurred on this pathological basis, and clinical manifestations are proteinuria and progressive renal dysfunction; some patients may eventually develop end-stage renal disease (ESRD) [2, 3].

CD36, also known as a fatty acid transporter protein, is a single-chain transmembrane surface glycoprotein and belongs to the class B scavenger receptor family [5, 6]. CD36 is a multifunctional receptor that binds to two types of ligands: one is lipid-related ligands, including long-chain free fatty acids (FFA), oxidative low-density lipoprotein (ox-LDL), and oxidized phospholipids, and the other is protein-related ligands, such as advanced oxidized protein

Table 1: Primer sequences for PCR analysis in animal and cellular experiments.

Target	Primer sequence (5′-3′)	Length (bp)
Nephrin	Forward GTCTGGGGACCCCTCTATGA	209
	Reverse CAGGTCTTCTCCAAGGCTGT	
Podocin	Forward CAGAAGGGGAAAAGGCTGCT	200
	Reverse GATGCTCCCTTGTGCTCTGT	
Desmin	Forward GTTTCAGACTTGACTCAGGCAG	106
	Reverse TCTCGCAGGTGTAGGACTGG	
ADRP	Forward CTGGTGAGTGGCCTGTGT TA	199
	Reverse AAGCACACGCCTTGAGAG AA	
CD36	Forward ATGGGCTGTGATCGGAACTG	60
	Reverse AGCCAGGACTGCACCAATAAC	
SREBP-1	Forward GCGTGGTTTCCA ACATGACC	188
	Reverse TAGTGCCTCCTTTGCCACTG	
PPARα	Forward CTGCAGAGCAACCATCCAGAT	70
	Reverse GCCGAAGGTCCACCATTTT	
NLRP3	Forward TCTGCACCCGGACTGTAAAC	131
	Reverse CATTGTTGCCCAGGTTCAGC	
Pro-caspase1	Forward ACAAGGCACGGGACCTATG	237
	Reverse TCCCAGTCAGTCCTGGAAATG	
IL-1β	Forward CGCAGCAGCACATCAACAAG	118
	Reverse GTGCTCATGTCCTCATCCTG	
GAPDH	Forward TGTGAACGGATTTGGCCGTA	202
	Reverse GATGGGCTTCCCGTTGATGA	

products (AOPPs), thrombin-sensitive protein-1 (TSP1), and amyloid protein [5]. In the kidney, CD36 is mainly expressed in podocytes, mesangial cells, and tubular epithelial cells. It binds to long-chain FFA ligands in podocytes and mediates lipid uptake, apoptosis, and release of reactive oxygen species (ROS) [5, 7–9].

Current studies have shown that CD36 may play an important role in kidney injury associated with metabolic diseases. The expression CD36 was upregulated in renal tissue of cases with diabetic nephropathy [7]. Sulfo-N-succimidyl oleate (SSO), an inhibitor of CD36, could alleviate albuminuria of high-fat diet-fed mice [10]. In *in vitro* experiments, after podocytes were stimulated by palmitate, a type of saturated FFA, the expression of CD36 was increased and a lipid droplet was formatted; the apoptosis of podocytes was also increased [7]. These studies suggest that CD36, which is related to lipid uptake, may play a role in podocyte injury of ORG. In addition, according to literature and our previous studies, inflammatory responses and activation of NOD-like receptor protein 3 (NLRP3) inflammasome may be involved in CD36-mediated lipid accumulation and podocyte injury [11, 12].

NLRP3 inflammasome is composed of NOD-like receptor protein 3 (NLRP3), apoptosis-related speck protein (ASC), and cysteine aspartate-1 precursor (pro-caspase-1) [13, 14]. Activation of NLRP3 results in the activation of caspase-1, which cleaves the proinflammatory cytokines IL-1β and IL-18 to their active forms. Mature IL-1β and IL-18 are released outside of the cell, resulting in a sterilized inflammatory response [14]. Numerous studies have shown that the activated NLRP3 inflammasome was involved in the pathogenesis of metabolic diseases, such as type 2 diabetes mellitus, atherosclerosis, and obesity [15, 16]. Our previous studies have observed the activation of NLRP3 inflammasome and podocyte injury in the ORG mouse model and cultured podocyte stimulated by leptin, and blocking one of the upstream receptors of NLRP3, purinergic ligand-gated ion channel 7 receptor (P2X7R), could ameliorate leptin-induced podocyte injury and inflammatory response [12].

CD36-mediated lipid accumulation may be associated with activation of the NLRP3 inflammasome. Such phenomenon was first reported in the study of arteriosclerosis [17]. OxLDL-induced IL-1β secretion promotes foam cell formation, which was mainly via CD36-mediated release of ROS production and activation of the NLRP3 inflammasome [18]. In terms of renal disease, Yang et al. reported that in a nephrotic syndrome animal model, increased expression of CD36 could mediate the apoptosis of podocyte through activating the NLRP3 inflammasome [11].

Is CD36-mediated lipid accumulation involved in podocyte injury of ORG? Is it associated with NLRP3 inflammasome activation? Our study showed that both in ORG mouse models and in leptin-stimulated cultured podocytes, formatted lipid droplets, increased expression of ADRP and CD36, activated NLRP3 inflammasome, and released IL-1β were found. In addition, blocking of CD36 reduced podocyte injury and activation of the NLRP3 inflammasome, while

blocking NLRP3 could alleviate podocyte injury, but did not decrease the expression of CD36 and adipose differentiation-related protein (ADRP). Taken together, our study suggests that CD36 mediated lipid accumulation and NLRP3 inflammasome activation, which may be one of the potential pathogeneses of ORG podocyte injury.

2. Materials and Methods

2.1. Animals and Grouping. Twenty 6-week-old male C57BL/6J mice (SPF Biotechnology, China) were housed in an animal room of specific-pathogen-free cleanliness grade with 50-60% humidity at temperature 20-26°C. Mice were randomly divided into 2 groups: control group ($n = 10$), which were fed a common diet *ad libitum* that contained fat accounting for 10% kcal (Beijing Huafukang Biological Technology Co. Ltd., Beijing, China), and ORG model group ($n = 10$), which were fed a high-fat diet that contained fat accounting for 60% kcal (Research Diet, USA) as described previously [4]. All mice were sacrificed after anesthesia with pentobarbital at the end of the 12th week. One fourth of renal tissue was fixed in 4% neutral formaldehyde solution for light microscopy; one fourth of renal tissue was fixed in 2.5% glutaraldehyde solution for electron microscopy; and the renal cortex of the remaining part was rapidly preserved in liquid nitrogen for real-time quantitative polymerase chain reaction (PCR) analysis and Western blot assay.

All animal care and experimental protocols complied with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (publication no. 85-23, 1996) and were approved by the Institutional Animal Care and Use Committee of Capital Medical University (Beijing, China).

2.2. Biological Parameters. Body weight was measured at baseline and every 4 weeks. Kidney weight was measured after mice were sacrificed. Nocturnal 12 h urine protein was collected and measured at the 0 and 12^{th} weeks by using the Bradford protein assay kit (Beyotime Biotechnology, China) according to the user's instruction. Following sacrifice, the blood samples were collected for the measurement of serum creatinine, serum triglyceride, serum cholesterol, and blood glucose levels, as well as urine creatinine levels. The measurement was carried out by using the Olympus AU5400 Chemistry Analyzer (Olympus, Japan). The calculation methods of Lee's index, visceral fat index, and creatinine clearance rate were described as previously [19].

2.3. Pathological Examination. The mouse renal cortical tissues were fixed, dehydrated, embedded, sectioned (3 μm), and stained with periodic acid-Schiff reagent as described [19] for light microscopy. Twenty images of glomerular maximal profiles with a vascular pole and/or urinary pole were taken under a high-power microscope (×400, Olympus, Japan) and were analyzed by Nikon NIS-Elements BR image analysis software (Nikon, Japan). The length (μm) of two longest perpendicular diameters in every glomerular capillary tuft without Bowman's space was measured, and then the mean value was calculated.

Table 2: Primary and secondary antibodies for Western blot assays.

Primary antibody	Secondary antibody
Rabbit anti-nephrin pAb (Abcam)	IRDye 800 conjugated goat anti- rabbit IgG antibody (LI-COR)
Rabbit anti-podocin pAb (Sigma)	Ditto
Rabbit anti-desmin pAb (Abcam)	Ditto
Rabbit anti-ADRP pAb (Abcam)	Ditto
Rabbit anti-CD36 pAb (Abcam)	Ditto
Rabbit anti-SREBP-1 pAb (Santa Cruz)	Ditto
Rabbit anti-PPARα pAb (Santa Cruz)	Ditto
Rabbit anti-NLRP3 pAb (Novus)	Ditto
Rabbit anti-caspase1 P10 pAb (Santa Cruz)	Ditto
Rabbit anti-IL-1β pAb (Abcam)	Ditto
Mouse anti-β-actin mAb (Sigma)	IRDye 700 conjugated goat anti-mouse IgG antibody (LI-COR)

Table 3: Biological parameters in the different groups at the 12th week ($\bar{x} \pm s$).

Group	Control	Model
Body weight (g)	28.9 ± 1.8	31.9 ± 1.9*
Kidney weight (mg)	0.34 ± 0.03	0.37 ± 0.02*
Lee's index (g/cm)	16.86 ± 0.38	17.64 ± 0.56*
Visceral fat index	0.02 ± 0.01	0.05 ± 0.02*
Urinary protein (mg/d)	801.5 ± 178.9	1001.7 ± 230.1*
Serum creatinine (μmol/L)	11.3 ± 0.8	10.4 ± 1.1
Creatinine clearance rate (mL/min)	0.4 ± 0.1	0.7 ± 0.2*
Serum triglyceride (mmol/L)	0.5 ± 0.1	0.9 ± 0.4*
serum cholesterol (mmol/L)	2.2 ± 0.4	3.6 ± 0.3*
Blood glucose (mmol/L)	9.5 ± 0.6	9.8 ± 1.3

*$P < 0.05$ vs. control group.

The ultra-thin section renal cortical tissues were stained with uranium acetate-lead citrate for electron microscopy. Briefly, for each specimen, ten photographs (×20 000 magnification) covering different regions in the glomerular cross section were taken separately. The length (μm) of the peripheral GBM was measured, and the number of slit pores overlying this GBM length was counted by Nikon NIS-Elements BR image analysis software (Nikon, Japan). The average width of the foot process was calculated as described [12].

2.4. Podocyte Culture and Grouping. The conditionally immortalized mouse podocyte cell line was kindly provided by Professor Maria Pia Rastaldi (S. Carlo Hospital, University of Milan). For the culture of podocytes, we followed the

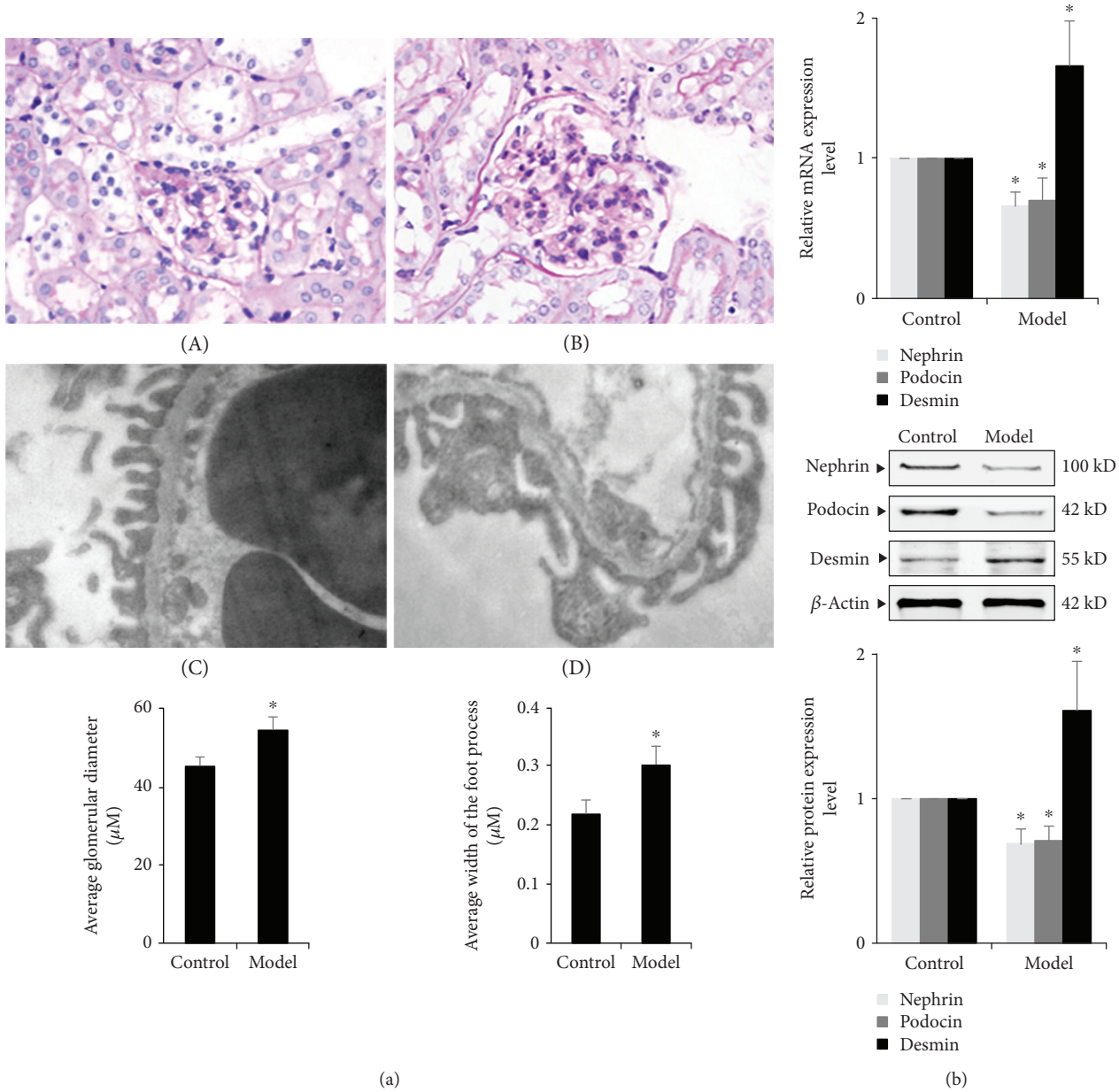


Figure 1: Changes in glomerular diameter and podocyte of renal tissue in the ORG model. (a) Histology of renal tissues of different groups. A and B Light microscope of PAS staining (×400). The average glomerular diameter was measured and compared. C and D, Electron microscope of glomeruli (×20000). The average width of the foot process was measured and compared. Values are represented as mean ± SD ($n = 10$). (b) The relative mRNA and protein expression levels of nephrin, podocin, and desmin of the renal cortex were measured by real-time quantitative PCR and Western blot assay. The relative protein expression level was expressed as the target protein/β-actin ratio. Values are represented as mean ± SD. $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. ORG model group.

methods of Wang et al. [20]. Podocytes were incubated in RPMI-1640 medium (Thermo Fisher Scientific, USA) containing 10% inactivated fetal bovine serum (FBS, Thermo Fisher Scientific, USA) and 10 μ/mL interferon-γ (IFN-γ, Cell Signaling Technology, USA) at 33°C in humidified air with 5% CO_2. When the cells reached 80-90% confluence, they were transferred to RPMI-1640 medium containing 10% inactivated FBS without IFN-γ and incubated at 37°C in humidified air with 5% CO_2 for 10-14 days to allow differentiation. Well-differentiated podocytes were used for experiments, and different stimulants were added to the cells for different times according to experimental requirements.

2.5. Reverse Transcription and Real-Time Quantitative PCR. Total RNA was extracted from the mouse renal cortex tissue or cultured podocytes using TRIzol reagent (Thermo Fisher Scientific, USA) following the manufacturer's instruction.

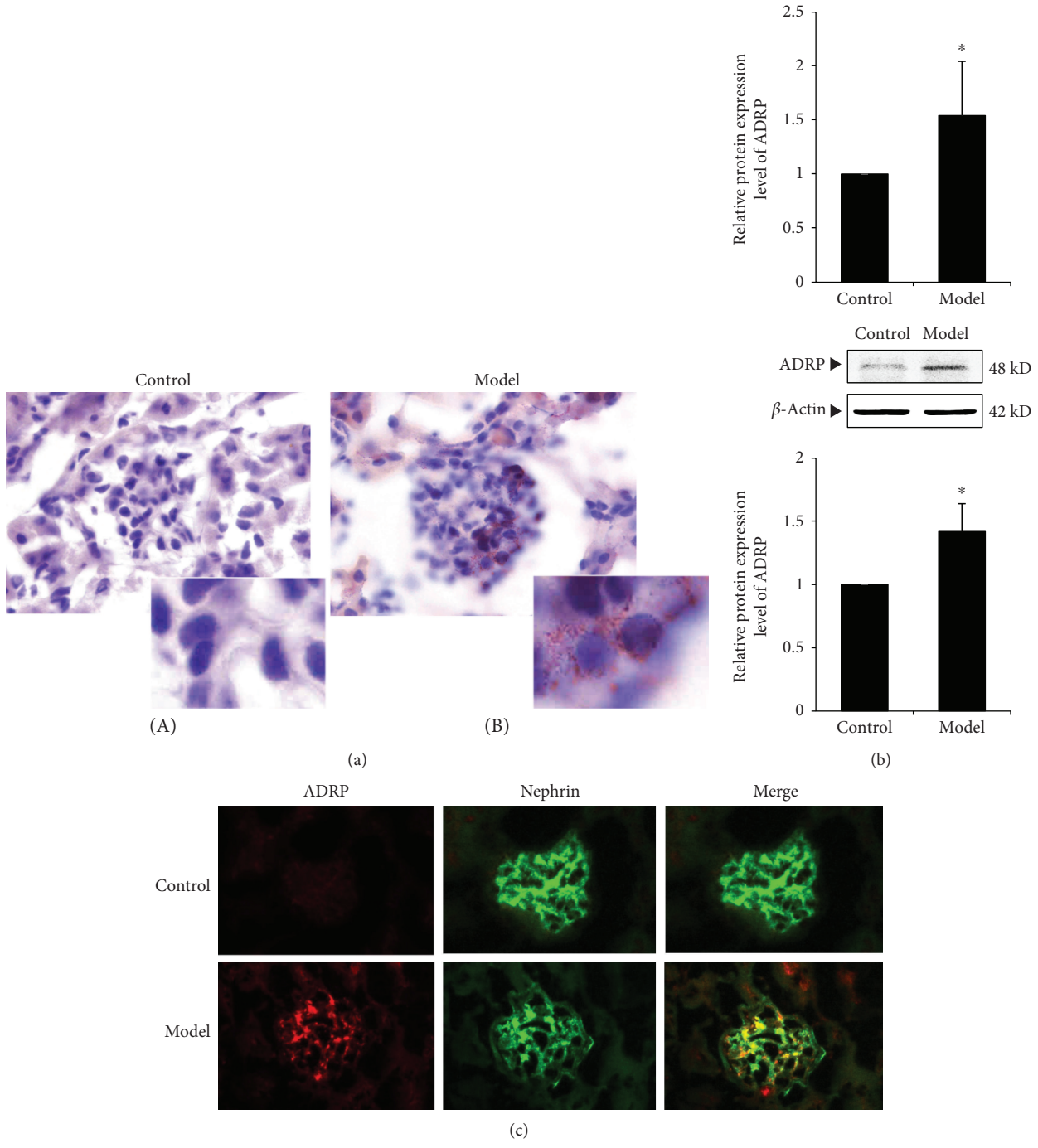


Figure 2: Changes of lipid accumulation of renal tissue in ORG model. (a) Representative Oil Red O staining images of renal tissue in different groups (magnification ×1000). (b) The relative mRNA and protein expression levels of ADRP of the renal cortex were measured by real-time quantitative PCR and Western blot assay. The relative protein expression level was expressed as the target protein/β-actin ratio. Values are represented as mean ± SD. $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. ORG model group. (c) Double immunofluorescence staining of ADRP and nephrin of the ORG model. The localization of ADRP (red spots), nephrin (green spots), and merged image (yellow spots) in the frozen section of renal tissue of the ORG model (×400) is shown as indicated.

2 μg total RNA from each sample was reverse-transcribed to cDNA with EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech). The gene-specific primers (SBS Genetech, China) are listed in Table 1. Real-time PCR was performed using SYBR Green RT-PCR Master Mix (TransGen Biotech, China) according to the manufacturer's instruction. GAPDH was set as the internal control gene in the animal and cellular experiments. The relative quantity of mRNA expression was calculated according to the formula $2^{-(\text{targetgeneCt}-\text{GAPDHCt})} \times 10^3$, in which Ct was the threshold cycle number. All assays were repeated at least in triplicate independently.

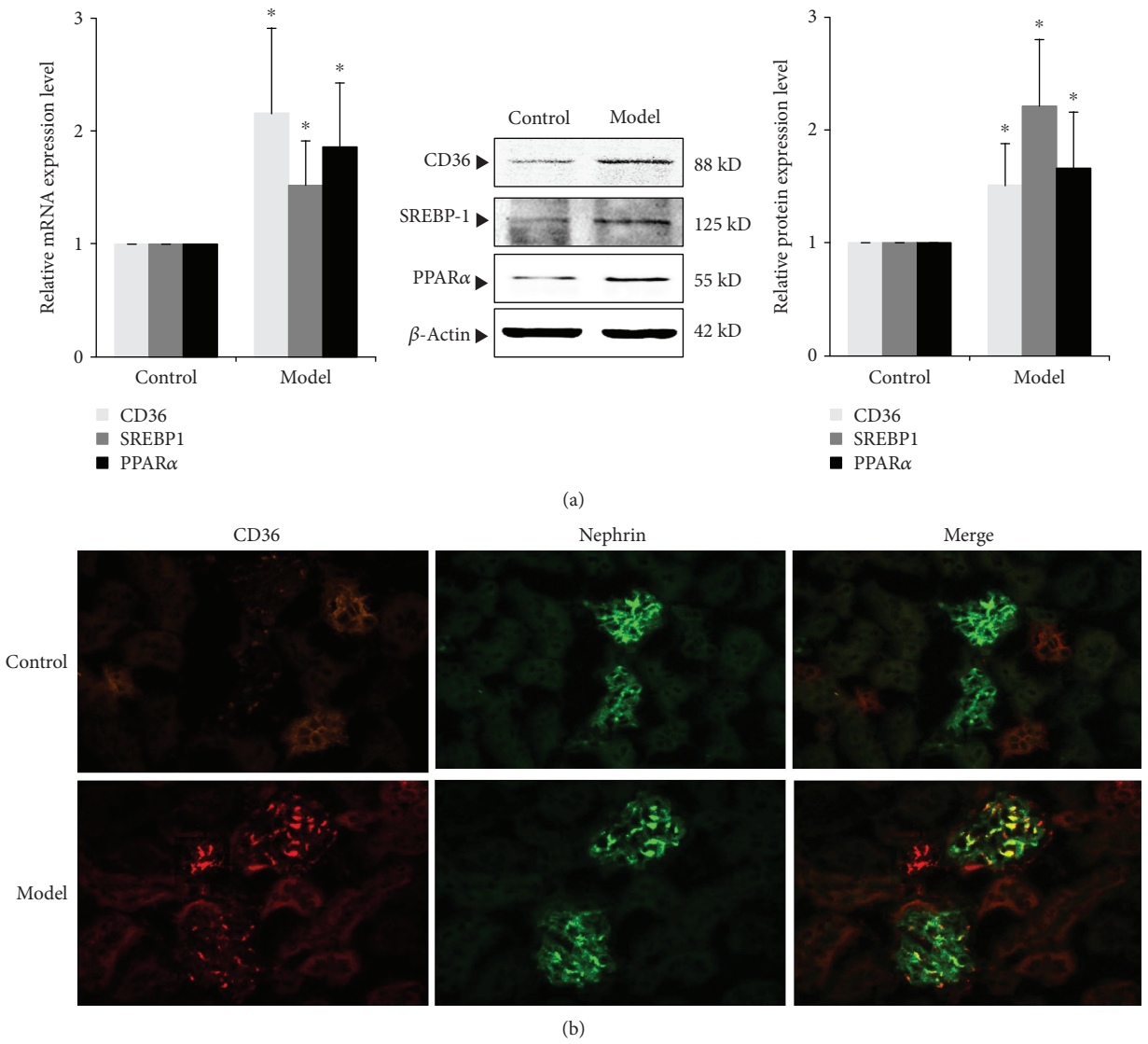


Figure 3: Expression of molecules related to lipid metabolism in renal tissue of ORG mice. (a) The relative mRNA and protein expression levels of CD36, SREBP1, and PPARα of the renal cortex were measured by real-time quantitative PCR and Western blot assay. The relative protein expression level was expressed as the target protein/β-actin ratio. Values are represented as mean ± SD. $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. ORG model group. (b) Double immunofluorescence staining of CD36 and nephrin of the ORG model. The localization of CD36 (red spots), nephrin (green spots), and merged image (yellow spots) in the frozen section of renal tissue of the ORG model (×400) is shown as indicated.

2.6. Western Blot Assay. Total protein lysates were extracted from the mouse renal cortex tissue or cultured podocytes using RIPA lysis buffer (ComWin Biotech, China). Protein samples were sonicated five times for 1 s each, centrifuged at 12 000 rpm for 10 min at 4°C, and then boiled for 5 min. Protein samples were separated by 10-12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (General Electric Co). After being blocked with 5% skim milk in phosphate-buffered saline with 0.1% Tween 20 for 1 h, the membranes were incubated with primary antibody at 4°C overnight and then incubated with secondary antibody at room temperature for 1 h. Details regarding primary and secondary antibodies are listed in Table 2. The blotted proteins were quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences). β-Actin was set as an internal control. The relative expression level of each target protein was displayed as a ratio of target protein/β-actin protein. All the assays were performed at least in triplicate independently.

2.7. Small Interfering RNA (siRNA) of CD36. Well-differentiated podocytes were transiently transfected with 50 pmol mouse CD36 siRNA (Santa Cruz, USA) or control siRNA-A (Santa Cruz, USA) using Lipofectamine 3000 (Life technologies, USA) according to the manufacturer's instruction. The transfected cells were cultured for 24 h. The efficiency of CD36 siRNA after 24 hours of transfection was confirmed by quantitative real-time PCR of CD36 mRNA. Then, cells

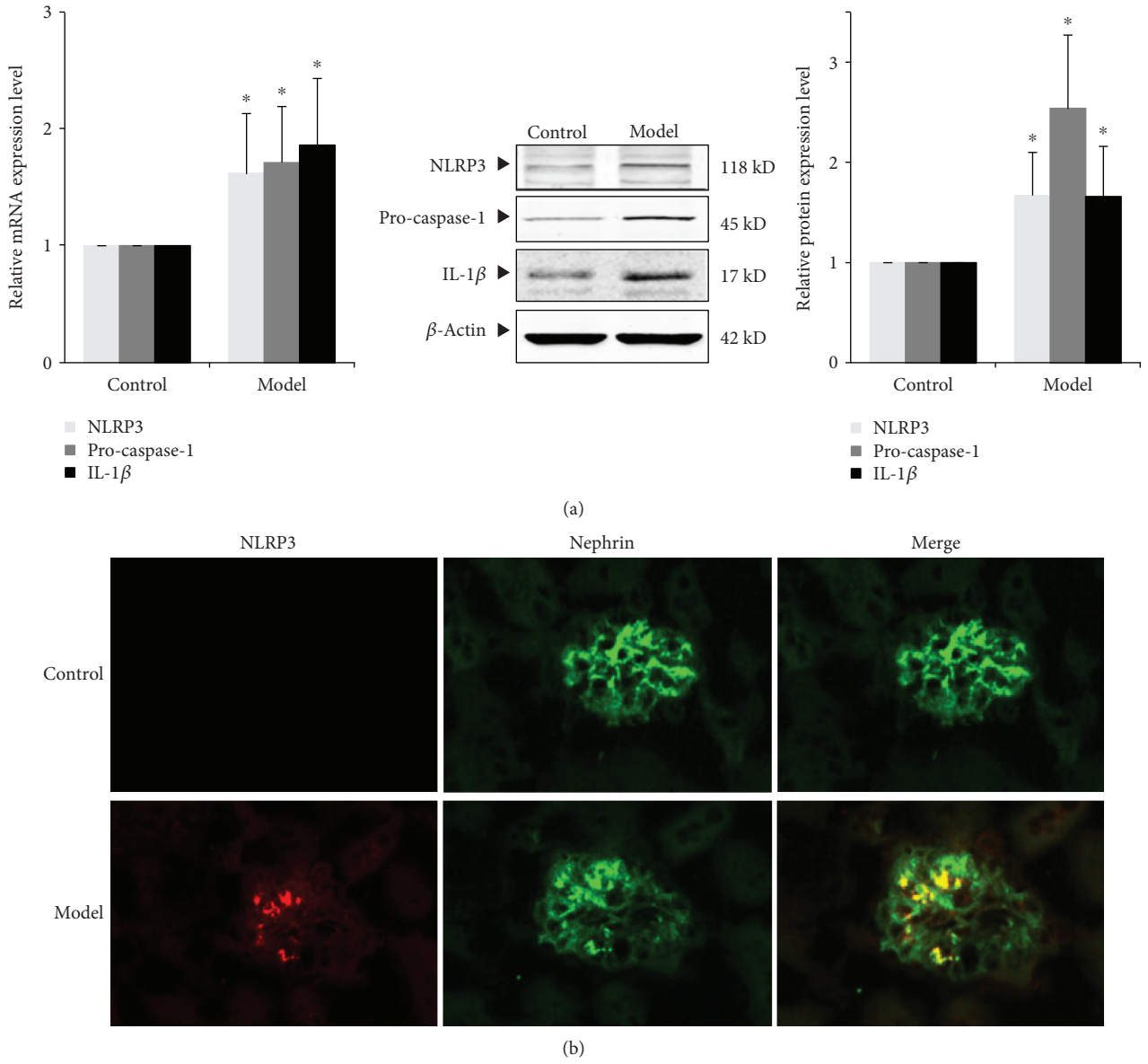


Figure 4: Expression of NLRP3 inflammasome and IL-1β in renal tissue of ORG mice. (a) The relative mRNA and protein expression levels of NLRP3, pro-caspase-1, and IL-1β of the renal cortex were measured by real-time quantitative PCR and Western blot assay. The relative protein expression level was expressed as the target protein/β-actin ratio. Values are represented as mean ± SD. $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. ORG model group. (b) Double immunofluorescence staining of NLRP3 and nephrin of the ORG model. The localization of NLRP3 (red spots), nephrin (green spots), and merged image (yellow spots) in the frozen section of renal tissue of the ORG model (×400) is shown as indicated.

were incubated with fresh medium with or without leptin for another 12 h or 24 h according to real-time quantitative PCR analysis or Western blot assay, respectively.

2.8. Oil Red O Staining. The lipid accumulation in mouse renal tissues and cultured podocytes was evaluated by Oil Red O staining. Briefly, the sections were fixed with 4% paraformaldehyde, rinsed with 60% isopropanol, stained with Oil Red O for 20 min, and rinsed with 60% isopropanol. Finally, the samples were counterstained with haematoxylin for 5 min. The results were examined by light microscopy (Nikon, Japan).

2.9. Double Immunofluorescence Staining. For double staining of an indirect immunofluorescence assay of proteins and podocyte marker nephrin, frozen renal tissues of mice were fixed in 4% paraformaldehyde, cut into 5 μm thick sections, permeabilized with 0.1% Triton X-100, and blocked with 2% BSA. After blocking, the sections were incubated overnight at 4°C with a rabbit monoclonal anti-ADRP (1 : 100, Abcam, UK), rabbit monoclonal anti-CD36 antibody (1 : 100, Abcam, UK), or rabbit monoclonal anti-NLRP3 (1 : 100, Novus, USA) and guinea pig polyclonal anti-nephrin (1 : 100, Progen Biotechnik, German), and then washed with PBS for three times. Next, the sections were

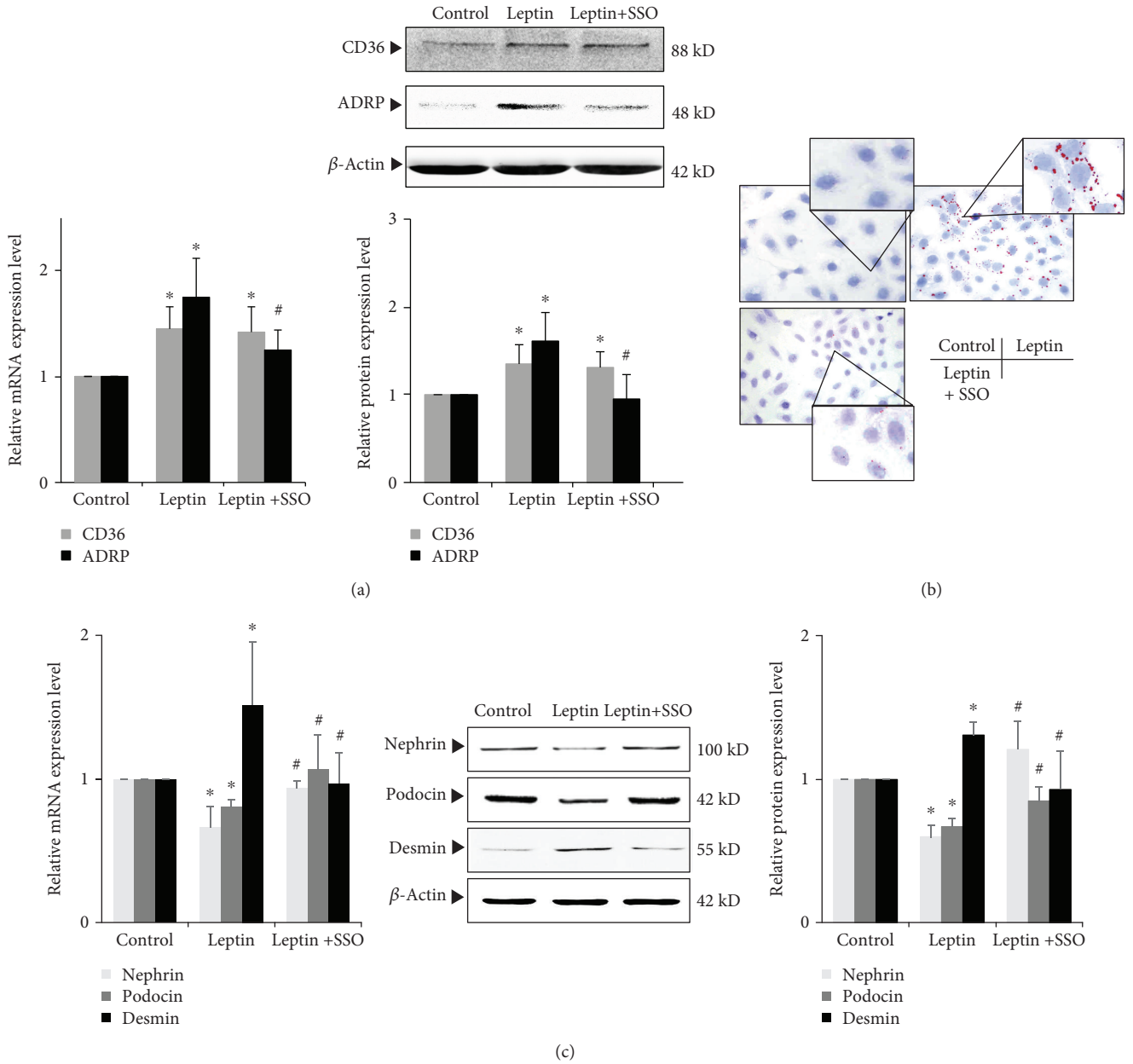


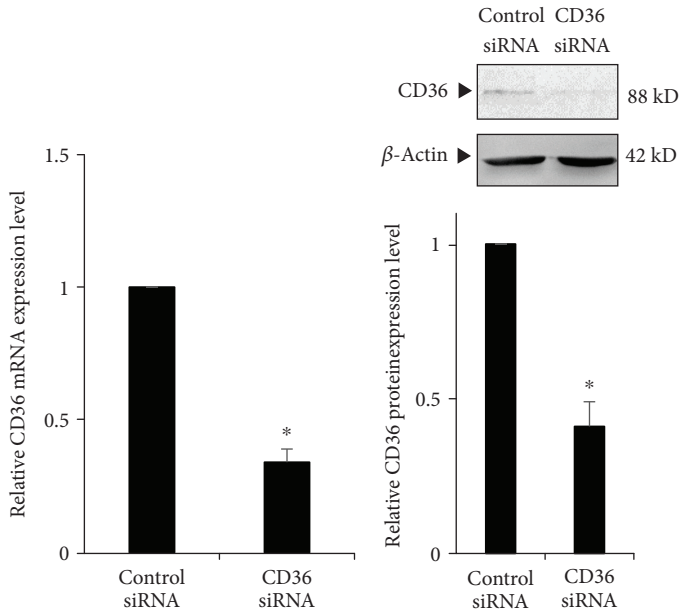
FIGURE 5: Effects of inhibiting CD36 by SSO on leptin-induced podocyte injury and lipid accumulation. (a) and (c) Differentiated podocytes were incubated in medium, medium containing 15 ng/mL leptin, or medium containing both 15 ng/mL leptin and 50 μM SSO, respectively. The relative mRNA and protein expression levels of CD36 and ADRP (a) and nephrin, podocin, and desmin (c) of podocytes were measured by real-time quantitative PCR and Western blot assay. (b) Representative Oil Red O staining images of podocytes in different groups as indicated (magnification ×400). Values are represented as mean ± SD ($n = 3$). $^*P < 0.05$ vs. control group, $^\#P < 0.05$ vs. leptin + SSO group.

incubated with rhodamine-labeled goat anti-mouse antibody (ZSBiO, China) and FITC-labeled rabbit anti-guinea pig antibody (Abcam, UK) for 2 h at room temperature as secondary antibodies, respectively. After staining, the tissue sections were observed with a fluorescent microscope (Nikon, Japan).

