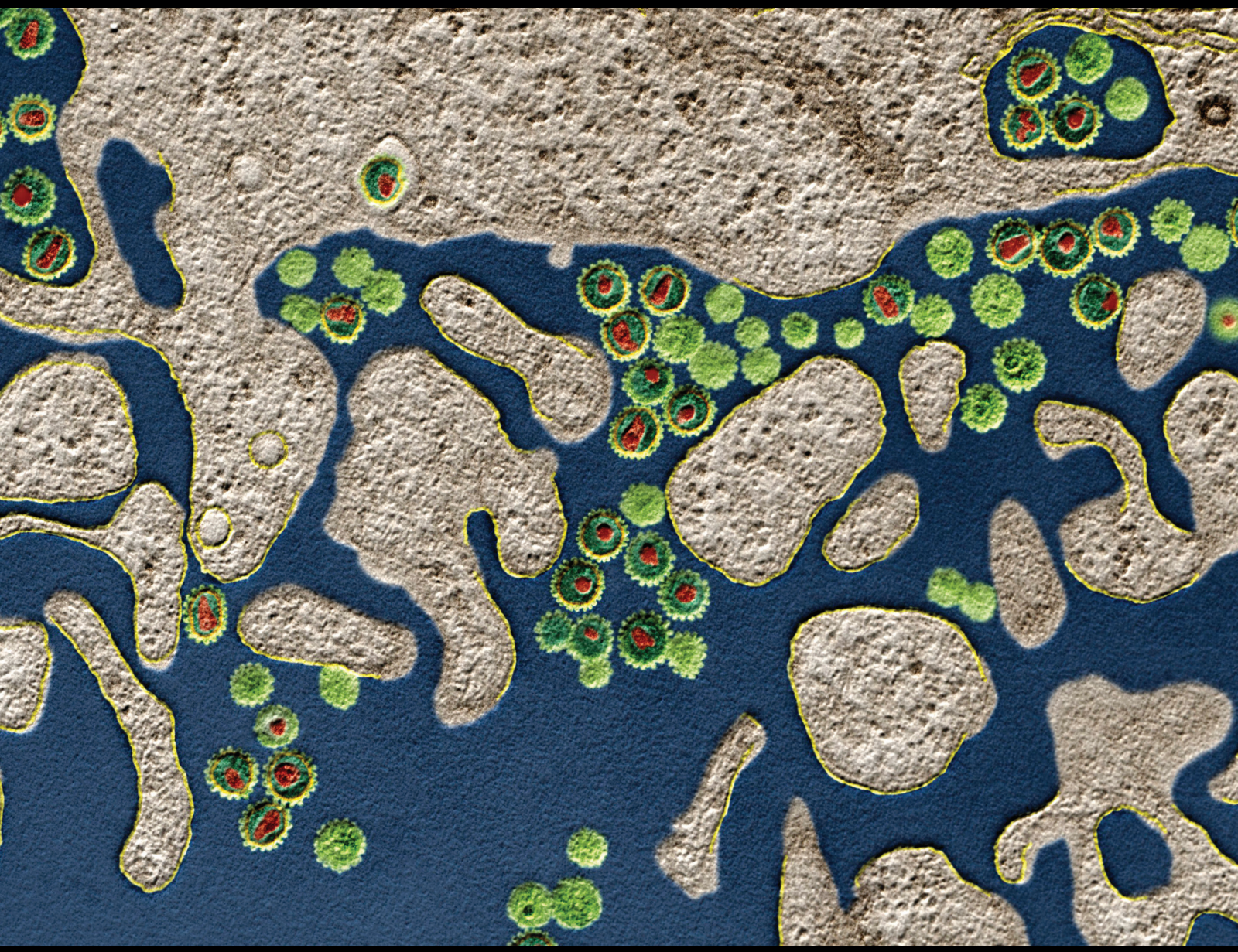
Journal of Immunology Research

Immune and Inflammatory Processes in Obesity, Insulin Resistance, Diabetes, and Related Cardiometabolic Complications

Guest Editors: Joseph Fomusi Ndisang, Sharad Rastogi, and Alfredo Vannacci



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Editorial Board

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Editorial

Immune and Inflammatory Processes in Obesity, Insulin Resistance, Diabetes, and Related Cardiometabolic Complications

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The high prevalence of obesity and diabetes in developed and developing nations poses a great health challenge [1, 2]. Obesity is one of the major causes of insulin resistance and type-2 diabetes [3]. Type-1 diabetes is primarily due to the autoimmune-mediated destruction of pancreatic beta-cell leading to insulin deficiency [4, 5]. This is generally accompanied by alterations in lipid metabolism, enhanced hyperglycemia-mediated inflammation and oxidative stress, endothelial cell dysfunction, and apoptosis. Similarly, type-2 diabetes is characterized by elevated inflammation, glucotoxicity, lipotoxicity, and apoptosis that leads to the progressive loss of beta cells and ultimately to insulin insufficiency at later stages of the disease [4, 5]. Thus, in diabetes, inflammation could be triggered by hyperglycemia and/or immune response. However, elevated inflammatory events not only may affect insulin production in type-2 and type-1 diabetes but also may affect insulin response in target tissues causing insulin resistance [3]. Although insulin resistance has traditionally been associated with type-2 diabetes, mounting evidence indicates that the incidence of insulin resistance in type-1 diabetes is increasing. Therefore, novel mechanistic approaches deciphering the role of inflammation in insulin resistance in type-1 and type-2 diabetes are needed. Many pathophysiological agents are implicated in insulin resistance. Although the exact nature of these factors is not completely understood, a high consensus of opinion suggests that inflammation, oxidative stress, and genetic, habitual, environmental, and epigenetic factors are implicated.

There has been significant advancement in elucidating the mechanisms implicated in insulin resistance, overt diabetes, and related cardiometabolic diseases [1–9]. However, novel mechanistic studies deciphering the role of inflammation in these chronic diseases are needed. Similarly, novel studies addressing the effect of inflammation on genetic and epigenetic factors that lead to insulin resistance, overt diabetes, and related cardiometabolic complications are needed. Therefore, this special issue highlights research and review papers that address a wide spectrum of inflammation-related mechanisms associated with insulin resistance, type-1 diabetes, type-2 diabetes, and related cardiometabolic complications. Accordingly, in an article featuring in this special issue, L. Zhang and coworkers investigated the pathophysiological role of tribbles homolog-3 (TRB3) in diabetic nephropathy, a common complication of diabetes. The authors reported that TRB3 may trigger renal fibrosis by regulating transforming growth factor β1 (TGF-β1) and collagen type-IV through a signaling pathway involving extracellular signal-regulated kinase and mitogen-activated protein kinase. TGF-β is a

glycoprotein and cytokine with diverse roles in many cellular events including reproduction [10]. On the other hand, gamma interferon (IFN-γ), another cytokine that is traditionally known for its role in innate and adaptive immunity, is increasingly reported to play a role in reproduction [11]. In this special issue, D. L. G. Fagundes et al. showed that IFN-γ and TGF-β modulate the phagocytic activity in the colostrum, maternal blood, and cord blood of pregnant diabetic women. There is an interesting reciprocal interaction between TGF-β and macrophage migration inhibitory factor (MIF) [12]. MIF is a proinflammatory cytokine that promotes immune cell recruitment following injury and polymorphism of MIF which has been associated with several diseases [13, 14]. In a related study by E. Valdés-Alvarado et al., the association of MIF gene polymorphism, a complication that is commonly associated with obesity, diabetes, and hypertension, and susceptibility to acute coronary syndrome was reported in a research article of this special issue. Furthermore, in a clinical study by N. A. Sinicato and coworkers, the role of cytokines such as tumor necrosis factor alpha, interleukin- (IL-) 6, and interleukin- (IL-) 10 in systemic lupus erythematosus, an autoimmune disease that is associated with a variety of different cardiovascular complications including atherosclerosis, was reported. Many cytokines are known to potentiate inflammatory cascades by modulating macrophage polarization [15]. The role of the different macrophage M1 and M2 phenotypes in obesity is becoming increasingly clear [3, 15, 16]. In a related article featuring in this special issue, K. Fjeldborg et al. have shed more light on the preponderance of macrophage M2 phenotype that was associated with a parallel reduction of the macrophage M1 phenotype in obese subjects. Macrophage-induced inflammation remains an important feature in insulin resistance and type-2 diabetes; thus, as an alternative strategy, A. L. Guadarrama-López et al. underscored the beneficial effects of polyunsaturated fatty acids and vitamin D in diabetes and related complications in a review article contained in this issue.

Diabetic retinopathy is another complication of diabetes and is amongst the leading causes of vision impairment [17] and a significant number of patients with diabetic retinopathy are also known to be affected by diabetic macular edema [18]. A common denominator between diabetic retinopathy and diabetic macular edema is the elevated levels of role of vascular endothelial growth factor (VEGF) [19, 20]. In this special issue, a review article by F. R. Stefanini and coworkers is featured highlighting the role of intravitreal injection of anti-VEGF as a therapeutic strategy against diabetic macular edema.

Collectively the articles featuring in this special issue constitute a cocktail of original research and reviews that would stimulate further research in this area given the increasing incidence of diabetes, obesity, hypertension, and the burden these chronic conditions pose to health care systems.

Joseph Fomusi Ndisang
Sharad Rastogi
Alfredo Vannacci

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Research Article

Tribbles 3 Regulates the Fibrosis Cytokine TGF-β1 through ERK1/2-MAPK Signaling Pathway in Diabetic Nephropathy

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To reveal the expression and possible role of tribbles homolog 3 (TRB3) in the incidence of type 2 diabetic nephropathy, we used immunohistochemistry, real-time quantitative PCR, western blot analysis, and enzyme-linked immunosorbent assay (ELISA) to study the expression of TRB3, extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase (ERK1/2 MAPK), transforming growth factor β1 (TGF-β1), and collagen type IV in kidneys of db/db diabetic mice and in murine renal mesangial cells stimulated with high glucose. The expression of TRB3, TGF-β1, and collagen type IV was increased in kidneys of db/db diabetic mice. TGF-β1 and collagen type IV regulated by high glucose through ERK1/2 MAPK were downregulated by silencing TRB3 in renal mesangial cells. TRB3 may be involved in diabetic nephropathy by regulating the fibrosis cytokine TGF-β1 and collagen type IV through the ERK1/2 MAPK signaling pathway.

1. Introduction

Diabetic nephropathy (DN) is an important diabetic microvascular complication and the major cause of disability and death. Morbidity and mortality with the disease are increasing every year [1]. DN is characterized by albuminuria, glomerular hypertrophy, and progressive accumulation of glomerular matrix, culminating in glomerulosclerosis, tubulointerstitial fibrosis, and progressive loss of renal function [2, 3].

Many studies have confirmed that glomerular sclerosis and interstitial fibrosis are the main pathologic characteristics in DN, especially in the midanaphase of DN. Deposition of extracellular matrix (ECM) such as collagens and fibronectin [4] regulated by transforming growth factor β1 (TGF-β1) is the core mechanism of glomerular sclerosis and interstitial fibrosis. The mesangial cells play important roles in DN, being responsible for the accumulation of ECM and mesangial expansion [5]. TGF-β1 is the core cytokine leading to the synthesis of ECM, which is responsible for mesangial fibrosis and hypertrophy under diabetic conditions [6]. The major components of the ECM proteins collagen types I–IV and their synthesis and immoderate deposition are consistently observed in multifarious renal disease processes affecting humans and experimental animals [7, 8].

Tribbles homolog 3 (TRB3) is an important member of the tribbles family. Combined with unphosphorylated Akt, TRB3 can prevent Akt activity and negatively regulate the insulin signaling pathway [9]. TRB3 and its gene polymorphism are associated with insulin resistance, a vital pathophysiologic characteristic of type 2 diabetes. TRB3 also serves as a scaffold protein and regulates the activation of the three classes of mitogen-activated protein kinases (MAPKs) [10]. As a member of the MAPK family, extracellular signal-regulated kinase 1/2 (ERK1/2) can be activated in mesangial cells exposed to high glucose (HG) [11]. Also, ERK activity may enhance the TGF-β1-dependent responses in human mesangial cells [12]. Therefore, TRB3 may be involved in DN through an ERK pathway.

In this study, we aimed to explore the role of TRB3 in DN and the possible regulating mechanism between TRB3, ERK, and TGF-β1 *in vivo* and *in vitro*.

2. Materials and Methods

2.1. Main Reagents. We obtained antibodies for TRB3 (Santa Cruz Biotechnology, Santa Cruz, CA), TGF-β1 (Abcam Biotechnology, CA), and PD98059, phospho-ERK1/2, and total ERK1/2 (Cell Signaling Technology, Beverly, MA). Trizol reagent and reagents for RT-PCR were from Takara Biotechnology (Dalian, China). ELISA kits for collagen types I and IV were from R&D systems (Minneapolis, MN).

2.2. Experimental Animals. SPF db/db diabetic mice (C57BL/KSJ) and their matched (12-week-old) controls (db/m) were obtained from Vital River Laboratory Animal Technology (Beijing). All animals were maintained on a normal diet under standard animal house conditions at the cardiovascular remodeling Laboratory Animal Center in Qilu Hospital of Shandong University. Animal experiments were conducted in accordance with guidelines established by the Animal Care and Use Committee of Shandong University.

Animals were divided into 3 groups ($n = 5$) and killed at 16, 20, and 25 weeks. Blood glucose and body weight were randomly monitored weekly; levels of urinary albumin excretion, serum creatinine, and blood urea nitrogen (BUN) were detected before death once. The left kidney pieces fixed in 4% paraformaldehyde were embedded in paraffin, sectioned at 4 μm thickness, and mounted on glass slides. The slides were used for morphological observation and immunohistochemical staining to detect the expression of TRB3; the right kidney was snap-frozen in liquid nitrogen and stored at −80°C for RT-PCR and western blot analysis.

2.3. Histology. Sections were stained with hematoxylin and eosin (H&E), periodic acid Schiff (PAS), and Masson trichrome for investigating renal pathological changes. Paraffin sections were dewaxed and washed in phosphate buffered saline (PBS) and then incubated in preheated 10 mmol/L sodium citrate buffer at 94°C for 15 min. Slides were washed and blocked with protein-blocking solution (10% normal goat serum) for 30 min and then incubated with rabbit anti-mouse TRB3 antibody (1 : 100) overnight at 4°C, biotin-labeled secondary antibody working solution for 30 min at 37°C, and then DAB color. Sections were preincubated with PBS as negative controls.

2.4. Cell Culture. Mycoplasma-free SV40 MES 13 cells (murine mesangial cells, MMCs) were purchased from China Center for Type Culture Collection. They were derived from glomerular explants of SV40 transgenic mice and showed both biochemical and morphological features of normal mesangial cells in culture. They were maintained in DMEM-F12 (3 : 1) containing 6 mM glucose and supplemented with 14 mM HEPES and 5% fetal bovine serum in 5% CO_2 and 95% humidified air at 37°C as described previously [13, 14]. Cells were synchronized by culturing in serum-free medium for 24 h before testing and all experiments were performed with cells between passages 30 and 40 to minimize the effects of phenotypic variation in continuous culture.

Mesangial cells were divided by glucose concentration into 3 groups: (1) normal glucose (NG, 5.6 mM glucose, and control); (2) HG (25 mM glucose); and (3) NG + high mannitol (HM, NG plus 19.5 mM mannitol). To investigate the effects of glucose on the expression of TRB3 and pERK1/2, cells were stimulated with HM or HG for 6, 12, 24, and 48 h. At the end of each time, total RNA and protein of the cells were extracted for TRB3 and pERK1/2 expression.

To examine the effect of the MAPK pathway on collagen expression by HG, a specific ERK inhibitor (PD98059, 10 μmol/L) was added 1 h before stimulation. To examine the effect of TRB3 on collagen expression by HG, TRB3 small interfering RNA (siRNA) was transfected 6 h before stimulation and cultured for 48 h in NG or HG medium; then, total RNA and protein were extracted from cells for analysis of TRB3, TGF-β1, and pERK1/2 expression and culture medium was collected for measurement of concentration of collagen types I and IV.

2.5. siRNA Transfection. Cells were replated and transfected in 6-well plates with 150 μL Opti-MEM (Invitrogen, CA) and 1.5 μL/well Lipofectamine 2000 (Invitrogen, CA) with 20 pmol siRNA and its controls. The sense and antisense sequences of the primers were 5′-GCACAGAGUACACCUGCAATT-3′ and 5′-UUGCAGGUGUACUCUGUGCTT-3′. As a negative control, we used randomly mixed sequences of TRB3 siRNA, 5′-UUCUCCGAACGUGUCACGUTT-3′, and 5′-ACGUGACACGUUCGGAGAATT-3′. The effect of siRNA knockdown of TRB3 on the expression of TGF-β1 and collagen types I and IV was evaluated by western blot analysis or ELISA at 48 h. All RNAi experiments were repeated at least 3 times.

2.6. Real-Time Quantitative PCR. Total RNA was extracted by use of Trizol. In total, 1 μg total RNA was reverse-transcribed in a 20 mL volume containing 0.5 mg oligo-dT primer, 1 mL dNTP mixture, 1.25 mL RNase inhibitor, and 4 U reverse transcriptase. Real-time quantitative PCR involved the SYBR-based method in a 20 mL reaction in a Roche light-cycler. Reaction specificity was confirmed by analyzing melting curves and by electrophoresis on 2.0% agarose gel analysis of products. The relative change in gene expression was analyzed by the $2^{-\Delta Ct}$ method and normalized to the expression of the housekeeping gene β-actin. Respective primer and product specifications are in Table 1.

2.7. Western Blot Analysis. Total protein was extracted from tissues and cells as described previously. The protein extracts were separated on 10% SDS-PAGE then transferred to PVDF membranes, and blocked in TBST with 5% skim milk at room temperature for 2 h. The blots were incubated with antibodies for TRB3 (1 : 200), TGF-β1 (1 : 200), pERK1/2 (1 : 2000), ERK1/2 (1 : 1000), and β-actin (1 : 1000) overnight at 4°C, washed, and then incubated with goat anti-rabbit antibody (1 : 10000) at room temperature for 1 h. Protein bands were analyzed by use of AlphaView SA software. All experiments were repeated at least 3 times.

Table 1: Primers used for RT-PCR.

Name	GenBank accession	Primers	Size (bp)
TRB3	NM 175093.2	Forward: 5′-GCTCTGAGGCTCCAGGACAA-3′ Reverse: 5′-TGTCATCAAACTCCAACGGTTTC-3′	91
TGF-β1	NM 011577.1	Forward: 5′-GTGTGGAGCAACATGTGGAACTCTA-3′ Reverse: 5′-CGCTGAATCGAAAGCCCTGTA-3′	174
Collagen type I α1	NM 007742.3	Forward: 5′-CAACAGTCGCTTCACCTACAGC-3′ Reverse: 5′-GTGGAGGGAGTTTACACGAAGC-5′	201
Collagen Type IV α1	NM 009931.2	Forward: 5′-TATGTCCAAGGCAACGAGC-5′ Reverse: 5′-AACCGCACACCTGCTAATG-3′	228
β-Actin	NM 007393.3	Forward: 5′-GTG ACG TTG ACA TCC GTA AAG A-3′ Reverse: 5′-GCC GGA CTC ATC GTA CTC C-3′	245

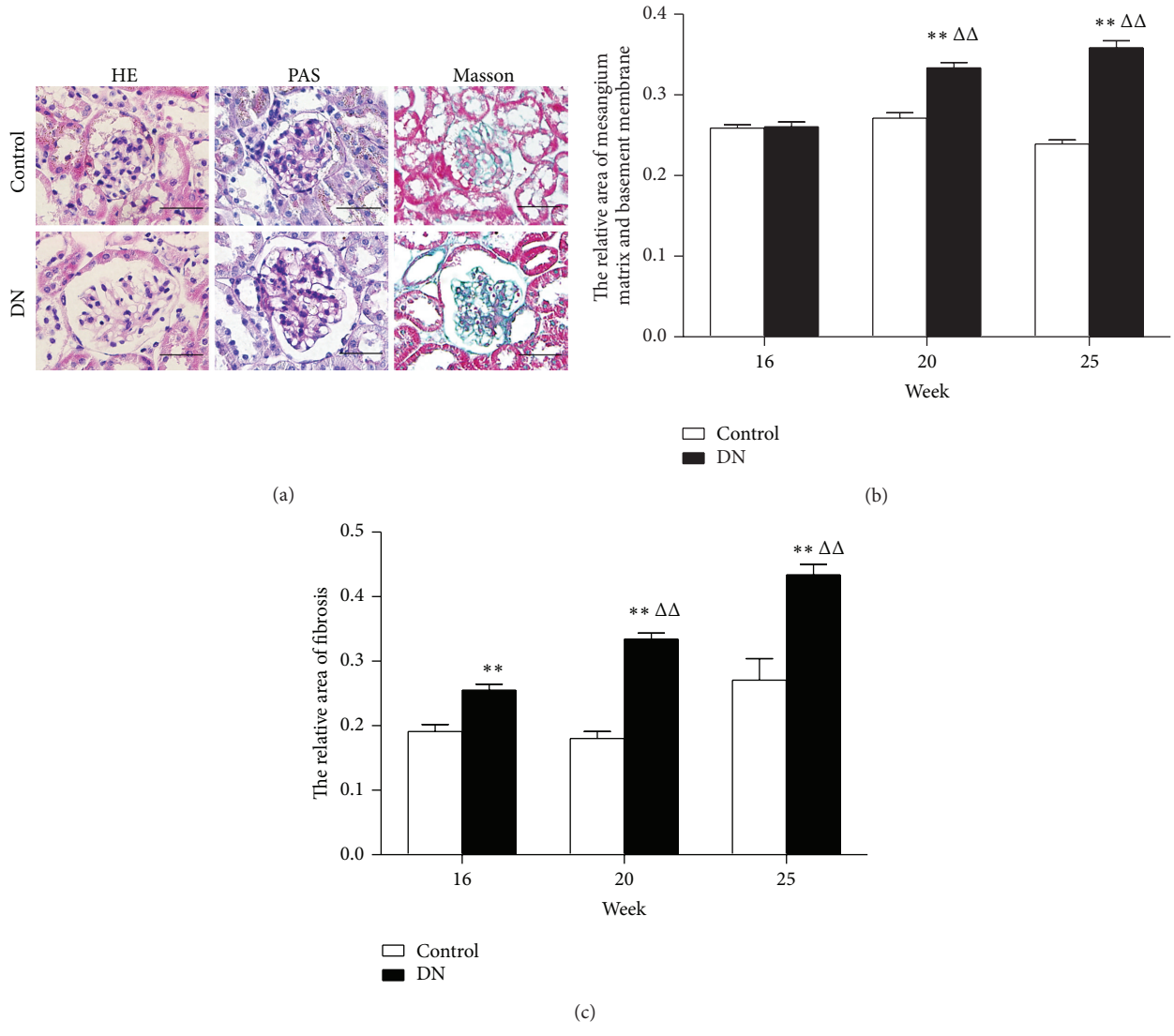


Figure 1: Glomerular pathological changes in mice with diabetic nephropathy (DN) and control mice. (a) Renal morphology and glycogen accumulation at 25 weeks evaluated by hematoxylin and eosin (HE) and periodic acid Schiff (PAS) staining, renal interstitial fibrosis detected by Masson trichrome staining. (b) Relative area of mesangium matrix and basement membrane at different times. (c) Relative area of renal interstitial fibrosis at different times. In both experiments, more than 12 glomeruli were evaluated in each mouse. Magnification in (a) HE, PAS, Masson: 400x. $^{*}P < 0.05$, $^{**}P < 0.01$ versus age-matched control mice. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ versus 16-week-old DN mice. Scale bars, 50 μm. $n = 5$ mice per group.