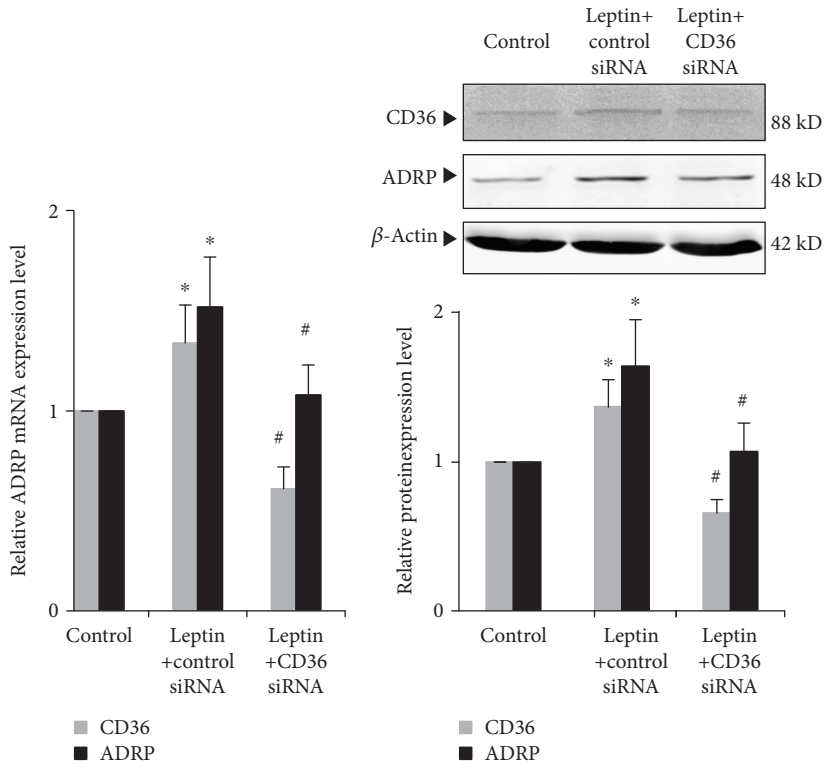
2.10. Statistical Analysis. All the data of continuous variables were represented as mean ± standard deviation (SD) and analyzed by using SPSS 21.0 statistical software (IBM, USA). Statistical significance between groups was analyzed by one-way ANOVA. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Biological Parameters and Renal Histological Changes in ORG Animal Models. The average body weight, kidney weight, Lee's index, visceral fat index, and 12 h urinary protein excretion were significantly increased in the ORG model group compared with the control group at the 12th week ($P < 0.05$), while there was no significance in serum

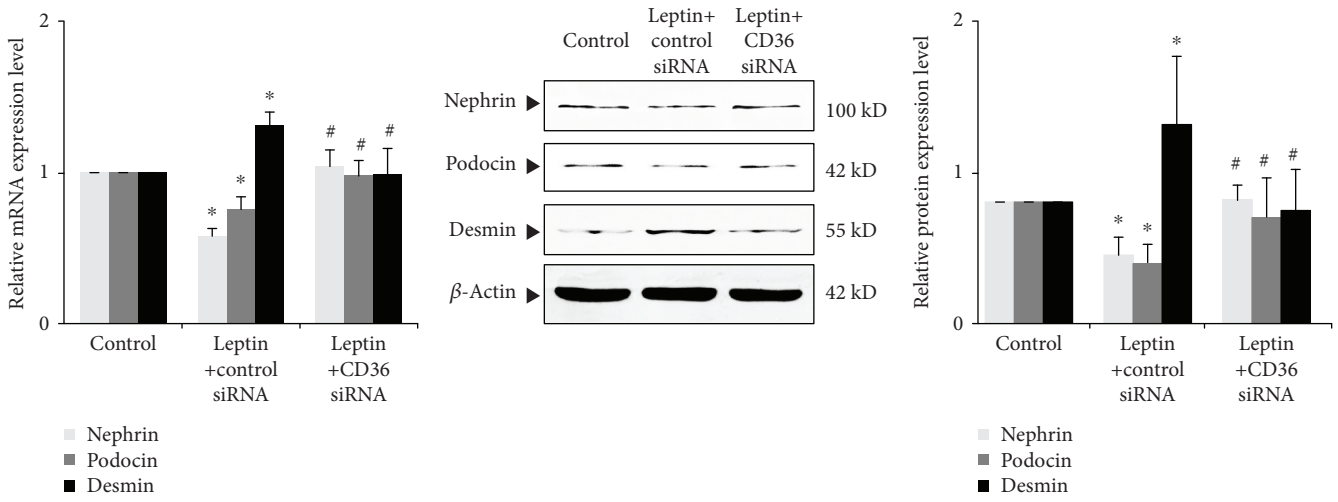


(a)



(b)

Figure 6: Continued.



(c)

Figure 6: Effects of inhibiting CD36 by small interfering RNA on leptin-induced podocyte injury and lipid accumulation. (a) Differentiated podocytes were transiently transfected with 50 pmol control siRNA or CD36 siRNA, respectively. After 24 h (for mRNA) or 36 h (for protein), cells were harvested and the relative mRNA and protein expression levels of CD36 were measured by real-time quantitative PCR and Western blot assay. (b) and (c) Differentiated podocytes were transiently transfected with 50 pmol control siRNA or CD36 siRNA, respectively. After 24 h, cells were incubated in medium or medium containing 15 ng/mL leptin as indicated, respectively. Following 24 h (for mRNA) or 36 h (for protein) stimulation by leptin, the relative mRNA and protein expression levels of CD36 and ADRP (b) or nephrin, podocin, and desmin (c) of podocytes were measured by real-time quantitative PCR and Western blot assay. Values are represented as mean ± SD ($n = 3$). $^{*}P < 0.05$ vs. control siRNA group (a) or control group (B and C), $^{\#}P < 0.05$ vs. leptin + control siRNA group.

creatinine levels among the two groups ($P > 0.05$). The creatinine clearance rate (CCr) of mice in the ORG model group significantly increased than that in the control group ($P < 0.05$) (Table 3).

Hyperlipidemia was found in ORG model mice. At the end of the 12th week, levels of serum triglyceride and cholesterol in the ORG model group were significantly higher than those in the control group ($P < 0.05$). There was no significant difference in blood glucose levels between the two groups ($P > 0.05$) (Table 3).

Renal tissue pathological examination showed that the mean glomerular diameter in the ORG model group was significantly longer than that in the control group at the end of the 12^{th} week ($P < 0.05$, Figure 1(a)). Under a transmission electron microscope, there was mild and segmental foot process effacement in the ORG model group, and the mean foot process width in the ORG model group was significantly wider than that in the control group at the end of the 12^{th} week ($P < 0.05$, Figure 1(a)).

3.2. Podocyte Injury of ORG Animal Models. We next investigated the changes of podocyte-associated molecules in renal tissues, including nephrin and podocin. The expression of desmin was also measured, which is a sensitive marker of podocyte injury [21]. Compared with the control group, the mRNA and protein expressions of podocyte-associated molecules in the ORG model group were significantly decreased, and expression of desmin was significantly increased in the renal cortical tissue of mice at the end of the 12^{th} week ($P < 0.05$) (Figure 1(b)).

3.3. Lipid Accumulation of Renal Tissues in ORG Animal Models. The results of Oil Red O staining revealed that there was an obvious lipid droplet formation in the glomeruli (Figure 2(a)). We also investigate the expression of adipose differentiation-related protein (ADRP), which is a major constituent located in the lipid droplet surface. Our results showed that the mRNA and protein expression of ADRP in the ORG model group was significantly upregulated ($P < 0.05$) (Figure 2(b)). Immunofluorescence staining of renal tissue showed that increased expression of ADRP in the glomerulus and its position overlapped with the podocyte marker protein nephrin, which suggested that there are lipid droplets in the podocytes during ORG (Figure 2(c)).

3.4. Expression of CD36 and Other Molecules Related to Lipid Metabolism of Renal Tissue in the ORG Model. We further examined the expression changes of CD36 and other molecules associated with lipid metabolism. At the end of the 12^{th} week, the expression of CD36 was significantly increased in the ORG model group compared with the control group ($P < 0.05$). The expression of sterol regulatory element-binding protein 1 (SREBP-1), which regulates genes required for fatty acid synthesis, was significantly upregulated ($P < 0.05$). The expression of peroxisome proliferator-activated receptor α (PPARα), the main regulator of FFA oxidation, was also upregulated ($P < 0.05$) (Figure 3(a)). The result suggests that hyperlipidemia of ORG leads to imbalance of renal lipid metabolism. Immunofluorescence staining of renal tissue showed high expression of CD36 in

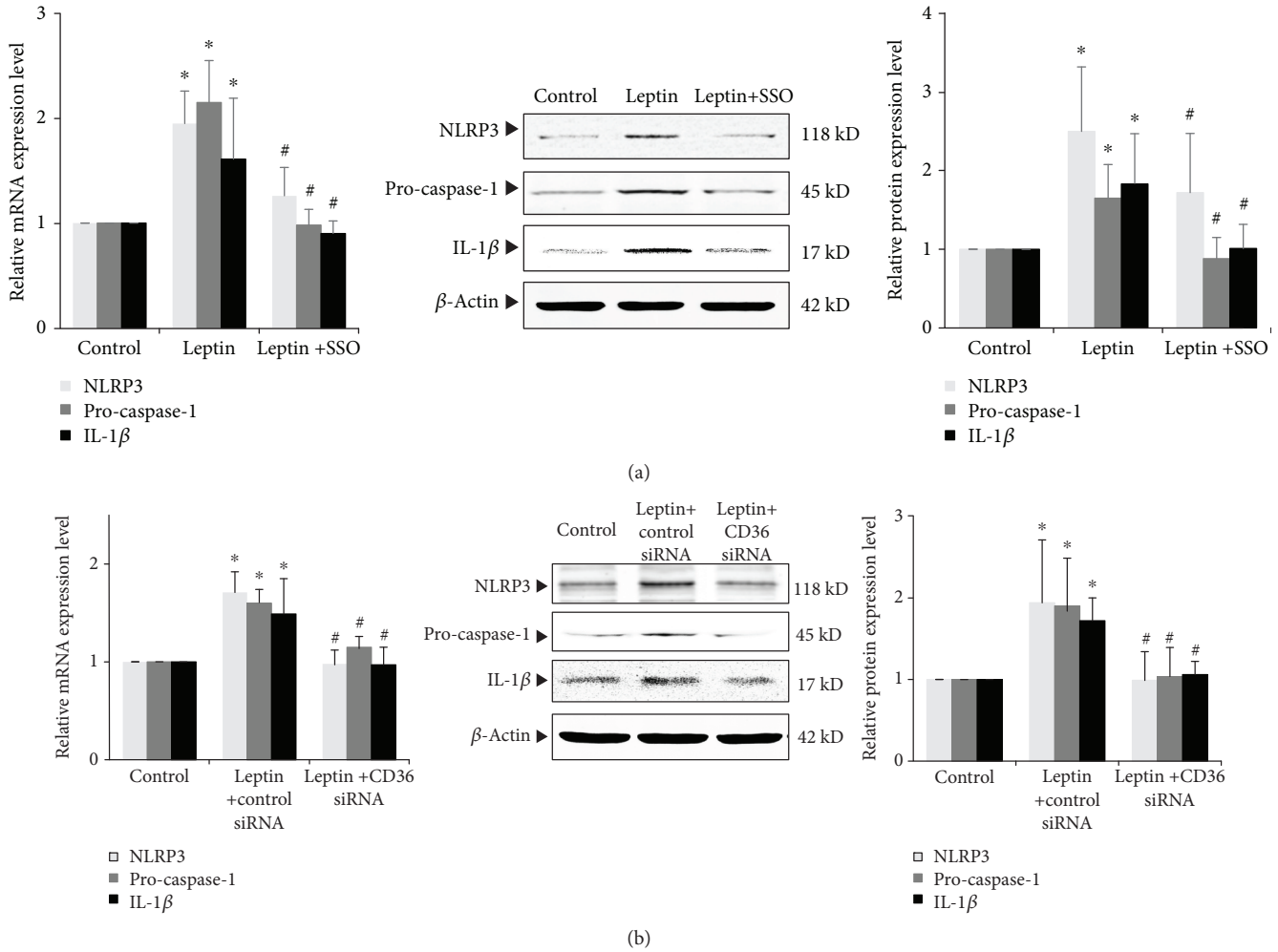


Figure 7: Effects of inhibiting CD36 on leptin-induced activation of NLRP3 inflammasome and IL-1β secretion of podocyte. (a) Differentiated podocytes were incubated in medium, medium containing 15 ng/mL leptin, or medium containing both 15 ng/mL leptin and 50 μM SSO, respectively. (b) Differentiated podocytes were transiently transfected with 50 pmol control siRNA or CD36 siRNA, respectively. After 24 h, cells were incubated in medium or medium containing 15 ng/mL leptin as indicated, respectively. (a) and (b) The relative mRNA and protein expression levels of NLRP3, pro-caspase-1, and IL-1β of podocytes were measured by real-time quantitative PCR and Western blot assay. Values are represented as mean ± SD ($n = 3$). $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. leptin + SSO group (a) or leptin + CD36 siRNA group (b).

both renal tubules and glomeruli (Figure 3(b)). Expression of CD36 in glomerulus was overlapped with nephrin, suggesting that CD36-midiated lipid uptake and lipid accumulation increased in podocytes during ORG (Figure 3(b)).

3.5. Expression of NLRP3 Inflammasome and IL-1β of Renal Tissue in the ORG Model. In order to detect renal inflammatory response in the ORG model, we measured the expression of components of NLRP3 inflammasome (NLRP3 and pro-caspase-1) and the downstream inflammatory factor, IL-1β. The results showed that the mRNA and protein expressions of NLRP3, pro-caspase-1, and IL-1β were significantly upregulated in the ORG model group ($P < 0.05$) (Figure 4(a)). Immunofluorescence staining of renal tissue showed a high expression of NLRP3 in the glomerulus (Figure 4(b)). Overexpressed NLRP3 overlapped with nephrin, suggesting that the overexpressed NLRP3 inflammasome exists in podocytes during ORG (Figure 4(b)).

Taken together, our results suggest that podocyte injury of ORG may be related with CD36-mediated lipid accumulation and activation of the NLRP3 inflammasome. We will next confirm the results in cellular experiments.

3.6. Effects of CD36 Inhibitor SSO on Leptin-Induced Podocyte Injury and Lipid Accumulation. To mimic podocyte injury of ORG, cultured podocytes were stimulated by 15 ng/mL leptin, a key adipocytokine that regulates satiety and body fat [4, 12, 19, 22]. After leptin stimulation, the expression of CD36 and ADRP was significantly increased, and Oil Red O staining showed intracellular lipid droplet formation in podocyte (Figures 5(a) and 5(b)). The expression of nephrin

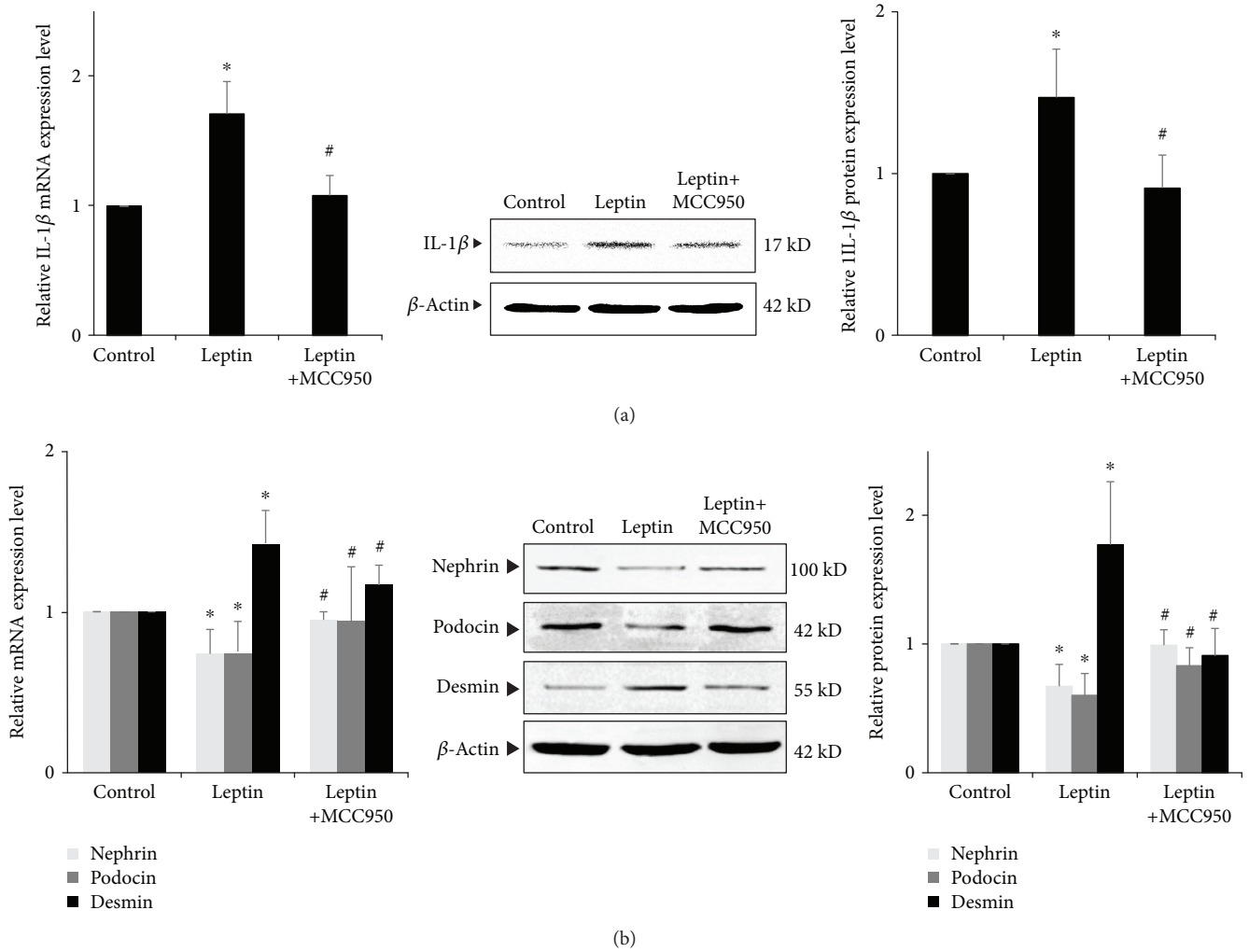


Figure 8: Effects of inhibiting NLRP3 by MCC950 on leptin-induced podocyte injury and IL-1β secretion. (a) and (b) Differentiated podocytes were incubated in medium, medium containing 15 ng/mL leptin, or medium containing both 15 ng/mL leptin and 1 μM MCC950, respectively. The relative mRNA and protein expression levels of nephrin, podocin, and desmin (a) and IL-1β (b) of podocytes were measured by real-time quantitative PCR and Western blot assay. Values are represented as mean ± SD ($n = 3$). $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. leptin + MCC950 group.

and podocin is significantly downregulated ($P < 0.05$), while the expression of desmin is significantly upregulated ($P < 0.05$) (Figure 5(c)).

While when a specific inhibitor of CD36, SSO, was added, the effects of leptin on podocyte were significantly inhibited. After SSO was added, although there was no significant change in CD36 expression in the leptin+SSO group compared with the leptin group, the expression of ADRP was downregulated in podocyte ($P < 0.05$, Figure 5(a)). In addition, Oil Red O staining showed the decreased intracellular lipid droplet formation (Figure 5(b)). Compared with the leptin group, the expression of nephrin and podocin in the leptin+SSO group was significantly upregulated, and desmin expression was significantly downregulated ($P < 0.05$) (Figure 5(c)). These results suggest that inhibition of CD36 by SSO could ameliorate lipid accumulation and podocyte injury.

3.7. Effects of CD36 siRNA on Leptin-Induced Podocyte Injury and Lipid Accumulation. We also used CD36 siRNA to inhibit the expression of CD36 in podocyte. Our results showed that CD36 siRNA could effectively inhibit the expression of CD36 in podocyte (Figure 6(a)). Compared with the leptin + control siRNA group, the expressions of podocin and nephrin in the leptin + CD36 siRNA group were significantly upregulated and the expression of desmin was significantly downregulated ($P < 0.05$); also, the expression of CD36 and ADRP was significantly downregulated by CD36 siRNA ($P < 0.05$) (Figure 6(b)).

3.8. Effects of Blocking CD36 on Leptin-Induced NLRP3 Inflammasome Activation and IL-1β Secretion. We also found that stimulation of leptin could increase the expression of NLRP3 inflammasome and secretion of inflammatory cytokine IL-1β (Figures 7(a) and 7(b)). Could inhibition of

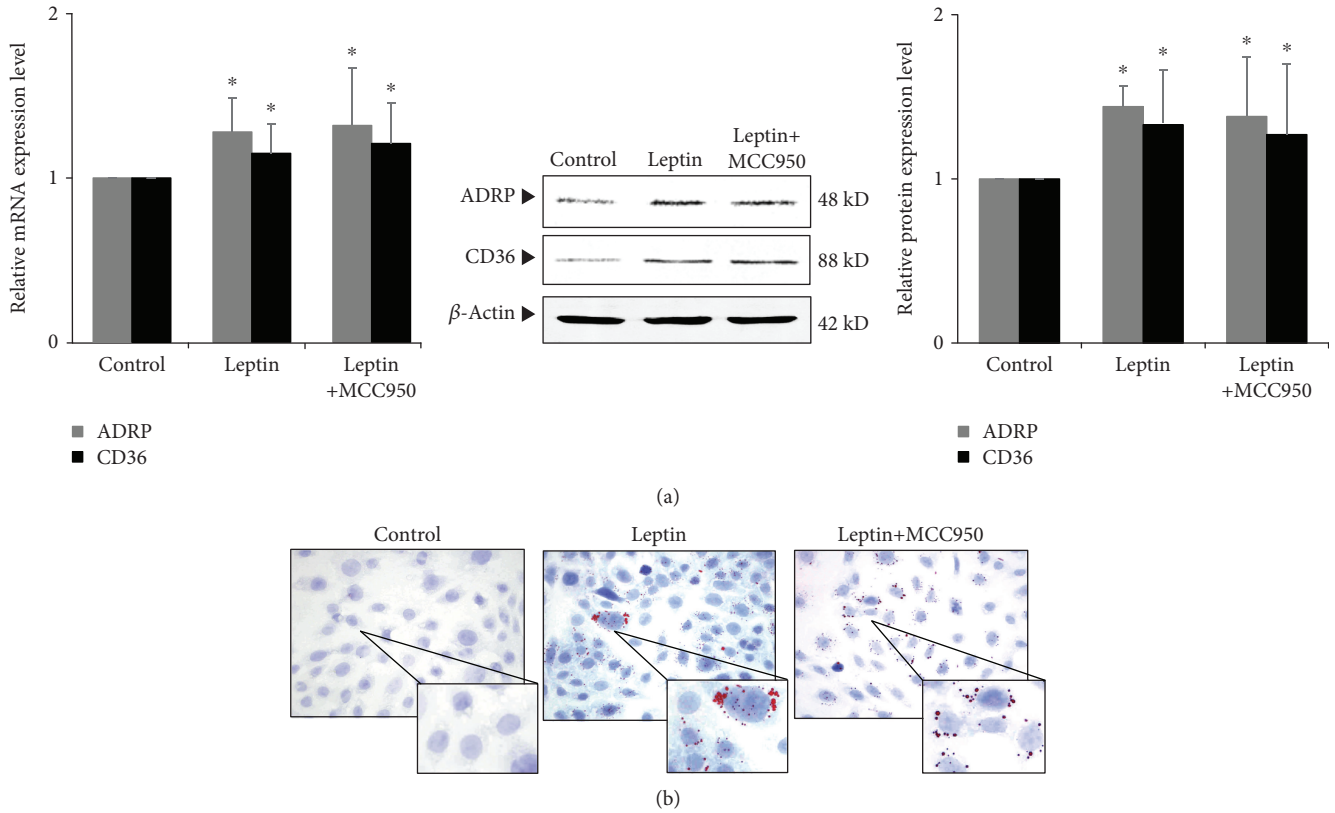


Figure 9: Effects of inhibiting NLRP3 by MCC950 on leptin-induced lipid uptake and accumulation of podocyte. (a) and (b) Differentiated podocytes were incubated in medium, medium containing 15 ng/mL leptin, or medium containing both 15 ng/mL leptin and 1 μM MCC950, respectively. (a) The relative mRNA and protein expression levels of ADRP and CD36 of podocytes were measured by real-time quantitative PCR and Western blot assay. (b) Representative Oil Red O staining images of podocytes in different groups as indicated (magnification ×400). Values are represented as mean ± SD ($n = 3$). $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. leptin + MCC950 group.

CD36 affect such effect? Our results showed that either SSO or CD36 siRNA could significantly inhibit leptin-induced podocyte NLRP3 inflammasome activation and inflammatory factor IL-1β secretion. After SSO or CD36 siRNA was given, mRNA and protein expressions of NLRP3, pro-caspase-1, and IL-1β were significantly downregulated ($P < 0.05$) (Figures 7(a) and 7(b)). This result suggests that leptin-induced podocyte NLRP3 inflammasome activation and IL-1β secretion might be mediated by CD36.

3.9. Effects of Blocking NLRP3 Inflammasome on Leptin-Induced Podocyte Injury and Lipid Accumulation. On the other hand, we used MCC950, a specific inhibitor of NLRP3, to observe the effects of blocking NLRP3 inflammasome on leptin-induced podocyte injury and lipid accumulation. Our results showed that after MCC950 was added, the secretion of IL-1β was reduced (Figure 8(a)) and leptin-induced podocyte injury was also alleviated. The expression of nephrin and podocin was significantly upregulated, and the expression of desmin was significantly downregulated ($P < 0.05$) (Figure 8(b)). However, there was no significant difference in the expression of mRNA and protein of CD36 and ADRP ($P > 0.05$) (Figure 9(a)), and Oil Red O staining also showed that lipid droplets in podocyte did not decrease (Figure 9(b)).

Our results show that although inhibition of the NLRP3 inflammasome attenuated inflammatory response and podocyte injury, it did not alter leptin-induced lipid uptake and lipid accumulation of podocytes. This result suggests that CD36-mediated lipid uptake and lipid accumulation play a vital role in podocyte injury of ORG, which might achieve such effects through CD36-mediated NLRP3 inflammasome activation and IL-1β secretion.

4. Discussion

The imbalance release of adipocytokine, including pathogenic adipocytokine (such as leptin) and protective adipocytokine (such as adiponectin), may be an important mechanism for ORG [23]. There were many studies on the role of adipocytokine in the pathogenesis of ORG [2, 23]. We also successfully established the ORG cell model by leptin-stimulated podocytes [4, 12, 19]. In recent years, the role of abnormal lipid metabolism in the pathogenesis of ORG has gradually attracted attention. This study is to observe the abnormal lipid metabolism and its pathogenesis in ORG.

ORG patients manifested metabolically unhealthy obesity, which means a central or visceral body fat distribution [24]. Verani found that obesity-associated focal segmental

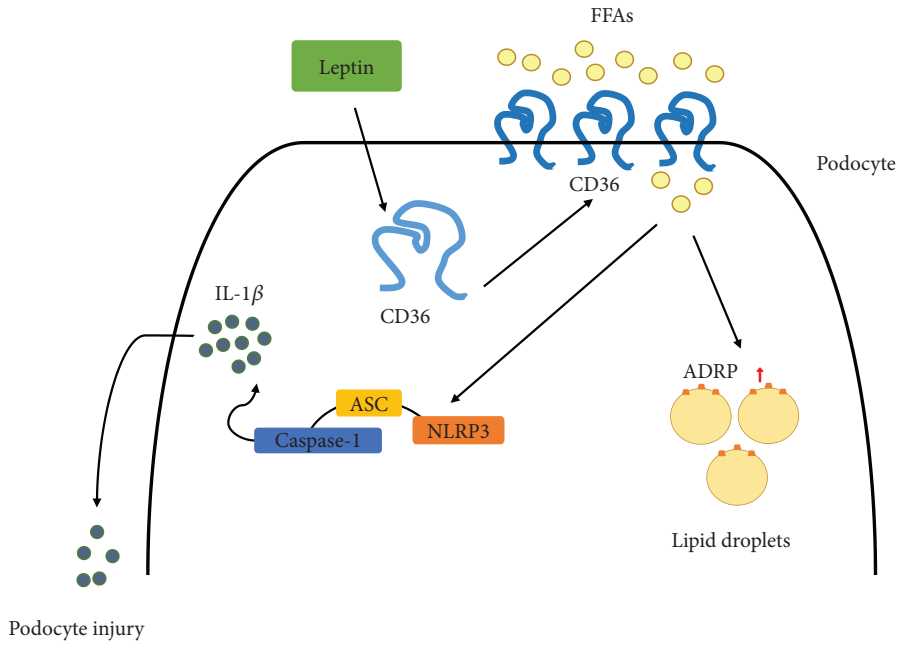


Figure 10: Schematic model depicting a possible mechanism that contributes to CD36-mediated podocyte injury of ORG. In the pathogenesis of ORG, after stimulation of adipocytokine such as leptin, the expression of CD36 increased, which is responsible for the FFA uptake in podocytes. Increased CD36 promotes lipid droplet formation in podocytes and further activates NLRP3 inflammasome; the cells release the mature form of inflammatory cytokines such as IL-1β, which causes the injury of podocytes. FFAs: free fatty acids.

glomerulosclerosis or glomerulomegaly was not associated with the amount of obesity per se, but rather with serum triglycerides and renal deposition of lipid [25]. Our study also found that serum triglyceride and cholesterol levels were significantly elevated in ORG mice.

Abdominal adipose tissue is thought to generate high concentrations of circulating FFAs. Excessive FFAs are transferred and accumulated in the liver and kidney through blood circulation and formed ectopic lipid deposits [24]. de Vries et al. reported that in ORG patients, there are significant lipid accumulation and lipid droplet formation in mesangial cells and podocytes of the glomerulus [24]. We also observed lipid droplet formation and upregulated ADRP in renal tissue and podocyte of ORG mice, which suggests that there are ectopic lipid deposits and lipid accumulation in ORG.

Is lipid accumulation the causative factor of ORG? The classical "lipotoxicity" emphasizes the important role of LDL and ox-LDL, while ORG patients mainly have an elevated TG level, which means there may be different pathogenic mechanisms of ORG [24, 26, 27]. We hypothesized that CD36 may play an important role in lipid accumulation and pathogenesis of ORG.

CD36 is the main receptor for lipid uptake of podocytes. In transgenic mice overexpressing CD36 in the kidney, lipid deposition of renal tissues was significantly increased [28]. When cultured podocytes were stimulated with palmitic acid, upregulated expression of CD36, formation of intracellular lipid droplet, and release of ROS were observed [7]. Our study showed that during ORG, the expression of CD36 in renal tissue was upregulated, which was consistent with lipid droplet formation and podocyte injury. When cultured podocytes were stimulated with leptin, expressions of CD36 and ADRP were also upregulated. In addition, after blocking CD36 with inhibitor SSO or CD36 siRNA, the lipid deposition of podocytes was significantly reduced, ADRP expression was downregulated, and podocyte injury was significantly reduced. This experiment initially confirmed the important role CD36 in podocyte injury of ORG.

Renal lipid metabolism includes fatty acid intake, fatty acid synthesis, and oxidation and utilization of fatty acids [3, 29]. In an ORG mouse model, we also observed an upregulated expression of SREBP-1 and PPARα. SREBP-1 is responsible for fatty acid synthesis, and PPARα regulates lipid oxidation utilization [3]. It has been reported that the expression of PPARα in kidney tissue is downregulated in the mouse model of high-fat diet and cases of stage IV of diabetic nephropathy [30, 31], which is inconsistent with our results. One of the possible reasons for inconsistency may be that the modeling time of ORG mice is about 12 weeks and the renal lesion seems to maintain in the early stage. So the lipid uptake and the oxidation utilization of lipid might be increased in synchronization.

We have previously observed an activation of the NLRP3 inflammasome in the ORG model and in leptin-induced podocyte injury [12]. Is there a relationship between CD36-mediated podocyte lipid accumulation and activation of NLRP3 inflammasome in ORG? Sheedy et al. reported that upregulation of CD36 promotes NLRP3 activation and IL-1β secretion in macrophages [17]. Liu et al. found that during the formation of foam cells in the pathogenesis of

atherosclerosis, lipids cause chronic inflammatory response through CD36-mediated release of ROS and activation of the NLRP3 inflammasome [18]. Therefore, we hypothesized that CD36-mediated lipid accumulation of podocyte may lead to podocyte injury through activating the NLRP3 inflammasome and releasing IL-1β.