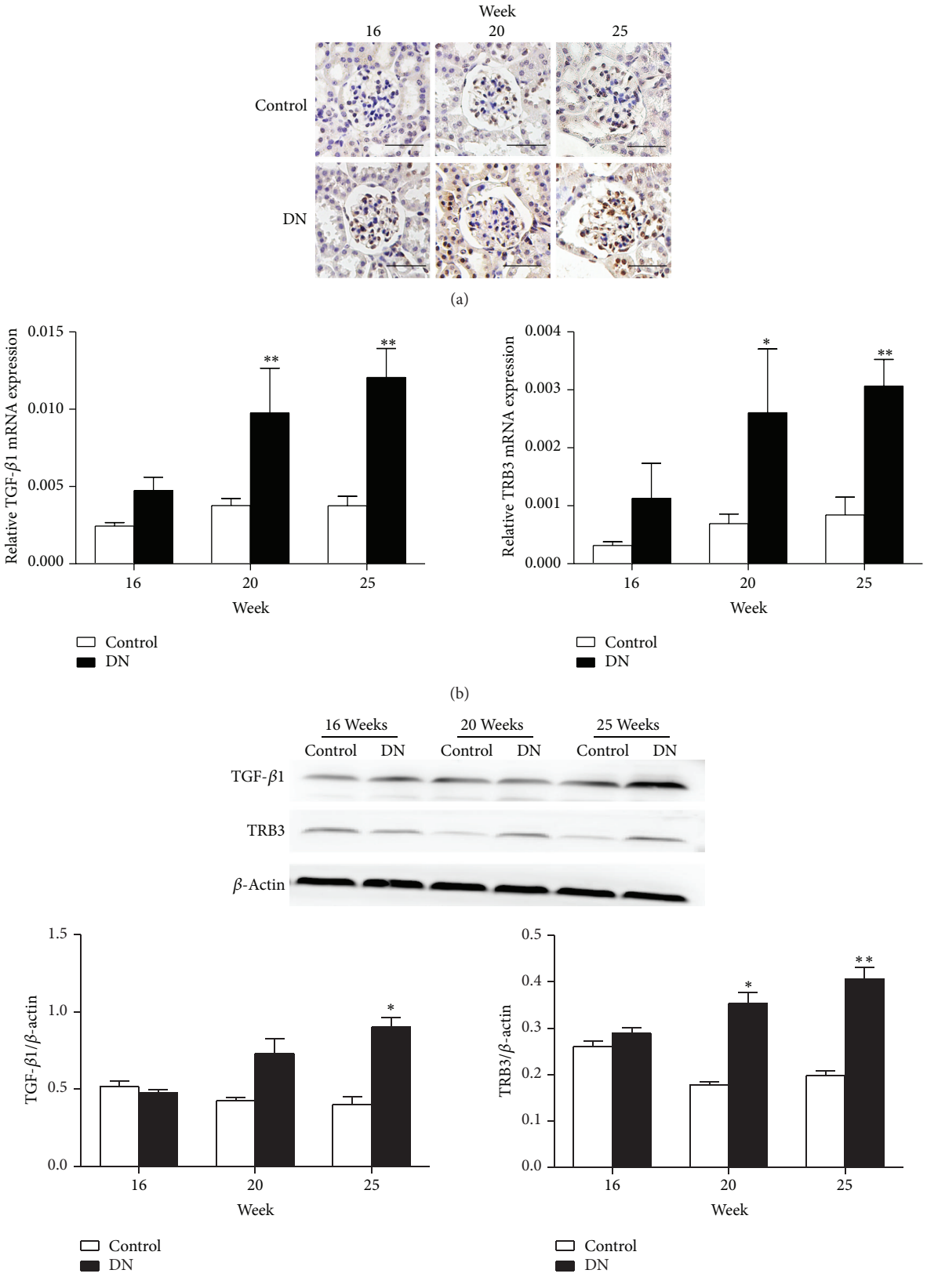


Figure 2: mRNA and protein expression of TGF-β1 and TRB3 upregulated in DN mice. (a) Protein TRB3 level in nucleus of intrinsic glomerular cells and tubular epithelial cells. Total TGF-β1 and TRB3 mRNA (b) and protein (c) levels by RT-PCR and western blot analysis, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ versus age-matched control mice. Scale bars, 50 μm. $n = 5$ mice per group.

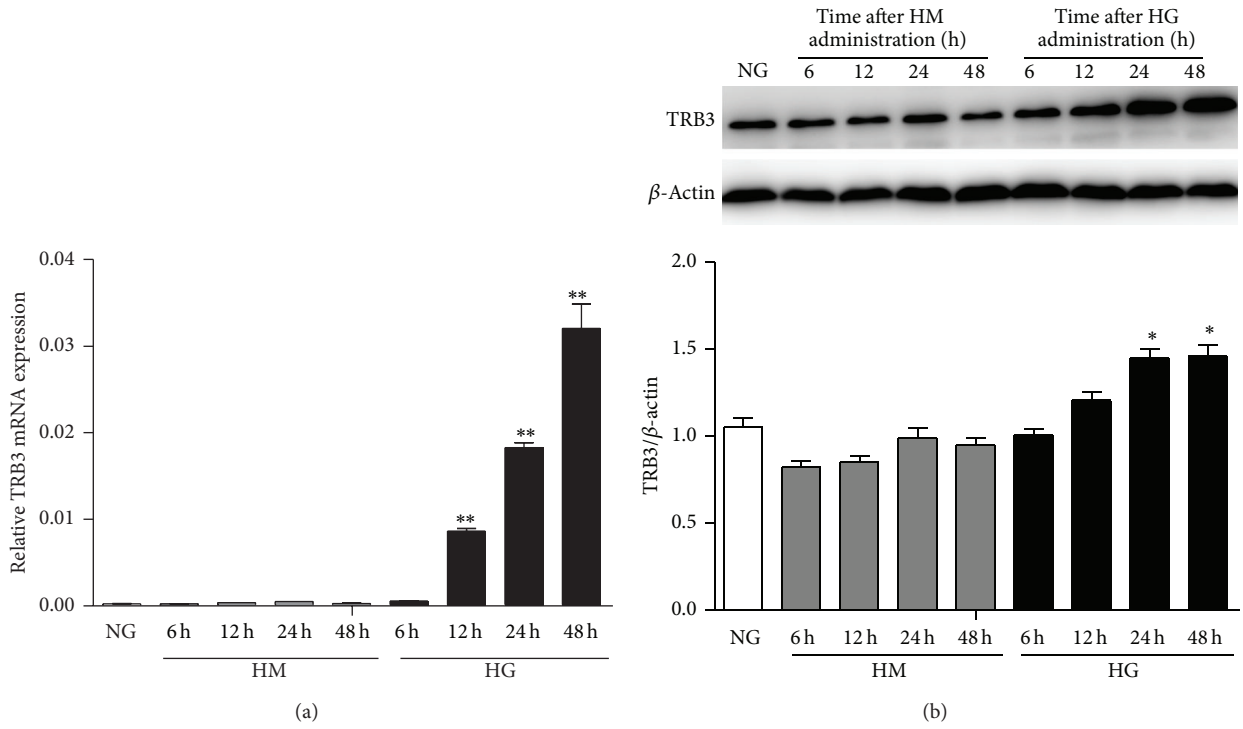


Figure 3: Effect of high glucose (HG) on TRB3 mRNA and protein levels in murine mesangial cells (MMCs) over time. MMCs were cultured in media containing NG and then stimulated with NG + high mannitol (HM) or HG for 6, 12, 24, and 48 h. (a) RT-PCR analysis of the mRNA level of TRB3. (b) Western blot analysis of the protein level of TRB3. Data are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus NG.

2.8. ELISA. Soluble collagen types I and IV proteins were determined by ELISA kits according to the manufacturer's protocol. The kits for mouse collagen types I and IV were species-specific and sensitive up to 1000, 10, and 20 ng/mL. All concentrations of proteins were normalized to the total protein amount.

2.9. Statistical Analyses. All values were expressed as means ± SEM. Images were analyzed by use of Image-Pro Plus 6.0 for semiquantitative analysis. Groups were compared by one-way ANOVA and correlation analysis involved Pearson correlation coefficient. Statistical analysis involved SPSS v17.0 for Windows (SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Evaluation of the DN Mouse Model. The blood glucose results for control mice remained stable, whereas DN mice showed hyperglycemia. Levels of UAE, Scr, and BUN and body weight were higher for db/db mice than age-matched controls ($P < 0.05$, $P < 0.01$) (Table 2). Mesangial matrix expansion and mesangial area were wider for DN than control mice. At 25 weeks, DN mice showed diffuse and nodular mesangial sclerosis (Figure 1(a)), and the area of mesangium matrix and basement membrane was increased (Figure 1(b)) as was the relative fibrosis area (Figure 1(c)).

3.2. TRB3 Expression Increased in Kidney of DN Mice. TRB3 was expressed mainly in the nucleus of intrinsic glomerular cells and tubular epithelial cells (Figure 2(a)). The expression of TRB3 was higher in DN than control mice. The mRNA and protein expression of TRB3 and TGF-β1 were higher in DN than control mice from 20 weeks and increased with time (Figures 2(b) and 2(c)). The protein level of TRB3 was positively correlated with TGF-β1 level ($r = 0.944$, $P < 0.01$) and renal interstitial fibrosis ($r = 0.857$, $P < 0.05$ in DN mice).

3.3. Effect of HG on the Expression of TRB3 in MMCs. To confirm the effect of glucose on the expression of TRB3, MMCs were stimulated with HG or HM for various times. The mRNA level of TRB3 was increased within 12 h after HG stimulation and peaked at 48 h ($P < 0.01$, versus NG; Figure 3(a)). TRB3 protein level was increased under HG at 12, 24, and 48 h (Figure 3(b)). This increase also peaked at 48 h ($P < 0.05$, versus NG). However, levels did not increase significantly under HM at different times. Thus, HG can upregulate the expression of TRB3 in MMCs.

3.4. HG Upregulated the Expression of TGF-β1 and Collagen Type IV in Cultured Cells. As compared with NG, HG time-dependently increased both the mRNA and protein expression of TGF-β1 and collagen type IV (Figure 4) within 48 h and 12 h, respectively, for mRNA and for both within 48 h for protein ($P < 0.01$, versus NG). However, collagen

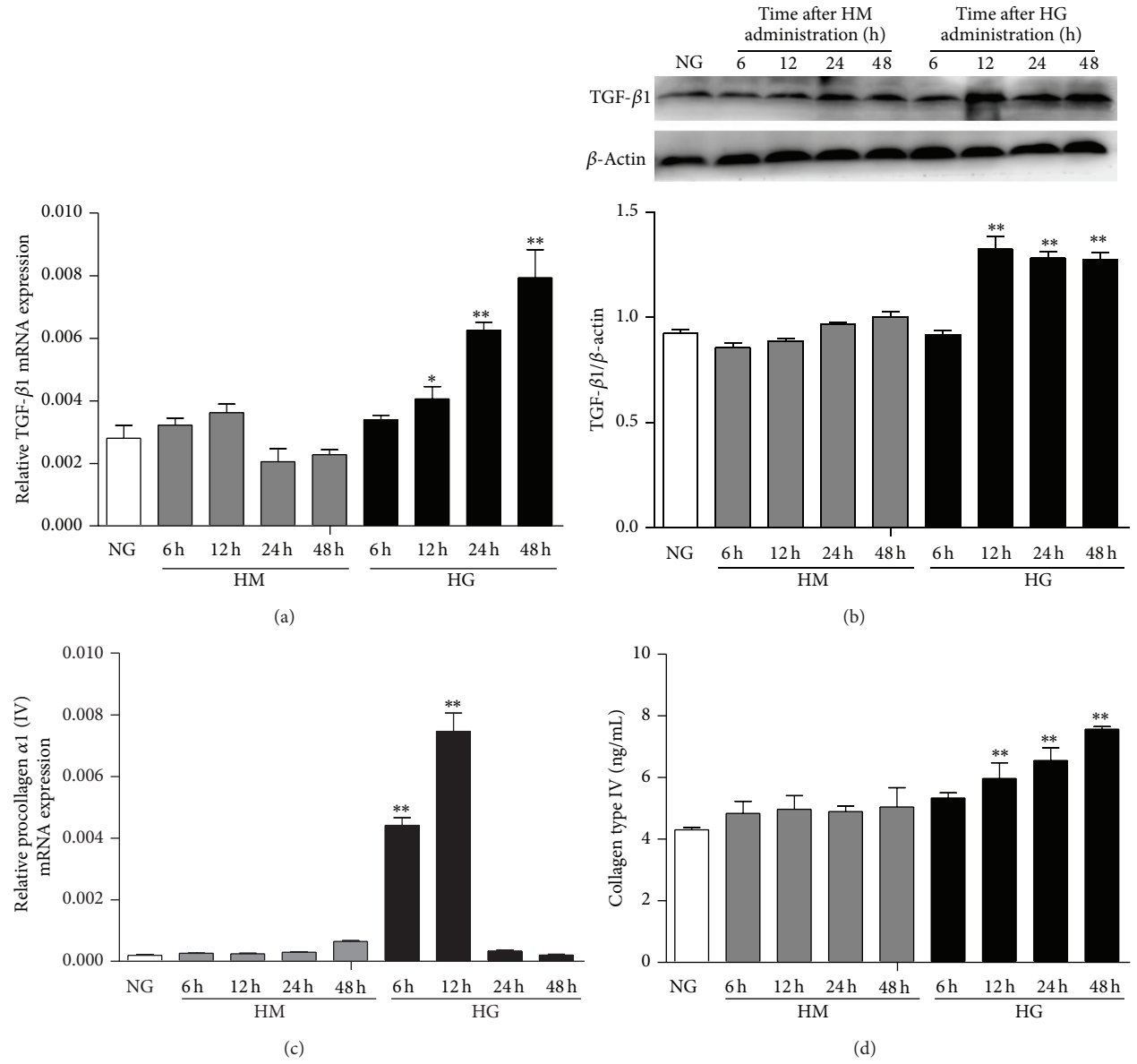


Figure 4: Effect of HG on mRNA and protein levels of TGF-β1 and collagen type IV in MMCs over time. MMCs were cultured in media containing NG and then stimulated with HM or HG for 6, 12, 24, and 48 h. (a) and (c) RT-PCR analysis of mRNA levels of TGF-β1 and collagen type IV, respectively. (b) Western blot analysis of protein level of TGF-β1. (d) ELISA of secretion of collagen type IV. Data are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus NG.