Our study showed that NLRP3 inflammasome activation and IL-1β secretion were increased in podocytes and renal tissue of ORG mice; after blocking CD36, lipid deposition in podocytes was reduced, activation of NLRP3 inflammasome and secretion of IL-1β were also inhibited, and podocyte injury was alleviated. This result suggests that CD36-mediated lipid accumulation leads to activation of the NLRP3 inflammasome and release of inflammatory factors, resulting in podocyte injury of ORG.

Previous studies have also shown that activation of the NLRP3 inflammasome may promote the expression of CD36, thereby promoting the uptake of lipids by cells. Yang et al. found that in the chronic inflammation model induced by injection of casein, the expression of CD36 in renal tissue was upregulated, and the deposition of renal fat in mice was aggravated [32]. In vitro experiments showed that mesangial cells stimulated with TNFα lead to upregulated expression of CD36, increased lipid uptake, and intracellular lipid accumulation [32]. Gnanaguru et al. found in macrophage studies that inhibition of NLRP3 inflammasome downregulates CD36 expression and reduces lipid uptake by cells [33]. However, our study showed that inhibition of the NLRP3 inflammasome could improve podocyte injury, but there was no significant change in CD36 and ADRP expression in podocytes, and lipid deposition was also not reduced. This result further suggests that CD36-mediated lipid uptake and lipid accumulation might be the initiating factors that promote inflammatory responses by activating NLRP3 inflammasome, leading to podocyte injury of ORG.

Taken together, we showed that obesity and maladjusted lipid metabolism in ORG lead to renal lipid accumulation and podocyte injury, which are partially mediated by CD36; CD36-mediated lipid accumulation activates NLRP3 inflammasome, releases inflammatory factor IL-1β, and induces podocyte injury of ORG; inhibition of CD36 also inhibits NLRP3 activation inflammasome and ameliorates podocyte injury (Figure 10).

Different from increased CD36 and activated NLRP3 inflammasome induced by LDL and ox-LDL in atherosclerosis and foam cell formation [33–35], high-level triglycerides and FFAs conduct CD36-mediated lipid accumulation, NLRP3 inflammasome activation, and podocyte injury of ORG. In summary, we believe that CD36 may play a central role that links the pathogenesis of ORG and abnormal lipid metabolism. CD36 may become one of the important targets for ORG treatment in the future.

Data Availability

The original data of the current study are available in the following website: https://figshare.com/s/75bd66bf6fb68b4b0106.

Conflicts of Interest

The authors declare that they have no conflict of interests.

Authors' Contributions

Jing Zhao and Hong-liang Rui contributed equally to this work.

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Research Article

Impact of Intensive Lifestyle Modification on Levels of Adipokines and Inflammatory Biomarkers in Metabolically Healthy Obese Women

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Background. For the metabolically healthy obese (MHO) subjects, it is unclear whether weight loss provides cardiometabolic benefits. Our objective was to evaluate whether changes in adipokine and inflammatory biomarker levels were related to lifestyle modification (with Mediterranean diet and physical exercise program). *Methods*. 115 women (35-55 years) with BMI of 30-40 kg/m^2 and ≤1 metabolic syndrome criteria were included. After a 2-year intervention, participants were classified by percent weight loss: Group 1, <5%; Group 2, ≥5%-<10%; and Group 3, ≥10%. Anthropometric data, inflammatory biomarker (IL-6, TNFa, and hsCRP) and adipokine levels (adiponectin and resistin), and lifestyle program adherence at baseline and 2 years were analyzed. *Results*. The final sample comprised 67 women. 23 (38.3%) lost <5%, 22 (36.7%) lost ≥5%-<10%, and 22 (36.7%) lost ≥10% of baseline weight. After 2 years, in Group 1, adiponectin, hsCRP, IL-6, and TNFa decreased (-1.2 ng/ml, $p = 0.003$; -2.1 mg/l, $p = 0.003$; -2.4 pg/ml, $p < 0.001$; and -2.4 pg/ml, $p = 0.001$, respectively) and resistin increased (+2.4 ng/ml, $p < 0.001$). In Group 2, hsCRP and IL-6 decreased (-2.0 mg/l, $p = 0.009$ and -2.6 pg/ml, $p = 0.001$) but TNFa increased (+0.2 pg/ml, $p = 0.02$). In Group 3, resistin increased (+3.5 ng/ml, $p < 0.001$) but hsCRP, IL-6, and TNFa decreased (-2.0 mg/l, $p = 0.009$; -2.5 pg/ml, $p < 0.001$; and -4.1 pg/ml, $p < 0.001$). Adiponectin, hsCRP, and physical exercise correlated significantly to subjects' dietary adherence. *Conclusion*. Weight loss reduces inflammatory biomarkers in the MHO but induces a deterioration in the adipokine profile, which does not improve with diet and exercise intervention. These findings allow us to clarify mechanisms behind inflammation and metabolic disorder genesis so as to prevent development of obesity-associated comorbidities.

1. Introduction

Obesity is a heterogeneous disease. The risk of developing complications associated with obesity, such as type 2 diabetes mellitus, obstructive sleep apnea, high blood pressure, cardiovascular disease, some types of neoplasms (endometrium, breast, and liver), and degenerative joint disease is well known. These complications vary widely among obese subjects. A subgroup of obese individuals, termed "metabolically healthy obese" (MHO), has a favorable metabolic profile characterized by high insulin sensitivity, low visceral adipose tissue content, less liver fat, normal blood pressure, and

favorable lipid, inflammatory, hormonal, and immunological profiles despite having excessive body fat [1]. MHO is an emerging phenotype with disease risks that are somewhere between those of healthy, normal-weight individuals, and unhealthy, obese individuals. In general, these subjects have a lower risk of diabetes and cardiovascular disease than obese individuals with metabolic abnormalities [2]. In regard to diabetes, 1 study found that the development of cardiometabolic abnormalities in one-third of the MHO subjects studied led to a significantly increased risk of diabetes. In regard to cardiovascular diseases (CVD), studies have found that MHO subjects have CVD risks similar to those of normal-weight subjects. Recently, the benign nature of the MHO phenotype in the long term has begun to be questioned [3].

Adipose tissue used to be considered as simply an energy deposit. However, nowadays, the function of adipose tissue in metabolism as an endocrine organ responsible for the secretion of bioactive molecules—adipokines—is known [4]. Adipokines contribute to the inflammatory process, which can lead to obesity-associated cardiometabolic complications. MHO individuals seem to be protected from or are more resistant to developing these complications. The mechanisms that may explain the favorable metabolic profile of these individuals are still unknown, and it is intriguing why some MHO individuals develop comorbidities and others do not. Characteristics of adipose tissue, such as the proinflammatory profile and expression of adipokines, may be involved [1]. Adipokines have a hormonal function, act as growth factors that modulate insulin resistance, influence fat and glucose metabolism, and play a role in pro- and anti-inflammatory responses. The different types of adipokines, such as adiponectin and resistin, are the most studied [5]. Furthermore, inflammatory biomarkers such as interleukin 6 (IL-6) [6], tumor necrosis factor-alpha (TNFa), and C-reactive protein (CRP) decrease with dietary intervention and weight loss [7].

Few studies have analyzed the metabolic effects of lifestyle interventions (LSI) involving a restrictive diet and/or exercise in MHO subjects. Studies that have been done show contradictory results [8]. Principally, prior studies have shown that combining a Mediterranean diet with moderate-to-high-intensity aerobic training is effective at improving body composition [9], but no studies have analyzed if adipokine levels and the inflammatory profile experience significant variations when MHO subjects undergo intensive lifestyle modifications. Therefore, our principal objective was to evaluate whether changes in adipokine and inflammatory biomarker levels observed after 2 years of a personalized intervention were related to a lifestyle modification program based on the Mediterranean diet and physical exercise.

2. Materials and Methods

2.1. Subjects and Study Design. An open-label, nonrandomized, interventional, and analytical study was performed on a population of MHO women to determine the effectiveness in 2 years of a weight loss intervention (≥5% of body weight). We conducted a study on a population of 115 MHO women belonging to 4 health centers in the Malaga District of the Andalusian Health Service. Participants were recruited by their general practitioners between June 2013 and April 2014. After obtaining written informed consent, the subjects underwent a clinical interview with a doctor of internal medicine, a nurse, and a nutritionist at the Regional University Hospital of Malaga (the Civil Hospital); these practitioners were their contact person during the study.

We followed the methods of Rodriguez-Garcia et al. [10]. The participants were considered to be MHO if they met ≤1 of these 4 metabolic syndrome criteria: fasting plasma glucose ≥ 5.5 mmol/l, blood pressure ≥ 135/85 mmHg (or use of blood pressure-lowering agents), HDL-cholesterol ≤ 1.30 mmol/l, or triglycerides ≥ 1.70 mmol/l (or use of lipid-lowering therapies).

2.2. Materials. All samples were managed and stored at IBIMA's Hospital Biobank of Malaga, which belongs to the Andalusian Public Health System Biobank, part of the Spanish National Biobank Network (project PT13/0010/0006). The informed consent form and protocols were approved by the institutional ethics committee (Comité Coordinador de Ética de la Investigación Biomédica de Andalucía). This study is listed on the ISRCTN registry with trial ID ISRCTN88315555.

The population underwent an intervention involving a hypocaloric Mediterranean diet and a physical exercise program. In addition, the participants filled out a validated food frequency questionnaire [11]. Adherence to the Mediterranean diet was measured using a validated questionnaire, as described by Trichopoulou et al. [12]. Patients were considered to have dropped out if there was lack of adherence to the scheduled visits with specialists from the study.

The physical activity program recommended daily exercise. To verify that participants met the proposed physical exercise goal, they were monitored using a pedometer (On Step PE12) and they were evaluated using the Rapid Assessment of Physical Activity (RAPA) questionnaire [13], a validated 7-item questionnaire. Sedentarism or light physical activity corresponded to a score of 1-3 points, moderate to a score of 4-5 points, and vigorous to a score of 6–7 points. In total, over the course of the study, participants completed 3 different questionnaires: 2 concerned nutrition and 1 concerned physical exercise. These 3 questionnaires were completed on 4 occasions (baseline, 3 months, 1st year, and 2nd year).

After 2 years of follow-up, the subjects were classified into 3 groups based on the percentage of weight loss with respect to their baseline body weight: <5%, ≥5% to <10%, and ≥10%.

2.3. Methods. 40 ml of peripheral blood was collected from each participant at 4 different periods (baseline, 3 months, 1st year, and 2nd year). Vacutainer® ethylenediaminetetraacetic acid (EDTA) spray-coated tubes were used for whole blood hematology measurements, and Vacutainer® Plus plastic serum tubes were used for serum determinations. These tubes were connected to a Vacutainer® Push Button Blood Collection Set and a tube and syringe holder for blood collection and for producing a vacuum inside the tube. They were placed on ice. The samples were immediately centrifuged at 2772 ×g for 15 minutes at 4°C (plasma) or

at room temperature (serum). Plasma and serum were aliquoted and stored at -80°C until analysis.

In the Clinical Analysis Department of the Regional University Hospital of Malaga, blood glucose was determined by the glucose oxidase method adapted to an autoanalyzer (Dimension®, Dade Behring, Germany) and HbA1c (%) was measured by high-performance liquid chromatography (HPLC). For the lipid profile, total cholesterol and triglycerides were measured by enzymatic methods using the commercial equipment (Dimension®, Dade Behring, Germany). High-density lipoprotein-linked cholesterol (HDL-c) and low-density lipoprotein-linked cholesterol (LDL-c) were both measured by homogeneous direct measurement methods that do not require any preliminary treatment of the sample.

In the Research Laboratory of IBIMA, adipokines and inflammatory parameters were measured. Serum adipokine and inflammatory biomarker levels (IL-6 and TNFa) were measured using an enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc., Minneapolis, MN, USA). For adiponectin levels, the minimum detectable concentration was 0.246 ng/ml. The intra- and interassay coefficients of variation were 3.5% and 6.5%, respectively. For resistin levels, the minimum detectable concentration was 0.026 ng/ml. The intra- and interassay coefficients of variation were 4.7% and 8.4%, respectively. For IL-6 levels, the minimum detectable concentration was 0.70 pg/ml. The intra- and interassay coefficients of variation were 2.6% and 4.5%, respectively. Lastly, for TNFa levels, the minimum detectable concentration was 1.6 pg/ml. The intra- and interassay coefficients of variation were 4.7% and 5.8%, respectively.

High-sensitivity CRP levels were measured using ELISA (DRG Instruments GmbH, Germany). The minimum detectable concentration was 0.1 mg/ml. The intra- and interassay coefficients of variation were 4.4% and 3.3%, respectively.

2.4. Statistical Analysis. We based our calculations to calculate the sample size on the previous MHO study [8]. A population sample of 115 MHO women was required.

Relationships between serum levels of adipokines and inflammatory biomarkers and adherence to the Mediterranean diet and physical exercise were examined by means of the Pearson correlation analysis. Quantitative variables were analyzed as mean ± standard deviation (SD), and qualitative variables were expressed as percentages. Student's t-test, one-way analysis of variance (ANOVA) test, and post hoc analysis were used to compare quantitative variables whereas the Chi-squared test, the Mantel-Haenszel test, and the Mann-Whitney test were used for nonparametric variables.

We used the SPSS® statistical program for Windows version 22.0 (IBM Corporation Inc., Somers, NY, USA) to analyze the results.

3. Results

This study began with 115 women. After 2 years of a hypocaloric diet (Mediterranean diet) and physical exercise, there were 55 dropouts (52.0%). Thus, the final sample included 67 women. They were classified into 3 groups according to the percentage of weight loss with respect to their baseline body weight: Group 1: <5% ($n = 23$), Group 2: ≥5% to <10% ($n = 22$), and Group 3: ≥10% ($n = 22$) ($p = 0.272$). The mean (±SD) age of the women was 44.5 (±3.6 years), with no significant differences between the 3 groups ($p = 0.389$).

After 2 years of intensive intervention in the population as a whole, we found a decrease in parameters, including body weight, BMI, and waist circumference (all $p < 0.0001$), with respect to baseline conditions. However, no statistically significant differences were found in blood pressure or in analytical parameters such as the glycemic profile, HbA1c, or the lipid profile, though these values did tend to decline. All analytical parameter mean values were within normal ranges at baseline.

According to the Kaplan-Meier estimator, the number of patients who reached the goal rate (weight loss ≥ 5%) was 67 participants (58.3%) during the intervention program (24 months). Of these, 57 participants (54.6%) reached the goal weight during the first 3 months and 67 participants (58.3%) reached the goal weight in the first year.

When analyzing the 3 weight loss groups individually after the intervention, significant differences were found between them. Differences were seen principally between Group 2 and Group 3 in regard to body weight ($p = 0.003$ and $p < 0.001$, respectively), BMI ($p = 0.005$ and $p < 0.001$, respectively), and waist circumference ($p = 0.002$ and $p > 0.001$, respectively) although all groups decreased their anthropometric parameters with respect to baseline conditions. However, parameters such as blood pressure, glucose, HbA1c, or the lipid profile did not show significant differences. Only glucose levels in Group 1 ($p = 0.012$) and triglyceride levels in Group 2 ($p = 0.003$) were significantly reduced. Table 1 shows changes in anthropometric and analytical parameters at baseline and after 2 years of intervention, according to the percentage of weight loss.

A positive association was found between adherence to the Mediterranean diet and amount of weight loss. At baseline, all groups showed moderate adherence to the Mediterranean diet, without significant differences between them ($p = 0.132$). After 2 years of intervention, adherence varied in accordance with the level of weight loss ($p = 0.004$, between Groups 1 and 3). Table 2 summarizes adherence to Mediterranean diet according to weight loss.

At the beginning of the study, participants had light (62 participants (53.6%)), moderate (35 participants (30.4%)), and vigorous (18 participants (16.1%)) levels of physical activity. After 2 years of training with a monitor, these levels of physical activity changed: 11 of the participants (16.1%) had light activity, 29 (42.9%) had moderate activity, and 27 (41.1%) had vigorous activity levels. This physical activity of the different weight loss groups during the study is summarized in Table 3.

The adipokine profile for these MHO women is summarized in Table 4. For the entire population, adiponectin levels decreased after the intervention ($p = 0.039$). When comparing the different weight loss groups, only Group 1 showed a significant reduction in adiponectin levels ($p = 0.003$). However, Group 3, the group which lost the most weight, saw an increase in adiponectin levels, though the difference was not statistically significant ($p = 0.911$).

Table 1: Parameters at baseline (n = 115 MHO participants) vs. after 2 years (n = 67 MHO participants) of lifestyle modification, according to percentage of weight loss.

	Weight loss group	Baseline	2 years	p
Age (years)	**<5%**	43.7 ± 3.4	—	
	≥5-<10%	45.0 ± 2.4	—	
	≥10%	45.4 ± 4.5	—	
	All	44.5 ± 3.6	46.5 ± 3.6	
Body weight (kg)	**<5%**	91.2 ± 13.8	86.7 ± 10.3	0.698
	≥5-<10%	95.0 ± 15.8	86.3 ± 17.8	0.003
	≥10%	90.8 ± 12.4	79.1 ± 12.4	<0.001
	All	92.7 ± 13.8	83.8 ± 13.4	<0.001
BMI (kg/m^2)	**<5%**	35.5 ± 3.6	34.3 ± 3.2	0.724
	≥5-<10%	37.2 ± 5.8	34.4 ± 6.5	0.005
	≥10%	36.0 ± 4.5	31.5 ± 4.6	<0.001
	All	36.3 ± 4.7	33.3 ± 4.8	<0.001
Waist circumference (cm)	**<5%**	111.6 ± 10.8	108.1 ± 9.5	0.831
	≥5-<10%	116.4 ± 11.4	108.8 ± 15.5	0.002
	≥10%	114.7 ± 11.2	100.9 ± 13.1	<0.001
	All	111.7 ± 11.1	105.6 ± 12.8	<0.001
SBP*/DBP** (mmHg)	**<5%**	113 ± 15/75 ± 9	114 ± 14/73 ± 12	0.285/0.302
	≥5-<10%	115±10/78±7	115 ± 12/76 ± 11	0.501/0.342
	≥10%	110±14/76±11	118 ± 20/74 ± 11	0.498/0.402
	All	114 ± 14/76 ± 9	116 ± 16/74 ± 11	0.532/0.199
Glucose (mmol/l)	**<5%**	4.89 ± 0.40	4.72 ± 0.43	0.012
	≥5-<10%	4.86 ± 0.54	4.77 ± 0.41	0.833
	≥10%	4.74 ± 0.44	4.62 ± 0.36	0.376
	All	4.85 ± 0.44	4.76 ± 0.39	0.214
HbA1c (%)	**<5%**	5.3 ± 0.2	5.4 ± 0.3	0.635
	≥5-<10%	5.4 ± 0.3	5.4 ± 0.3	0.333
	≥10%	5.3 ± 0.3	5.3 ± 0.3	0.801
	All	5.4 ± 0.3	5.4 ± 0.3	0.645
Total cholesterol (mmol/l)	**<5%**	5.05 ± 0.765	5.08 ± 0.80	0.578
	≥5-<10%	4.89±0.77	4.81 ± 0.62	0.098
	≥10%	5.15 ± 0.71	4.97 ± 0.82	0.478
	All	5.04 ± 0.73	4.98 ± 0.76	0.941
LDL cholesterol (mmol/l)	**<5%**	3.13 ± 0.69	3.17 ± 0.76	0.445
	≥5-<10%	2.89 ± 0.63	2.92 ± 0.56	0.902
	≥10%	3.08 ± 0.79	3.04 ± 0.66	0.789
	All	3.05 ± 0.69	3.06 ± 0.67	0.501
HDL cholesterol (mmol/l)	**<5%**	1.41 ± 0.32	1.37 ± 0.21	0.112
	≥5-<10%	1.53 ± 0.28	1.53 ± 0.22	0.584
	≥10%	1.54 ± 0.32	1.54 ± 0.31	0.897
	All	1.48 ± 0.31	1.47 ± 0.26	0.632

Table 1: Continued.

	Weight loss group	Baseline	2 years	p
Triglycerides (mmol/l)	**<5%**	0.98 (0.76–1.29)	0.98 (0.77–1.47)	0.545
	≥5-<10%	0.88 (0.66–1.39)	0.70 (0.62–0.99)	0.003
	≥10%	0.93 (0.69–1.18)	0.85 (0.72–1.08)	0.497
	All	0.95 (0.75–1.27)	0.89 (0.70–1.23)	0.132

Group 1: weight loss <5% (baseline, $n = 47$; 2nd year, $n = 23$); Group 2: weight loss ≥5-<10% (baseline, $n = 27$; 2nd year, $n = 22$); Group 3: weight loss ≥10% (baseline, $n = 30$; 2nd year, $n = 22$). *SBP: systolic blood pressure; **DBP: diastolic blood pressure. Normal values (NV): glucose NV: 3.89-6.16; HbA1c NV: 4.0-6.0; total cholesterol NV: <5.18; LDL cholesterol NV: <3.37; HDL cholesterol NV: >1.30; triglycerides NV: <1.70.

Table 2: Adherence to Mediterranean diet at baseline ($n = 115$ MHO participants) vs. after 2 years ($n = 67$ MHO participants) of lifestyle modification, according to percentage of weight loss.

	Weight loss group	Baseline	2 years	p
Very low adherence (n (%))	**<5%**	3 (6.4)	0 (0.0)	—
	≥5-<10%	2 (7.4)	0 (0.0)	—
	≥10%	4 (13.3)	0 (0.0)	—
	All	9 (8.0)	0 (0.0)	—
Low adherence (n (%))	**<5%**	16 (34.0)	6 (27.3)	0.006
	≥5-<10%	13 (48.1)	0 (0.0)	—
	≥10%	11 (36.7)	1 (5.0)	<0.001
	All	40 (41.6)	7 (12.7)	0.001
Moderate adherence (n (%))	**<5%**	28 (59.6)	14 (63.6)	0.01
	≥5-<10%	12 (44.4)	12 (92.3)	0.006
	≥10%	15 (50.0)	10 (50.0)	0.824
	All	55 (50.4)	36 (65.5)	0.003
High adherence (n (%))	**<5%**	0 (0.0)	2 (9.1)	—
	≥5-<10%	0 (0.0)	1 (7.7)	—
	≥10%	0 (0.0)	9 (45.0)	—
	All	0 (0.0)	12 (21.8)	—

Group 1: weight loss <5% (baseline, $n = 47$; 2nd year, $n = 23$); Group 2: weight loss ≥5-<10% (baseline, $n = 27$; 2nd year, $n = 22$); Group 3: weight loss ≥10% (baseline, $n = 30$; 2nd year, $n = 22$). Adherence to Mediterranean diet was measured as follows: very low adherence <5 points, low adherence ≥5-<8 points, moderate adherence ≥8-<12 points, and high adherence ≥12 points.

Table 3: Physical activity levels at baseline ($n = 115$ MHO participants) vs. after 2 years ($n = 67$ MHO participants) of lifestyle modification, according to percentage of weight loss.

	Weight loss group	Baseline	2 years	p
Sedentarism or light level (n (%))	**<5%**	23 (50.0)	5 (22.7)	0.01
	≥5-<10%	15 (55.6)	3 (23.1)	0.002
	≥10%	17 (56.7)	1 (4.8)	<0.001
	All	55 (53.5)	9 (16.0)	0.001
Moderate level (n (%))	**<5%**	16 (34.8)	12 (54.5)	0.006
	≥5-<10%	8 (29.6)	5 (38.5)	0.068
	≥10%	6 (20.0)	7 (33.3)	0.010
	All	30 (30.4)	24 (42.9)	0.002
Vigorous level (n (%))	**<5%**	7 (15.2)	5 (22.7)	0.01
	≥5-<10%	4 (14.8)	5 (38.5)	0.006
	≥10%	7 (23.3)	13 (61.9)	<0.001
	All	18 (16.1)	23 (41.1)	0.003

Group 1: weight loss <5% (baseline, $n = 47$; 2nd year, $n = 23$); Group 2: weight loss ≥5-<10% (baseline, $n = 27$; 2nd year, $n = 22$); Group 3: weight loss ≥10% (baseline, $n = 30$; 2nd year, $n = 22$). Physical activity levels according to the Rapid Assessment of Physical Activity (RAPA) questionnaire are as follows: sedentarism or light level RAPA = 1-3 points, moderate level RAPA = 4-5 points, and vigorous level RAPA = 6-7 points.

Table 4: Adipokine and inflammatory biomarker levels at baseline vs. after 2 years of lifestyle modification, according to percentage of weight loss. MHO participants: baseline, $n = 115$; 3 months, $n = 104$; 1st year, $n = 75$; and 2nd year, $n = 67$.

	Weight loss	Baseline	3 months	1 year	2 years	p (baseline vs. 2 y)
Adiponectin (ng/ml)	**<5%**	7.0 ± 2.1	7.1 ± 2.5	7.7 ± 3.5	5.8 ± 2.4	0.003
	≥5-<10%	7.8 ± 3.0	9.0 ± 4.3	12.6 ± 5.1	6.7 ± 3.1	0.699
	≥10%	7.7 ± 3.1	7.4 ± 3.4	12.6 ± 5.5	8.6 ± 4.8	0.911
	All	7.4 ± 2.7	7.7 ± 3.4	10.7 ± 5.2	7.05 ± 3.7	0.039
Resistin (ng/ml)	**<5%**	5.5 ± 2.4	4.6 ± 1.2	6.1 ± 2.8	7.9 ± 3.4	<0.001
	≥5-<10%	5.2 ± 2.2	5.2 ± 1.6	5.5 ± 1.9	7.5 ± 4.4	0.08
	≥10%	5.2 ± 1.8	5.8 ± 2.3	5.4 ± 1.9	8.8 ± 4.3	<0.001
	All	5.3 ± 2.2	5.1 ± 1.8	5.7 ± 2.4	8.1 ± 2.9	<0.001
hsCRP (mg/l)	**<5%**	5.4 ± 2.9	5.0 ± 2.7	4.1 ± 2.1	3.3 ± 3.1	0.003
	≥5-<10%	5.2 ± 2.5	4.6 ± 2.4	3.4 ± 2.4	3.2 ± 2.8	0.010
	≥10%	5.2 ± 2.6	4.4 ± 2.3	3.8 ± 3.04	3.2 ± 3.3	0.097
	All	5.3 ± 2.7	4.7 ± 2.5	3.8 ± 2.5	3.3 ± 3.1	<0.001
IL-6 (pg/ml)	**<5%**	4.9 ± 0.9	4.8 ± 1.2	7.4 ± 1.9	2.5 ± 0.7	<0.001
	≥5-<10%	5.1 ± 1.2	6.0 ± 2.6	6.9 ± 2.0	2.5 ± 0.4	0.001
	≥10%	4.5 ± 0.8	5.5 ± 2.7	7.5 ± 3.2	2.4 ± 0.7	<0.001
	All	4.8 ± 1.0	5.3 ± 2.2	7.3 ± 2.5	2.4 ± 0.6	<0.001
TNFa (pg/ml)	**<5%**	13.3 ± 1.0	12.5 ± 1.4	11.3 ± 2.7	10.9 ± 3.4	0.001
	≥5-<10%	13.6 ± 0.6	11.8 ± 2.3	11.4 ± 2.1	13.8 ± 1.6	0.019
	≥10%	13.9 ± 2.5	12.7 ± 2.7	10.4 ± 2.3	9.8 ± 0.8	<0.001
	All	13.6 ± 1.5	12.4 ± 2.1	11.0 ± 2.5	11.1 ± 2.9	<0.001

Group 1: weight loss <5% (baseline: 3 months, $n = 47$; 1st year, $n = 20$; and 2nd year, $n = 23$); Group 2: weight loss ≥5-<10% (baseline: 3 months, $n = 27$; 1st year, $n = 27$; and 2nd year, $n = 22$); Group 3: weight loss ≥10% (baseline: 3 months, $n = 30$; 1st year, $n = 28$; and 2nd year, $n = 22$). Normal values (NV): adiponectin NV: 0.8–21; resistin NV: 6.1–26.4; hsCRP NV: 0.07-8.2; IL-6 NV: 3.13–12.5; TNFa NV: <15.6.

Resistin levels increased significantly in the entire study population following the intervention ($p < 0.001$). However, when analyzing each group, only the increases in serum resistin levels in Group 1 ($p < 0.001$) and Group 3 ($p < 0.001$) were statistically significant.

There were significant decreases in hsCRP, IL-6, and TNFa levels in the entire population following the intervention ($p < 0.001$ for all parameters). Serum hsCRP levels decreased in all groups (Group 1: $p = 0.003$, Group 2: $p = 0.010$, and Group 3: $p = 0.097$). Serum IL-6 levels also decreased in all groups (Group 1: $p < 0.001$, Group 2: $p = 0.001$, and Group 3: $p < 0.001$), and serum TNFa levels decreased in Group 1 ($p = 0.001$) and Group 3 ($p < 0.001$), but increased slightly in Group 2 ($p = 0.019$).

In this study, our aim was to verify whether different adipokines (adiponectin and resistin) and inflammatory biomarkers (CRP, IL-6, and TNFa) correlated with adherence to Mediterranean diet and physical exercise in our MHO population. Upon analyzing adipokines and inflammatory biomarkers, it was found that only CRP and adiponectin were shown to be correlated with adherence to the Mediterranean diet after 2 years of intervention. CRP showed a negative correlation ($r = -0.292$, $p = 0.030$), and adiponectin showed a positive correlation ($r = 0.340$, $p = 0.011$). Moreover, as expected, physical exercise was shown to be correlated with adherence to the Mediterranean diet after 2 years of intervention ($r = 0.414$, $p = 0.002$).

4. Discussion

Our study focuses on analyzing adipokine and inflammatory biomarker profiles involved in the pathogenesis of inflammation found in metabolically healthy obese individuals after 2 years of lifestyle modification based on a calorie-restricted Mediterranean diet and physical exercise. We provide evidence that clinically significant weight loss in MHO women leads to limited improvement in serum levels of adipokines and inflammatory biomarkers. We provide further evidence for the concept of the MHO phenotype as a subgroup that is resistant to the metabolic disorders associated with obesity and that weight loss does not substantially improve this phenotype's metabolic profile.

The intervention in this study produced different effects on energy metabolism and improved health parameters, such as BMI, in the MHO subjects evaluated. In this study, the weight loss resulting from the Mediterranean diet and exercise had effects on all metabolic parameters after the intervention. Though some results were not statistically significant (glucose and lipid profile), changes in the levels

of these parameters may be clinically relevant in the management of future obesity-related pathologies.

In the adipokine profile, adiponectin has a wide spectrum of metabolic and anti-inflammatory effects by its inhibition of monocyte adhesion to endothelial cells, macrophage transformation to foam cells, and endothelial cell activation. We found a significant decrease in serum adiponectin levels in the study population as a whole, though they remained within normal range. These data were in accordance with Rondanelli et al. [14]. However, in adiponectin levels measured according to the different weight loss groups, we found that this significant difference was not noted in groups with a greater weight loss. In fact, in the group with ≥10% of weight loss, serum adiponectin levels increased. This group had a high adherence to the Mediterranean diet and a vigorous physical exercise score after 2 years of intervention. It has been demonstrated that exercise promotes a decrease in the body mass and body mass index along with an increase in insulin sensitivity and adiponectin concentration [15]. Adiponectin is mostly expressed in subcutaneous adipose tissue. Its expression and blood concentration decrease as adiposity increases. However, some authors have shown that individuals with higher subcutaneous rather than visceral adipose tissue distribution have higher levels of adiponectin. It is not known whether MHO subjects have predominantly peripheral adiposity along with higher adiponectin levels [16]. MHO women have an adipokine profile somewhere between at-risk obese and metabolically benign normal-weight women and have higher adiponectin levels than at-risk obese women [17]. Although adiponectin levels are lower in obese subjects, MHO subjects might present a paradoxical hyperadiponectinemia that could be a clue in explaining their favorable metabolic profile.

Adiponectin binds to its AdipoR1 or AdipoR2 receptors in muscle, liver, and adipose tissue, increasing the activity of adenosine monophosphate kinase (AMPK) or peroxisome proliferator-activated receptor- (PPAR-) α. Reducing adipose tissue mass, through weight loss in association with physical exercise, can increase adiponectin concentrations as occurred in our participants who lose more than 10% of their body weight. On the other hand, the effect of TNFa on obesity increased the release of fatty acid by adipocytes, reduced adiponectin synthesis, and impaired insulin signalling [18].

It has also been shown that the adipose tissue of MHO individuals has a favorable inflammatory profile associated with high levels of adiponectin [19]. In contrast, in other studies, no differences in adiponectin levels among MHO individuals were found [20].

The role of resistin in human obesity is unclear. There are few studies on the profile of resistin in the MHO, though studies have shown that the level of resistin does not significantly change between metabolic phenotypes [17]. However, other studies have shown that the MHO phenotype is characterized by low levels of resistin. Our results showed a significant increase in resistin levels in both the total study population and in the groups of participants who had moderate adherence to the Mediterranean diet and moderate-vigorous physical exercise, data in concordance with findings of Gómez-Ambrosi et al. [21]. The effects of resistin may be mediated by the paracrine and endocrine ways, probably via binding of resistin to a surface receptor on target cells. Resistin can activate cytokines such as CRP, IL-6, IL-12, and TNF via the NFκB pathway [22].

Obesity as well as BMI and waist circumference are associated with CRP levels [23]. Postmenopausal women with the MHO phenotype have lower levels of CRP. It has been theorized that a lower-grade state of inflammation could play a role in the protective metabolic profile of the MHO individuals [24]. Evidence from interventional studies has demonstrated that weight loss by caloric restriction reduces several inflammatory biomarkers, including CRP, IL-6, and TNFa [25]. Our population saw a significant reduction in CRP levels, a finding that was independent of the percentage of weight loss but which was significantly associated with a decrease in waist circumference. These results are in accordance with the findings of Nakamura et al. [26].

IL-6 is an inflammatory biomarker that is positively correlated with obesity, glucose intolerance, and insulin resistance [27]. The production of IL-6 is 3 times higher in visceral adipose tissue than in subcutaneous adipose tissue [27], which could explain the decrease in IL-6 levels in MHO women after weight loss. Both adipocyte hypertrophy and inflammatory stimuli, such as TNF, favor an increase in IL-6. It has been reported that weight loss reduces both circulating levels and adipose tissue expression of IL-6 [4]. Similar IL-6 levels in lean and MHO individuals have been found [28], data that are in agreement with those found in our study, as our population maintained normal levels of both IL-6 and TNFa. MHO subjects have lower levels of TNFa and a reduced proinflammatory profile in comparison with other obese phenotypes [18]. TNF is also involved in the synthesis of proinflammatory cytokines, such as IL-6 and TNF itself, through NFκB activation. TNF is a factor involved in diet-induced obesity and insulin resistance as well as in low-grade inflammation related to adipose tissue expansion. We found improved CRP, IL-6, and TNFa serum levels after dietary intervention in both the total population and in the different weight loss groups, data in accordance with Poelkens et al. [29].

Studies assessing the short-term effect of the Mediterranean diet [30] and exercise [31] in obese subjects have shown reductions in inflammatory parameters. However, in contrast with other authors [8], we did not find that weight loss improved the profile of several inflammatory markers (CRP, IL-6, and TNFa) in subjects who lost ≥10% of their baseline body weight.

5. Limitations

Our study has some limitations. Our population comprised middle-aged Caucasian women; thus, we cannot extrapolate our conclusions to MHO patients of other races, sexes, or ages. Another important limitation was the losses during follow-up, perhaps due to participants growing tired of the restrictive diet.

Although public health messaging should continue to promote healthy lifestyle habits for all obese patients, the debatable results of lifestyle modifications in MHO individuals

would justify, for reasons of cost efficiency, prioritizing these types of intensive interventions in metabolically abnormal obese individuals and monitoring MHO subjects for early detection of the development of metabolic abnormalities.

6. Conclusion

Weight loss reduces inflammatory biomarkers in the MHO but induces a deterioration in the adipokine profile, which does not improve with diet and exercise intervention. These findings allow us to clarify mechanisms behind inflammation and metabolic disorder genesis so as to prevent the development of obesity-associated comorbidities.

Data Availability

Data availability will be provided previous requeriment to the corresponding authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

RGH and MRBL contributed to conception and design, analysis and interpretation of data, drafting, reviewing, and revising the article critically for important intellectual content and final approval of the version to be published. JRN and SSF contributed to conception, design, and acquisition of data analysis. AVAM contributed to acquisition of data analysis. FJT contributed to conception, design, and interpretation of data as well as revising the article critically for important intellectual content.

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Research Article

Association between Irisin, hs-CRP, and Metabolic Status in Children and Adolescents with Type 2 Diabetes Mellitus

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Proinflammatory cytokines and the novel myokine irisin, a cleavage product of FNDC5, have been found to play a role in obesity and type 2 diabetes mellitus (T2DM). Irisin has been shown to increase browning of adipose tissue, thermogenesis, energy expenditure, and insulin sensitivity, yet its association with inflammatory markers is still limited. Circulating irisin has been found to be increased in obesity, while in adult subjects with T2DM decreased levels have been found. However, data establishing the association of circulating irisin in children and adolescents with T2DM has not been described in the literature. The objective of this study was to determine irisin plasma concentration and its association with metabolic and adiposity markers and with hs-CRP, a surrogate marker of inflammation used in clinical practice, in a pediatric population with T2DM. A cross-sample of 40 Mexican children and adolescents aged 7-17 were recruited, 20 diagnosed with T2DM and 20 healthy controls. Plasma irisin levels were found to be lower in the T2DM group compared with controls, which could be attributed to a reduced PGC-1α activity in muscle tissue with a consequent decrease in FNDC5 and irisin expression. Irisin concentration was found to be positively correlated with HDL-c, LDL-c, and total cholesterol, while negatively correlated with BMI, waist circumference, and triglycerides. However, after multiple regression analysis, only HDL-c correlation remained significant. hs-CRP was higher in the T2DM group and positively associated with adiposity markers, unfavorable lipid profile, insulin levels, and HOMA-IR, but no association with irisin was found. Given the favorable metabolic effects attributed to irisin, the low plasma levels found in children and adolescents with T2DM could exacerbate the inflammatory and metabolic imbalances and the intrinsic cardiovascular risk of this disease. We propose an "irisin-proinflammatory/anti-inflammatory axis" to explain the role of irisin as a metabolic regulator in obesity and T2DM.

1. Introduction

Overweight and obesity are well established risk factors for the development of hypertension, atherogenic dyslipidemia, insulin resistance, and glucose intolerance, all of which carry an increased risk of developing cardiovascular disease and type 2 diabetes mellitus (T2DM) [1, 2] with subsequent increased morbidity and premature mortality. Over the last three decades, overweight and obesity in children and adolescents have become increasingly prevalent worldwide [3]. Both Mexico and the United States present the highest obesity rates in the world [4]. According to the national survey

ENSANUT 2016, 34% of children and 36% of adolescents in Mexico were identified as either overweight or obese [5]. While in the United States the prevalence of T2DM in children and adolescents is 4% [6], no data is available for Mexican children. However, approximately half of all pediatric diabetes mellitus cases are estimated to be T2DM [7]. Obesity is considered as a state of systemic low-grade subclinical inflammation, which results in insulin resistance, metabolic abnormalities, and eventually T2DM [8]. Indeed, increased markers of inflammation have been found in serum of obese adults [9–11], but accumulating evidence supports the hypothesis that the obesity-related proinflammatory state begins at and is perpetuated in early childhood [12]. During the last decade, research has also focused on circulating factors involved in the metabolic and inflammatory derangements observed not only in obesity but in T2DM also including inflammatory markers [13–16] and novel myokines, such as irisin, among others [17]. Furthermore, there is overwhelming evidence that T2DM also has an important inflammatory component in its pathogenesis [18]. Some researchers have found increased inflammatory markers in the serum of T2DM children [19] and adolescents [20], which suggests that this inflammatory phenomenon occurs at all ages and justifies further investigation in the less studied pediatric population.

Irisin, a novel myokine discovered in 2012 by Boström and colleagues [21], is a 112-amino acid cleavage product of fibronectin type III domain-containing protein 5 (FNDC5), whose expression is induced by peroxisome proliferator-activated receptor gamma (PPAR-γ) and its coactivator peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α). Irisin was shown to be released by muscle during exercise and to upregulate genes involved in thermogenesis and browning of white adipose tissue (WAT), such as uncoupling protein 1 (UCP-1), which resulted in increased energy expenditure and weight loss in mice fed with a high-fat diet [21]. It was also demonstrated to substantially decrease serum levels of glucose and insulin, thus indicating an improvement in insulin resistance [21]. It was later shown that irisin can also be secreted by subcutaneous adipose tissue [22], being therefore considered an adipomyokine.

Irisin has been proposed to play a role in the pathophysiology of obesity and the consequent metabolic diseases [23, 24]. Most studies have reported a positive correlation between irisin concentration and adiposity and biochemical markers of obesity in adult populations [25–29]. However, the information in children is scarce and rather confusing showing strong contradictory findings [30]. Some studies have shown a positive correlation between irisin and anthropometric and cardiovascular disease markers in obese children [31–34], while others have found an inverse association [35]. Concerning T2DM, 2 meta-analyses have determined that irisin levels are lower in adult patients with T2DM [36, 37], but data establishing the association of irisin concentration in the pediatric population with T2DM has not been described in the literature [19]. Furthermore, there is no available data regarding the association of irisin with an important inflammatory marker in clinical practice such as high-sensitivity C-reactive protein (hs-CRP) in the pediatric population with T2DM. Although hs-CRP has been used clinically as an inflammatory marker in this population [38], its relationship with irisin concentration has not been studied. Therefore, the objective of this study was to investigate irisin concentration and its association with hs-CRP, as well with metabolic and anthropometric parameters in children and adolescents with T2DM compared with healthy controls.

2. Materials and Methods

2.1. Population. A cohort of 40 Mexican children and adolescents aged 7-17 (20 boys and 20 girls) were recruited, 20 children and adolescents diagnosed with T2DM and 20 healthy controls. Inclusion criteria for the T2DM group included to have been previously diagnosed with T2DM according to the American Diabetes Association (ADA) criteria. Children were diagnosed by fasting plasma glucose $\geq$ 126 mg/dL (7.0 mmol/L) [39]. Healthy controls were required to have normal weight according to the Centers for Disease Control and Prevention (CDC) criteria (body mass index percentile (BMIp) $\geq$5th and <85th) [40], no metabolic abnormalities on blood samples, and no previously diagnosed cardiometabolic diseases, as well no antihypertensive, lipid, or sugar lowering medications use. Written informed consent was obtained from legal guardians prior to the inclusion in this study. Approval by the Ethics and Research Committees of the School of Medicine, Tecnologico de Monterrey was obtained.

2.2. Vital Signs and Anthropometric Parameters. Anthropometric variables and blood pressure were measured by qualified medical personnel. Blood pressure measurements were obtained by triplicate with a mercury sphygmomanometer, using an appropriate cuff size and with the patient in sitting position. Anthropometric parameters were obtained according to standardized protocols [41]. Weight in kilograms (kg) was rounded to the nearest decimal point and measured with an age-appropriate scale (TANITA® BF-689; TANITA Corporation of America Inc., Arlington Heights, Illinois, USA); height in centimeters (cm) was measured with a stadiometer (SECA® 217, SECA Mexico, Mexico City, Mexico); body mass index (BMI) was calculated as kg/m^2; waist circumference (WC) and hip circumference (HC) in cm were obtained with a standard fiber optic measuring tape.

2.3. Laboratory Studies. Blood samples were taken from each subject by peripheral venipuncture after an overnight 12-hour fast. Samples were then centrifuged to obtain plasma and serum and were then frozen at −80°C for further processing. Fasting serum glucose levels were measured by the hexokinase (HK)/glucose-6-phosphate dehydrogenase (G-6-PDH) method, using the Glucose 3L82 (304772/R02; DENKA SEIKEN Co. Ltd., Tokyo, Japan) reagent kit on the Architect *c*Systems™. Serum insulin concentrations were obtained by chemiluminescent microparticle immunoassay, using the ARCHITECT Insulin Reagent kit 8K41-27 (G6-2892/R03; Abbot Laboratories Diagnostic Division, IL, USA). Total cholesterol was measured using the Cholesterol

Reagent kit 7D62 (304796/R02; Abbot Laboratories Diagnostic Division, IL, USA) on the Architect *c*Systems™ through enzymatic methodology. High-density lipoprotein cholesterol concentrations were measured by the accelerator selective detergent method using the Ultra HDL 3K33-21 assay (306571/R03; Abbot Laboratories Diagnostic Division, IL, USA). Quantitation of triglycerides in plasma was obtained through the glycerol-phosphate-oxidase reaction, using the Triglyceride 7D74-20 (30-3140/R3; Abbot Laboratories Diagnostic Division, IL, USA) reagent kit on the Architect *c*Systems™ and the AEROSET system. High-sensitivity C-reactive protein levels were determined by quantitative immunoturbidimetric methodology, using the CRP Vario 6k26-30 and 6k26-41 kits (306731/R04; Abbot Laboratories Diagnostic Division, IL, USA) on the Architect *c*Systems™. Irisin was measured by sandwich enzyme-linked immunosorbent assay (ELISA) method with an irisin (human) ELISA kit (SK00170-08) following the manufacturer's instructions (Avisera Bioscience Inc., Santa Clara, California, USA). The sensitivity of this assay is 0.1 ng/mL; its standard curve linear range is 0.8-51.2 ng/mL, and intra- and interassay variations are 4-6% and 8-10%, respectively. For cytokine measurement, human TNF-α (catalogue number 430306) and human IL-6 ELISA MAX (catalogue number 430503) sets, both from BioLegend (San Diego, CA, USA), were used following the manufacturer's instructions. Sensitivity for the assay is 2 pg/mL.

2.4. Statistical Analyses. Statistical analyses were performed using Microsoft Excel® (version 16.17, Microsoft Corporation, Redmont, Washington, USA), IBM SPSS® (version 21.0; SPSS Inc., Armonk, New York, USA), and GraphPad Prism (version 6.0, GraphPad Software, La Jolla California, USA). D'Agostino-Pearson tests were used to evaluate normality of the sample distribution. Unpaired *t*-tests and Mann-Whitney tests were conducted for parametric and nonparametric data, respectively, to compare anthropometric and metabolic variables between the two groups. Chi-square and Fisher's exact tests, when deemed appropriate, were conducted to evaluate differences in proportions of clinical categorical variables between groups. Spearman's rank correlation test was used to assess the relation between levels of irisin and all clinical and biochemical parameters. Stepwise multiple linear regression analysis was also performed to adjust for gender, age, and BMI. A p value of <0.05 was considered statistically significant for all analyses.

3. Results

3.1. Demographic and Clinical Parameters. Table 1 shows the demographic and clinical characteristics of the pediatric population classified into two groups: T2DM ($n = 20$) and healthy controls ($n = 20$) with an equal distribution of male and female participants. The subject's mean age was 12.58 (±2.47) years. Ninety percent of the T2DM group were found to be either obese (70%) or overweight (20%), while none of the healthy control group subjects were. The mean age of the T2DM children was significantly greater (13.9 (11-17)) compared with that of the control group (11.2 (7-16)) ($p = 0.0002$). Acanthosis nigricans was found in ninety-five percent of the T2DM patients, but in none of the subjects from the control group. All T2DM subjects presented Tanner developmental stages 3-5, while controls showed equal distribution between Tanner 1-2 and 3-5 stages.

Table 1: Demographic and clinical characteristics of the population.

Parameter	T2DM ($n = 20$)	Healthy controls ($n = 20$)	p value
Male	10 (50)	10 (50)	1.00
Female	10 (50)	10 (50)	
Age in years	13.9 ± 1.52	11.2 ± 2.67	**<0.001**
Obesity[a]	14 (70)	0 (0)	
Overweight[b]	4 (20)	0 (0)	**<0.001**
Normal weight	2 (10)	20 (100)	
Acanthosis nigricans	19 (95)	0 (0)	**<0.001**
Tanner 1-2	0 (0)	11 (55)	**<0.001**
Tanner 3-5	20 (100)	9 (45)	

T2DM = type 2 diabetes mellitus. [a]Obesity was defined as BMI ≥ 95th percentile according to CDC criteria. [b]Overweight was defined as BMI ≥ 85th percentile and <95th percentile according to CDC criteria. Data are presented as absolute number and percentage (%) unless specified otherwise.

3.2. Anthropometric and Metabolic Parameters. Table 2 describes the anthropometric, metabolic, and clinical variables for the T2DM and healthy control groups. Body weight (kg) (68.40), height (cm) (1.59), BMI (27.8), and BMI percentiles (96.4) were significantly higher in the T2DM group compared with the control group (34.90, 1.45, 17.70, and 54.15), respectively. As well, waist circumference (96.10), hip circumference (102.2), and waist-height ratio (0.596) were also significantly higher in the T2DM group compared with the healthy group (63.60, 65.40, and 0.439), respectively.

Regarding metabolic and clinical variables, fasting glucose (114.00 (92.00-158.00)), triglycerides (157.00 (135.20-199.50)), insulin (23.3 (10.63-30.93)), HOMA-IR (8.35 (2.85-12.78)), and systolic blood pressure (112.00 (104.30-120.00)) were significantly higher and HDL-c (37.50 (31.25-44.75)) significantly lower in the T2DM group compared with the control group. As well, hs-CRP was significantly higher in T2DM subjects (1.32 ± 0.62 (1.03-1.61)) compared with the control subjects (0.83 ± 0.39 (0.64-1.02)) ($p = 0.006$). The normal insulin range (mIU/L) reported by the laboratory department is 5.0-20.0 (mIU/L).

3.3. Irisin Plasma Levels. Figure 1 shows irisin plasma levels in the T2DM group compared with the control group. Irisin concentration was significantly lower for the T2DM group (6.84 (2.07-23.72)) compared with the healthy control group (27.35 (8.91-50.95)) ($p = 0.014$).

3.4. Correlation between Plasma Irisin Levels and hs-CRP, Anthropometric, Metabolic, Clinical, and Inflammatory

Table 2: Anthropometric, metabolic, clinical, and inflammatory parameters for the T2DM and healthy control groups.

Parameter	T2DM ($n = 20$)	Healthy controls ($n = 20$)	p value
Weight (kg)	68.4 (61.60-90.85)	34.90 (26.50-44.80)	**<0.001**
Height (m)	1.59 (1.55-1.66)	1.45 (1.33-1.60)	**0.002**
BMI (kg/m^2)	27.8 (24.73-34.68)	17.70 (16.35-18.95)	**<0.001**
BMI%	96.4 (89.73-99.10)	54.15 (25.45-70.20)	**<0.001**
WC (cm)	96.13 ± 16.72	63.6 ± 6.14	**<0.001**
WC%	85.00 (50.00-93.75)	15 (10.00-25-00)	**<0.001**
HC (cm)	102.2 ± 16.89	65.4 ± 6.73	**<0.001**
WHI	0.939 ± 0.07	0.968 ± 0.01	0.081
W/ht	0.596 ± 0.093	0.439 ± 0.016	**<0.001**
SBP (mmHg)	112.00 (104.3-120.00)	98.00 (96.00-104.00)	**<0.001**
SBP%	58.00 (31.2-89.00)	39.00 (26.50-42.00)	**0.027**
DBP (mmHg)	67.50 (62.25-78.75)	64.00 (63.00-67.00)	0.249
DBP%	57.00 (43.25-90.75)	65 (53.00-67.50)	0.970
Glc (mg/dL)	114.5 (92.00-205.30)	84.00 (71.50-88.50)	**<0.001**
Serum insulin (mIU/L)	23.30 (10.63-30.93)	6.60 (4.83-7.98)	**<0.001**
HOMA-IR	8.35 (3.85-12.78)	1.20 (1.00-1.65)	**<0.001**
TC (mg/dL)	149.50 ± 29.24	153.4 ± 22.93	0.658
HDL-c (mg/dL)	37.50 (31.25-44.75)	50.50 (43.25-58.75)	**<0.001**
LDL-c (mg/dL)	74.90 ± 20.63	100.3 ± 24.02	**0.001**
TG (mg/dL)	157.00 (112.8-195.30)	87.50 (70.75-99.50)	**<0.001**
hs-CRP (mg/L)	1.32 ± 0.62	0.83 ± 0.39	**0.006**
TNF-α (pg/mL)	2.12 (1.92-2.51)	2.58 (2.25-2.85)	0.167
IL-6 (pg/mL)	3.38 (2.90-3.84)	4.13 (2.95-4.68)	0.104

Values are presented as median and interquartile range for nonparametric data and as mean and standard deviation for parametric data. BMI = body mass index; DBP = diastolic blood pressure; Glc = fasting glucose; HC = hip circumference; HDL-c = high-density lipoprotein cholesterol; hs-CRP = high sensitivity C-reactive protein; LDL = low-density lipoprotein cholesterol; HOMA-IR = homeostatic model assessment of insulin resistance; SBP = systolic blood pressure; T2DM = type 2 diabetes mellitus; TC = total cholesterol; TG = triglycerides; WC = waist circumference; WHI = waist-hip index; W/ht = waist-to-height ratio; % = percentile for age and gender.

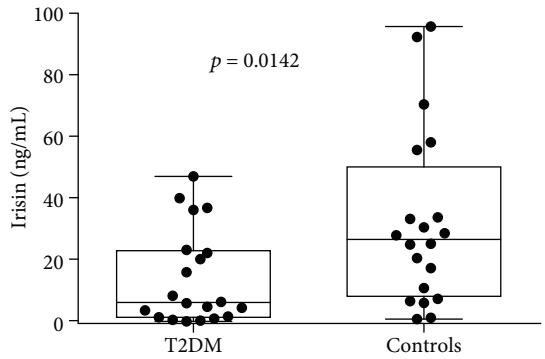

Figure 1: Irisin levels in studied groups. T2DM = type 2 diabetes mellitus.

Parameters. Table 3 shows Spearman's correlation coefficients between irisin concentration and the anthropometric and metabolic parameters. Irisin levels were found to be inversely correlated with height ($r = -0.380$), weight ($r = -0.356$), BMI ($r = -0.356$), WC ($r = -0.383$), and HC ($r = -0.391$) ($p < 0.05$). Regarding metabolic and clinical parameters, plasma irisin levels were found to be positively correlated with total cholesterol ($r = 0.431$), LDL-c ($r = 0.596$), HDL-c ($r = 0.447$) ($p < 0.01$), and DBP percentile ($r = 0.350$, $p < 0.05$). However, after multiple linear regression analysis adjusting for age, gender, and BMI, only HDL-c showed a positive correlation with irisin ($B = 0.813$, $p = 0.009$). Scatter plots for anthropometric and metabolic data are shown in Figures 2 and 3, respectively. Of note, subjects with acanthosis showed significantly greater insulin levels (23.7 (10.2-31.3)) than those without acanthosis (7.0 (5.05-8.20)) ($p < 0.001$), alike for HOMA-IR (8.00 (3.80-12.9) and 1.20 (1.00-1.75), respectively.

3.5. Correlation between hs-CRP, Irisin, Anthropometric, and Metabolic Parameters. hs-CRP was found to be significantly associated with anthropometric markers of obesity, such as weight ($r = 0.509$), BMI ($r = 0.435$), BMI% ($r = 0.518$), WC% ($r = 0.542$), W/ht ratio ($r = 0.463$) ($p < 0.01$), and WC ($r = 0.415$, $p < 0.05$). hs-CRP was also found to be positively correlated with metabolic markers such as triglycerides ($r = 0.374$, $p < 0.05$) and negatively correlated with HDL-c ($r = -0.327$, $p < 0.05$). Interestingly, we found hs-CRP to be positively associated with serum insulin levels ($r = 0.394$,

Table 3: Correlation between irisin concentration and anthropometric, metabolic, and inflammatory parameters.

	Height	Weight	BMI	BMI%	WC	WC%	HC	WHI	W/ht	SBP	SBP%	DBP	DBP%	TC	TG	HDL-c	LDL-c	Glc	Insulin	HOMA	hs-CRP
Irisin	**-0.380***	**-0.313***	**-0.356***	-0.250	**-0.383***	-0.208	**-0.391***	0.168	-0.268	-0.166	0.051	0.263	**0.350***	**0.431****	**-0.334***	**0.447****	**0.596****	-0.173	-0.040	-0.052	0.036
Height		**0.786****	**0.605****	**0.474****	**0.711****	**0.497****	**0.703****	-0.183	**0.355***	**0.536****	-0.064	0.092	-0.272	-0.183	0.349*	**-0.374***	**-0.499****	0.254	0.281	0.243	0.141
Weight			**0.956****	**0.882****	**0.934****	**0.808****	**0.946****	-0.240	**0.735****	**0.628****	0.154	0.118	-0.186	-0.134	**0.523****	**-0.573****	**-0.506****	**0.624****	**0.575****	**0.589****	**0.509****
BMI				**0.941****	**0.907****	**0.794****	**0.935****	-0.261	**0.788****	**0.598****	0.209	0.099	-0.151	-0.098	**0.563****	**-0.634****	**-0.435****	**0.601****	**0.656****	**0.634****	**0.435****
BMI%					**0.812****	**0.836****	**0.838****	-0.173	**0.782****	**0.524****	0.261	0.056	-0.086	-0.058	**0.588****	**-0.537****	**-0.398***	**0.659****	**0.629****	**0.655****	**0.518****
WC						**0.896****	**0.969****	-0.140	**0.871****	**0.720****	0.322	0.157	-0.121	-0.122	**0.548****	**-0.612****	**-0.465****	**0.555****	**0.662****	**0.608****	**0.415***
WC%							**0.861****	-0.061	**0.952****	**0.649****	**0.468****	0.125	-0.006	-0.085	**0.562****	**-0.490****	**-0.376***	**0.582****	**0.635****	**0.634****	**0.542****
HC								**-0.334***	**0.846****	**0.639****	0.227	0.117	-0.177	-0.151	**0.558****	**-0.641****	**-0.478****	**0.604****	**0.679****	**0.653****	**0.393***
WHI									-0.053	0.047	0.298	0.173	0.320	0.200	-0.050	0.181	0.163	-0.198	-0.180	-0.265	0.187
W/Ht										**0.606****	**0.490****	0.123	0.009	-0.139	**0.554****	**-0.547****	**-0.349***	**0.520****	**0.706****	**0.656****	**0.463****
SBP											**0.735****	**0.498****	0.289	-0.061	**0.441****	**-0.412***	-0.295	0.295	**0.388***	0.287	0.210
SBP%												**0.531****	**0.596****	0.073	0.362*	-0.192	-0.002	0.151	0.252	0.167	0.258
DBP													**0.860****	**0.366***	0.162	0.163	0.171	0.100	0.083	0.026	0.028
DBP%														0.288	0.076	0.263	0.266	0.006	-0.081	-0.109	-0.098
TC															0.099	**0.377***	**0.678****	-0.037	0.205	0.115	0.105
TG																**-0.497****	-0.299	**0.516****	**0.629****	**0.566****	**0.374***
HDL-c																	**0.444****	**-0.500****	**-0.395***	**-0.410***	**-0.327***
LDL-c																		**-0.432****	-0.162	-0.238	-0.159
Glc																			**0.602****	**0.742****	0.257
Insulin																				**0.958****	**0.394***
HOMA																					**0.393***

Spearman's correlation coefficients. Significant correlations are shown in bold ($^{*}p < 0.05$, $^{**}p < 0.01$). BMI = body mass index; WC = waist circumference; HC = hip circumference; WHI = waist-hip index; W/Ht = waist-height ratio; SBP = systolic blood pressure; DBP = diastolic blood pressure; TC = total cholesterol; TG = triglycerides; HDL-c = high-density lipoprotein cholesterol; LDL-c = low-density lipoprotein cholesterol; Glc = glucose; HOMA-IR = homeostatic model assessment of insulin resistance.

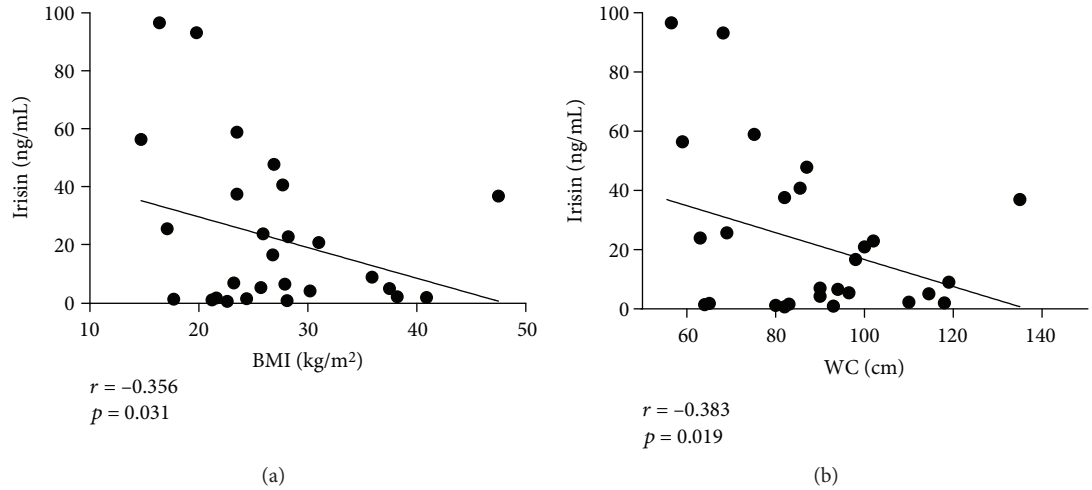

Figure 2: Correlation between irisin plasma levels and anthropometric parameters. Association of plasma irisin levels with BMI (a) and waist circumference (b). BMI = body mass index; WC = waist circumference.

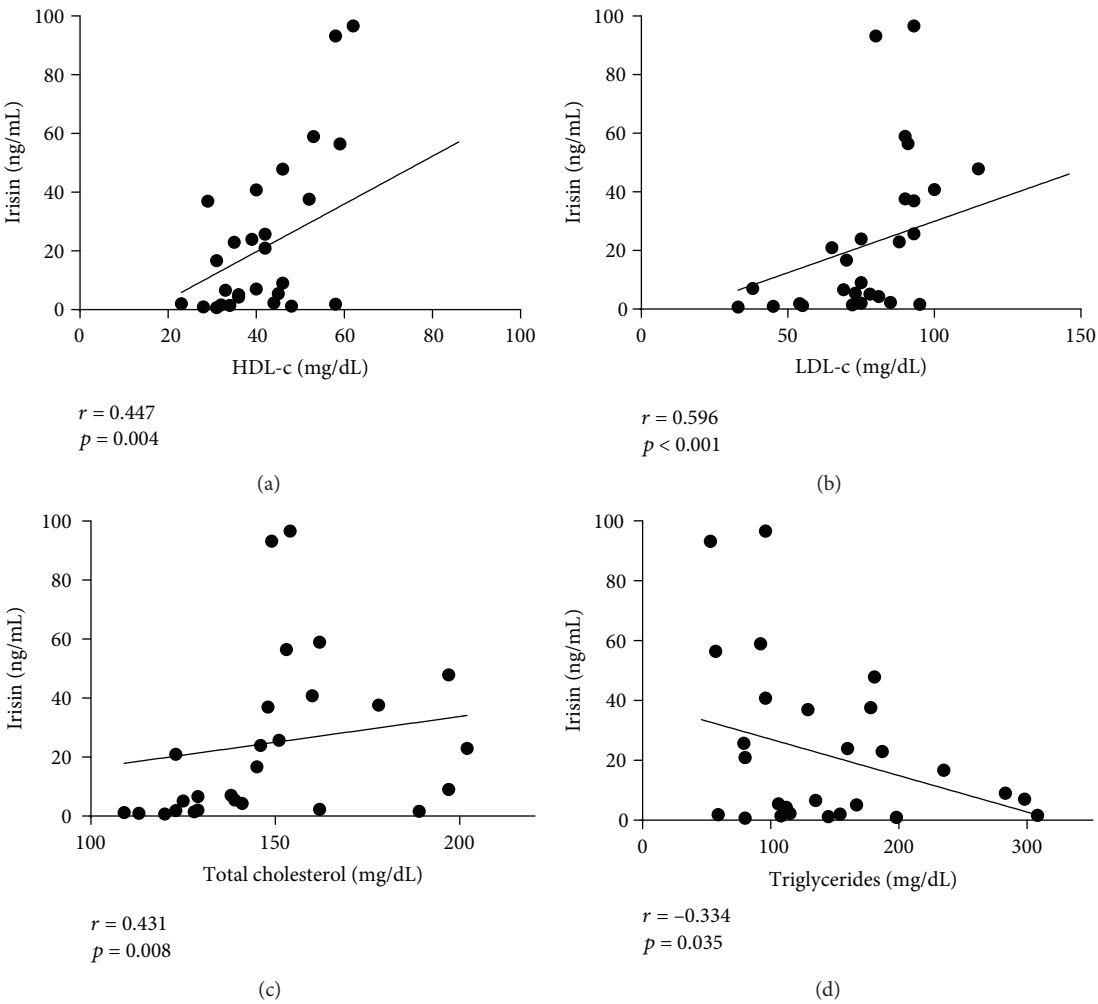

Figure 3: Irisin plasma level correlations with metabolic parameters. Association of plasma irisin levels with high-density lipoprotein cholesterol (a), low-density lipoprotein cholesterol (b), total cholesterol (c), and triglycerides (d).

$p < 0.05$) and with HOMA-IR ($r = 0.393$, $p < 0.05$). No association was found between hs-CRP and irisin levels.

4. Discussion

4.1. Irisin Levels in Children and Adolescents with T2DM. Irisin was initially described as a myokine in transgenic mice overexpressing *Ppargc1a*, a gene that encodes the transcription cofactor peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α) [21], a protein whose expression has been found to be increased by exercise. Irisin has been described to be involved in the regulation of mitochondrial biogenesis and function in muscle cells, FNDC5 expression, and other metabolic pathways [23]. Irisin has also been linked to an array of clinical entities, especially to metabolic diseases including obesity, T2DM, and cardiovascular risk factors [24].

To our knowledge, this is the first study to evaluate irisin levels in the pediatric population with T2DM. We found irisin levels to be decreased in children and adolescents with T2DM compared with control subjects. Similar results have been shown in adults with T2DM. [42–45]. The higher irisin levels found in our normal weight group support the original role described for irisin related to an increased thermogenesis and energy expenditure mediated through UCP1 [21], leading to browning of adipose tissue. *In vitro* and animal studies have shown irisin to have both direct and indirect effects on metabolic pathways, acting mainly on adipose tissue, muscle, and the liver. [23]. To begin with, in adipose tissue, irisin has been able to enhance glucose uptake [46], to stimulate lipolysis, and to inhibit lipid accumulation [47]. In addition, in muscle, irisin appears to modulate metabolic processes by activating AMP-activated protein kinase (AMPK), thereby increasing glucose uptake, lipid uptake, and metabolism and decreasing glycogenolysis and gluconeogenesis [48]. Finally, in hepatocytes, irisin has been shown to reduce oxidative stress [49, 50], to promote glycogenesis, to inhibit gluconeogenesis [51], and to reduce lipogenesis and lipid accumulation [49]. Overall, considering these favorable metabolic effects, the decreased levels of irisin observed in our T2DM pediatric population could potentially exacerbate the decreased glucose uptake in muscle and other metabolic derangements observed in peripheral tissues of T2DM subjects. The low irisin levels could also alter lipid uptake and metabolism, promoting lipogenesis and lipid accumulation, which may well increase the cardiovascular risk in children and adolescents with T2DM.

Although the mechanisms underlying the lower levels of irisin observed in T2DM subjects are not clearly understood, some hypotheses have been described. Lower irisin concentration in T2DM patients could be explained by a reduced PGC-1α activity in muscle tissue of T2DM individuals, which was observed long before the discovery of irisin [52]. Lower levels of circulating irisin have been found in a sample of 96 Asian adults with T2DM compared with 60 nondiabetic controls [44]. Consequently, the reduction in PGC-1α activity in muscle tissue of T2DM patients would lead to a resultant decrease in FNDC5 and irisin expression. As well, insulin resistance, which ultimately results in hyperglycemia and increased circulating free fatty acids [53], has also been proposed as responsible for the decrease in PGC-1α activity [54]. In addition, free fatty acids and glucose, in turn, could be the direct mediators of decreased irisin expression, as indicated by a study conducted by Kurdiova and colleagues in a cohort of 99 European sedentary adults, divided into lean ($n = 29$), obese ($n = 29$), prediabetic ($n = 25$), and T2DM ($n = 16$) subjects. Hyperglycemia and triglyceridemia were found to be negatively associated with adipose tissue FNDC5 mRNA expression and with circulating irisin. This finding was further reinforced by the observation that FNDC5 mRNA expression in muscle cells obtained from these subjects was found to be lower after an *in vitro* treatment with palmitate (a saturated fatty acid) and with glucose [42]. Therefore, since there is no previous data associating irisin plasma levels in T2DM in children and adolescents and considering the findings in the adult population, the lower irisin levels found in our cohort could be attributed to a reduced PGC-1α activity in muscle tissue with a consequent decrease in FNDC5 and irisin expression. Furthermore, the hyperglycemia and hypertriglyceridemia found in our T2DM group might also be negatively associated with adipose tissue FNDC5 mRNA expression and circulating irisin.

Patients with T2DM have metabolic imbalances in which irisin may play a role [24]. Relationships between irisin concentration and specific common metabolic parameters in the clinical setting have been scarcely studied in children resulting in contradictory findings [30]. Our results show irisin concentration to be positively correlated with HDL-c, total cholesterol, and LDL-c levels, and negatively correlated with TG. However, after multiple linear regression analysis, only HDL-c correlation remained significant, which might be attributed to the higher levels shown by the normal weight group compared with the T2DM group. Since HDL-c has been found to have a protective anti-inflammatory role against cardiovascular disease and has been associated with preserved endothelial function [55], higher irisin levels found in the control group may favor the maintenance of vascular function in normal weight children. On the other hand, endothelial function has been shown to be impaired in adolescents with T2DM [56]. Interestingly, a positive correlation between irisin and endothelial progenitor cells (EPCs) has been found in a cohort of 24 nondiabetic overweight and obese children. The authors hypothesized that irisin could represent a compensatory protective effect against endothelial damage in obesity through EPC activation and/or mobilization [57]. Given that lower irisin levels were found in our cohort of T2DM children and adolescents, the compensatory role of irisin on vascular damage through the EPC pathway may be lost, further increasing their cardiovascular risk.