Table 2: Metabolic data of mice in different groups by time.

	16 weeks		20 weeks		25 weeks	
	C	DN	C	DN	C	DN
BW (mg)	22.80 ± 2.52	47.86 ± 0.83**	22.54 ± 1.24	52.26 ± 2.47**	23.26 ± 0.67	47.52 ± 2.56**
RBG (mmol/L)	4.48 ± 0.71	12.33 ± 1.67**	4.78 ± 0.84	24.15 ± 1.62$^{**\triangle}$	5.92 ± 0.87	33.08 ± 1.54$^{**\triangle}$
UAE (μg/24 h)	27.68 ± 1.65	386.12 ± 16.30**	24.20 ± 1.45	452.12 ± 32.26$^{**\triangle}$	27.24 ± 1.52	551.78 ± 29.19$^{**\triangle\triangle}$
BUN (mmol/L)	8.32 ± 0.37	9.38 ± 0.29*	8.56 ± 0.43	9.94 ± 0.20**	8.44 ± 0.45	10.48 ± 0.18$^{**\triangle}$
Scr (mmol/L)	14.80 ± 0.58	28.2 ± 1.7	18.00 ± 1.30	33.60 ± 3.37**	16.60 ± 1.67	25.40 ± 2.77**

Data are means ± SEM. C: control; DN: diabetic nephropathy; BW: body weight; UAE: urinary albumin excretion; BUN: blood urea nitrogen; Scr: serum creatinine.

$^{*}P < 0.05$, $^{**}P < 0.01$ versus age-matched control mice.

$^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ versus 16-week-old db/db mice.

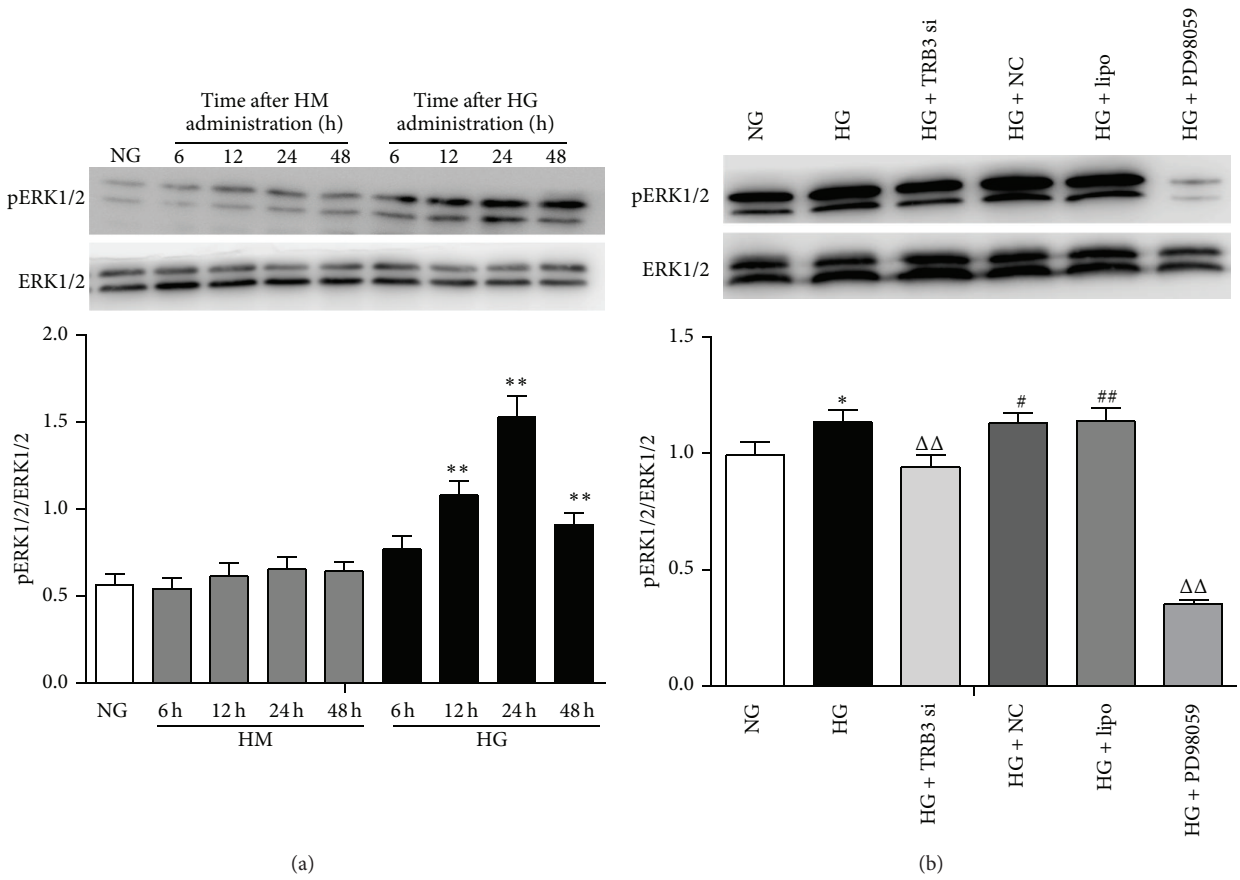


Figure 5: Effect of TRB3 on phosphorylated ERK1/2 (pERK1/2) expression in cells. (a) Western blot analysis of pERK1/2 level in cells incubated with HM or HG for 6, 12, 24, and 48 h. (b) Cells were transfected with TRB3 siRNA or corresponding negative control. Western blot analysis of pERK1/2 level with HG stimulation for 48 h. Data are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus NG, $^{\Delta\Delta}P < 0.01$ versus HG, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus NG +TRB3 siRNA.

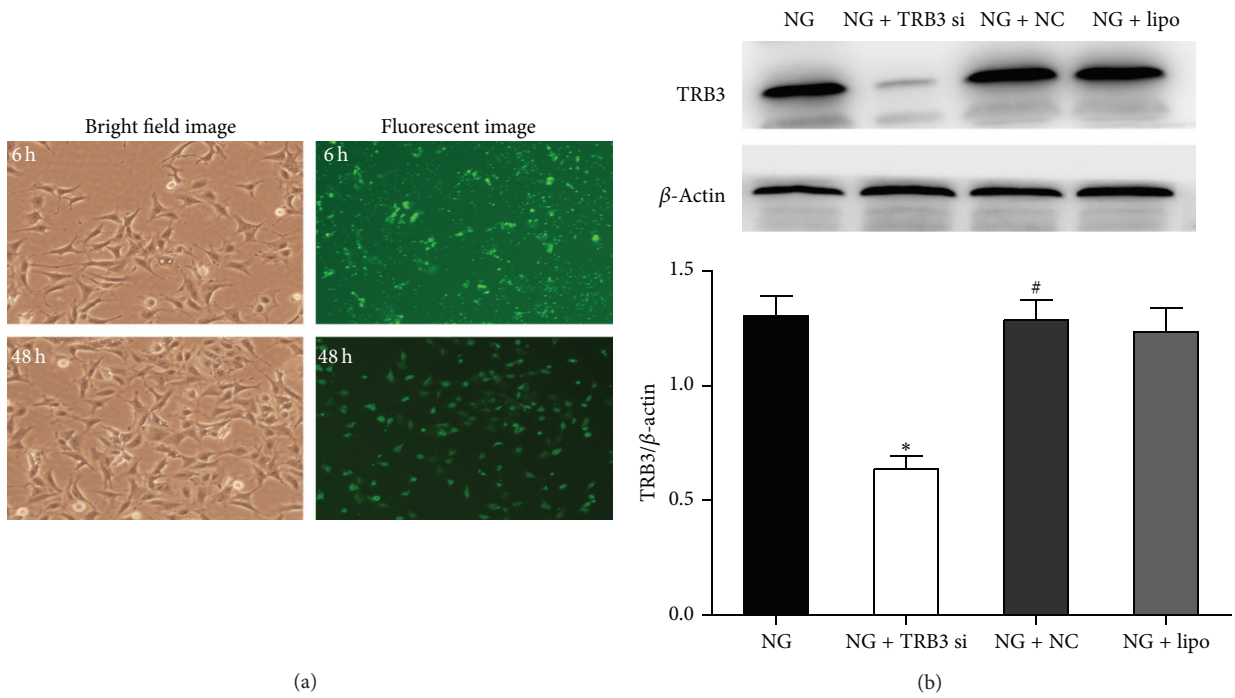
type I expression did not change under any conditions within 48 h (data not shown). Therefore, HG increased TGF-β1 and collagen type IV secreted from cultured MMC cells.

3.5. Effect of TRB3 on Activation of the ERK1/2 MAPK Pathway in MMCs. To verify the effect of glucose on the activation of the ERK1/2 MAPK pathway in MMCs, cells were cultured in NG medium and then stimulated with HG or HM for various times. The level of pERK1/2 increased during the first 6 h ($P < 0.01$, versus NG) after HG stimulation and peaked at 24 h ($P < 0.01$, versus NG) (Figure 5(a)). However, stimulation with HM had no effect on the activation of this pathway. Therefore, HG can activate the ERK1/2 pathway in MMCs. To confirm the effect of TRB3 on this pathway, we transfected TRB3 siRNA into MMCs exposed to HG medium for 24 h and evaluated pERK1/2 levels. Transient transfection of siRNA into MMCs induced FAM expression, which indicated successful transfection. Expression of FAM increased at 6 h and peaked at 48 h (Figure 6(a)). To test the efficacy of the selected siRNA sequence, we measured the protein level of TRB3 after 48 h transfection with TRB3 siRNA. TRB3 protein expression was lower in MMCs with TRB3 siRNA than in cells with control siRNA ($P < 0.05$, versus NG + NC) (Figure 6(b)). pERK1/2 expression was decreased in MMCs transfected with TRB3 siRNA ($P < 0.05$, versus HG + NC; Figure 5(b)). TRB3 may activate the ERK1/2 MAPK pathway in MMCs.

3.6. TRB3 is Involved in the Expression of TGF-β1 and Collagen Type IV Regulated by High Glucose in DN by Activating ERK1/2 MAPK. The expression of pERK1/2 was markedly reduced by blocking ERK1/2 MAPK signaling by a specific ERK inhibitor (PD98059, 10 μmol/L) (Figure 5(b)). The mRNA and protein levels of TGF-β1 and collagen type IV were decreased with the ERK1/2 pathway blocked (Figures 7(a)–7(d), $P < 0.01$ versus HG) as was the pERK1/2 level (Figure 5(b), $P < 0.01$ versus HG), which suggests that HG can regulate TGF-β1 and collagen type IV through the ERK1/2 MAPK pathway. The protein expression of TRB3 was markedly increased after blocking the ERK1/2 MAPK pathway (Figure 7(e), $P < 0.01$ versus HG). This finding indicates a possible feedback regulation between TRB3 and some downstream cytokines of ERK1/2 MAPK.

4. Discussion

Here, we found augmented expression of TRB3 in kidneys of DN mice, which was positively related to TGF-β1 expression



(a) (b)

Figure 6: Effect of siRNA on the expression of FAM and TRB3 in cells. Transient transfection of cells with TRB3 siRNA induced FAM expression, indicating successful transfection. FAM expression was observed at 0 and 48 h after transfection. Cells were transfected with TRB3 siRNA or its corresponding negative control and then incubated with NG (5.5 mM glucose) for 48 h. (a) FAM expression was observed by bright field and fluorescent imaging (200x). (b) Western blot analysis of the protein expression of TRB3 at 48 h after TRB3 siRNA transfection. Data are mean ± SEM. $^{*}P < 0.05$ versus NG; $^{\#}P < 0.05$ versus NG +TRB3 siRNA.

and renal interstitial fibrosis. HG upregulated the expression of TRB3, TGF-β1, collagen type IV, and phosphorylated-ERK1/2 MAPK in MMCs. After inhibiting TRB3 with TRB3 siRNA, the HG-induced level of pERK1/2 MAPK was attenuated and HG-induced expression of TGF-β1 and collagen type IV decreased. Moreover, the protein expression of TRB3 was increased after blocking the ERK1/2 MAPK pathway. TRB3 may be involved in DN by regulating the fibrosis cytokine TGF-β1 and collagen type IV via ERK1/2 MAPK signaling.

Tribbles was first discovered to regulate Drosophila embryogenesis, and it has the same location as the type 2 diabetes gene, so TRB3 may have a natural link with diabetes. Compared with wild-type mice, db/db diabetic mice were found to have increased TRB3 mRNA and protein expression in liver; TRB3 expression increased in the db/db mouse liver promoted blood glucose and increased glucose tolerance [9]. However, few studies have reported the expression and role of TRBs in DN. We examined the expression of TRB1, TRB2, and TRB3 in the DN mouse kidney and found only TRB3 expressed differently between DN and control groups, so TRB3 could be a potential cytokine to widen the current knowledge of DN. Our further study revealed that the mRNA and protein levels of TRB3 were higher in the DN than normal kidney, which was also positively correlated with TGF-β1 protein level and content of collagen. Previous study showed that TRB3 was positively correlated with kidney tissue fibrosis, which may play a role in promoting the progression of fibrosis by inducing the transformation between epithelial and mesenchymal tissue [15]. Therefore, TRB3 may be involved in DN by inducing interstitial fibrosis, in which TGF-β1 plays a key role.

We wondered about the role of TRB3 in the fibrosis of DN and the relationship between TRB3 and TGF-β1. Several studies supported that TRB3 may function as a scaffold protein to control MAPK activity [10, 16, 17]. Among the several MAPK signal pathways, the ERK1/2 pathway is activated under HG in mesangial cells, followed by the complicated synthesis of TGF-β1 [18]. The activation of the ERK1/2 pathway is necessary for HG-induced production of TGF-β1 and connective tissue growth factor (CTGF) in MMCs [19]. Our preliminary study excluded the involvement of the p-38 and JNK MAPK pathway. Therefore, we focused on the ERK MAPK pathway. Silencing TRB3 decreased ERK1/2 activation, followed by decreased mRNA and protein levels of TGF-β1 and collagen type IV in MMCs, so TRB3 may participate in renal fibrosis of DN by upregulating TGF-β1 and collagen type IV in MMCs via ERK MAPK signaling. To our knowledge, our study is the first to reveal the interaction between TRB3, ERK1/2, and TGF-β1 in renal tissue of DN.

Recently, TRB3 was found stimulated in diabetic kidneys, regulated by the ER stress marker CHOP, and inhibited the podocyte expression of monocyte chemoattractant protein 1, which first suggested that TRB3 plays a protective role in diabetic kidney disease [20]. These findings that differ from our results may be due to cell specificity [21]; we used mesangial cells, but the previous study used podocytes.

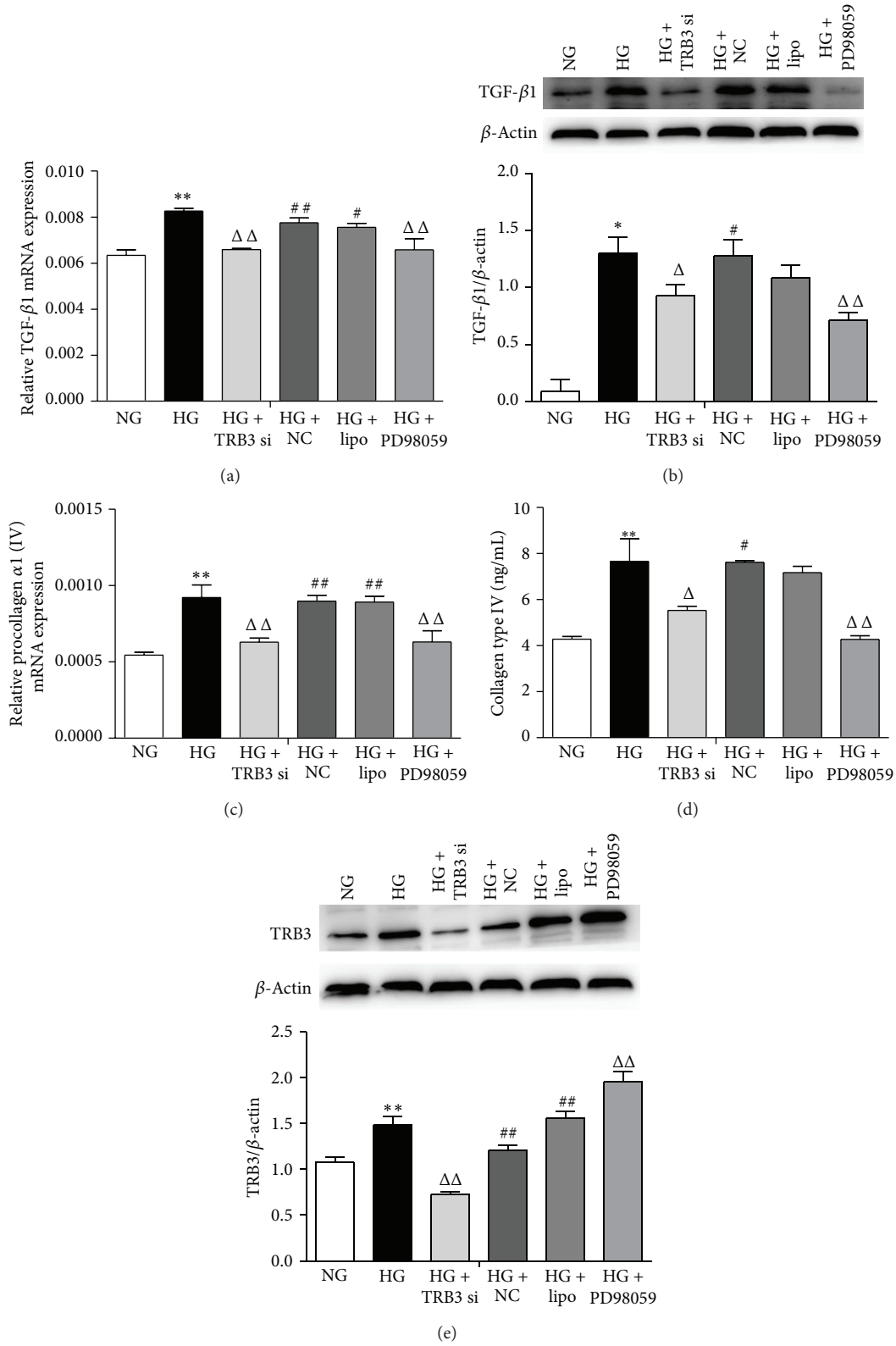


Figure 7: Effect of silencing TRB3 protein or blocking ERK1/2 MAPK pathway by a specific ERK inhibitor (PD98059, 10 μmol/L) on HG induction of TGF-β1, collagen type IV, and TRB3 level in MMCs. (a) and (b) mRNA level of TGF-β1 at 12 h and protein level at 48 h; (c) and (d) mRNA level of collagen type IV at 12 h and protein level at 48 h. MMCs were stimulated with NG or HG after blockade of TRB3 with TRB3 siRNA. (e) Protein expression of TRB3 at 48 h. Data are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus NG; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ versus HG; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus NG + TRB3 siRNA.

Moreover, signal transduction *in vivo* is a complex system. Numerous cytokines may interact through different pathways, and different signal pathways may have "crosstalk." So, the role of TRB3 in DN can also be studied from other signal pathways. TRB3 plays a role in the pathogenesis of DN by participating in insulin resistance, functioning as a negative modulator of Akt [9, 22, 23]. Another study showed that TRB3 may cause renal cell apoptosis by participating in the NF-κB pathway and thus participate in the process of renal fibrosis in DN [24, 25]. Even Smad3 is considered to mediate TRB3 enhancing TGF-β signaling [26]. There must be some interactions between ERK, Akt, NF-κB, Smad3, and other unknown pathways. More detailed and systemic studies are needed to establish the complete theory on TRB3 involved in DN.

In this study, we found that the expression of TRB3 increased after blocking the ERK1/2 MAPK pathway, which may indicate a negative feedback regulation between TRB3 and some downstream cytokines of ERK1/2 MAPK or special "crosstalk" with other signal pathways, which requires further exploration.

5. Conclusions

TRB3 expression is upregulated in renal tissue of DN mice *in vivo* and MMCs *in vitro*. TRB3 may be involved in DN by regulating the fibrosis cytokine TGF-β1 and collagen type IV via ERK1/2 MAPK signaling.

Conflict of Interests

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

Authors' Contribution

Jinhang Zhang and Luwei Zhang contributed equally to this work.

Acknowledgments

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Research Article

Association between the −794 $(CATT)_{5–8}$ *MIF* Gene Polymorphism and Susceptibility to Acute Coronary Syndrome in a Western Mexican Population

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The macrophage migration inhibitory factor (*MIF*) is related to the progression of atherosclerosis, which, in turn, is a key factor in the development of acute coronary syndrome (ACS). *MIF* has a CATT short tandem repeat (STR) at position −794 that might be involved in its expression rate. The aim of this study was to investigate the association between the −794 $(CATT)_{5–8}$ *MIF* gene polymorphism and susceptibility to ACS in a western Mexican population. This research included 200 ACS patients classified according to the criteria of the American College of Cardiology (ACC) and 200 healthy subjects (HS). The −794 $(CATT)_{5–8}$ *MIF* gene polymorphism was analyzed using a conventional polymerase chain reaction (PCR) technique. The 6 allele was the most frequent in both groups (ACS: 54% and HS: 57%). The most common genotypes in ACS patients and HS were 6/7 and 6/6, respectively, and a significant association was found between the 6/7 genotype and susceptibility to ACS (68% versus 47% in ACS and HS, resp., $P = 0.03$). We conclude that the 6/7 genotype of the *MIF* −794 $(CATT)_{5–8}$ polymorphism is associated with susceptibility to ACS in a western Mexican population.

1. Introduction

Acute coronary syndrome (ACS) is characterized by acute, regional reductions in coronary blood flow and myocardial ischemia [1]. ACS describes a spectrum of clinical manifestations, including unstable angina (UA), non-ST-segment elevation myocardial infarction (NSTEMI), and ST-segment elevation myocardial infarction (STEMI) [1–3]. The vast majority of ACS is triggered by disruption of an atherosclerotic plaque [1]. Atherosclerosis is a chronic inflammatory disease of the arterial wall, characterized by endothelial dysfunction, intimal hyperplasia, and smooth muscle proliferation, as well as deposition of lipids and formation of microvessels within the vascular wall [1, 4–6]. Endothelial dysfunction is accompanied by the expression of adhesion molecules (such as the vascular cell adhesion molecule-(VCAM-) 1) [7, 8] and chemokines [4, 9]. The recruitment of inflammatory cells is triggered by the production of cytokines (such as IL-1β, IL-6, IL-8, TNF-α, and CCL2) [6] within the plaque microenvironment. Chemokines are released from

endothelial cells, mast cells, platelets, macrophages, and lymphocytes [10].

The macrophage migration inhibitory factor (*MIF*) is a molecule that consists of 115 amino acids [11]; it was described as the main cytokine involved in attracting immune cells such as macrophages and T and B cells [12, 13]. The secretion of *MIF* by inflammatory cells can be induced by exposure to oxidized low-density lipoprotein or other cytokines, such as TNF-α and interleukin-c [13, 14]. *MIF* activates the expression of various proinflammatory cytokines and chemokines and recruits macrophages to the site of atherosclerosis [12]. *MIF* participates in the pathogenesis of inflammatory and atherosclerosis processes [12, 13, 15–17].