4.2. Irisin and Inflammation in Children and Adolescents with Obesity and T2DM. One of the features that characterizes most of the population with T2DM is central obesity. Regarding anthropometric markers of adiposity, most studies in nondiabetic obese adults have shown increased circulating levels of irisin [24] and a positive association with BMI, WC, and body fat percentage [28, 29, 58–60], but in

the pediatric population findings are still controversial and attributed to differences in body composition and its variations during growth and development [30]. In our study, we found irisin levels to be negatively correlated with BMI and WC. In agreement with our findings, Shim et al. found irisin plasma levels to be higher in normal weight children compared with nondiabetic overweight/obese subjects [35], while some authors have found an increased irisin concentration and a positive correlation with BMI, WC, fat mass, and % body fat [31–33, 61, 62] in obese children and still others have found no correlation at all [63]. The increase in irisin levels observed in obesity may simply be the result of an excessive adipose tissue as an important source of irisin, which may increase insulin sensitivity and energy expenditure as a compensatory mechanism to counteract the deleterious metabolic effects of excess adiposity, namely, insulin and catecholamine resistance [23, 64]. On the other hand, the increased circulating irisin may represent an "irisin resistance" state, similar to the insulin and leptin resistance that results in hyperinsulinemia and hyperleptinemia observed in obese individuals [24]. Considering that virtually all overweight and obese subjects in our study were diabetic, while none of the children in the control group were, the diabetic condition itself could be accountable for the inverse relationship between irisin levels and BMI and WC.

Obesity has long been considered as a state of low-grade chronic inflammation [65]. Recent evidence has shown that this inflammatory state begins and is maintained early in childhood [12], but evidence of this association in children with T2DM is limited. Although the precise stimuli that promote obesity-associated inflammation are poorly understood, it has been hypothesized that hypertrophic adipose tissue induces adipocyte apoptosis, hypoxia, and mechanical stress that activate proinflammatory pathways, with a resultant increase in proinflammatory cytokine and chemokine expression in adipocytes, which results in recruitment of macrophages to adipose tissue and their polarization to the M1 proinflammatory phenotype [64]. Furthermore, the increased circulating levels of free fatty acids associated with obesity may also directly initiate an inflammatory reaction [13, 66]. In turn, the inflammatory response induces insulin resistance through several mechanisms [67]. For instance, proinflammatory cytokines such as TNF-α, secreted locally by adipose tissue macrophages, have been found to be likely mediators of insulin resistance [64, 67]. High-sensitivity C-reactive protein (hs-CRP), synthesized in the liver in response to circulating proinflammatory cytokines [68], has been extensively used as a surrogate marker of nonspecific inflammation in a variety of clinical settings [69] in which evaluation of the cytokine profile is not always available and is rather expensive. Noteworthy, evidence of the association of a proinflammatory profile in children and adolescents with T2DM is scarce. The only available evidence to date has shown higher concentrations of TNF-α, IL-1β, and hs-CRP in obese Caucasian adolescents with T2DM compared with obese controls without T2DM [20]. In addition, high levels of C-reactive protein (CRP) and IL-6 have been found to predict the onset of T2DM [15]. As expected, our results show hs-CRP levels to be higher in the T2DM group compared with the normal weight group. hs-CRP levels were also found to be positively associated with WC, W/ht ratio, and triglycerides, while a negative association with HDL-c was found, all markers of central adiposity and the metabolic imbalance characteristic of both obesity and T2DM. Finally, hs-CRP was found to be positively associated with insulin levels and HOMA-IR, the hallmark of T2DM. Thus, the increased inflammatory response found in the T2DM children might be attributed to the hypertrophic adipose tissue and increased circulating free fatty acids, among other stimuli.

An interesting possibility of an "irisin-inflammatory/anti-inflammatory axis" could arise, supported by evidence related to obesity and diabetes [68]. At the anti-inflammatory side of the axis, after treatment with irisin, a decrease in the expression of TNF-α, IL-1β, IL-6, and MCP-1 by LPS-activated murine macrophages [70] and by adipocytes [71] has been demonstrated. Similarly, decreased M1 polarization and proinflammatory cytokine expression after treating murine macrophages with irisin's precursor, FNDC5, has been reported [72]. As well, irisin has been shown to promote polarization of adipose tissue macrophages to the alternative M2 anti-inflammatory phenotype [73]. In studies in humans, mRNA expression of FNDC5 was found to be negatively associated with TNF-α expression in visceral adipose tissue, while positively associated with the expression of IL-10, an anti-inflammatory cytokine, in subcutaneous adipose tissue. These findings were reported in a cohort of obese and normal weight Caucasian individuals with and without T2DM [43], a finding that could represent the interaction of irisin with adipose tissue macrophages. At the proinflammatory side of this axis, TNF-α and IL-1β have been shown to inhibit FNDC5 expression in murine myotubes [74], possibly decreasing irisin secretion. A positive association between irisin and hs-CRP might suggest that irisin is secreted as a counterregulatory mechanism against inflammation, while a negative association could indicate that irisin expression is being suppressed by ongoing inflammation. However, we did not find any association between hs-CRP and plasma irisin levels, which could be attributed among other factors, to our small sample size. To our knowledge, the association between irisin and inflammatory markers in the pediatric population is greatly limited. Viitasalo et al. [75] found irisin to be positively associated with TNF-α and IL-6 in nondiabetic Caucasian children aged 6-8 years. The authors attributed the observed positive correlation between irisin and proinflammatory cytokines to a compensatory increase of irisin to limit lipid accumulation and inflammatory changes in the liver of children at increased risk of NAFLD. In another study conducted by Sarac et al., higher levels of irisin as well as of hs-CRP were found in children with acute appendicitis compared with controls [76]. This finding might suggest that irisin secretion increases in conditions characterized by a state of inflammation.

4.3. The Role of Irisin in the Inflammatory State and Metabolic Imbalances of T2DM. Figure 4 shows the hypothesis for our findings correlating the integration between the possible irisin-inflammatory/anti-inflammatory axis, as well as the role of irisin in T2DM. In summary, adipose tissue

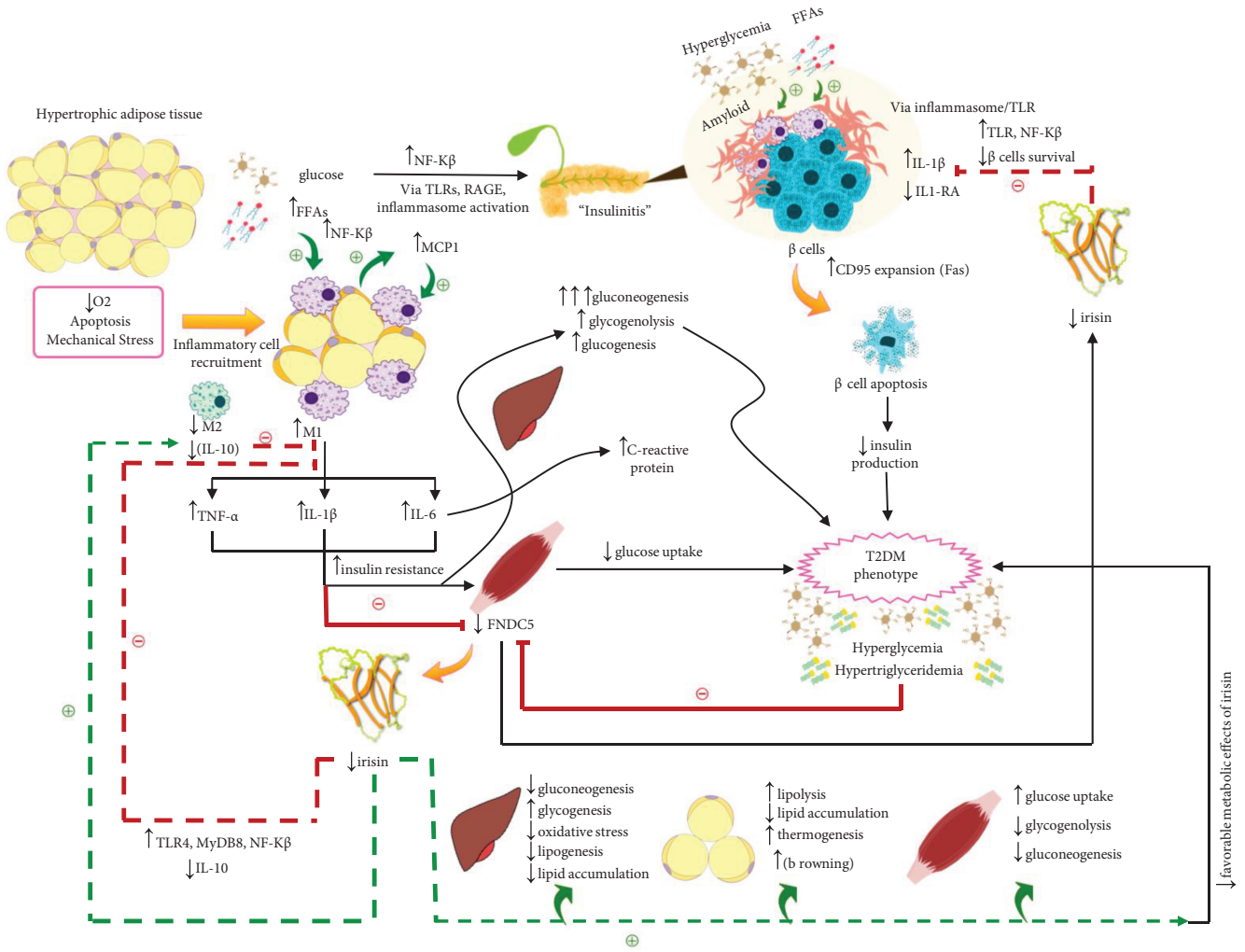

Figure 4: Possible irisin-inflammatory cross talk in overt T2DM and exacerbation of metabolic derangements due to hypoirisinemia. Dotted lines indicate the theoretical compensatory effects of higher irisin levels in context of obesity, which may be impaired in patients with T2DM who have decreased levels of irisin. Continuous lines indicate an effect that could be observed in context of T2DM. Green lines indicate stimulation and red lines indicate inhibition. FFAs = free fatty acids; FNDC5 = fibonectin type III domain-containing protein 5; IL-1RA = interleukin-1 receptor antagonist; IL-1β = interleukin-1β; IL-6 = interleukin-6; IL-10 = interleukin-10; LPS = lipopolysaccharide; M1 = classically activated (proinflammatory) macrophage; M2 = alternatively activated (anti-inflammatory) macrophage; MCP1 = monocyte chemoattractant protein 1; MyD88 = myeloid differentiation primary response 88; NF-κB = nuclear factor kappa B; TLR = toll-like receptor; RAGE = receptor for advanced glycation end products; T2DM = type 2 diabetes mellitus; TNF-α = tumor necrosis factor-alpha.

hypertrophy may lead to hypoxia, adipocyte apoptosis, and mechanical stress, all of which activate an inflammatory response in adipocytes that causes the release of cytokines and chemokines, including MCP-1. This is followed by recruitment and differentiation of monocytes to proinflammatory M1 classically activated macrophages [64]. M1 macrophages secrete the proinflammatory cytokines TNF-α, IL-1β, and IL-6, which promote insulin resistance and the accompanying deleterious metabolic effects [67], as well as synthesis of C-reactive protein [77]. Irisin expression early in obesity, before development of overt diabetes, may be increased in adipose tissue as a compensatory response to counteract insulin resistance and to increase energy expenditure [24]. As well, increased irisin levels inhibit the secretion of proinflammatory cytokines and promote secretion of the anti-inflammatory cytokine IL-10 by interacting with infiltrating immune cells in adipose tissue and stimulating macrophage polarization from M1 to M2 phenotype [43, 70, 72, 73, 78].

However, at the other end of the spectrum, in obese type 2 diabetic individuals, irisin may be suppressed due to either insulin resistance itself, which could indirectly inhibit the secretion of irisin by glucotoxic or lipotoxic mechanisms [42]. Alternatively, an overwhelming proinflammatory milieu directly inhibits irisin production in muscle [74]. Both may further exacerbate the deleterious inflammatory and metabolic imbalances present in T2DM. Furthermore, decreased irisin levels might exacerbate the pancreatic islet infiltration of immune cells ("insulinitis") observed in T2DM [18]. This effect might be attributed to a decreased

inhibitory effect over the local production of IL-1β by interacting with local macrophages [70], which the presence of continued hyperglycemia, elevated free fatty acids, and amyloid deposition [79] leads to continued islet inflammation, β-cell apoptosis, and consequent decreased insulin secretion, observed late in the evolution of T2DM [18]. In addition, irisin has been found to be positively associated with circulating insulin and HOMA-β even in healthy subjects, indicating that it may play a role in the regulation of β-cell function [23, 80].

The study has several limitations. The sample size was small and all subjects are from Hispanic ethnicity; thus, the generalizability of the results is limited. Due to the small sample size and transversal design, direct establishment of a correlation of irisin with inflammatory markers is not possible. However, the study presents several strengths. This is the first study to evaluate irisin concentration in a children and adolescents with T2DM and its association with the metabolic and anthropometric markers in this T2DM population. The role of irisin in inflammation, a well-known element in T2DM patients, has been proposed. In addition, the novel figure highlights the role of irisin in inflammation and as a metabolic regulator in obesity and T2DM based on literature findings.

5. Conclusions

This is the first study to demonstrate decreased levels of irisin in children and adolescents with T2DM compared with healthy controls. Based on experimental evidence, decreased circulating irisin in the pediatric population with T2DM could be attributed to decreased muscle secretion of FDNC5. After adjustment for possible confounders, we found a positive correlation between irisin and HDL-c, which could suggest that irisin has a potential protective mechanism against endothelial damage and vascular disease. Importantly, we observed hs-CRP to be higher in the T2DM group and to be positively associated with anthropometric and metabolic markers of T2DM, which indicates that a systemic inflammatory state is present in the pediatric population with T2DM. Irisin lower concentrations in this population could potentially exacerbate the metabolic and inflammatory components of this disease. Larger clinical studies are needed to elucidate the relationship between circulating inflammatory markers and irisin in children with T2DM, as well as experimental studies to elucidate the mechanisms of hypoirisinemia in T2DM, as irisin may ultimately play a role as a therapeutic agent in obesity and in T2DM.

Data Availability

The values used to build the tables and graphs that support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Supplementary Materials

In order to address the detailed methodology, supplementary material is provided with a more extensive description of each laboratory study. *(Supplementary Materials)*

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Research Article

Circulating Monocyte and Lymphocyte Populations in Healthy First-Degree Relatives of Type 2 Diabetic Patients at Fasting and during Short-Term Hyperinsulinemia

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Aim. The development of type 2 diabetes (T2DM) is associated with disturbances of immune status that may be reflected by alterations of the profile of circulating immune cells. In order to study whether there exists genetic predisposition to these alterations, we investigated the relative content of circulating monocyte and lymphocyte subpopulations at fasting condition and upon stimulation by short-term hyperinsulinemia in nondiabetic first-degree relatives (FDR) of T2DM patients and in control subjects. *Materials and Methods*. 19 nondiabetic (FDR) and 19 control subjects without a family history of diabetes (all men) matched for age and BMI underwent 2-hour hyperinsulinemic-euglycemic clamp. Blood samples taken before and at the end of the clamp were used for the flow cytometry analysis of lymphocyte and monocyte populations and for the assessment of cytokine levels. *Results*. At fasting conditions, FDR showed a higher CD4/CD8 ratio of peripheral lymphocytes, a higher percentage of Th17 lymphocytes, and a lower content of intermediate monocytes when compared to controls. The CD4/CD8 ratio correlated with fat mass, insulin, and HOMA-IR in the entire group of subjects. Hyperinsulinemia decreased a relative content of peripheral CD4+ and increased a relative content of CD8+ T lymphocytes, thus decreasing the CD4/CD8 ratio by 18-22% in both groups of subjects. In FDR but not in controls, the decrease of CD4+ T lymphocyte content was partially based on the decrease of T_H2 and T_H17 lymphocyte subpopulations. In control subjects but not in FDR, the number of intermediate monocytes has declined in response to hyperinsulinemia. *Conclusion*. The alterations of the CD4/CD8 lymphocyte ratio, relative content of T_H17 cells, and intermediate monocytes in FDR are features of genetic predisposition to T2DM and may play a role in pathogenesis of T2DM. Short-term hyperinsulinemia affected mostly the immune cell populations deregulated in FDR subjects, which suggests important interplay between immune system homeostasis and insulin levels.

1. Introduction

Type 2 diabetes (T2DM) and its complications are associated with a systemic low-grade inflammation manifested by higher systemic levels of proinflammatory cytokines, such as IL-6, IL-1β, or TNFα [1]. The increased levels of cytokines can originate from metabolically challenged tissues such as liver, pancreas, and adipose tissues or from circulating immune cells, whose phenotype and numbers (or relative content) are sensitive to metabolic challenge. Higher fasting glucose concentration and occurrence of T2DM were positively associated with higher abundance of memory CD4+ T-lymphocytes [2] and T_H1 and T_H17 lymphocytes in the blood (reviewed in [3]). Furthermore, a recent meta-

analysis showed that T2DM patients have decreased the content of peripheral CD4/CD25/Foxp3 regulatory T-cells and concomitantly decreased circulating levels of anti-inflammatory cytokine IL-10 [4]. Thus, the dysregulation of immune response might be associated and contribute to T2DM development.

It was suggested previously that the primary trigger of immune cell activation and thus low grade inflammation could be both acute and prolonged periods of hyperglycemia in obese patients and other patients at risk of T2DM development [5, 6]. Insulin, a hormone released in response to increased glucose levels, is one of the factors, which may alleviate the detrimental effects of hyperglycemias on immune cell phenotypes. Indeed, insulin was shown to inhibit the NFκB pathway [7] or downregulate circulating levels of acute-phase proteins [8], and thus, it is believed to have anti-inflammatory effects. In *in vitro* treatment of peripheral blood mononuclear cells (PBMC) with insulin, it induced a differentiation of lymphocytes to T-helper type 2 (T_H2) phenotype and decreased the ratio of T_H1 to T_H2 cells [9]. In response to acute hyperinsulinemia, a decrease of TNFα, IL-8, and IL-18 circulating levels was observed in healthy lean subjects, while in first-degree relatives (FDR) of type 2 diabetic patients, this anti-inflammatory response was blunted [10]. Thus, it seems likely that the dysregulation of the immune response to insulin/nutritional stimuli represents one part of the genetic predisposition to T2DM.

To test this hypothesis, we aimed to investigate the changes in lymphocyte and monocyte populations in association with genetic predisposition to T2DM and the response of these cells to short-term hyperinsulinemia in healthy FDR of T2DM when compared to control subjects.

2. Material and Methods

2.1. Subjects, Dietary Protocol, and Clinical Examination. 38 lean men participated in the study. Two groups of subjects matched for age and BMI were recruited: (1) nondiabetic first-degree relatives of T2DM patients (FDR; $n = 19$)—family history of diabetes was considered as follows: two first-degree relatives (parents, siblings) or one first-degree and one or more second-degree relatives (grandparents, uncle, aunt) were diagnosed with T2DM; and (2) control group—subjects without any family history of diabetes (CON; $n = 19$).

All subjects were generally healthy and nonobese and did not use any prescription drugs, as determined by medical history and laboratory findings. The exclusion criteria for both groups were weight change more than 3 kg within 3 months preceding the study, smoking, hypertension, diabetes, hyperlipidemia, illicit drug, or alcohol abuse. Subjects were examined at a certified laboratory starting at 8 am after an overnight fast. Body weight and waist and hip circumferences were measured, and body composition was assessed by bioimpedance (QuadScan 4000, Bodystat, Douglas, British Isles). The study was approved by the Ethical Committee of the Third Faculty of Medicine, Charles University, in Prague, and all subjects gave their informed consent before the start of the study.

2.2. Euglycemic-Hyperinsulinemic Clamp. The euglycemic-hyperinsulinemic clamp was performed according to the de Fronzo method [11]. A catheter for insulin and glucose infusion was inserted into an antecubital vein, and the second catheter for blood sampling was placed in the same location of the ipsilateral arm. The forearm was kept wrapped in a heated blanket to provide arterialization of venous blood. Priming dose plus continuous infusion of crystalline human insulin (Actrapid Human, Novo, A/S, Bagsvaerd, Denmark), 40 mU/m^2 body area/min, was given for 120 min. Euglycemia (at the level of the individual fasting blood glucose concentration) was maintained by a variable 20% glucose infusion. The infusion rate was adjusted according to arterialized plasma glucose levels measured every 5 minutes (Beckman Glucose analyzer, Beckman Instruments, Fullerton, CA, USA).

2.3. Flow Cytometry Analysis of Immune Cell Populations. The subpopulation of blood cells representing lymphocytes and monocytes was analyzed according to their size and granularity. To detect specific surface antigens, the whole blood samples were stained with fluorescence-labelled monoclonal antibodies (FITC-conjugated antibody CD4, CD14; PE-conjugated antibody CD3, and CD163; PerCP-conjugated antibody CD45; APC-conjugated antibody CD8, CD36, TLR2, and CD196; APC-Cy7-conjugated antibody CD16; PE-Cy7-conjugated antibody CCR2 and CD25; BV421-conjugated antibody CD183; and CD194; all except CD163 from BD Bioscience, US; CD163 from Exbio, CZ) for 30 min at room temperature. After staining, erythrocytes were lysed by erythrocyte lysis buffer for 15 min at room temperature. The cells were then washed with PBS and analyzed on a FACSVerse flow cytometer and by BD FACSuite Software 1.0.6 (BD Biosciences). The number of immune cells in the analyzed populations was expressed as the percentage of gated events related to CD45, CD45+/14+ (monogate), or CD3+ (lymphogate) events. Background was set up to 5% of positive cells of isotype control.

T cells were distinguished by standard FSC/SSC position "lymphogate" and by positivity for pan T cell marker CD3. CD4-positive T cells were considered T helper (T_H) cells and CD8-positive cells as T cytotoxic (Tc). T_H1 cells were defined as CD4+/194-/196-/183+ (positive for CXCR3), T_H2 were defined as CD4+/194+/196-/183- (positive for CCR4), and T_H17 were defined as CD4+/194+/196+/183- (positive for CCR4/CCR6). Regulatory T cells (Tregs) were identified as CD4/CD25high/CD127low cells. Monocytes were distinguished by standard FSC/SSC position and by positivity for CD45, CD14, and CD16 markers.

2.4. Analysis of Plasma. Plasma concentrations of glucose, insulin, lipids, and nonesterified fatty acids (NEFA) were determined using standard biochemical methods. Cytokines in the plasma were analyzed using xMAP technology (High Sensitivity Human T-cell Kit: IL-4, IL-6, IL-10, IL-12, IL-17A, and TNFα; Merck-Millipore, USA) on the MagPIX

instrument or using ELISA (Duoset MCP1, R&D Systems, Minneapolis, USA).

2.5. Statistical Analysis. Data are presented as the means ± SEM. Statistical analysis was performed using GraphPad Prism 7.0 for Windows (La Jolla, USA). Differences between the baseline values and between the responses to hyperinsulinemia in the two groups of subjects, the FDR and control group, were analyzed by two-way ANOVA, with Bonferroni post hoc analysis. Correlations at the baseline were expressed as Pearson's correlation coefficient. The level of significance was set at $p < 0.05$.

3. Results

3.1. Baseline Characteristics of the Subjects and Immune Cell Populations. The anthropometric and biochemical parameters of the subjects are listed in Table 1. The groups were not different in age, BMI, fat mass, waist circumference, and lipid parameters. FDR had a higher baseline glucose, fasting insulin, and insulin resistance as evaluated by the HOMA-IR index.

At the fasting state (baseline), the T lymphocyte populations in the blood were not different between the groups, except for T_H17 lymphocytes, which were higher in FDR (Figure 1). Moreover, the ratio of CD4/CD8 T lymphocytes was higher in the FDR group when compared to controls (2.29 ± 0.24 vs. 1.61 ± 0.14, $p = 0.04$, respectively). The relative content of CD4+ T cells and CD4/CD8 ratio correlated positively with FM in the whole group of subjects (FDR + controls) (Table 2). The CD4/CD8 ratio correlated positively to insulin and HOMA-IR and negatively to IL-4 cytokine (Table 2).

The monocyte populations in the blood did not differ between the groups at fasting conditions (Figures 2(a) and 2(b)), except for intermediate monocytes (CD45/14+/16+), which were lower in FDR compared to controls (Figure 2(a)). Moreover, in FDR, expression levels of CD163 (MFI) in CD163+ monocytes were lower compared to control subjects (Figure 2(c)). The relative content of the CD163+ monocytes was positively correlated to insulin sensitivity as evaluated by hyperinsulinemic-euglycemic clamp (M_{FFM}), while the relative content of classical monocytes correlated to fasting insulin levels and HOMA-IR in the whole group of subjects (Table 3).

3.2. Effect of Hyperinsulinemia on Circulating Lymphocytes. In response to hyperinsulinemia, a decrease in the relative content of CD4+ T helper cells and an increase of relative content of CD8+ cytotoxic T cells were detected in both groups of subjects. Thus, the CD4/CD8 ratio decreased from 2.29 ± 0.24 to 1.90 ± 0.24 ($p < 0.0001$) in FDR and from 1.61 ± 0.14 to 1.37 ± 0.16 ($p = 0.004$) in the control group, with no significant difference in this response between the groups. The relative content of T_H2 and T_H17 lymphocytes dropped in FDR (Figure 1), while no change in these T_H-cell subpopulations was observed in control subjects (Figure 1).

Table 1: The anthropometrical and biochemical parameters of the 2 groups of subjects.

	Controls ($n = 19$)	FDR ($n = 19$)	p value
Age (years)	36 ± 1.1	37 ± 1.3	0.469
Weight (kg)	81.9 ± 2.2	83.6 ± 1.1	0.515
BMI (kg/m^2)	24.7 ± 0.5	25.2 ± 0.4	0.401
Waist (cm)	84.2 ± 1.3	86.0 ± 1.3	0.341
Fat mass (%)	16.6 ± 0.9	18.6 ± 0.9	0.117
Fat-free mass (kg)	68.1 ± 1.6	67.9 ± 0.8	0.948
HDL (mmol/l)	1.36 ± 0.06	1.26 ± 0.07	0.313
TG (mmol/l)	1.06 ± 0.23	1.15 ± 0.27	0.808
Total cholesterol (mmol/l)	4.3 ± 0.3	4.7 ± 0.2	0.209
Ureic acid (μmol/l)	346 ± 16	334 ± 12	0.725
Glucose (mmol/l)	5.2 ± 0.1	5.6 ± 0.1	0.019
Insulin (mU/l)	5.2 ± 0.6	8.4 ± 1.1	0.018
HOMA-IR	1.2 ± 0.2	2.1 ± 0.3	0.015
M_{FFM} (mg/kgFFM/min)	8.1 ± 0.5	7.4 ± 0.7	0.461

Data are presented as the mean ± SEM. Statistical difference between the groups evaluated by a nonpaired t-test, $p < 0.05$. BMI: body mass index; HDL: high-density cholesterol; TG: triglycerides; HOMA-IR: homeostasis model assessment of insulin resistance; M_{FFM}: glucose disposal related to fat-free mas.

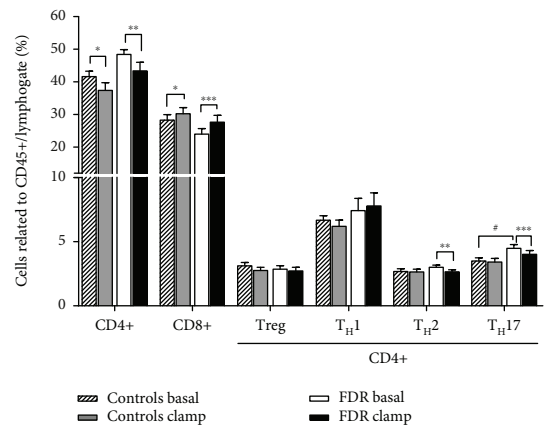

Figure 1: Effect of hyperinsulinemia on lymphocyte populations in the blood in FDR and in control subjects. Lymphocyte populations are expressed as the percentage of positive cells related to CD45/lymphogate. $^{***}p < 0.001$. $^{**}p < 0.01$. $^{*}p < 0.05$, significant change during the euglycemic-hyperinsulinemic clamp. $^{\$}p < 0.05$, significant difference between FDR and in control subjects at baseline. Striped bars: baseline preclamp levels in controls. Grey bars: postclamp levels at the end (2 hours) of the clamp in controls. White bars: baseline preclamp levels in FDR. Black bars: postclamp levels at the end (2 hours) of the clamp in FDR. FDR: first-degree relatives.

3.3. Effect of Hyperinsulinemia on Circulating Monocytes. No change in the relative content of classical (CD45/14+/16-) and nonclassical monocytes (CD45/14^{low}/16+) was observed in response to hyperinsulinemia in either group.

Table 2: Correlations between clinical parameters and circulating immune cells at baseline in all subjects.

Clinical parameter	Immune population	p value	Correl. coef.
FM	CD45/3/4+	0.016	0.399
	CD4/CD8 ratio	0.020	0.387
Insulin	Classical monocytes	0.026	0.376
	CD4/CD8 ratio	0.048	0.387
HOMA-IR	Classical monocytes	0.029	0.369
	CD4/CD8 ratio	0.035	0.348
M_{FFM}	CD45/14/163+	0.045	0.341
IL-4	CD45/3/4+	0.014	-0.422
	CD4/CD8 ratio	0.010	-0.451
	Intermediate monocytes	0.020	0.418

Data are presented as Pearson's correlation coefficient and p value. FM: fat mass; HOMA-IR: homeostasis model assessment of insulin resistance; M_{FFM}: glucose disposal related to fat-free mas; IL-4: interleukin 4.

Intermediate monocyte (CD45/14+/16+) count decreased after 2 h of sustained hyperinsulinemia in the control group only (Figure 2(a)). No changes in monocyte subpopulations expressing TLR2, CD163, CCR2, and CD36 were observed (Figure 2(b)).

An increased of expression (expressed as MFI) of the TLR2 scavenger receptor was detected on monocytes in response to hyperinsulinemia in both groups of subjects (Figure 2(c)).

3.4. Plasma Cytokines. No difference in the plasma levels of IL-4, IL-6, IL-10, IL-12, IL-17, TNFα, and MCP-1 was observed in baseline and in response to hyperinsulinemia in either group of subjects (Table 3). Baseline IL-4 concentrations correlated positively with the relative content of intermediate monocytes and negatively with CD4+ cells and with the CD4/CD8 ratio when analyzed in all subjects (Table 2).

4. Discussion

In this study, we investigated the relative content and polarization of circulating monocyte and T lymphocyte populations and the effect of hyperinsulinemia on these immune cells, in respect to genetic predisposition to T2DM.

Compared to control subjects without genetic predisposition to T2DM, FDR entering our study exhibited mildly higher glucose and insulin fasting levels (and concomitantly higher HOMA-IR index) representing early risk factors for the development of T2DM. These findings were in agreement with the previously described difference between nonobese FDR and control subjects [12–14]. Notably, insulin and HOMA-IR levels correlated positively to the CD4/CD8 lymphocyte ratio. Previously, the CD4/CD8 ratio was found to be increased in the new-onset streptozocin-treated diabetic mice [15] and also in T1DM patients and their first-degree relatives [16]. Moreover, in humans, the genetic predisposition to T1DM was associated with genetic variation at MHC locus, which seems to be responsible for peripheral blood CD4+/CD8+ T lymphocyte homeostasis [17]. In T1DM patients, the β-cell destruction is caused by the autoimmune attack of CD8+ activated by cytokines released by CD4/T_H1 cells and macrophages [18]. Interestingly, the increased infiltration of immune cells together with increased expression of proinflammatory cytokines, such as IL-1β, TNFα, was found also in islets in T2DM subjects [19, 20]. Thus, it was suggested that the development of the local proinflammatory state is an important pathophysiological mechanism in autoimmune-induced apoptosis of pancreatic β-cells not only in T1DM [18] but also in T2DM subjects as well [21, 22]. The findings observed in our study with FDR of T2DM subjects and other studies with T1DM patients and their FDR may suggest that the imbalance in the CD4/CD8 lymphocyte ratio might be figured among factors associated with diabetes development.

Moreover, the higher percentage of baseline Th17 lymphocytes found in the FDR group fits to the concept of the proinflammatory state preceding/contributing to diabetes onset, since T_H17 T-cells producing IL-17 were associated with proinflammatory reactions and diabetic complications [23] and increased levels of T_H1 and T_H17 circulating T-cells were reported also in T2DM subjects [4, 24, 25].

On the other hand, there was no difference between the two groups in relative distribution of classical and nonclassical monocyte populations, while the "intermediate" CD14+/16+ monocytes were lower in the FDR group. Intermediate monocytes express more than 80% of genes and surface markers at levels between classical and nonclassical monocytes [26]. They are considered proinflammatory and were associated with several inflammatory diseases [27, 28]. However, their role in metabolic diseases is not fully understood. In several studies with obese subjects, no difference or increased levels of peripheral intermediate monocytes were reported [29, 30]. On the other hand, in women with gestational diabetes, intermediate monocytes were shown to be lower when compared to healthy controls [31]. This monocyte subpopulation was shown to produce the anti-inflammatory cytokine IL-10, and thus, the function of this monocyte population might be considered immunomodulatory [31]. In agreement with this view, the content of intermediate monocytes correlated positively with anti-inflammatory cytokine IL-4 in our study. Nevertheless, the role of intermediate monocytes in diabetes development should be warranted in future studies.

In order to investigate further regulation of circulating lymphocytes and monocytes, we investigated responses of these immune cell populations to insulin action; namely, we studied these populations during short-term hyperinsulinemia induced by hyperinsulinemic-euglycemic clamp. The decrease of the CD4/CD8 ratio of T-lymphocytes evoked by short-term hyperinsulinemia in both groups of subjects suggests a marked role of insulin in the balance of these two cell populations. In fact, the insulin-stimulated decrease of CD4+ T-cells was accompanied by a decline of the relative content of T_H2 and T_H17 subpopulations in the FDR group to the levels seen in the control group. This could suggest that in the absence of elevated glucose, hyperinsulinemia is able to normalize the balance between T_H subsets, while under more

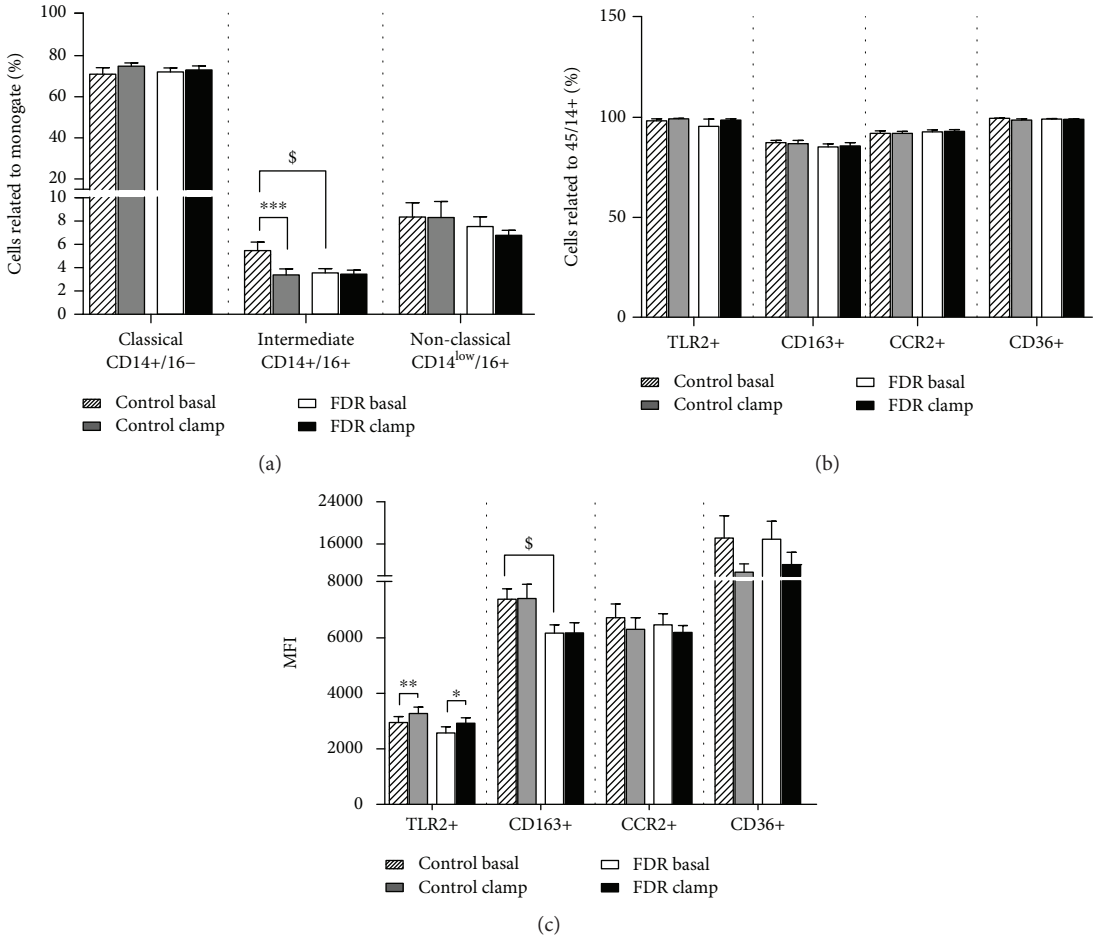

Figure 2: Effect of hyperinsulinemia on monocyte populations in the blood in FDR and in control subjects: (a) classical, intermediate, and nonclassical monocytes expressed as the percentage of positive cells related to CD45/14+; (b) monocyte populations expressing markers TLR2, CD163, CCR2, and CD36 expressed as the percentage of positive cells related to CD45/14+; (c) expression of markers TLR2, CD163, CCR2, and CD36 on monocyte subsets expressed as MFI $^{**}p < 0.01$; $^{*}p < 0.05$, significant change during the euglycemic-hyperinsulinemic clamp; $^{\$}p < 0.05$, significant difference between FDR and control subjects at baseline; striped bars—baseline preclamp levels in controls; grey bars—postclamp levels at the end (2 hours) of the clamp in controls; white bars—baseline preclamp levels in FDR; black bars—postclamp levels at the end (2 hours) of the clamp in FDR. FDR: first-degree relatives; MFI: mean fluorescence intensity.

Table 3: The concentrations of cytokines in the plasma before and after the hyperinsulinemic clamp in the 2 groups of subjects.

	Controls ($n = 18$)		FDR ($n = 18$)	
	Basal	Clamp	Basal	Clamp
IL-4 (pg/ml)	20.1 ± 2.3	21.4 ± 2.1	17.4 ± 1.5	18.0 ± 1.4
IL-6 (pg/ml)	0.99 ± 0.15	1.19 ± 0.16	0.89 ± 0.10	0.96 ± 0.14
IL-10 (pg/ml)	4.2 ± 0.6	4.6 ± 0.8	3.9 ± 0.7	3.4 ± 0.7
IL-12 (pg/ml)	1.6 ± 0.2	1.8 ± 0.2	1.7 ± 0.2	1.7 ± 0.2
IL-17 (pg/ml)	6.6 ± 0.7	6.9 ± 0.8	5.4 ± 0.6	5.5 ± 0.6
TNFα (pg/ml)	1.7 ± 0.1	1.9 ± 0.2	1.6 ± 0.2	1.5 ± 0.2
MCP-1 (pg/ml)	42.6 ± 5.8	34.8 ± 5.6	36.7 ± 5.0	30.8 ± 5.1

Data are presented as the mean ± SEM. IL: interleukin; TNFα: tumor necrosis factor alpha; MCP1: monocyte chemotactic protein 1.

physiological or chronic conditions, this protective role of insulin may be masked or overridden by the proinflammatory effects of glucose and other metabolites/factors. On the other hand, hyperinsulinemia drove the decline of intermediate monocytes in control subjects to the level seen in FDR. Although the function of intermediate monocytes in metabolism is questionable as mentioned above, this result could suggest that both acute and chronic hyperinsulinemia is an important regulator of this monocyte subtype.

Further, an increase of expression (MFI) of TLR2, a well-characterized immune scavenger receptor, on monocytes was observed in response to hyperinsulinemia. TLR2 was shown to trigger low-grade chronic inflammation and activation of macrophages, present in obesity, T2DM, or atherosclerosis [32], and a higher TLR2 expression was shown in monocytes of T2DM patients compared with control subjects [33]. Thus, insulin exerts complex regulation of monocytes and T lymphocytes, which warrants further studies in this issue.

Finally, our study did not show any effect of the genetic predisposition on fasting plasmatic levels of various cytokines similarly as described previously [10, 34]. This suggests that low-grade inflammation on the systemic level is not induced before the T2DM development in FDR. However, contrary to our results, the study of Ruotsalainen et al. [10] demonstrated the differential response of cytokines to hyperinsulinemia in controls and FDR (i.e., decrease of IL10, TNFα, IL8, and IL18 levels selectively in control subjects). The lack of the acute effect of hyperinsulinemia on circulating levels of analyzed cytokines in our study could imply the gender effect on this response, as we investigated only men, while the study of Ruotsalainen et al. [10] included both genders.

5. Conclusions

In conclusion, we demonstrated a shift in the peripheral CD4/CD8 lymphocytes ratio, T_H17 cells, and intermediate monocytes in subjects genetically predisposed to T2DM in comparison to controls. The imbalance in these immune cell populations might be features of genetic predisposition to T2DM and may play a role in pathogenesis of T2DM. Interestingly, short-term hyperinsulinemia affected mostly the immune cell populations deregulated in FDR subjects, which suggests an important interplay between immune system homeostasis and insulin levels.

Data Availability

The (lymphocyte and monocyte populations measured by flow cytometry and plasma cytokine levels) data used to support the findings of this study are included within the article. The original (raw) data of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interest.

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Research Article

High-Fat Diet Alters Immunogenic Properties of Circulating and Adipose Tissue-Associated Myeloid-Derived CD45$^+$DDR2$^+$ Cells

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Chronic inflammation is evident in the adipose tissue and periphery of patients with obesity, as well as mouse models of obesity. T cell subsets in obese adipose tissue are skewed towards Th1- and Th17-associated phenotypes and their secreted cytokines contribute to obesity-associated inflammation. Our lab recently identified a novel, myeloid-derived CD45$^+$DDR2$^+$ cell subset that modulates T cell activity. The current study sought to determine how these myeloid-derived CD45$^+$DDR2$^+$ cells are altered in the adipose tissue and peripheral blood of preobese mice and how this population modulates T cell activity. C57BL/6 mice were fed with a diet high in milkfat (60%·kcal, HFD) *ad libitum* until a 20% increase in total body weight was reached, and myeloid-derived CD45$^+$DDR2$^+$ cells and CD4$^+$ T cells in visceral adipose tissue (VAT), mammary gland-associated adipose tissue (MGAT), and peripheral blood (PB) were phenotypically analyzed. Also analyzed was whether mediators from MGAT-primed myeloid-derived CD45$^+$DDR2$^+$ cells stimulate normal CD4$^+$ T cell cytokine production. A higher percentage of myeloid-derived CD45$^+$DDR2$^+$ cells expressed the activation markers MHC II and CD80 in both VAT and MGAT of preobese mice. CD4$^+$ T cells were preferentially skewed towards Th1- and Th17-associated phenotypes in the adipose tissue and periphery of preobese mice. *In vitro*, MGAT from HFD-fed mice triggered myeloid-derived CD45$^+$DDR2$^+$ cells to induce CD4$^+$ T cell IFN-γ and TNF-α production. Taken together, this study shows that myeloid-derived CD45$^+$DDR2$^+$ cells express markers of immune activation and suggests that they play an immune modulatory role in the adipose tissue of preobese mice.

1. Introduction

Obesity is a complex disease that contributes to the development of type 2 diabetes (T2D), cardiovascular disease, and various cancers [1–6]. An increase of 5 kg/m^2 in body mass index is associated with a 30% increase in all-cause mortality [4]. The pathology of obesity is multifold and includes aberrant insulin growth factor/insulin signaling, altered steroid production, and chronic systemic and local inflammation [4, 6]. However, the full view of immune dysfunction in obesity is unclear.

Mouse models of high-fat diet- (HFD-) induced obesity are typically characterized by at least a 30% increase in total body weight and closely mimic human disease [7–9]. C57BL/6 mice fed with a HFD *ad libitum* for 16-20 weeks exhibit adipocyte hyperplasia, increased fat mass, hypertension, and impaired glucose sensitivity leading to T2D [7, 10, 11]. Overall, less is known about the molecular and immune changes that occur before obesity is fully established. There is some evidence to suggest that short-term HFD feeding in mice results in hyperglycemia and changes in NK T cell and macrophage populations [12, 13]. The current study is focused on the inflammatory changes that occur in the adipose tissue of HFD-fed preobese mice, which are characterized by a 20% increase in total body weight and more closely represent an overweight, or preobese condition vs. obese condition [14].

In obesity, hypertrophied adipose tissue is comprised of a myriad of cell types, including adipocytes, preadipocytes, fibroblasts, and infiltrating immune cells. Previous studies have shown that monocyte-derived macrophages comprise a significant population in obese adipose tissue, where they become classically activated and skewed towards

a proinflammatory, "M1" phenotype [15, 16]. Obese adipose tissue-associated F4/80$^+$CD11c$^+$ "M1" macrophages produce inflammatory cytokines such as interleukin- (IL-) 12 and tumor necrosis factor- (TNF-) α and elicit the abnormal production of adipokines/cytokines such as leptin and IL-6 from surrounding adipocytes [15, 17–23]. This cycle of inflammation becomes self-sustaining and, over time, contributes to the reduced insulin sensitivity and metabolic dysfunction observed in patients with obesity and mouse models of obesity [24–27]. In addition to classically activated "M1" macrophages, populations of F4/80$^+$CD11c$^-$CD206$^-$ "M0" macrophages and alternatively activated F4/80$^+$CD11c$^-$CD206$^+$ "M2" macrophages have also been observed in obese adipose tissue, suggesting that the macrophage phenotype is highly heterogeneous [22, 28, 29]. Interestingly, in patients with obesity, adipose tissue is characterized by a large population of CD11c$^+$CD206$^+$ "M2"-like macrophages, which retain their remodeling capacity but also secrete proinflammatory cytokines and have been associated with insulin resistance [30]. Accumulating evidence suggests that the skewing of monocyte-derived macrophages in obese adipose tissue is a highly complex and diverse process that depends on a number of factors, including the stroma and metabolic signature (i.e., fatty acid accumulation) of the specific adipose depot, as well as the severity of obesity [22, 31, 32].

There is a growing appreciation for the role of T cells in the obese adipose tissue environment. Adipocytes and other stromal cell subsets in obese adipose tissue secrete proinflammatory mediators (e.g., IL-6, MCP-1) that directly activate and skew T cells, even before a dramatic increase in mature tissue macrophages is observed [17, 33–36]. Resultant production of interferon- (IFN-) γ, IL-17A, and IL-6 by adipose tissue-activated T cells impacts adipocytes, muscle cells, and hepatocytes by disrupting glucose and lipid homeostasis and contributing to insulin resistance [37–43]. In mice with HFD-induced obesity, increased T cells are evident in the adipose tissue and have been linked to abnormal glucose homeostasis [8, 37, 44, 45]. However, there remains a critical gap in knowledge of how CD4$^+$ T cells become skewed towards Th1 vs. Th17 subsets in adipose tissue and how this changes as obesity is established.

Our lab has previously identified a novel population of hematopoietic stem cell- (HSC-) derived discoidin domain receptor 2- (DDR2-) expressing cells called circulating fibroblast precursors (CFPs), defined by their expression of CD45 and DDR2. DDR2, a tyrosine kinase receptor that binds collagens I and III, is known for its role in cell migration and extracellular matrix sensing [46]. There is some evidence to suggest that DDR2 may also play a role in immune cell activation; its expression on a subset of myeloid-derived immune cells was shown to mediate activation and cytokine production [47]. Previous studies in our lab have shown that CD45$^+$DDR2$^+$ cells are a circulating progenitor population that home to normal tissue as well as pathological environments, including inflammatory tissue and tumor microenvironments [48–50]. Depending on the local milieu, CD45$^+$DDR2$^+$ cells can differentiate into a spectrum of cell types, including fibroblasts and immune cells [48–50]. While the fibroblastic phenotype of these cells has been studied, considerably less is known about the immunogenic potential of these cells. Recently, we showed that myeloid-derived CD45$^+$DDR2$^+$ cells isolated from lung tissue of mice with pulmonary fibrosis expressed high levels of CD80 and MHC II and were capable of stimulating an inflammatory T cell cytokine response [49]. This suggests that a subset of myeloid-derived CD45$^+$DDR2$^+$ cells may play a role in regulating the immune response in inflammatory tissue.

Given that myeloid-derived CD45$^+$DDR2$^+$ cells home to sites of inflammation and contribute to both myeloid and stromal populations, the present study sought to determine how HFD impacts on the contribution of myeloid-derived CD45$^+$DDR2$^+$ cells to adipose tissue and their role as immune modulators in the adipose tissue environment of HFD-fed mice. Specifically, we sought to determine how this cell population may be modulating adipose tissue-associated inflammation in a preobese setting, when immune-based therapies may be more effective. To model preobesity, C57BL/6 mice were fed with a milkfat-based HFD or normal diet (ND) *ad libitum* for 8-10 weeks, until HFD-fed mice reached a 20% increase in total body weight compared to ND-fed mice. Myeloid-derived CD45$^+$DDR2$^+$ cells and CD4$^+$ T cells from peripheral blood (PB), mammary gland-associated adipose tissue (MGAT), and visceral adipose tissue (VAT) were analyzed by flow cytometric analysis. We discovered that myeloid-derived CD45$^+$DDR2$^+$ cells were more activated in the adipose tissue of HFD-fed preobese mice, characterized by increased expression of MHC II and CD80. CD4$^+$ T cells in the adipose tissue of HFD-fed mice were preferentially skewed towards proinflammatory Th1- and Th17-type T cells. CD45$^+$DDR2$^+$ cells cultured in media conditioned by adipose tissue from HFD-fed mice elicited increased production of IFN-γ and TNF-α by CD4$^+$ T cells, suggesting that HSC-derived CD45$^+$DDR2$^+$ cells play a role in modulating T cell skewing in the adipose tissue environment of HFD-fed mice.

2. Materials and Methods

2.1. Mouse Model of HFD-Induced Preobesity. Six-week-old female C57BL/6 mice (B6.SJL-Ptprc^{a}Pepc^b/BoyJ, Jackson Laboratories, Bar Harbor, ME) were fed with a HFD (60.3%·kcal from milkfat) or normal diet (ND, 18%·kcal from fat) for 8-10 weeks, until HFD-fed mice exhibited a 20% increase in total body weight and were characterized as preobese (Envigo Teklad Diets, Madison, WI; Table S1). Body weight was recorded weekly. At endpoint, mice were euthanized and peripheral blood (PB) and mammary gland-associated adipose tissue (MGAT) and visceral adipose tissue (VAT) were collected. MGAT consisted of the subcutaneous adipose tissue surrounding the 4th mammary glands. Visceral adipose tissue (VAT) consisted of the adipose tissue in the gonadal region [51]. Mice were maintained at the Animal Research Facility of the Veterans Affairs Medical Center. The study was carried out in accordance with the principles of the Basel Declaration and was reviewed and approved by the Institutional Animal Care and Use Committee at the Ralph H. Johnson VA Medical Center.

2.2. Cell and Tissue Culture Medium. For adipose cell and tissue culture, Dulbecco's modified Eagle's medium nutrient mixture F-12 (DMEM-F12, Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA) was used. For CFP and $CD4^+$ T cell coculture, DMEM containing 10% FBS was used.

2.3. Spleen Processing and Isolation of $CD4^+$ T Cells. The spleens were harvested from 12-16-week-old control female C57BL/6 mice and homogenized in Hank's buffered saline solution (HBSS). Cells were passed through a 70 μm cell strainer and rinsed with HBSS and red blood cells were lysed (ACK Lysing Buffer; Lonza, Walkersville, MD, USA; 3 min). Splenocytes were washed twice with HBSS. $CD4^+$ T cells were isolated using a magnetic-based negative selection kit per manufacturer's instructions (Miltenyi Biotec Inc., Auburn, CA). Isolation purity of $CD4^+$ T cells based on flow cytometric analysis was 97.37%+0.24.

2.4. Blood and Adipose Tissue Processing for Flow Cytometric Analysis. Blood was incubated with PharmLyse (15 min, room temperature) and remaining white blood cells were washed 3X in phosphate-buffered saline (PBS). VAT and MGAT were dissected, finely minced into 1 mm pieces, and digested in PBS containing 1% bovine serum albumin (BSA) with 2 mg/ml type I collagenase (Sigma, St. Louis, Missouri) on a rotary shaker (100 rpm, 1 h, 37°C). Cells were filtered (40 μm), washed (10% FBS/DMEM-F12), and centrifuged (10,000 rpm, 5 min). The upper layer was decanted and the remaining stromal vascular fraction was washed in PBS, incubated with PharmLyse (10 min, room temperature), and washed in PBS before analysis.

2.5. Adipose Tissue Explant Culture. Dissected, minced VAT and MGAT were cultured in 10% FBS/DMEM-F12 media in inverted 25 mm^2 cell culture flasks using a modified organ culture method (37°C, 5% CO_2, 48 h) [52]. After 48 h of culture, fresh media was added and cells were cultured an additional 48 h. Supernatants were then collected for cytokine analysis and coculture experiments.

2.6. Flow Cytometric Analysis of $CD45^+DDR2^+$ Cells and T Cells. Antibodies and reagents used for flow cytometric analysis were from BD Biosciences, unless noted. Cells (1×10^6cells/tube) were washed, resuspended in 1 ml PBS with near-IR dead cell stain (1 : 1000, Invitrogen), and incubated (30 min, 4°C). After washing, cells were resuspended in 10 μl Fc block, containing anti-CD16/CD32 antibody in PBS (1 : 100, 10 min, 4°C). For $CD45^+DDR2^+$ cell analysis, cells were incubated (20 minutes, 4°C) with equal concentrations of the following antibodies or isotype controls: PE-CD45 (Clone 30-F11), APC-DDR2 (N-20, Santa Cruz Biotechnology, Dallas, TX), FITC-CD11b (M1/70), BV421-F4/80 (T45-2342), BV711-CD80 (16-10A1), and PE-Cy7-MHC II (M5/114.15.2, eBioscience, San Diego, CA). Cells were washed and resuspended in 250 μl stain buffer for analysis. For T cell analysis, cells were incubated with 1X cell stimulation cocktail (eBioscience, 5 h, 37°C). Golgi stop was added at the recommended dilution after 1 h of stimulation. Cells were processed as above and stained with equal concentrations of the following antibodies or isotype controls: FITC-CD3e (145-2C11) and APC-CD4 (RM4-5). After washing, cells were resuspended in 1 ml cold Cytofix and incubated (20 min, 4°C). Cells were washed, resuspended in 50 μl Perm/Wash Buffer, and incubated (30 min, room temperature) with equal concentrations of the following antibodies or isotype controls: BV421-IL-17A (TC11-18H10) and PE-Cy7-IFN-γ (XMG1.2). Cells were washed in Perm/Wash Buffer and resuspended in 400 μl stain buffer for analysis. Analysis was conducted using a BD Fortessa X-20 flow cytometer.

2.7. Coculture of $CD45^+DDR2^+$ Cells and $CD4^+$ T Cells. $CD45^+DDR2^+$ cells sorted from the peripheral blood of control mice were cultured at 1×10^4 cells/well in 96-well tissue culture plates precoated with anti-CD3ε (1 : 100, Clone 145-2C11, R&D Systems, Minneapolis, MN, USA) in media conditioned by MGAT from HFD- or ND-fed mice (1 : 4, 48 h, 37°C). After 48 h, adipose tissue-conditioned media was replaced with fresh 10% FBS/DMEM. Isolated $CD4^+$ T cells from control C57BL/6 mice were added at 1×10^5 cells/well for coculture (72 h, 37°C). Controls consisted of $CD4^+$ T cells or $CD45^+DDR2^+$ cells cultured alone in conditioned or fresh media. After 72 h of coculture, selected wells were stimulated with 1X cell stimulation cocktail (eBioscience, 5 h, 37°C). Supernatants were collected for cytokine analysis.

2.8. Cytometric Bead Array. The levels of IL-2, IL-4, IL-6, IFN-γ, TNF-α, IL-17A, and IL-10 were determined using a mouse Th1/Th2/Th17 cytometric bead array kit (BD Biosciences). Levels of MCP-1, GM-CSF, G-CSF, MIP-1α, MIP-1β, MIG, and RANTES were determined using cytometric bead array flex sets. Relative amounts of each cytokine/chemokine were analyzed using a FACSCanto (BD Biosciences) flow cytometer and FCAP Array Software (Soft Flow Hungary Ltd. for BD Biosciences, St. Louis Park, MN, USA).

2.9. Statistical Analyses. Data were reported using the mean as a measure of central tendency ± standard error of the mean. To compare one variable condition between 2 groups, a 2-tailed Student *t*-test was used. To compare one variable condition between 3 or more groups, a one-way ANOVA with Bonferroni correction was used (GraphPad Prism version 5.03). Significance was reported in the 95% confidence interval.

3. Results and Discussion

3.1. Increased Total Body Weight and Expansion of MGAT and VAT in Female C57BL/6 Mice Fed with a HFD or ND for 8-10 Weeks. A diet high in milkfat (60%·kcal) results in consistent weight gain and increased adipose tissue in C57BL/6 mice [8, 11]. Most previous studies have focused on the long-term metabolic and inflammatory impacts of this diet, at 12-16 weeks and beyond, when mice were defined as obese and exhibited at least a 30% increase in total body weight compared to control mice [7–9]. To determine the more immediate impacts of HFD, our study focused on a shorter time point, 8-10 weeks, when mice exhibited a 20%

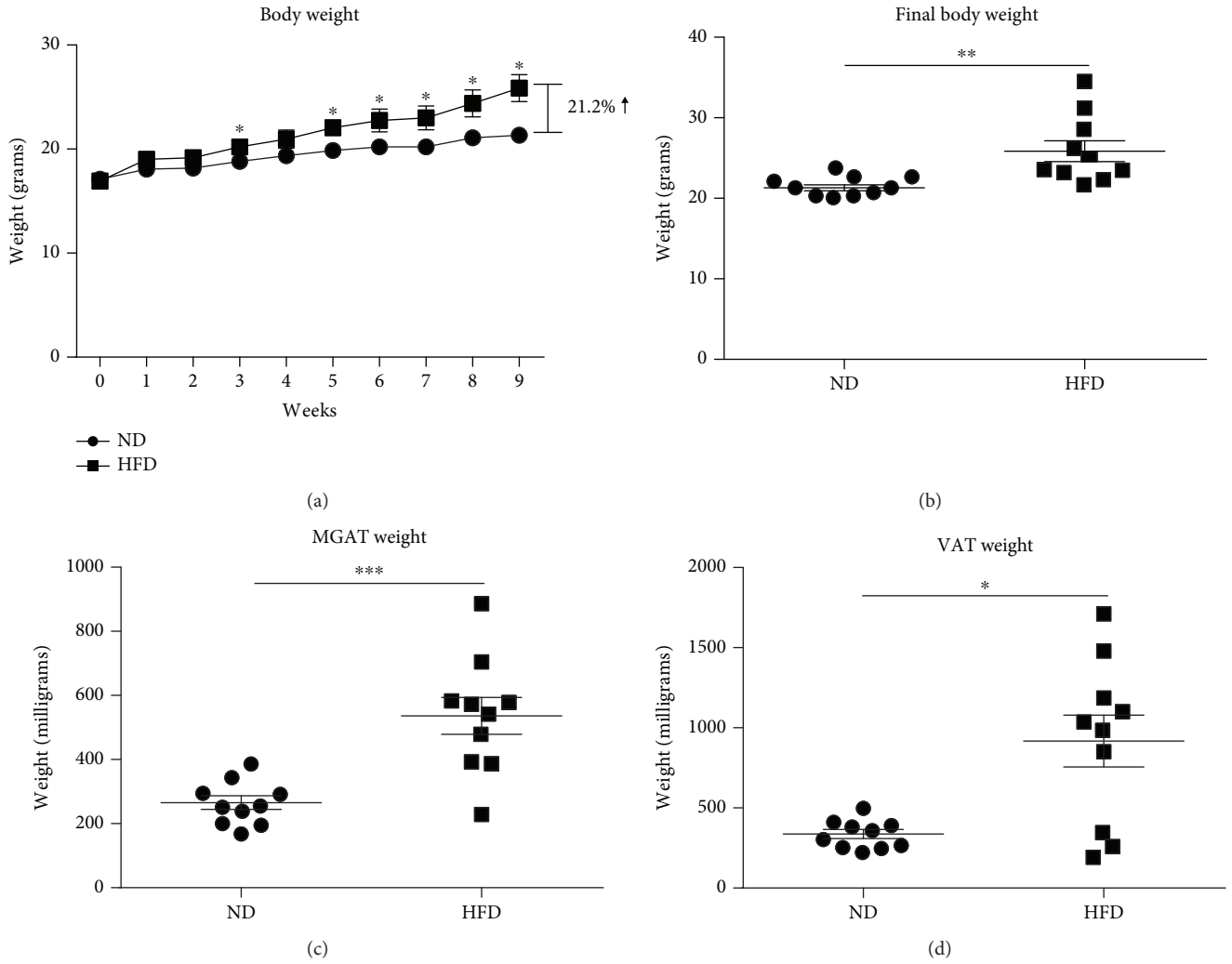

Figure 1: Increased overall body weight and adipose tissue in mice fed with a high-fat diet for 9 weeks compared to lean mice. (a) Total body weight of female C57BL/6 mice fed with a high-fat diet (HFD, 60%·kcal from milkfat) or normal diet (ND) over 9 weeks. (b) Graphical representation of total body weight of HFD- or ND-fed mice at endpoint, when HFD-fed mice reached a ~20% increase in total body weight compared to ND-fed mice. (c, d) Total weight of mammary gland-associated adipose tissue (MGAT) or visceral adipose tissue (VAT) isolated from HFD- or ND-fed mice at endpoint. Data are presented as mean ± SEM of 10 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

increase in total body weight compared to control mice, to reflect a preobese condition. As shown in Figure 1(a), HFD-fed mice gained more weight than ND-fed mice over 9 weeks. This difference remained statistically significant 5 weeks of postinitiation of diet. At 9 weeks of postinitiation of diet, the mean total body weight of HFD-fed mice was 21.2% greater than that of ND-fed mice (Figure 1(b)), indicating that the preobese condition (20% increase in total body weight) had been met and signifying study endpoint. Examination of adipose tissue showed that HFD-fed, preobese mice were characterized by increased MGAT and VAT by weight at study endpoint (Figures 1(c) and 1(d)).

3.2. Increased Percentage of Myeloid-Derived CD45^{+}DDR2^{+} Cells Express MHC II and CD80 in MGAT and VAT of Preobese Mice Compared to Lean Mice. Previous studies in our lab have shown that myeloid-derived CD45^{+}DDR2^{+} cells home to pathological environments, where they differentiate into fibroblasts and other stromal subsets [48, 50]. Recently, myeloid-derived CD45^{+}DDR2^{+} cells were also shown to express markers of immune activation, including CD80 and MHC II, in an inflammatory lung model [49]. These findings led us to examine if a subset of CD45^{+}DDR2^{+} cells contributes to inflammation in the adipose tissue of preobese mice. This was accomplished by isolating and phenotyping myeloid-derived CD45^{+}DDR2^{+}cells from MGAT, VAT, and PB of HFD- vs. ND-fed mice. Both MGAT and VAT were analyzed to provide a more complete view of the phenotype and activation status of CD45^{+}DDR2^{+} cells in a local, subcutaneous adipose tissue environment (MGAT) as well as a central, gonadal adipose tissue environment (VAT) [51, 53].

Myeloid-derived CD45^{+}DDR2^{+} cells were present in both MGAT and VAT, although there was no difference in overall percentage in MGAT (Figures 2(a) and 2(d)), VAT (Figures 2(b) and 2(d)), or PB (Figures 2(c) and 2(d))

between HFD- and ND-fed mice. However, because total fat pad size was increased in HFD-fed mice, we observed an increase in total $CD45^+DDR2^+$ cells in MGAT of HFD-fed mice compared to ND-fed mice (Figure S2). In both MGAT and VAT, a large subset of $CD45^+DDR2^+$ cells expressed CD11b, suggesting that these cells are of the myeloid lineage. The percentage of CD11b-expressing $CD45^+DDR2^+$ cells in MGAT, VAT, or PB was not altered by HFD (Figure 2(d)). However, the total number of $CD45^+DDR2^+$ cells expressing CD11b was increased in MGAT of HFD-fed vs. ND-fed mice (Figure S2). Of note, in PB of both HFD- and ND-fed mice, the majority of $CD45^+DDR2^+$ cells expressed CD11b (Figures 2(c) and 2(d)), suggesting that they represent a progenitor population close to the monocyte lineage.

A higher percentage of myeloid-derived $CD45^+DDR2^+$ cells expressed markers of immune activation in the adipose tissue of HFD-fed mice. In both MGAT and VAT, an increased percentage of myeloid-derived $CD45^+DDR2^+$ cells expressed MHC II and CD80 compared to levels in adipose tissue of ND-fed mice (37.0% vs. 21.6%, $^*p = 0.0375$ and 41.0% vs. 24.2%, $^{***}p = 0.0002$, Figure 2(d)). The total number of myeloid-derived $CD45^+DDR2^+$ cells expressing MHC II and CD80 in MGAT of HFD-fed was also increased compared to what was observed in ND-fed mice (Figure S2). Analysis of the mean fluorescence intensity (MFI) of activation marker expression showed that MHC II expression on myeloid-derived $CD45^+DDR2^+$ cells was increased in both MGAT and VAT of HFD-fed mice compared to ND-fed mice ($^*p = 0.0169$ and $^{***}p < 0.001$, Figure 2(d)), indicating that MHC II was more highly expressed on this population in the adipose tissue of HFD-fed mice. The MFI of CD80 expression on myeloid-derived $CD45^+DDR2^+$ cells was also increased in MGAT and VAT of HFD-fed mice compared to ND-fed mice, although not statistically significant in MGAT ($p = 0.0923$ and $^{**}p = 0.0048$, respectively). Overall, these data suggest that the myeloid-derived $CD45^+DDR2^+$ cell population may be skewed towards an activated immune cell phenotype in the adipose tissue of HFD-fed mice, characterized by increased expression of markers of antigen presentation and activation.

There was no statistically significant difference in the percentage of myeloid-derived $CD45^+DDR2^+$ cells expressing MHC II or CD80 in PB of HFD- vs. ND-fed mice, which was not surprising given that $CD45^+DDR2^+$ cells are a circulating progenitor population. However, the MFI of CD80 expression on myeloid-derived $CD45^+DDR2^+$ cells in PB of HFD-fed mice was increased compared to myeloid-derived $CD45^+DDR2^+$ cells in ND-fed mice ($^*p = 0.0184$, Figure 2(d)), suggesting that a subset of these cells may become activated even before reaching adipose tissue.

Analysis of myeloid-derived $CD45^+DDR2^-$ cells in MGAT of HFD-fed mice showed that these cells were not as activated as the $DDR2^+$ subset, characterized by significantly lower levels of expression of both MHC II and CD80. In MGAT of HFD-fed mice, the MFI of MHC II expression on myeloid-derived $CD45^+DDR2^-$ cells was significantly lower than that of $DDR2^+$ cells (1124 vs. 6279, $^{***}p = 0.0002$) and was not significantly different than what was observed in ND-fed mice ($p = 0.7766$, Figure S3). The MFI of CD80 expression of myeloid-derived $CD45^+DDR2^-$ cells in MGAT of HFD-fed mice was also significantly lower than that of $DDR2^+$ cells (382.2 vs. 667.2, $^{***}p = 0.0005$, Figure S3).

As was seen in MGAT, $CD45^+DDR2^-$ cells in VAT and PB of HFD-fed mice were not as highly activated as $CD45^+DDR2^+$ cells. In VAT of HFD-fed mice, the expression of both MHC II and CD80 on $CD45^+DDR2^-$ cells was significantly lower than that of $DDR2^+$ cells (1290 vs. 8040, $^{***}p < 0.0001$ and 376.4 vs. 718.4, $^{***}p < 0.0001$). As was observed in MGAT, the levels of MHC II and CD80 expression on $CD45^+DDR2^-$ cells in HFD-fed mice were not significantly different than the levels observed on $CD45^+DDR2^-$ cells in ND-fed mice ($p = 0.1508$ and $p = 0.2586$, Figure S3). In PB of HFD-fed mice, the MFI of both MHC II and CD80 expression on $CD45^+DDR2^-$ cells was lower than what was observed on $CD45^+DDR2^+$ cells (169.4 vs. 280.8, $p = 0.0653$ and 182.8 vs. 254.0, $^{***}p < 0.0001$, Figure S3). Overall, myeloid-derived $CD45^+DDR2^-$ cells were not as highly activated as the $CD45^+DDR2^+$ subset in the adipose tissue or PB, and more importantly, do not exhibit increased activation in the adipose tissue of HFD-fed mice.

Taken together, these data show that myeloid-derived $CD45^+DDR2^+$ cells are a component of adipose tissue that become highly activated in HFD-fed, preobese mice and suggest that this cell subset contributes to the inflammatory adipose tissue environment.

3.3. Increased Percentage of T Cells Produce IL-17A in MGAT and PB of Preobese Mice Compared to Lean Mice. Adipose tissue of obese mice is inflammatory and marked by the infiltration of activated immune cells, including macrophages and Th1-type cells [15, 44]. The contribution of other proinflammatory T cell subsets, including Th17 cells, to adipose tissue in obese or preobese mice has not been well defined. To determine how $CD4^+$ T cells are skewed in the adipose tissue and periphery of preobese mice, IL-17A and IFN-γ expression in T cells isolated from MGAT, VAT, and PB of HFD- vs. ND-fed mice was analyzed.