The *MIF* gene is located at 22q11.2 [18] and contains several polymorphisms, including the rs5844572. This genetic marker is a CATT short tandem repeat (STR) at position −794, with five to eight length variants (alleles 5 to 8). There is an association between the length of the repeats and the expression of the genetic marker; the higher alleles (CATT6, CATT7, and CATT8) show higher expression of the gene [13]. Since *MIF* is an inflammatory mediator and given the role of genetic factors that modify its expression, *MIF* could contribute to atherosclerosis and susceptibility to ACS. The aim of this study was to investigate the association between the −794 $(CATT)_{5-8}$ *MIF* gene polymorphism and susceptibility to ACS in a western Mexican population.

2. Materials and Methods

2.1. Subjects. The study group included 200 ACS unrelated patients recruited from Hospital de Especialidades del Centro Médico Nacional de Occidente del Instituto Mexicano del Seguro Social (CMNO-IMSS) and classified according to the criteria of the American College of Cardiology (ACC) [19]. As a control group, 200 unrelated healthy subjects (HS) were recruited from the general population of western Mexico. We applied a standardized questionnaire to the HS group and applied routine laboratorial clinical assessments to detect any potential alterations, and those subjects with any clinical alterations were excluded of the study. We considered Mexican Mestizo subjects, only those individuals who for three generations, including their own, had been born in western Mexico. The participation of the subjects was voluntary and all signed a written informed consent. The study conforms to the ethical principles contained in the declaration of Helsinki, and ethical approval was obtained from Centro Universitario de Ciencias de la Salud, CUCS, UdeG (C.I. 069-2012).

2.2. Genotyping of the MIF −794 $(CATT)_{5-8}$. Genomic DNA was extracted from peripheral blood leukocytes using Miller's Technique [20]. Genotyping of the STR −794 $(CATT)_{5-8}$ polymorphism was achieved by conventional PCR and polyacrylamide gel electrophoresis using the primers reported by Radskate et al. [21]. Cycling conditions were as follows: an initial denaturation at 95°C for 4 min followed by 30 cycles of 30s at 95°C, 30s at 60°C, and 30s at 72°C and then a final extension of 2 min at 72°C. Amplification products were further electrophoresed on a 19:1 10% polyacrylamide gel. Allele identification was done by using a 10-bp (Invitrogen) and homemade allelic ladders containing pooled samples. In addition, as genotyping control, automatized sequencing of one random sample of each homozygote genotype allowed confirmation of results (ABI PRISM 377 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA).

Table 1: Clinical characteristics in the study population.

Parameter	ACS *n* (%)	HS *n* (%)	
Age(years)	63 (37–91)*	61 (26–91)*	
Gender			
Male	152 (76)	136 (68)	
Female	48 (24)	64 (32)	
ACS diagnosis		ACS risk factor *n* (%)	
UA	26 (13)	Obesity	79 (61)
NSTEMI	22 (11)	DM2	50 (50)
		DYS	93 (47)
STEMI	152 (76)	HBP	119 (60)

Non-ST-segment elevation myocardial infarction (NSTEMI), unstable angina (UA), and ST-segment elevation myocardial infarction (STEMI). DM2: type 2 diabetes mellitus, DYS: dyslipidemia HBP: high blood pressure.
*Minimum–maximum.

2.3. Statistical Analysis. The Hardy-Weinberg equilibrium test and genotype and allele frequencies were calculated by the chi-square test or Fisher's exact test, when applicable. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated to test the probability that the genotype and allele frequencies were associated with ACS. A P value <0.05 was considered to be statistically significant. All the statistical analyses were done with the Stata 9.0 software.

In this study the sample size was calculated according to the minor allele frequency of the −794 $(CATT)_{5-8}$ *MIF* gene polymorphism reported in Mexican Mestizo population by Llamas-Covarrubias et al. [16] and we obtained at least 164 alleles (22.14%) using the Kelsey formula. It means that in order to detect differences, we needed at least 82 individuals each group with 95% confidence interval and statistical power of 80%. In this respect 200 individuals were included exceeding by far the required sample size.

3. Results

3.1. Clinical Characteristics. All clinical characteristics are shown in Table 1. The median age of HS and ACS groups was 61 and 63 years, respectively. The gender distribution among ACS individuals was 76% male and 24% female. The most prevalent clinical diagnosis in the ACS group was STEMI (76%), while obesity was the most common risk factor (61%), followed by high blood pressure (HBP: 60%). Also, in the ACS group other clinical parameters such as troponin I (TnI), troponin T (TnT), creatine phosphokinase (CPK), and creatine kinase (CK) were evaluated in order to find a possible genetic association. However, we did not find any statistical association with −794 $(CATT)_{5-8}$ *MIF* gene polymorphism.

3.2. Distribution of the MIF −794 $(CATT)_{5-8}$ Polymorphism. No deviation from the Hardy-Weinberg equilibrium was

Table 2: Allele and genotype distributions of −794 $(CATT)_{5–8}$ *MIF* polymorphisms in ACS and HS.

Polymorphism	ACS $n = 200$ (%)	HS $n = 200$ (%)	OR (CI 95%); P^*
−794 $CATT_{5–8}$ *MIF*			
Genotype			
5,5	9 (4)	6 (3)	2.76 (0.71–12.86); 0.09
5,6	41 (21)	52 (26)	0.93 (0.52–1.66); 0.79
5,7	19 (9)	18 (9)	1.45 (0.64–3.35); 0.33
6, 6[§]	53 (27)	65 (33)	1
6,7	**68 (34)**	**47 (23)**	**1.77 (1.05–2.98); 0.03**
7,7	10 (5)	12 (6)	1.02 (0.36–2.81); 0.96
Allele			
5	78 (19)	82 (20)	1.01 (0.70–1.45); 0.94
6[§]	215 (54)	229 (57)	1
7	107 (27)	89 (23)	1.28 (0.91–1.79); 0.15

ACS: acute coronary syndrome, HS: healthy subjects, OR: odd ratio, and CI: confidence interval. $^*P < 0.05$.
MIF: macrophage migration inhibitory factor.

detected in the −794 $(CATT)_{5–8}$ *MIF* polymorphism ($P = 0.16$). The allele and genotype frequencies in both the ACS and HS groups are shown in Table 2. Allele 6 was the most frequent in both groups (ACS: 54% and HS: 57%). The most common genotypes in ACS and HS subjects were 6/7 and 6/6, respectively. A significant association was found between the 6/7 genotype and susceptibility to ACS (68% versus 47% in ACS and HS, resp., $P = 0.03$).

3.3. Risk Factors in the ACS Group Related to the Genotypes of the −794 $(CATT)_{5–8}$ MIF Polymorphism. We also stratified the main risk factors for development of ACS by genotype (Table 3). Despite no significant differences, we observed that the 6/7 genotype was the most frequent in each subgroup and that the 5/5 genotype was the less common.

4. Discussion

Acute coronary syndrome is a multifactorial disease arising through a combination of both environmental and genetic risk factors. Several studies have reported a relationship between atherosclerosis and ACS [8, 22]. Inflammation regulates the stability of the atherosclerotic plaque [8]. Activated endothelial cells express adhesion molecules and favor the recruitment of monocytes to the endothelium. These in turn release proinflammatory cytokines including IL-1, IL-6, TNF-α, and *MIF*. Several researches have focused on the role of *MIF* in the atherosclerosis process. Ayoub et al. report that *MIF* enhances macrophage uptake of oxidized LDL in the progression of atherosclerosis [23]. Zernecke et al. reported a correlation between the expression of *MIF* and an increased intima-media thickness and also with lipid deposition in carotid artery plaques [4]; White et al. reported a proinflammatory role for *MIF* in acute myocardial infarction [24]; Müller et al. demonstrated that the expression of *MIF* is significantly higher in ACS patients [15].

The *MIF* promoter has a CATT STR polymorphism at position −794. This genetic marker has five to eight variants $(CATT)_{5–8}$. A higher repeat number is associated with an increase in the gene expression [13]. In the present research, we reviewed the association of the −794 $(CATT)_{5–8}$ *MIF* polymorphism with ACS in Mexican Mestizo individuals. The genotype and allele frequencies were distributed in a similar way to a previous study with a Mexican Mestizo population by Llamas-Covarrubias et al. [16]. We also compared the frequencies with those of Caucasian American and African American populations [25] and observed that the genotype frequencies in our population differed from them; they found that the genotype 6/6 was the most frequent, while the 5/8 and 6/8 genotypes were less frequent in Caucasian Americans and African Americans, respectively. In addition, a statistical difference was found in genotypic and allelic distributions when compared with an African ethnic group where the 5/6 genotype and the 5 allele showed the highest frequency [26]; these differences could be attributed to the ancestry of the population. The Mexican Mestizo population is a crossbreed of Amerindian, European, and African populations, with an estimated contribution of 21–25%, 60–64%, and 15%, respectively (Rubi-Castellanos, 2009).

In this study, we found an association between the 6/7 genotype of the *MIF* −794 $(CATT)_{5–8}$ polymorphism and susceptibility to ACS in a western Mexican population. The 6/7 carriers present 1.77 more susceptibility to develop ACS. We did not find any association with respect to the allelic frequencies. There is also another single nucleotide polymorphism in the promoter of the *MIF* gene at the position −173 that has been previously analyzed and related to the inflammatory response in coronary bypass surgery [27] and coronary alterations in children with Kawasaki disease [28].

Recent studies support the fact that *MIF* is a pleiotropic cytokine mainly released from macrophages that has been shown to be increasingly expressed during atherogenesis,

Table 3: Risk factors in the ACS group related to the genotypes of the −794 $(CATT)_{5-8}$ *MIF* polymorphism.

Phenotype	Genotypes of the −794 $(CATT)_{5-8}$ *MIF* polymorphism								
	ACS (*n*)	5,5	5,6	5,7	6,6	6,7	7,7	OR (CI 95%)	*P*
Obesity	80	4 (5.06)	15 (18.99)	6 (7.59)	22 (27.85)	25 (31.65)	7 (8.86)	3.29 (0.65–21.48)	0.10
DM2	100	3 (3)	20 (20)	10 (10)	27 (27)	34 (34)	6 (6)	0.48 (0.07–2.57)	0.33
DYS	93	5 (5.38)	22 (23.76)	6 (6.45)	24 (25.81)	30 (32.26)	6 (6.45)	0.56 (0.15–1.89)	0.30
HBP	119	6 (5.04)	26 (21.85)	9 (7.56)	32 (26.89)	37 (31.09)	9 (7.56)	5.91 (0.71–270.94)	0.07

DM2: type 2 diabetes mellitus, DYS: dyslipidemia, and HBP: high blood pressure.

oxLDL being a major inducer [14, 23, 24]. ACS is a pathology where inflammation favors the recruitment of monocytes to the atherosclerotic plaque; otherwise, new studies have described the functional effect of *MIF* −794 $(CATT)_{5-8}$, showing that the $CATT_5$ allele has the lowest transcriptional activity and the $CATT_{6-8}$ alleles increase the expression rate. This evidence validates the association found between the 6/7 genotype of the *MIF* −794 $(CATT)_{5-8}$ polymorphism and susceptibility to ACS. The molecular mechanisms that regulate *MIF* expression have been poorly studied. Recently, Chen et al. showed that the transcriptional repressor HBP1 (HMG box-containing protein 1) negatively regulates *MIF* expression, but this is still under investigation [29].

We also stratified the risk factors in the ACS group regarding the genotypes of the −794 $(CATT)_{5-8}$ *MIF* polymorphism. As we mentioned, *MIF* has an important function in inflammatory processes (Kleemann et al. [30]), suggesting that *MIF* controls the development of metabolic pathologies associated with ACS risk factors such as obesity and type 2 diabetes mellitus. Nevertheless, we did not find any association between the genotypes of the −794 $(CATT)_{5-8}$ *MIF* polymorphism and the risk factors in the ACS group. However a trend is shown between the 6/7 genotype and obesity, type 2 diabetes mellitus, dyslipidemia, and high blood pressure. We want to highlight that, as a limitation of the study, the sample size in each subgroup is not enough to find an association, but there is a marked tendency with the higher alleles, which are responsible for a higher expression.

It is worth noting that the −794 $(CATT)_{5-8}$ promoter polymorphism of *MIF* has not been studied in relation to susceptibility to ACS.

5. Conclusion

The 6/7 genotype of the *MIF* −794 $(CATT)_{5-8}$ polymorphism is related with susceptibility to ACS in a western Mexican population.

Conflict of Interests

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

Acknowledgment

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Research Article

Human Adipose Tissue Macrophages Are Enhanced but Changed to an Anti-Inflammatory Profile in Obesity

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Objective. Adipose tissue (AT) macrophages are increased in obesity and associated with low grade inflammation. We aimed to characterize the phenotype of AT macrophages in humans in relation to obesity and insulin resistance.*Design*. Gene-expression levels of general macrophage markers (CD68 and CD14), proinflammatory markers/M1 (TNF-α, MCP-1, and IL-6), and anti-inflammatory markers/M2 (CD163, CD206, and IL-10) were determined by RT-PCR in subcutaneous AT samples from lean and obese subjects. Insulin resistance was determined by HOMA-IR.*Results*. All the macrophage markers were elevated in the AT from obese compared to lean subjects ($P < 0.001$). To determine the phenotype of the macrophages the level of CD14 was used to adjust the total number of macrophages. The relative expression of CD163 and IL-10 was elevated, and TNF-α and IL-6 were reduced in AT from obese subjects (all $P < 0.05$). In a multivariate regression analysis CD163 was the only macrophage marker significantly associated with HOMA-IR (β: 0.57; $P < 0.05$). Conclusion. Obesity is associated with elevated numbers of macrophages in the AT. Unexpectedly, the macrophages change phenotype by obesity, with a preponderance of M2 and a decrement of M1 markers in AT from obese subjects. Moreover, CD163 was the only macrophage marker associated with HOMA-IR after multiple adjustments.

1. Introduction

In obese subjects there are an increased number of macrophages in the adipose tissue (AT), which produce several cytokines that contribute to local AT inflammation and to systemic low grade inflammation [1–3]. It is believed that adipocyte hypertrophy and local hypoxia due to adipocyte expansion are two important contributing factors to the increased accumulation of macrophages in AT in the obese state [4, 5]. The adipose tissue macrophages (ATMs) of obese subjects are often located in "crown like structures" (CLS) surrounding dead adipocytes [6] and they are also found in elevated numbers in fibrotic areas in the AT [7]. ATMs may have a scavenger function in response to necrotic adipocytes, but the role in regard to fibrosis is unclear.

Macrophage phenotypes are often divided into pro- (M1) or anti-inflammatory (M2) subpopulations. The M1 or classical activated macrophages are induced by, for example, LPS and TNF-α and produce proinflammatory cytokines. The M2 or alternative activated macrophages are induced by, for example, glucocorticoids, IL-4, and IL-10 and produce anti-inflammatory cytokines [5, 8–10]. An intermediate phenotype has also been described, which resembles anti-inflammatory M2 markers but at the same time secretes large amounts of proinflammatory cytokines [11, 12]. In obese mice an increased number of macrophages in the AT are observed which is similar to the human situation. In rodent models diet-induced obesity generally leads to a shift in the phenotype in ATMs from a M2-polarized state in lean animals to a M1-polarized state in obese animals [13, 14]. In humans the results concerning changes in polarization in obese subjects are less clear. Some studies have shown that the levels of proinflammatory markers in subcutaneous ATMs are elevated in obese subjects compared with lean subjects [3, 15]. And it has been shown that weight loss induced by either very low energy diet (VLED) or gastric bypass

induces an increased level of anti-inflammatory macrophage markers and reduced level of proinflammatory macrophage markers in subcutaneous AT [3, 15, 16]. However, one study has shown that subcutaneous ATMs change to a more anti-inflammatory phenotype by obesity [7].

Chronic low grade inflammation in obesity is associated with insulin resistance, which predispose to the development of type 2 diabetes. Several studies have described an association between ATMs and insulin resistance [2] and a recent study has described a positive association between proinflammatory macrophages in the AT and systemic insulin resistance measured by HOMA-IR [17].

With the present study we wanted to examine whether the phenotype (polarization) of ATMs is changed in the obese state in humans. As proinflammatory markers we used the gene-expression level of monocyte chemoattractant protein-1 (MCP-1), tumor necrotic factor-α (TNF-α), and interleukin 6 (IL-6). MCP-1 is involved in the recruitment of macrophages and we have earlier found that mRNA MCP-1 levels in human AT samples correlate with measures of adiposity [18]. Both TNF-α and IL-6 are known proinflammatory mediators, which have been used as proinflammatory markers in other studies [19, 20]. As anti-inflammatory markers we used the gene expression of CD163, CD206, and interleukin 10 (IL-10), all of them frequently used as anti-inflammatory markers in the literature [3, 11, 19, 20]. CD206 is a mannose receptor and CD163 is a scavenger receptor involved in clearance of haptoglobin-hemoglobin complexes, from ruptured red blood cells [21, 22]. Soluble CD163 (sCD163) is the extracellular part of CD163 and it is found to be shed of the receptor during proinflammatory conditions and in obesity [23–26].

We hypothesized that there is a shift in phenotype towards an increased level of proinflammatory macrophage markers in AT from obese subjects compared with AT from lean subjects as generally found in rodent models. Furthermore, we wanted to study the relationship between insulin resistance at the whole body level measured by HOMA-IR and pro- and anti-inflammatory macrophage markers in AT.

2. Material and Methods

2.1. Subjects. Fat biopsies were obtained from the subcutaneous abdominal AT and originated from two previously performed studies. Only baseline fat biopsies were used in the present study. Study 1: AT samples were obtained from 21 lean, healthy controls and 21 obese and otherwise healthy subjects who participated in a weight loss program with VLED for 12 weeks, as previously described [27]. Study 2: AT samples were obtained from 14 lean, healthy controls and 36 obese and healthy subjects, who participated in a 12-week weight loss intervention program with exercise alone, VLED, or a combination of exercise and VLED [28]. Both studies took place at the research laboratory at Aarhus University Hospital. All subjects were recruited via advertisements in local newspapers. None of the subjects had type 2 diabetes or took medicine that could affect inflammation or adipose tissue metabolism. Inclusion and exclusion criteria are previously described [27, 28]. The subjects were all Caucasian and had a sedentary life style. The study was approved by the local ethics committee in the county of Aarhus and followed the principles of the Declaration of Helsinki.

2.2. Anthropometrics and Blood Samples. Anthropometrics and fasting blood samples were collected after an overnight fast. All participants were asked not to engage in excessive physical exercise or alcohol intake the day before or in the morning of clinical investigations. Venous blood samples were collected and serum frozen at −80°C. The homeostasis model assessment insulin resistance index (HOMA-IR) was calculated using the formula of serum fasting insulin (μU/mL) $*$ fasting glucose (mmol/L)/22.5 [29, 30]. Soluble CD163 was quantified in serum samples using an in-house enzyme-linked immunosorbent assay, as previously described [31]. IL-6 was measured with a high sensitivity ELISA (R&D Systems, USA) and MCP-1 was measured with a human ELISA DuoSet (R&D systems). sCD163, MCP-1, and IL-6 were measured and described in a previous study [23].

2.3. AT Biopsy. Subcutaneous abdominal adipose tissue was obtained from 57 obese subjects and 35 lean subjects in total. The biopsies were obtained by liposuction from the subcutaneous abdominal adipose depot in local anesthesia under sterile conditions. Immediately after removal, the adipose tissue sample was washed in isotonic NaCl, snap-frozen in liquid nitrogen, and kept at −80°C until RNA extraction. The procedures are described in detail elsewhere [27, 28].

2.4. mRNA Isolation and RT-PCR Analysis. RNA was isolated using TRIzol reagent (GIBCO-BRL Life Technologies, Roskilde, Denmark), and cDNA was synthesized using random hexamer primers using the Verso cDNA Kit (Applied Biosystems). All analyses were performed simultaneously and the mRNA levels of the target genes were expressed relative to the house-keeping gene low-density lipoprotein receptor related protein-10 (LRP10). The PCR reactions were performed in duplicate using the KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Inc., Woburn, MA, USA) in a LightCycler 480 (Roche Applied Science) using the following protocol: one step at 95°C for 3 min, then 95°C for 10 s, 60°C for 20 s, and 72°C for 10 s. The increase in fluorescence was measured in real time during the extension step. The relative gene expression was estimated using the default "Advanced Relative Quantification" mode of the software version LCS 480 1.5.0.39 (Roche Applied Science). The primers were designed using QuantPrime software [32]. The specificity was tested and amplification efficiency determined (between 1.9 and 2.0). Before analysis of target genes, the house-keeping gene was tested for stability and found to be stable comparing both groups and displaying comparable number of C_T cycles. The primer pairs are listed in Table 1.

2.5. Statistical Analysis. Descriptive statistics for anthropometrics and HOMA-IR are presented as mean ± SD. Baseline unpaired data were analyzed with an unpaired t-test or a Wilcoxon Mann-Whitney rank sum test for those variables,

Table 1: Primer pairs used for mRNA determination.

	Sense primer	Antisense primer
CD68	5′-GCTACATGGCGGTGGAGTACAA-3′	5′-ATGATGAGAGGCAGCAAGATGG-3′
CD14	5′-AGCCAAGGCAGTTTGAGTCC-3′	5′-TAAAGGACTGCCAGCCAAGC-3′
CD163	5′-CGG CTG CCT CCA CCT CTA AGT-3′	5′-ATG AAG ATG CTG GCG TGA CA-3′
CD206	5′-TTC GGA CAC CCA TCG GAA TTT-3′	5′-CAC AAG CGC TGC GTG GAT-3′
IL-10	5′-AGG GAA GAA ATC GAT GAC AGC-3′	5′-TCA AGG CGC ATG TGA ACT C-3′
IL-6	5′-TTTTGTACTCATCTGCACAGC-3′	5′-GGATTCAATGAGGAGACTTGC-3′
MCP-1	5′-GTCTTGAAGATCACAGCTTCTTTGG-3′	5′-AGCCAGATGCAATCAATGCC-3′
TNF-α	5′-TTGAGGGTTTGCTACAACATGGG-3′	5′-GCTGCACTTTGGAGTGATCG-3′
LRP10	5′-AGGTTGCCCAGCACTGAGTTATC-3′	5′-TGCCATCCCACCTGTAGAAGAC-3′

which were not normally distributed. To analyze the association between the cytokines, HOMA-IR, and the different macrophage markers we used a Spearman's correlation test. Multivariate linear regression was performed on a log scale to adjust the total number of macrophages and to find the macrophage marker with the closest association to HOMA-IR. The chosen significance level was a two-tailed P value of <0.05. The statistical software packet SPSS (SPSS, Chicago, IL, USA) was used for all calculations. Graphs were made in SigmaPlot.

3. Results

Characteristics of the lean and obese group are given in Table 2. Mean BMI of the obese subjects was $35.6 \pm 3.8\,kg/cm^2$ and the lean subjects $23.2 \pm 1.8\,kg/cm^2$. Age was between 18 and 49 years and was similar between the two groups. The level of HOMA-IR was significantly higher in the obese subjects than in the lean subjects (3.8 ± 1.8 versus 1.8 ± 0.8, $P < 0.001$). Furthermore, there was a significantly higher level of sCD163, MCP-1 (for both, $P < 0.001$), and IL-6 ($P < 0.05$) in blood samples from obese subjects compared with the lean subjects (Table 2).

3.1. Expression of Macrophage Markers in AT. The general macrophage markers CD68 and CD14 were taken as close correlates to the total number of macrophages, and both markers were significantly elevated in AT from obese subjects compared with lean subjects ($P < 0.001$) (Figure 1(a)). The levels of CD68 and CD14 were not significantly different between the genders and were not associated with age (data not shown). The gene expression of the proinflammatory markers IL-6, MCP-1, and TNF-α were all significantly elevated in the AT from obese subjects compared with lean subjects (for all, $P < 0.001$) (Figure 1(b)). Likewise there was a significantly higher level of the anti-inflammatory markers CD163, CD206, and IL-10 in the AT from obese subjects compared with AT from lean subjects (for all, $P < 0.001$) (Figure 1(c)). The gene expression of CD14 and CD68 were positively and significantly associated with the anti-inflammatory markers: CD163 (r: 0.76; r: 0.73), CD206 (r: 0.86; r: 0.78), and IL-10 (r: 0.73; r: 0.67), and with the proinflammatory markers: TNF-α (r: 0.45; r: 0.40), MCP-1 (r: 0.73; r: 0.74), and IL-6 (r: 0.56; r: 0.47)(for all, $P < 0.001$).