The percentage of $CD3e^+$ T cells in VAT of HFD-fed mice was increased compared to levels in ND-fed mice (39.6% vs. 28.3%, $^{**}p = 0.0049$, Figure 3(b)), but this difference was not seen in MGAT or peripheral blood. An increased percentage of total $CD3e^+CD4^+$ T cells was also observed in VAT of HFD-fed mice ($^*p = 0.0126$, Figure 3(c), Figure S4). No difference in the percentage of total $CD3e^+CD4^+$ T cells was observed in MGAT or peripheral blood of HFD-fed vs. ND-fed mice.

Analysis of T cell subsets showed an increased percentage of $CD4^+$ T cells expressing IL-17A in MGAT of HFD-fed mice compared to $CD4^+$ T cells in MGAT of ND-fed mice (18.3% vs. 5.5%, $^{**}p = 0.0023$, Figure 3(d)). Although a large percentage of $CD4^+$ T cells expressed IFN-γ in MGAT, no difference was observed between HFD- and ND-fed mice (Figure 3(e)). A small population of $CD4^+$ T cells in MGAT expressed both IL-17A and IFN-γ, but no difference was observed between groups (Figure 3(f)).

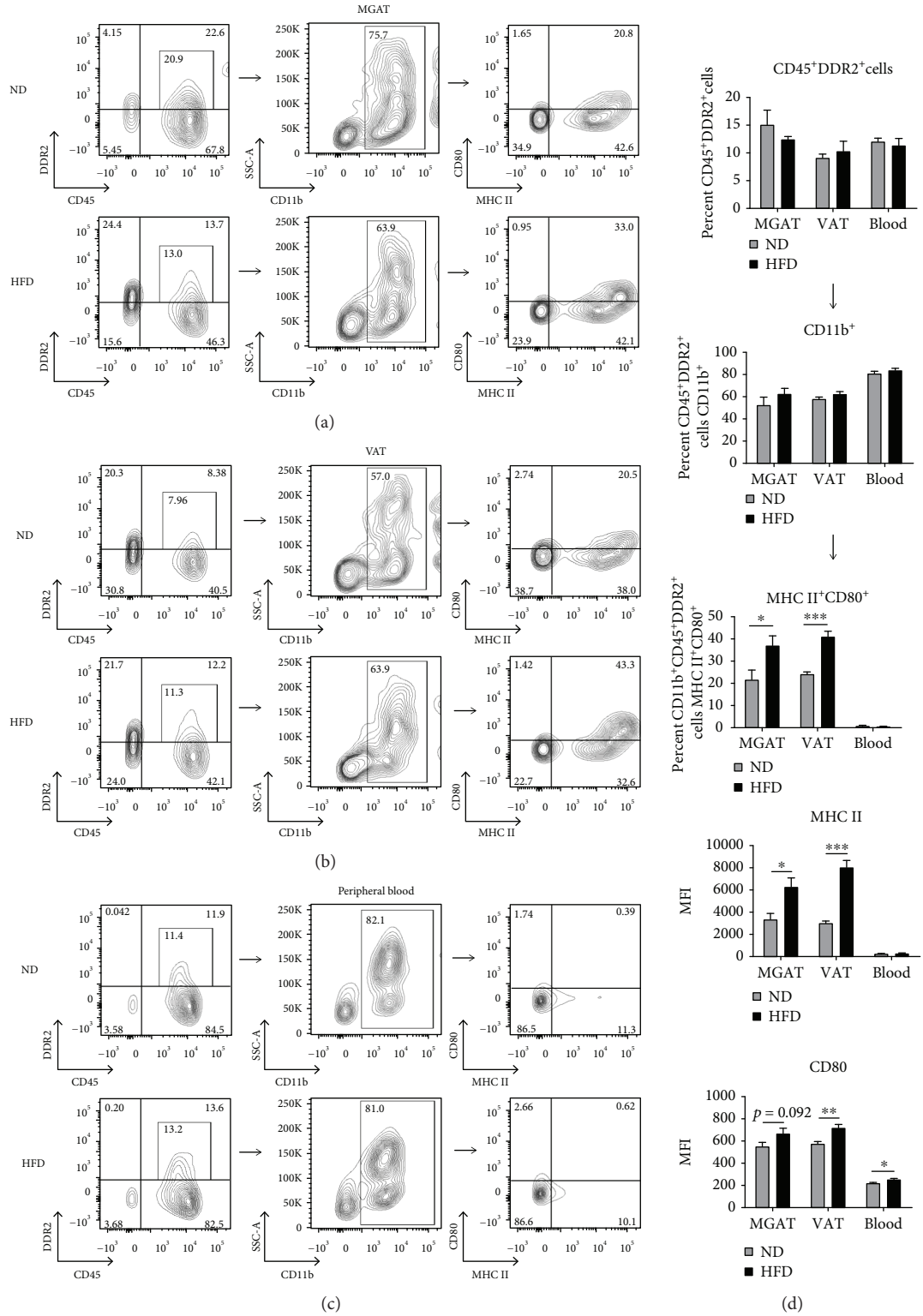

Figure 2: Increased percentage of myeloid-derived $CD45^+DDR2^+$ cells express MHC II and CD80 in mammary gland-associated adipose tissue (MGAT) and visceral adipose tissue (VAT) of preobese mice compared to myeloid-derived $CD45^+DDR2^+$ cells in MGAT and VAT of lean control mice. (a–c) Flow cytometric analysis of a representative mouse from each group, showing MHC II and CD80 expression in CD11b-expressing $CD45^+DDR2^+$ cells from MGAT, VAT, and peripheral blood (PB) of HFD- and ND-fed C57BL/6 mice at endpoint (9 weeks of postinitiation of diet). For flow cytometric analysis, cells were first gated on single, live cell populations. (d) Graphical representation of flow cytometric analysis, showing the percentage of total $CD45^+DDR2^+$ cells, CD11b-expressing $CD45^+DDR2^+$ cells, and MHC II and CD80 expression on CD11b-expressing $CD45^+DDR2^+$ cells in MGAT, VAT, and PB of HFD- vs. ND-fed C57BL/6 mice. Data are presented as mean ± SEM of 5 mice per group. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

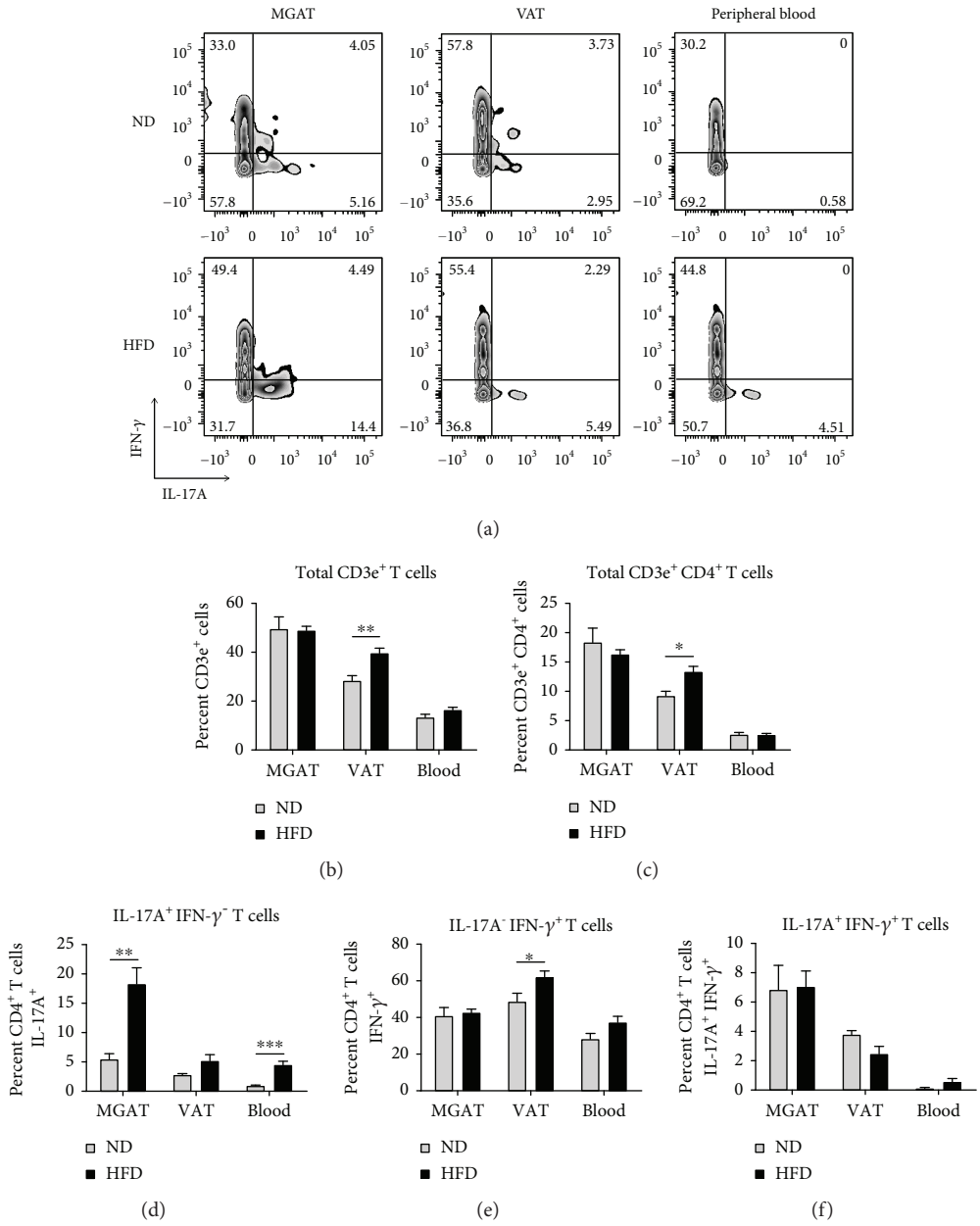

Figure 3: Increased percentage of $CD4^+$ T cells express IL-17A in MGAT and blood of preobese mice compared to $CD4^+$ T cells in MGAT and blood of lean control mice. (a) Flow cytometric analysis of a representative mouse from each group, showing IL-17A and IFN-γ cytokine expression in $CD3e^+CD4^+$ T cell populations from MGAT, visceral adipose tissue (VAT), and peripheral blood (PB) of HFD- and ND-fed C57BL/6 mice at endpoint (9 weeks of postinitiation of diet). Cells were stimulated with PMA/ionomycin cocktail + protein transport inhibitor for 5 h at 37°C prior to fixation, permeabilization, and staining. For flow cytometric analysis of cytokine expression, cells were first gated on single, live, $CD3e^+$, and $CD4^+$ T cell populations (Figure S4). (b–f) Graphical representation of the percentage of total $CD3e^+$ T cells, total $CD3e^+CD4^+$ T cells, $IL\text{-}17A^+CD4^+$ T cells, IFN-γ^+CD4^+ T cells, and $IL\text{-}17A^+$IFN-γ^+CD4^+ T cells in MGAT, VAT, and PB of HFD- vs. ND-fed mice. Data are presented as mean ± SEM of 5 mice per group. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

In VAT of HFD-fed mice, an increased although not statistically significant percentage of $CD4^+$ T cells expressed IL-17A compared to ND-fed mice, and the total population of IL-17A-expressing cells was lower compared to levels in MGAT ($p = 0.0605$, Figure 3(d)). VAT of HFD-fed mice was characterized by an increased percentage of IFN-γ-expressing $CD4^+$ T cells compared to ND-fed mice (62.0% vs. 48.6%, $^*p = 0.0483$, Figures 3(a) and 3(e)). A small population of $CD4^+$ T cells expressed both IL-17A and IFN-γ in VAT, but no difference was observed between HFD- and ND-fed mice (Figure 3(f)). In PB, an increased percentage of $CD4^+$ T cells expressed IL-17A in HFD- vs. ND-fed mice ($^{***}p < 0.0004$, Figure 3(d)). A large proportion of $CD4^+$ T cells also expressed Th1-associated IFN-γ, although no stastically significant difference between HFD- and ND-fed mice was observed ($p = 0.0788$, Figure 3(e)).

Together, these data show that a greater percentage of $CD4^+$ T cells is stimulated in the adipose tissue and PB of

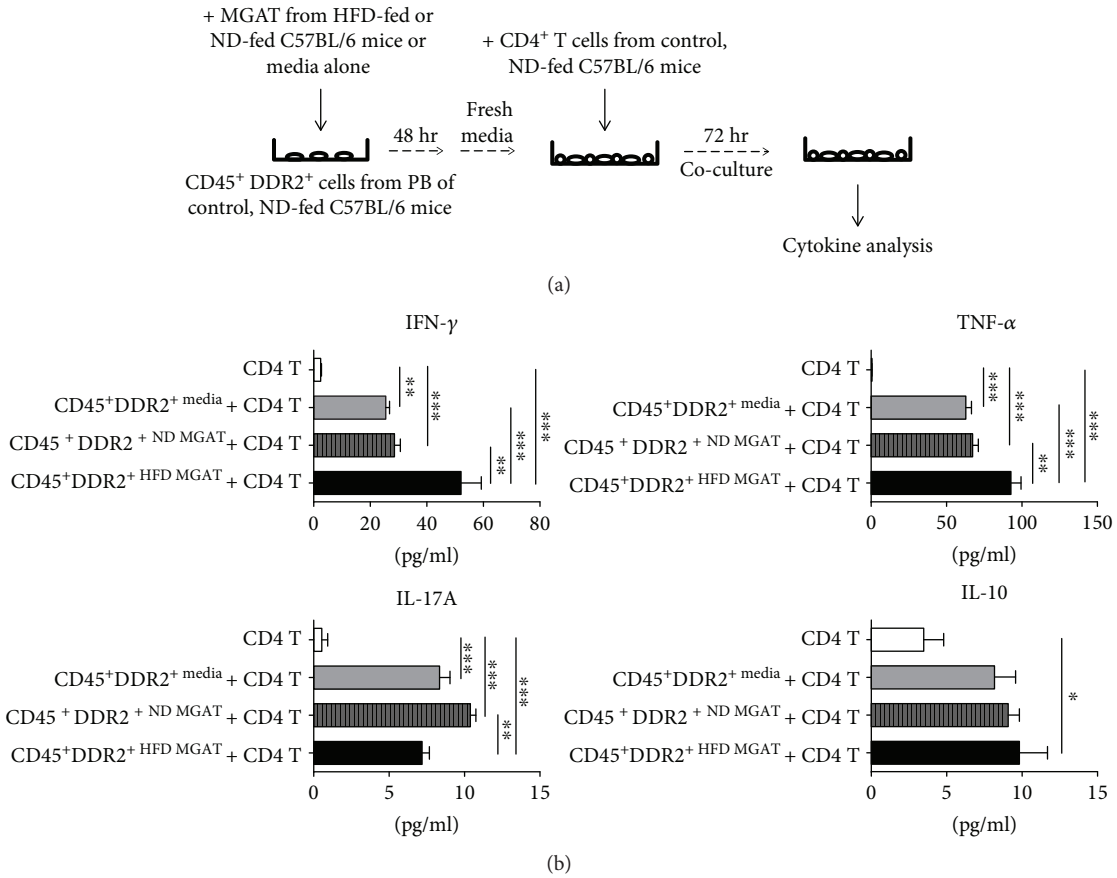

Figure 4: MGAT from HFD-fed mice triggers $CD45^+DDR2^+$ cells to elicit increased production of IFN-γ and TNF-α from $CD4^+$ T cells. (a) Experimental overview: sorted $CD45^+DDR2^+$cells from peripheral blood of control, ND-fed C57BL/6 mice were cultured in media conditioned by MGAT from HFD- or ND-fed mice at 1×10^4 cells/well for 48 h at 37°C. For T cell coculture, media was replaced and splenic $CD4^+$ T cells from control, ND-fed C57BL/6 mice were added to $CD45^+DDR2^+$ cell cultures at 1×10^5 cells/well for 72 h. Supernatants were collected and levels of Th1-, Th2-, and Th17-associated cytokines were analyzed by cytometric bead array. (b) Graphical representation of levels of IFN-γ, TNF-α, IL-17A, and IL-10 detected in supernatants. Data are presented as mean ± SEM of triplicate wells analyzed in duplicate. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

HFD-fed preobese mice. In MGAT and PB, the increase in levels of $CD4^+$ T cells expressing IL-17A suggests that they are skewed towards a Th17 phenotype. In VAT, the increase in levels of $CD4^+$ T cells expressing IFN-γ suggests that they are skewed towards a Th1 phenotype.

3.4. MGAT from HFD-Fed Mice Triggers $CD45^+DDR2^+$ Cells to Activate T Cell Production of IFN-γ and TNF-α. Previously, we showed that a subset of activated $CD45^+DDR2^+$ cell shaves the capacity to skew $CD4^+$ T cells towards a Th17 phenotype [49]. To determine if adipose tissue from HFD-fed mice triggers $CD45^+DDR2^+$ cells to stimulate T cell cytokine production, sorted $CD45^+DDR2^+$ cells from peripheral blood of control, ND-fed mice were primed in media conditioned by MGAT from HFD- or ND-fed mice and then cultured with $CD4^+$ T cells from control, ND-fed mice. Following coculture, Th1, Th2, and Th17 cell-associated cytokines produced by $CD4^+$ T cells were analyzed.

$CD45^+DDR2^+$ cells cultured in media conditioned by MGAT from HFD-fed mice ($CD45^+DDR2^{+HFD/MGAT}$) stimulated increased production of the Th1-associated cytokine IFN-γ from $CD4^+$ T cells compared to $CD45^+DDR2^+$ cells cultured in media conditioned by adipose tissue from ND-fed mice ($CD45^+DDR2^{+ND/MGAT}$, $^{**}p < 0.01$) or media alone ($CD45^+DDR2^{+media}$, $^{***}p < 0.001$, Figure 4). $CD45^+DDR2^{+HFD/MGAT}$ also stimulated increased production of the proinflammatory mediator TNF-α from $CD4^+$ T cells compared to $CD45^+DDR2^{+ND/MGAT}$($^{**}p < 0.01$) or CFP^{media} ($^{***}p < 0.001$). These data suggest that $CD45^+DDR2^{+HFD/MGAT}$ skew T cell cytokine production and promote a Th1-type, inflammatory response.

In contrast, $CD45^+DDR2^{+ND/MGAT}$ stimulated increased production of the Th17-associated cytokine IL-17A from $CD4^+$ T cells compared to $CD45^+DDR2^{+HFD/MGAT}$ ($^{**}p < 0.01$, Figure 4). However, it is important to note that both $CD45^+DDR2^{+HFD/MGAT}$ and $CD45^+DDR2^{+media}$ stimulated increased IL-17A production from $CD4^+$ T cells compared to $CD4^+$ T cells cultured in the absence of $CD45^+DDR2^+$ cells ($^{***}p < 0.001$). Taken together, this data shows that $CD45^+DDR2^+$ cells alone elicit production of inflammatory IL-17A from $CD4^+$ T cells, although to a lesser degree than IFN-γ *in vitro*.

Since IL-10 is a known immune inhibitory mediator, its expression was also measured to more completely assess the

immune regulatory capacity of $CD45^+DDR2^+$ cells. There were no statistically significant differences in the production of Th2 cell-associated cytokine IL-10 by $CD4^+$ T cells cultured with $CD45^+DDR2^{+HFD/MGAT}$ vs. $CD45^+DDR2^{+ND/MGAT}$, although $CD45^+DDR2^{+HFD/MGAT}$ elicited more IL-10 production from $CD4^+$ T cells compared to T cells cultured in the absence of $CD45^+DDR2^+$ cells ($^*p < 0.05$). Overall, the levels of IL-10 produced by T cells were significantly lower than the levels of IFN-γ and TNF-α. Taken together, the data show that $CD45^+DDR2^+$ cells preconditioned in the HFD MGAT environment preferentially skew T cells towards a proinflammatory phenotype.

As a negative control, $CD45^+DDR2^+$ cells were cultured alone in fresh media or media conditioned by adipose tissue from HFD- or ND-fed mice; there were no detectable levels of Th1, Th2, or Th17 cell-associated cytokines in these cultures (data not shown). We found that $CD45^+DDR2^+$ cells primed in media conditioned by VAT from HFD mice stimulated T cell cytokine production of IFN-γ and TNF-α, but to a lesser degree (~8- and ~2-fold less, respectively, data not shown), suggesting that $CD45^+DDR2^+$ cells differentially modulate T cells in different adipose environments and that these cells may play a more significant proinflammatory role in MGAT of HFD-fed preobese mice. Overall, our data suggest that $CD45^+DDR2^+$ cells exposed to MGAT of HFD-fed mice preferentially skew T cells towards a proinflammatory, Th1-type phenotype.

4. Conclusions

To our knowledge, this is the first study examining $CD45^+DDR2^+$ cells in the adipose tissue of HFD-fed mice at the preobese stage and suggests a novel role for myeloid-derived $CD45^+DDR2^+$ cells in modulating the inflammatory immune response in adipose tissue.

The expression of DDR2 on immune cells and the role it plays in immune cell function has not been well defined. DDR2, a tyrosine receptor kinase, binds collagens I and III and is traditionally involved in extracellular matrix sensing and cell migration [46]. Previous studies in human vascular smooth muscle cells and mouse fibroblasts showed that DDR2 regulates expression and/or activation of MMP-1 and MMP-2, respectively [54, 55]. We have previously shown that HSC-derived $DDR2^+$ cells home to tumor via the CCR2/MCP-1 axis and are capable of differentiating into fibroblasts in the local tumor environment [48]. However, the immune phenotype of $CD45^+DDR2^+$ cells has not been extensively investigated. One previous study showed that DDR2 is expressed on a subset of dendritic cells and that binding of collagen I to DDR2 leads to increased expression of the activation marker CD86 and increased production of IL-12, a proinflammatory cytokine involved in Th1-type skewing [47]. This suggests that DDR2 expression on a subset of myeloid-derived immune cells may play a role in activation and inflammatory cytokine production in these cells. Our previous work in a mouse model of silica-induced pulmonary fibrosis showed that a subset of myeloid-derived $CD45^+DDR2^+$ cells honed to lung tissue, expressed markers of immune activation, and were capable of skewing T cell cytokine production [49]. These prior studies suggest that myeloid-derived $CD45^+DDR2^+$ cells play a role in modulating the immune response and led us to evaluate their role in promoting inflammation in the preobese adipose tissue environment.

Many models of HFD-induced obesity consider 16-20 weeks of feeding as study endpoint, when mice are characterized as obese and exhibit at least a 30% increase in total body weight [7–9, 11]. Our study is focused on immune changes that are occurring much earlier in the progression to obesity—what we are characterizing as a preobese state—when mice exhibit a 20% increase in total body weight compared to ND-fed mice. As defined in the current study, HFD-fed preobese mice may more closely mirror the overweight condition in human patients [14] and may also offer an ideal model to study immune-based therapies. In the current study, C57BL/6 mice were fed with a diet high in milkfat (60.3% kcal from fat, Table S1), as this diet has been shown to induce metabolic, cardiac, and inflammatory changes in C57BL/6 mice that closely mirror what is observed in obese patients [56–59]. This milkfat-based diet was shown to induce metabolic and inflammatory changes in C57BL/6 mice at earlier time points than a lard-based diet (8 weeks vs. 20 weeks and beyond), suggesting that it is ideal for the current preobese model [56, 60]. It is important to note that HFD feeding has differential effects on weight gain, metabolism, and inflammation in male vs. female mice [61–63]. However, many of these studies utilized lard- or soybean-based HFDs, and overall less is known about the effects of a milkfat-based HFD. We chose to focus on female mice in the current study to provide a baseline for future work examining the role of myeloid-derived $CD45^+DDR2^+$ cells in obesity-associated breast cancer. We have previously observed changes in circulating IL-6 levels and insulin and glucose metabolism in female C57BL/6 mice following 12 weeks of milkfat-based HFD feeding, although to a lesser extent than what was observed in male mice (data not published). The effects of a milkfat-based HFD on $CD45^+DDR2^+$ cell activation and T cell skewing at the preobese state (after ~8 weeks of feeding) have not been previously described and are the focus of the current study.

In our preobese model, $CD45^+DDR2^+$ cells and T cells were examined in two adipose tissue regions: VAT and MGAT. Previous reports have shown that VAT from the gonadal region of obese mice, which often represents the largest and most accessible fat pad, is characterized by elevated oxidative stress, altered glucose metabolism, and increased expression of inflammatory markers [53, 64–67]. There is growing evidence to suggest that MGAT in overweight or obese patients is characterized by increased levels of inflammatory immune cells and adipocytes, which have been shown to contribute to the development and progression of breast cancer [68–72]. Given that considerably less is known about the immune alterations that occur in subcutaneous MGAT in HFD-fed preobese mice, the current study sought to address this question.

In control mice, we found that $CD45^+DDR2^+$cells are present in both MGAT and VAT. While the overall percentage of myeloid-derived $CD45^+DDR2^+$ cells in adipose tissue

was unchanged in HFD-fed preobese mice, a significantly higher percentage of myeloid-derived $CD45^+DDR2^+$ cells expressed MHC II and CD80 in both MGAT and VAT. Increased expression of MHC II, which presents antigen/-peptide to a T cell via the T cell receptor, and CD80, a costimulatory molecule that binds to CD28 on an interacting T cell, are characteristics of an activated antigen-presenting cell [73–75]. In the adipose tissue of HFD-fed preobese mice, myeloid-derived $CD45^+DDR2^+$ cells were characterized by significantly increased expression of both MHC II and CD80 compared to myeloid-derived $CD45^+DDR2^+$ cells in the adipose tissue of ND-fed mice, indicating that these cells were more activated in the HFD-fed adipose environment.

Changes in the activation of myeloid-derived $CD45^{+-}DDR2^+$ cells were also observed in PB of HFD-fed mice, although to a lesser extent than what was observed in adipose tissue. Previous studies have shown that $CD45^+DDR2^+$ cells differentiate into activated fibroblasts and immune cells in inflammatory tissue environments (i.e., tumor, inflammatory lung) [48–50]. In the current study, the intensity of CD80 expression was increased on myeloid-derived $CD45^+DDR2^+$ cells in the peripheral blood of HFD-fed mice, demonstrating that a subset of this population expresses markers of activation even before they reach the adipose tissue. Thus, at least in a model of preobesity, the activation status of myeloid-derived $CD45^+DDR2^+$ cells may be a useful biomarker for immune activation in the progression to obesity. Further, as they are a circulating progenitor population, $CD45^+DDR2^+$ cells may represent an important target for therapies aimed at reducing inflammation by redirecting their skewing away from an activated immune state.

Analysis of myeloid-derived $CD45^+DDR2^-$ cells in the adipose tissue of HFD-fed mice showed that these cells were not as highly activated as the $DDR2^+$ population; furthermore, the extent of activation of $DDR2^-$ cells, as defined by the MFI of MHC II and CD80 expression, was not altered in HFD-fed mice vs. ND-fed mice (Figure S3). Taken together, our data show that myeloid-derived $CD45^+DDR2^+$ cells are highly activated in HFD-fed preobese mice and suggest that a subset of these cells may be modulating the inflammatory immune response before obesity is established.

Future studies will focus on defining the differentiation potential of myeloid-derived $CD45^+DDR2^+$ cells in the adipose tissue of HFD-fed preobese mice, using the expression of macrophage, dendritic cell, and fibroblast markers. It will be important to compare the progenitor $CD45^+DDR2^+$ cells presented herein to traditional monocyte-derived macrophages in the adipose tissue and blood of preobese vs. control mice, based on expression of specific macrophage markers such as F4/80, CD11c, CD64, CD206, and Ly6c. Previous studies have shown that the skewing of monocyte-derived macrophages in obese adipose tissue is a highly diverse process and results in the differentiation of both $F4/80^+CD11c^+$ "M1"-like macrophages as well as $F4/80^+CD11c^-CD206^+$ "M2"-like macrophages [15, 22, 28, 29]. Previous studies in our lab have shown that a subset of myeloid-derived $CD45^{+-}DDR2^+$ cells migrates in response to monocyte-derived chemoattractant protein- (MCP-) 1 [48] and express CD11c in inflammatory lung tissue [49]. It will be important to determine if the myeloid-derived $CD45^+DDR2^+$ cells presented herein contribute to "M1" or "M2" macrophage populations, or a unique macrophage population in adipose tissue, and how this changes as obesity is established. Preliminary analysis of F4/80 expression on myeloid-derived $CD45^+DDR2^+$ cells in MGAT of HFD-fed, preobese mice has shown ~61% of this population expresses F4/80 and was not statistically different than what was observed in ND-fed mice (data not shown). Together, these data suggest that a subset of $CD45^+DDR2^+$ cells differentiates into macrophages or macrophage-like cells in tissue and may represent a progenitor population from which activated tissue macrophages arise. Further defining how $CD45^+DDR2^+$ cells differentiate into immune cell subsets in inflammatory tissue will help guide future studies aimed at targeting this circulating progenitor population.

T cell skewing in adipose tissue contributes to inflammation and may also impact obesity-related metabolic dysfunction. In patients with obesity, adipose tissue-associated and circulating T cells are preferentially skewed towards inflammatory Th1- and/or Th17-type T cells and studies have shown that IFN-γ and IL-17A contribute to aberrant glucose and lipid homeostasis in several cell types, including adipocytes [38–40, 76]. In the current study, we show that changes in total T cells and T cell skewing are evident in HFD-fed preobese mice, which are characterized by a 20% increase in total body weight. In both MGAT and PB of HFD-fed mice, the increase in $CD4^+$ T cells expressing IL-17A suggests skewing towards a proinflammatory Th17 phenotype. In MGAT of HFD-fed mice, we found 3.4X IL-17A-expressing $CD4^+$ T cells compared to MGAT of ND-fed mice, paralleling what is observed in the subcutaneous adipose tissue of patients with obesity-associated T2D [39]. To our knowledge, this is the first report showing increased IL-17A-expressing cells in MGAT of HFD-fed preobese mice and suggests that our model may be a clinically relevant tool for investigating immune alterations associated with the overweight, or preobese, state. In VAT of preobese mice, we observed an increase in $CD4^+$ T cells expressing IFN-γ, suggesting a skewing towards a Th1 phenotype. This observation supports previous studies which showed a preferential skewing of $CD4^+$ T cells towards Tbet-expressing Th1-type cells in VAT of patients with obesity and HFD-induced mouse models of obesity [36, 43, 77]. Of note, an $IL\text{-}17A^+IFN\text{-}\gamma^+$ T cell population was observed in both MGAT and VAT and may represent a T cell in transition from a Th17 to Th1 phenotype or a highly activated Th17 cell [78]. Future studies examining the expression of transcription factors such as RORγt and Tbet in adipose tissue-associated T cells may further delineate these populations. Overall, the data show a differential skewing of T cells in different adipose tissue environments and suggest that HFD promotes both Th1- and Th17-type skewing, even before obesity is established.

While our studies focused on the impact of HFD in female animals, given the sex-specific response to HFD feeding [61–63], it will be important to investigate $CD45^+DDR2^+$ and T cell populations in the peripheral blood and adipose tissue of preobese, male C57BL/6 mice in future studies. There is evidence to suggest that total T cells are also

increased in the adipose tissue of HFD-fed, male mice, although reports of how $CD4^+$ T cells are skewed in specific adipose depots, particularly at earlier time points, are limited. One study showed that the percentage of total $CD3e^+$ T cells was increased in epididymal (i.e., visceral) adipose tissue of HFD-fed, male C57BL/6 mice after 22 weeks of HFD feeding [44]. Increased levels of IFN-γ were detected in epididymal adipose tissue isolated from HFD-fed mice after 12 weeks of HFD feeding, suggesting that T cells may be skewed towards a Th1-type phenotype in this adipose depot at an earlier time point [44]. We observed a similar increase in the percentage of total $CD3e^+$ T cells and IFN-γ-expressing T cells in visceral adipose tissue of HFD-fed, female C57BL/6 mice, though at an earlier time point (8-10 weeks). A few studies in HFD-fed, male C57BL/6 mice have also shown that IL-17A-expressing $CD4^+$ T cells are preferentially increased in inguinal (i.e., subcutaneous) adipose tissue compared to ND-fed mice, after 8 weeks of feeding [43]. We observed similar increases in IL-17A-expressing $CD4^+$ T cells in MGAT (i.e., subcutaneous) of HFD-fed, female C57BL/6 mice, suggesting that HFD may promote Th17 cell skewing in subcutaneous adipose tissue, independent of sex.

Because we observed a ~3-fold increase in the percentage of inflammatory IL-17A-expressing T cells in MGAT of HFD-fed, preobese mice compared to ND-fed mice, we next sought to hone in on the mechanism by which activated $CD45^+DDR2^+$ cells impact immune activation in this environment. For these studies, MGAT-conditioned media was derived using the organ culture method, to preserve the cellular composition and paracrine interactions within the adipose tissue [52]. In this way, we could examine the impact of the MGAT environment, as a whole, on $CD45^+DDR2^+$ cell-mediated T cell skewing. We found that $CD45^+DDR2^+$ cells from control C57BL/6 mice exposed to MGAT from HFD-fed mice had a greater capacity to stimulate normal $CD4^+$ T cell production of IFN-γ and TNF-α. This suggests that, in the adipose environment of HFD-fed mice, $CD45^+DDR2^+$ cells promote Th1-type skewing. $CD45^+DDR2^{+ND/MGAT}$ and $CD45^+DDR2^{+media}$ also induced increased production of IFN-γ and TNF-α, although to a lesser extent than $CD45^+DDR2^{+HFD/MGAT}$. These data indicate that a $CD45^+DDR2^+$ cell alone has the capacity to activate a T cell but becomes more activated in the HFD-associated adipose tissue environment, resulting in increased Th1 cell-associated cytokine production. One mechanism by which $CD45^+DDR2^+$ cells may be stimulating inflammatory T cell cytokine production in the HFD environment is via production of proinflammatory mediators such as MIG, MIP-1α, and RANTES(Figure S5). Future studies using direct vs. indirect coculture systems will further delineate the mechanism by which $CD45^+DDR2^+$ cells skew T cell cytokine production.

While a stark increase in the percentage of IL-17A-producing $CD4^+$ T cells was observed in MGAT of HFD-fed mice compared to $CD4^+$ T cells in ND-fed mice, we did not observe a dramatic increase in IL-17A production by $CD4^+$ T cells cocultured with $CD45^+DDR2^+$ cells preconditioned in the HFD- vs. ND-fed MGAT environment. These differences could be due to the limitations of the *in vitro* system; the coculture assay includes only preconditioned $CD45^+DDR2^+$ cells and $CD4^+$ T cells and we cannot exclude the possibility that $CD45^+DDR2^+$ cells may rely on other cell types in the mammary gland, including preadipocytes, adipocytes, and stromal cells, to elicit Th17 cell skewing. Further, while the percentage of IL-17A-expressing $CD4^+$ T cells was increased in MGAT of HFD-fed mice, the level of IL-17A produced by these cells ex vivo was not assessed (precluded by intracellular staining) and therefore we cannot directly compare the results. It is important to note that we observed significantly higher levels of IFN-γ production by $CD4^+$ T cells compared to IL-17A production *in vitro*. One interpretation of this result is that $CD45^+DDR2^+$ cells preconditioned in the HFD-fed MGAT environment skew $CD4^+$ T cells more strongly towards a Th1-type phenotype. It is also possible that a population of $CD4^+$T cells producing both IFN-γ and IL-17A is induced by coculture with $CD45^+DDR2^{+HFD/MGAT}$ *in vitro*. These cells may represent a population in transition from a Th1 to Th17 phenotype or a highly activated Th17 cell [78]. This is supported by the *in vivo* data, which showed a population of $CD4^+$ T cells expressing both IFN-γ and IL-17A in MGAT of HFD- and ND-fed mice.

The level of the Th2 cell-associated, inhibitory mediator IL-10 produced by T cells cultured with $CD45^+DDR2^{+HFD/MGAT}$ was increased compared to that produced by T cells alone or T cells cultured with $CD45^+DDR2^{+ND/MGAT}$ or $CD45^+DDR2^{+media}$. This was a puzzling observation in light of the concurrent Th1 cell skewing and may indicate that $CD45^+DDR2^+$ cells in adipose tissue of HFD-fed mice also promote a Th2-type response, although to a lesser degree. Alternatively, the increased IL-10 production may reflect a compensatory "toning down" of the inflammatory response. Overall, the current study demonstrates, for the first time, that $CD45^+DDR2^+$ cells have the capacity to skew T cell cytokine production in the MGAT environment of HFD-fed mice and points to a novel role for $CD45^+DDR2^+$ cells in promoting inflammation in this adipose depot.

The mechanism by which adipose tissue from HFD-fed mice activates $CD45^+DDR2^+$ cells to stimulate T cell cytokine production remains to be elucidated; one possibility is via the production of inflammatory mediators such as IL-6, MCP-1, G-CSF, MIP-1α, and MIP-1β which have been shown to be upregulated in the adipose environment of HFD-fed mice and were detected in adipose tissue explant cultures in the current study (data not shown) [17, 33, 34, 36]. Future studies using blocking antibodies to secreted inflammatory mediators will help to define this mechanism *in vitro*. It may also be important to investigate how $CD45^+DDR2^+$ cells preconditioned in the MGAT environment of HFD-fed mice modulate $CD4^+$ T cells from HFD- vs. ND-fed mice, as there may be differences in the activation and functional profile of splenic T cells in these mice. *In vivo* manipulation of this population will be an important focus of future studies. Because specific inhibitors of activators of DDR2 are not currently commercially available, these cells are difficult to target *in vivo*. Furthermore, because the $CD45^+DDR2^+$ cell population we have identified is a progenitor population that has the capacity to differentiate into a wide spectrum of cells,

including fibroblasts [48, 50] and immune cells [49], the experimental parameters of when to transfer and if/when they home to adipose tissue, in a way that is analogous to their homeostatic arrival, remains to be determined and will be the focus of future studies. While the *in vitro* system used in the current study is a simplified model of the *in vivo* adipose tissue environment, it suggests that $CD45^+DDR2^+$ cells exposed to the HFD-fed adipose environment contribute to T cell activation and provide important insight for future experiments in the preobese mouse model.

Our current understanding of inflammation in preobesity and obesity—and the network of events leading to T cell activation in adipose tissue—is not complete. Recent evidence points to a direct link between Th1 and Th17 cells in the adipose tissue and aberrant glucose homeostasis, suggesting that T cell skewing may play a critical role not only in obesity-related inflammation but also metabolic dysfunction [37–44]. The current study shows, for the first time, that myeloid-derived $CD45^+DDR2^+$ cells express markers of immune activation in the adipose tissue of HFD-fed preobese mice and suggests that they promote Th1-type skewing and the production of inflammatory cytokines. Because myeloid-derived $CD45^+DDR2^+$ cells are a circulating progenitor population, they may represent an important target for therapies aimed at reducing inflammation in overweight or obese patients.

Abbreviations

CCR2: Chemokine receptor 2
CFP: Circulating fibroblast precursor
DDR2: Discoidin domain receptor 2
G-CSF: Granulocyte colony-stimulating factor
GM-CSF: Granulocyte-macrophage colony-stimulating factor
HFD: High-fat diet
IFN-γ: Interferon-gamma
IL-: Interleukin-
MCP-1: Monocyte chemoattractant protein-1
MGAT: Mammary gland-associated adipose tissue
MFI: Mean fluorescence intensity
MHC II: Major histocompatibility complex II
MIG: Monokine induced by gamma interferon
MIP-1α: Macrophage inflammatory protein-1 alpha
MIP-1β: Macrophage inflammatory protein-1 beta
ND: Normal diet
PB: Peripheral blood
RANTES: Regulated upon activation normal T cell expressed and secreted
T2D: Type 2 diabetes
TNF-α: Tumor necrosis factor-alpha
VAT: Visceral adipose tissue.

Data Availability

The flow cytometric data used to support the findings of this study are included within the article and supplementary data file.

Disclosure

The contents of this publication do not represent the views of the Department of Veterans Affairs or the United States Government.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

SS, RY, and AL conceived the experiments. YX assisted in establishing experimental methodologies. SS carried out the experiments, analyzed the data, and wrote the manuscript. All authors were involved in editing the manuscript and gave final approval of the submitted and published versions.

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Supplementary Materials

Supplementary Table 1: comparison of high-fat diet (HFD) and normal diet (ND). Supplementary Figure 1: flow cytometric controls (unstained cells) from MGAT, VAT, and peripheral blood of ND-fed, control female C57BL/6 mice. Supplementary Figure 2: total myeloid-derived $CD45^+DDR2^+$ cells in MGAT and VAT of ND- vs. HFD-fed mice. Supplementary Figure 3: flow cytometric analysis of $CD45^+DDR2^-$ cells in MGAT, VAT, and peripheral blood of ND- vs. HFD-fed mice. Supplementary Figure 4: flow cytometric analysis of total $CD3e^+$ T cells and $CD3e^+CD4^+$ T cells in MGAT, VAT, and peripheral blood of ND- vs. HFD-fed mice. Supplementary Figure 5: mediators produced by $CD45^+DDR2^+$ cells cultured in media conditioned by MGAT from HFD- or ND-fed mice. *(Supplementary Materials)*

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Research Article

Soluble Receptor for Advanced Glycation End Products: A Protective Molecule against Intramyocardial Lipid Accumulation in Obese Zucker Rats?

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Most of the obesity-related complications are due to ectopic fat accumulation. Recently, the activation of the cell-surface receptor for advanced glycation end products (RAGE) has been associated with lipid accumulation in different organs. Nevertheless, the role of RAGE and sRAGE, the soluble form that prevents ligands to activate RAGE, in intramyocardial lipid accumulation is presently unknown. To this aim, we analyzed whether, in obesity, intramyocardial lipid accumulation and lipid metabolism-related transcriptome are related to RAGE and sRAGE. Heart and serum samples were collected from 10 lean (L) and 10 obese (OB) Zucker rats. Oil red staining was used to detect lipids on frozen heart sections. The lipid metabolism-related transcriptome (84 genes) was analyzed by a specific PCR array. Heart RAGE expression was explored by real-time RT-PCR and Western blot analyses. Serum levels of sRAGE (total and endogenous secretory form (esRAGE)) were quantified by ELISA. Genes promoting fatty acid transport, activation, and oxidation in mitochondria/peroxisomes were upregulated in OB hearts. Intramyocardial lipid content did not differ between OB and L rats, as well as RAGE expression. A slight increase in epicardial adipose tissue was observed in OB hearts. Total sRAGE and esRAGE concentrations were significantly higher in OB rats. sRAGE may protect against obesity-induced intramyocardial lipid accumulation by preventing RAGE hyperexpression, therefore allowing lipids to be metabolized. EAT also played a protective role by working as a buffering system that protects the myocardium against exposure to excessively high levels of fatty acids. These observations reinforce the potential role of RAGE pathway as an interesting therapeutic target for obesity-related complications, at least at the cardiovascular level.

1. Introduction

Obesity is one of the leading risk factors for cardiovascular diseases [1]. Most of the obesity-related complications may deal with fat accumulation in tissues different from the adipose one, among which are the liver, muscle, and pancreas [2–5]. This can take place also in the heart where lipid deposition may promote organ damage and dysfunction by inducing abnormalities in cardiac cell metabolism as well as structural adaptation of the cardiovascular system [6]. Intramyocardial lipid accumulation has been observed in different animal models of obesity [7, 8]. Human studies also demonstrated an existing association between myocardial fat content and adiposity [9–12]. Although preclinical studies described some potential cellular and molecular mechanisms linking obesity to heart steatosis [13–16], the identification of

additional pathways and potential targets that could be useful to prevent and/or reverse the detrimental effects of obesity at the cardiovascular level is a compelling need.

Recent insights, also from our group, demonstrated the involvement of the cell membrane receptor for advanced glycation end products (receptor for AGEs (RAGE)), a known trigger of inflammation and oxidative stress [17–19], in inducing adipocyte hypertrophy, adipose tissue expansion, and also ectopic lipid accumulation in different organs, such as the liver [20–24]. Contrarily, its corresponding soluble form, sRAGE, seems to work as a decoy receptor. By binding RAGE ligands in the circulation, sRAGE can prevent membrane RAGE activation and related detrimental effects. Among the different forms that compose the circulating sRAGE pool, namely, cRAGE and esRAGE, the former is the most abundant, but the real decoy receptor seems to be the latter. The circulating levels of total sRAGE and the different forms have also been suggested as biomarkers of different cardiometabolic complications [25–28].

Nevertheless, the role of RAGE and sRAGE in heart steatosis is presently unknown. In this study, we aimed to analyze whether, in obesity, intramyocardial lipid accumulation and lipid metabolism-related transcriptome are related to RAGE and sRAGE forms by using Zucker rats as a model of obesity.

2. Materials and Methods

2.1. Animal Model and Tissue Collection. Ten obese nondiabetic male Zucker rats (OB) (fa/fa, 10 weeks of age) and 10 lean littermates (L) (Fa/?) were purchased from Charles River Laboratories (Calco, Lecco, Italy). The rats were housed at constant room temperature ($22 \pm 2^{\circ}$C) and humidity ($60 \pm 5\%$) with a light-dark cycle of 12 hours each and fed a standard rodent chow (10% fat) and water ad libitum. At the age of 25 weeks, the rats were anesthetized with zoletil (20 mg/kg) and sacrificed by cervical dislocation. Ten hearts (five L and five OB) were stored in Allprotect Tissue Reagent (QIAGEN, Hilden, Germany) at -20°C until RNA and protein extraction. The remaining hearts were fresh frozen in OCT for cryosectioning. Blood was obtained by cardiac puncture, and after cloating, serum was isolated by centrifugation at 1500 g for 15 min. The Italian Ministry of Health approved the procedures of animal care, anesthesia, euthanasia, and tissue collections for this study (Ministerial Authorization 325/2015PR of 2015/04/05).

2.2. Heart Lipid Staining. Staining of lipids was performed with Oil Red O (ORO) dye (Sigma-Aldrich, Milan, Italy). Briefly, 10 μm cryostat sections were air dried, formalin fixed (5 minutes in 10% ice-cold formalin), and washed with running tap water. After rinsing with 60% isopropanol, the sections were stained with freshly prepared ORO working solution, obtained by diluting ORO with deionized water in a ratio of 3 : 2, for 15 minutes. A second wash with 60% isopropanol and a counterstaining with hematoxylin were performed. A coverslip was applied by using an aqueous medium. The lipid resulted in a red stain, while the nuclei in blue. Slides were visualized with a Nikon Eclipse 80i microscope, and images were captured with the attached digital camera and image acquisition software.

2.3. RNA Extraction and Reverse Transcription. Total RNA was isolated from rat hearts using the RNeasy Lipid Tissue Mini Kit (QIAGEN), according to the manufacturer's instructions. Elution was performed with 30 μL of RNase-free water, and the RNA concentration was quantified with NanoDrop (Thermo Fisher Scientific, Waltham, MA). RNA samples (1 μg) were first treated with a genomic DNA elimination step (42°C for 5 min) and then reversely transcribed in 20 μL using the RT^2 First Strand Kit according to the manufacturer's instructions (QIAGEN).

2.4. Real-Time PCR Assay. The lipid metabolism-related transcriptome was evaluated by real-time PCR using the ready-to-use rat *Fatty Acid Metabolism* RT^2 Profiler PCR Array (PARN-007Z, QIAGEN) which profiles the expression of 84 key gene transcripts involved in lipid metabolism. Each cDNA sample was diluted with nuclease-free water and mixed with the RT^2 SYBR green Mastermix (QIAGEN). Twenty-five μL of the experimental mixture was added to each well of the rat *Fatty Acid Metabolism* array (one array for each cDNA). Real-time PCR was performed on the CFX96 thermocycler (Bio-Rad, Milan, Italy) using the following cycling conditions: (1) 95°C/10 min and (2) 40 cycles: 95°C/15 sec followed by 60°C/1 min. Dissociation curves were then performed to verify PCR specificity using the default melting curve program of the instrument. Data were analyzed using the QIAGEN RT^2 Profiler PCR Array Data Analysis Web Portal. RAGE gene expression was carried out according to the manufacturer's instructions using specific rat primers (PPR44508A, QIAGEN) and the same mastermix, thermocycler, and conditions previously indicated. Each sample was run in triplicate, and the fold difference between groups was evaluated by the $\Delta\Delta$Ct method.

2.5. Analysis of Real-Time PCR Array. Each array contained 5 housekeeping genes for normalization, 1 genomic DNA control, 3 reverse transcription controls, and 3 positive PCR controls. Following real-time PCR, data from all the arrays were analyzed using the same threshold. Cycle threshold (Ct) values > 35 were considered a negative call. Ct for genomic DNA controls > 35 and Ct of 20 ± 2 for the positive PCR controls confirmed the lack of DNA contamination and efficient PCR amplification, respectively. According to the manufacturer's protocol, normalization of the expression data of the analyzed samples can be performed using one of the housekeeping genes or any other of the 84 genes, provided that the Ct values of the gene used for normalization does not differ more than 1.5 cycles in all samples (plates). Among the housekeeping and tested genes, SLC27A4 was identified as the most stable gene between our groups and therefore selected as the housekeeping gene for our study. Normalization was performed by calculating the ΔCt for each gene in the plate. The RT^2 Profiler PCR Array Data Analysis Web Portal was used to calculate the fold change based on the $\Delta\Delta$Ct method. For fold-change values greater than 1, the results were reported as fold upregulation. For

fold-change values less than 1, the negative inverse of the results are reported as fold downregulation. The p values were calculated using the QIAGEN RT^2 Profiler PCR Array Data Analysis Web Portal and were based on Student's t-test (two-tail distribution and equal variances between the two samples) on the replicate 2-ΔCt values for each gene in each treatment group compared to the control group and considered significant for values < 0.05.

2.6. Western Blot Analysis. Samples of heart tissues were homogenized with TissueLyser II (QIAGEN) in ice-cold RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.5% Na-deoxycholate, and 50 mM sodium fluoride) containing 1% protease inhibitor cocktail (Sigma-Aldrich, Milan, Italy). The homogenate was kept on ice for 30 min, centrifuged at 500 rpm for 10 min at 4°C, and the resulting supernatant centrifuged at 13,200 rpm for 15 min at 4°C. Protein concentration was determined with the Quantum Protein Assay Kit, based on the BCA reagent (EuroClone, Milan, Italy). Equal amounts of protein samples (50 μg) were suspended in Laemmli sample buffer and separated using 4-20% Mini-PROTEAN TGX Stain-Free Gels and Tris/Glycine/SDS running buffer (Bio-Rad). After SDS-electrophoresis, TGX Stain-Free Gels were activated for 1 min and imaged using the ChemiDoc Touch System and the Image Lab 5.2.1 software (Bio-Rad). The separated proteins were then transferred from the gel to a nitrocellulose membrane using the Trans-Blot Turbo Mini Nitrocellulose Transfer Packs and the Trans-Blot Transfer System (Bio-Rad). The membranes were blocked with 5% dry milk in Tris-buffered saline/0.1% Tween 20 for 1 h at room temperature, and the blots were then incubated overnight at 4°C with a diluted solution of the primary anti-RAGE antibody (1 μg/mL) (Abcam, Cambridge, UK). The subsequent incubation with a secondary antibody conjugated with peroxidase was performed at room temperature for 2 h. Immunoreactivity was detected by a working solution (Clarity Western ECL Substrate, Bio-Rad) and the ChemiDoc Touch System. Analysis included the determination of total stain-free fluorescence and signal for RAGE of each sample/lane on the blots using the Image Lab software. RAGE signals were normalized with stain-free total lane volumes [29].

2.7. sRAGE and esRAGE Enzyme-Linked Immunosorbent Assays (ELISA). Circulating levels of sRAGE and esRAGE were quantified on serum samples according to the manufacturer's directions, with the following assays: rat sRAGE duo set ELISA (DY1616, R&D System, Minneapolis, MN) and rat esRAGE ELISA (E-EL-R2497, Elabscience, Houston, TX). The GloMax®-Multi Microplate Multimode Reader was used for photometric measurements (Promega, Milan, Italy).

2.8. Statistical Analysis. Data are expressed as mean ± SD. The normality of data distribution was assessed with the Kolmogorov-Smirnoff test. t-test or Mann-Whitney tests were used for group comparisons. Data were analyzed using the GraphPad Prism 5.0 biochemical statistical package (GraphPad Software, San Diego, CA). A p value < 0.05 was considered significant.

3. Results

3.1. Fat Staining on Heart Tissues. In the myocardium, no lipid accumulation was found, either in the L (Figure 1(a)) or in the OB rats (Figure 1(b)). In both the L and OB animals, a small amount of fat (red staining) was visible in the atrioventricular groove underneath the epicardium (epicardial adipose tissue (EAT)). Compared to the L animals (Figure 1(c)), the accumulation was slightly more consistent in the OB rats (Figure 1(d)).

3.2. Evaluation of Heart RAGE Expression. We compared OB and L hearts in term of RAGE expression. RAGE did not differ between the two animal groups at both the gene (fold change: -1.3, $p > 0.05$) and the protein level (Figure 2, $p > 0.05$).

3.3. Evaluation of Lipid Metabolism-Related Transcriptome in Hearts. The rat *Fatty Acid Metabolism* RT^2 Profiler PCR Array allowed us to assess the expression of 84 genes involved in fatty acid metabolism, fatty acid transport, fatty acid biosynthesis regulation, ketogenesis and ketone body metabolism, and triacylglycerol metabolism. Among the 84 genes, 16 had a Ct between 30 and 35 in both OB and L rats, which means low levels of expression, and 68 had a Ct below 30. As shown in Figure 3, 16 genes were upregulated in OB hearts. Among these, 9 genes are involved in fatty acid metabolism. In detail, one (*Acaa2*) is an acetyl-CoA transferase, 4 (*Acad11*, *Acad9*, *Acadl*, and *Acadm*) are acetyl-CoA dehydrogenases, 3 (*Acot12*, *Acot2*, and *ACot9*) are acetyl-CoA thioesterases, and 1 (*Acsbg2*) is an acetyl-CoA synthetase. One gene (*Bdh2*) is involved in ketogenesis and ketone body metabolism, 1 (*Gpd1*) is involved in triacylglycerol metabolism, 1 (*Prkag2*) participates in the regulation of fatty acid biosynthesis, and 2 are involved in fatty acid transport (*Crat* and *Slc27a2*). The other two genes (*Decr*1 and *Eci2*) are also involved in metabolic pathways that regulate fatty acid metabolism. A statistically significant difference was also observed for two of these genes (*Prkag2* and *Slc27a2*) (p value < 0.05, OB vs. L).

3.4. Evaluation of sRAGE and esRAGE Plasma Levels. Circulating levels of total sRAGE and esRAGE were quantified on serum samples. cRAGE was calculated as the difference between the total and the esRAGE form instead. As shown in Figure 4, sRAGE levels (Figure 4(a)) were higher in OB than in L (1869.00 ± 296.90 vs. 1115.00 ± 166.90 pg/mL, respectively, $p < 0.05$) as well as esRAGE (Figure 4(b)) (115.90 ± 43.85 vs. 37.89 ± 21.67 pg/mL, respectively, $p < 0.001$). Levels of cRAGE (Figure 4(c)) did not differ between the two groups instead (1403.00 ± 588.30 vs. 1169.00 ± 526.80 pg/mL, respectively, $p = 0.373$).

4. Discussion

The main finding of this study is the observation that intramyocardial lipid accumulation does not occur in OB rats

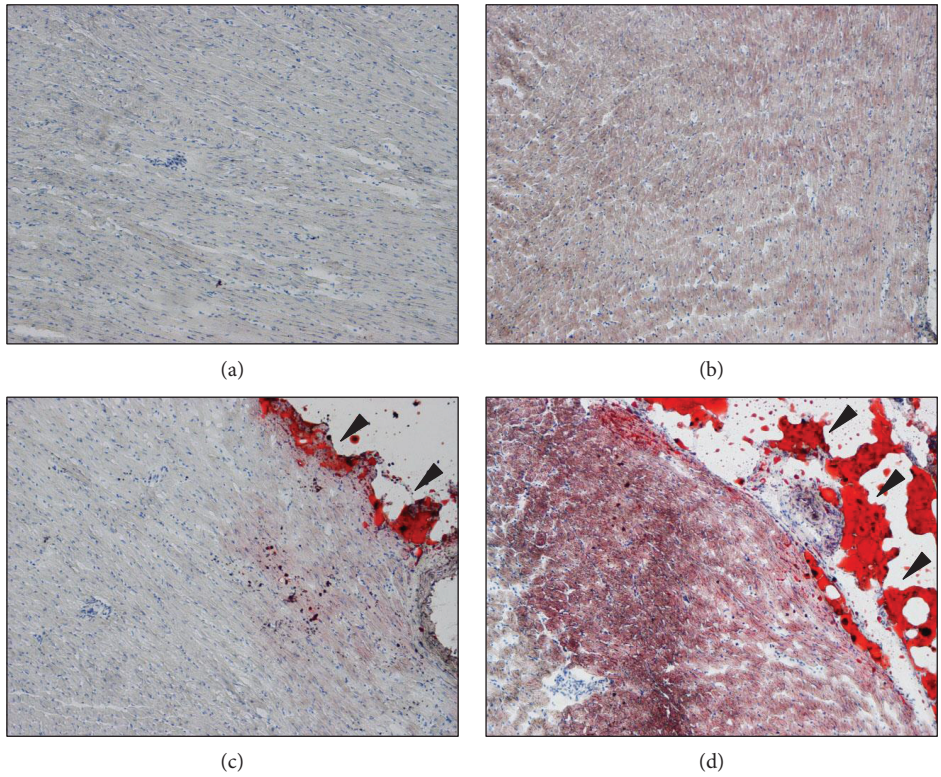

Figure 1: Fat staining on heart tissues. Ten μm heart cryostat sections were stained with Oil Red O dye. (a) and (c) are representative pictures of lean heart tissues and (b) and (d) of obese heart tissue. Red staining in (c) and (d) indicates (arrowheads) epicardial adipose tissue. Images were all captured at 10x magnification.

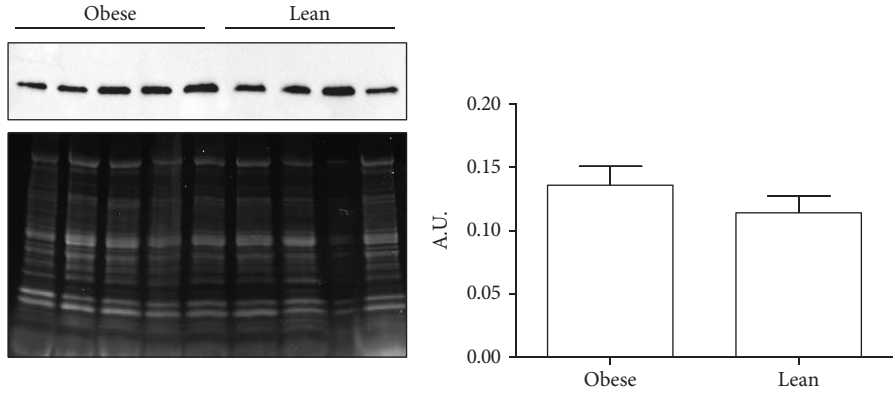

Figure 2: Western blot analysis of RAGE expression in obese and lean hearts. RAGE protein levels were quantified in 5 obese and 4 lean heart samples by Western blot analysis. The fifty μg protein extract/lane was analyzed with the anti-RAGE antibody. A representative blot, the corresponding stain-free gel utilized for protein normalization, and the semiquantitative analysis are shown. Data are expressed as mean ± SD.

that display increased circulating levels of sRAGE, namely, the esRAGE form, and that there is no change in the RAGE expression in the heart. The reasons for exploring the association between RAGE, sRAGE, and heart steatosis in obesity were many, but there were two immediate ones: a previously described role of RAGE in promoting lipid accumulation and the lack of information about RAGE and heart steatosis in obesity [21, 22, 30–34].

According to previous findings [35, 36], we expected to observe an increased intramyocardial lipid deposition in OB rats. Differently, fat did not accumulate ectopically in the myocardium, while EAT was slightly increased. Fatty acids are the main fuel for the heart, and intramyocardial fat accumulation may occur when fatty acid availability and oxidation are not properly balanced. In obesity, visceral fat displays an increased level of lipid turnover that, together

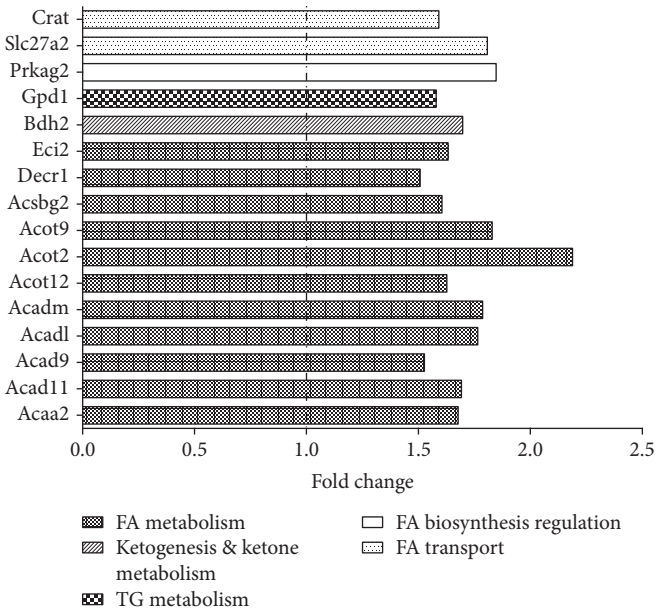

Figure 3: Lipid metabolism-related transcriptome in obese and lean hearts. Gene expression profile was evaluated using the rat *Fatty Acid Metabolism* RT^2 Profiler PCR Array. Genes that showed a fold change > 1.5 in obese vs. lean groups are represented and grouped according to their biological function.

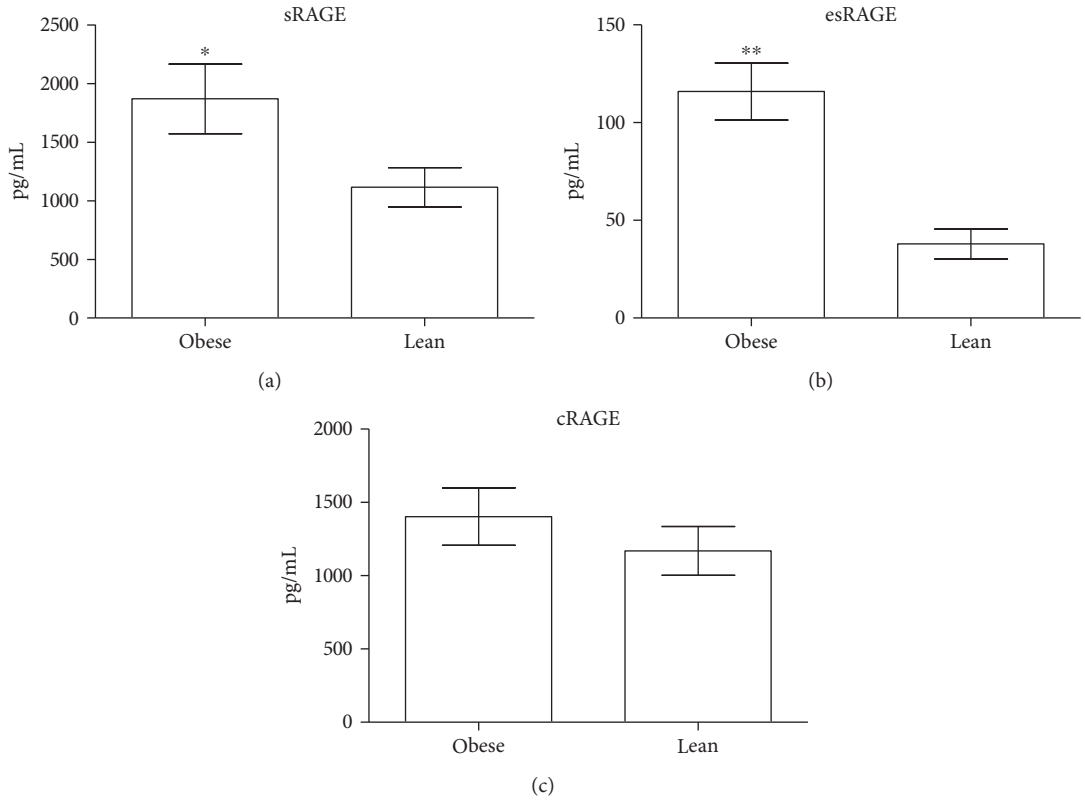

Figure 4: Serum levels of sRAGE, esRAGE, and cRAGE in obese and lean rats. Serum levels of total sRAGE (a) and esRAGE (b), quantified in serum samples, were higher in obese than in lean animals. Levels of cRAGE were calculated as the difference between the total and the esRAGE form and did not differ between the two groups (c). Data are expressed as mean ± SD. $^*p < 0.05$ and $^{**}p < 0.001$ vs. the lean group.

with an increased release of proinflammatory molecules from adipocytes and/or infiltrating macrophages, gives rise to metabolic complications in different organs [37–39]. The lack of intramyocardial lipid accumulation in OB rats may result from an increased lipid utilization by cardiac cells and storage in EAT. Although the expansion of EAT in

obesity led to considering it as a pathological organ, this depot may also play important protective effects on heart metabolism. In fact, EAT has been proposed to provide fatty acids for the myocardium and to function as a buffering system that protects the heart against exposure to excessively high levels of fatty acids [40]. In our obesity model, its slight increase seems thus to play a protective role in terms of heart lipid metabolism. Moreover, from a molecular point of view, data obtained from the lipid metabolism-related transcriptome also suggested the activation of specific metabolic pathways that promote lipid utilization instead of deposition in OB hearts. In particular, the increased expression of *Acaa2* (acetyl-CoA transferase), *Acad11*, *Acad9*, *Acadl* and *Acadm* (acetyl-CoA dehydrogenases), *Acot12*, *Acot2* and *ACot9* (acetyl-CoA thioesterases), *Crat* and *Slc27a2* (fatty acid transporters), *Decr*1 and *Eci2* (accessory enzymes which participate in betaoxidation), and *Acsbg2* (acetyl-CoA synthetase) confirmed the activation of metabolic pathways that, by promoting fatty acid transport, activation, and oxidation in mitochondria/peroxisomes, represent a protective mechanism against fat accumulation. On the other side, the upregulation of *Gpd1*, a key element that connects carbohydrate and lipid metabolism, and *Prkag2*, the noncatalytic subunit of AMP-activated protein kinase that in response to increased intracellular ATP levels activates energy-producing pathways (i.e., biosynthesis), might contribute to increase the synthesis of triacylglycerol. However, the increase in fatty acid oxidation which reduces fatty acids necessary for glycerol 3-phosphate esterification protects against fat accumulation [41].

Which is the role of RAGE in promoting fat accumulation? Previous studies demonstrated a link between increased RAGE expression and activation of pathways that can promote lipid accumulation in many cell types/tissues and suggested that therapies preventing RAGE activation are able to reduce these effects [21, 30, 33, 34, 42]. Results obtained in this study emphasized the potential association between RAGE, lipid accumulation, and genes involved in lipid metabolism in the heart; however, they did not prove a direct cause-effect relationship. Just the manipulation of RAGE expression in *in vitro* studies will be able to confirm the role of the receptor in affecting specific lipid metabolic pathways that can finally lead to lipid accumulation. Another interesting question is why RAGE levels, different from what was expected, did not increase. Obesity is a condition characterized by increased levels of many RAGE ligands, such as AGEs and ALEs (advanced lipoxidation end products). Formation of these products is a naturally occurring process and is the result of normal metabolism. However, their production and accumulation is enhanced under chronic inflammation and oxidative stress, two conditions accompanying obesity, and this may occur before obesity-related complications become manifest. A significant accumulation of RAGE ligands has been described in murine models of obesity, in which these products can promote engagement of the RAGE pathway and RAGE upregulation and detrimentally impact organ function [32, 43–45]. Any mechanism preventing RAGE engagement and activation may thus protect against detrimental effects. sRAGE just plays this protective role, and its upregulation may be acknowledged as a mechanism that prevents RAGE activation and RAGE-mediated ectopic lipid deposition. With regard to sRAGE, it is also important to point out that this soluble form is a pool composed by cRAGE, derived by the proteolytic cleavage of the membrane-bound molecule RAGE, and esRAGE, the endogenous secretory form. Among these forms, an increase in the cRAGE level is considered a surrogate marker of inflammation. In fact, the activation of RAGE and its proinflammatory signaling promotes the expression of membrane RAGE and inflammation-related enzymes, such as the matrix metalloproteinase 9, which upregulate RAGE cleavage and release into the blood [46]. esRAGE, instead, is endogenously secreted. Since it works as a decoy receptor, keeping its levels high is a protective mechanism against RAGE activation, upregulation, and related damaging effects [47–50]. According to our results, the observed increase in esRAGE may be considered a counterregulatory strategy activated by the body to reduce the obesity-related damaging effects. In our animal model, thus, until esRAGE levels are high, no intramyocardial lipid deposition occurs and EAT plays a protective role for the heart too. How long esRAGE levels are kept high and how esRAGE levels potentially protect against heart steatosis are two questions that can be answered by just using animal models of different ages that experience obesity for a longer time and already display different obesity-related cardiometabolic complications. Furthermore, reduction of esRAGE levels may also impact on EAT by promoting a significant enlargement of this depot, as previously described in our human studies [28], and its transformation into pathological organs with a well-documented role in the onset and progression of cardiovascular diseases.

5. Conclusions

In conclusion, increased levels of sRAGE, namely, esRAGE, seem to protect against ectopic lipid accumulation in the myocardium by preventing RAGE hyperexpression, promoting fatty acid storage in EAT and their oxidation. These observations reinforce the potential role of RAGE pathway as an interesting therapeutic target for obesity-related complications, at least at the cardiovascular level.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

The research idea and study design were developed by ED, FB, and MMCR; data acquisition was handled by ED, EV, EL, and SB; data analysis/interpretation was handled by ED

and MN; the manuscript was drafted by ED; and mentorship was handled by MMCR. All authors revised and approved the final version of the article.

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