Table 2: Characteristics of lean and obese subjects.

	Lean	Obese
Number, n	35	57
Age, year	36.7 ± 10.3	37.4 ± 7.5
Gender, female %	51.4	49.1
BMI, kg/m^2	23.2 ± 1.8	$35.6 \pm 3.8^{**}$
HOMA-IR	1.8 ± 0.8	$3.8 \pm 1.8^{**}$
sCD163, mg/L	1.6 ± 0.4	$2.2 \pm 0.8^{**}$
IL-6, pg/mL	2.0 ± 1.4	$2.9 \pm 1.6^{*}$
MCP-1, pg/mL	90.9 ± 62.8	$196.3 \pm 102.4^{**}$

Data are given for the lean and obese group. Data are mean ± SD or relative frequency (%). Comparison of lean and obese subjects by unpaired t-test or Wilcoxon Mann-Whitney rank sum test were appropriated. $^{*}P < 0.05$; $^{**}P < 0.001$.

3.2. Differences in the Macrophage Phenotypes. To determine the changes in the polarization of the macrophages, the ratio of the different anti- and proinflammatory markers relative to CD14 was examined. As shown in Figures 2(a)–2(c) the level of the anti-inflammatory markers, CD163 and IL-10 adjusted for CD14, was significantly elevated in AT from obese compared to lean subjects ($P < 0.05$). The ratio of the proinflammatory markers, TNF-α and IL-6 adjusted for CD14, was significantly lower in AT from obese subjects compared to lean individuals ($P < 0.05$) (Figures 2(d)–2(f)). There was no difference in the level of CD14+ macrophages expressing CD206 and MCP-1 between lean and obese subjects. Using CD68 showed similar results as with CD14 (data not shown).

3.3. Association between Protein Levels and Gene-Expression Levels. The serum level of sCD163 was positively and significantly associated with the gene-expression level of mRNA CD163 (r: 0.37, $P < 0.001$) (Table 3). The level of sCD163 was also found to be significantly associated with the gene-expression levels of IL-10, CD206, and CD68 ($P < 0.001$) and with CD14, TNF-α, and MCP-1 ($P < 0.05$). The serum levels of IL-6 and MCP-1 were also significantly associated with all the macrophage markers ($P < 0.05$) (Table 3).

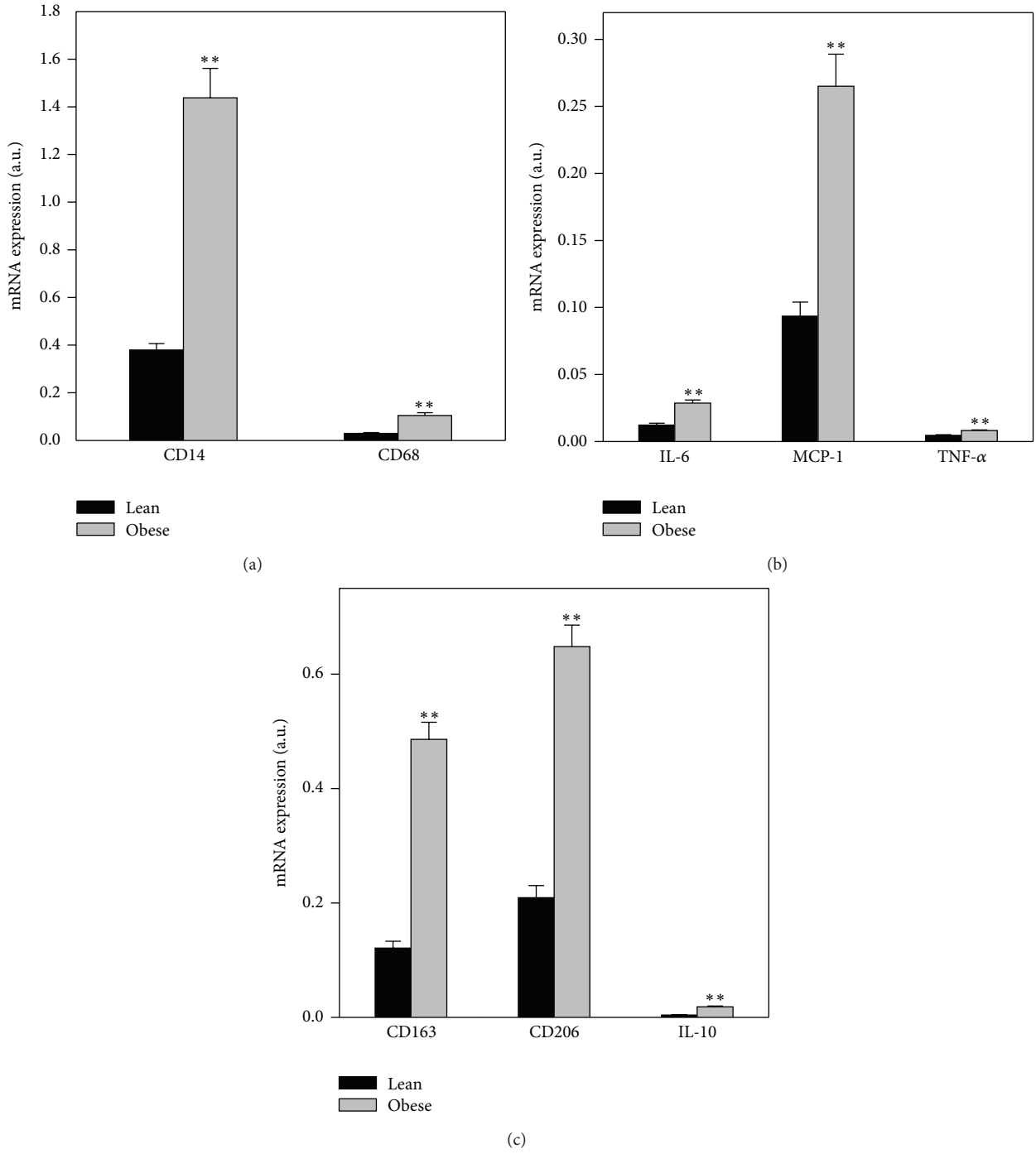

Figure 1: Difference in macrophage markers in AT between lean and obese subjects. Subcutaneous abdominal adipose tissue samples from lean ($n = 35$) and obese ($n = 57$) subjects. Gene-expression levels of different macrophage markers measured by real time PCR. (a) The general macrophage markers CD14 and CD68 in ATMs from lean and obese subjects. (b) Proinflammatory markers, IL-6, MCP-1, and TNF-α, in ATMs from lean and obese subjects. (c) Anti-inflammatory markers CD163, CD206, and IL-10 in ATMs from lean and obese subjects. Wilcoxon Mann-Whitney rank sum test. $^{**}P < 0.001$.

3.4. Macrophage Markers and Insulin Resistance. In an univariate correlation analysis a positive and significant association was found between CD68 and HOMA-IR (r: 0.34, $P < 0.05$) and CD14 and HOMA-IR (r: 0.37, $P < 0.001$) (Figures 3(a)-3(b)). The anti-inflammatory markers, CD163 (r: 0.47) (Figure 3(c)), CD206 (r: 0.37), and IL-10 (r: 0.40), were also found to be positively and significantly associated with HOMA-IR (all $P < 0.001$), and similar association between

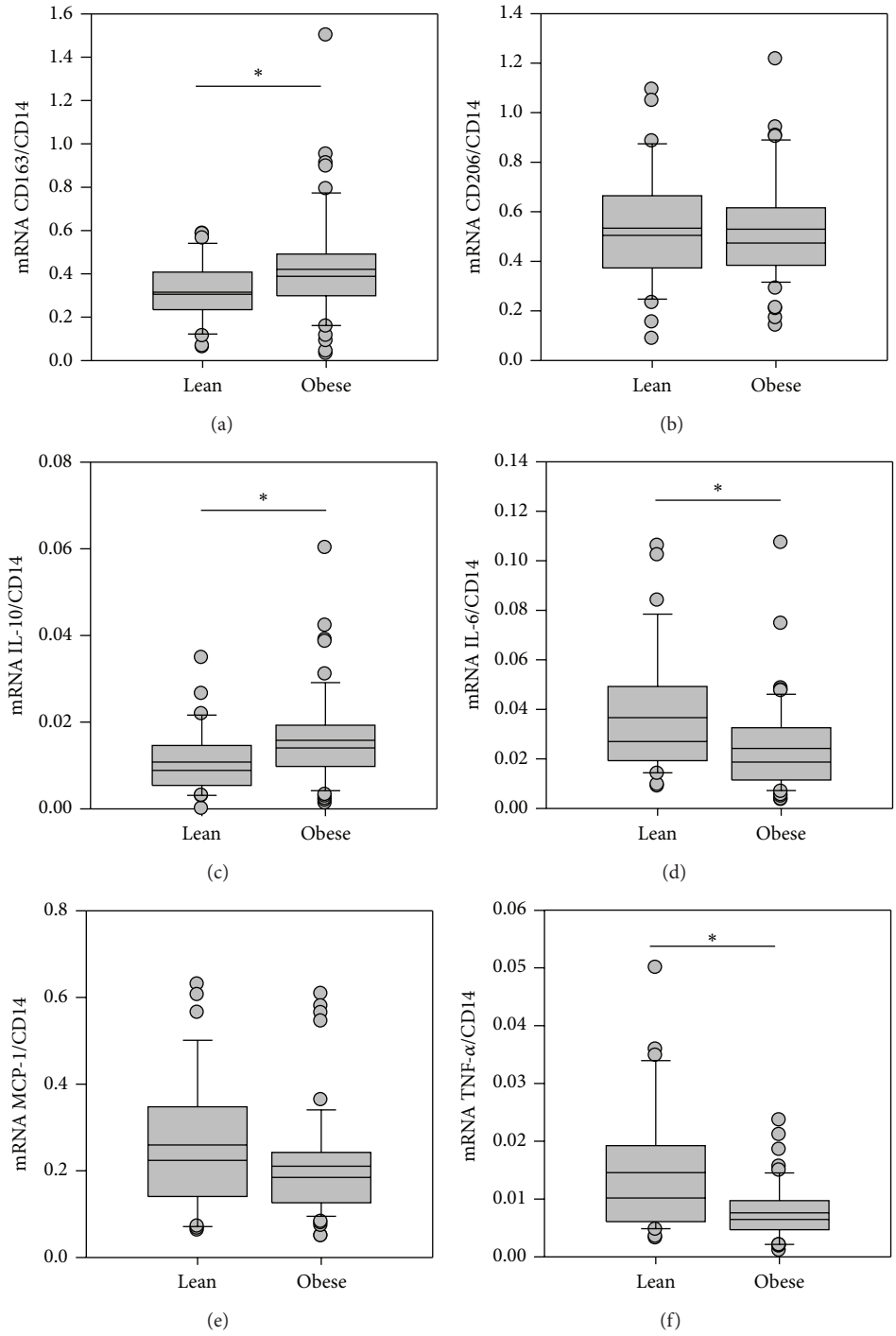

Figure 2: Polarization of anti- and proinflammatory macrophage markers in lean and obese subjects. Difference in phenotype of macrophage markers is expressed in subcutaneous abdominal adipose tissue samples from lean ($n = 35$) and obese ($n = 57$) subjects. Gene-expression levels of macrophage markers are presented relative to CD14, measured by RT-PCR. Lean versus obese analyzed by a Wilcoxon Mann-Whitney rank sum test. The ratio between the anti-inflammatory markers CD163, CD206, and IL-10 ((a)–(c)). The ratio between the proinflammatory markers IL-6, MCP-1, and TNF-α ((d)–(f)). $^{*}P < 0.05$. Graph showing each outliner and mean value with a solid line.

HOMA-IR and the proinflammatory markers TNF-α (r: 0.24), MCP-1 (r: 0.28), and IL-6 (r: 0.27) was found (for all, $P < 0.05$). In a multivariate linear regression analysis CD163 was the only macrophage marker that remained significantly associated with HOMA-IR, also after adjusting the total number of macrophages by CD68 (β: 0.57, $P < 0.05$) (Table 4).

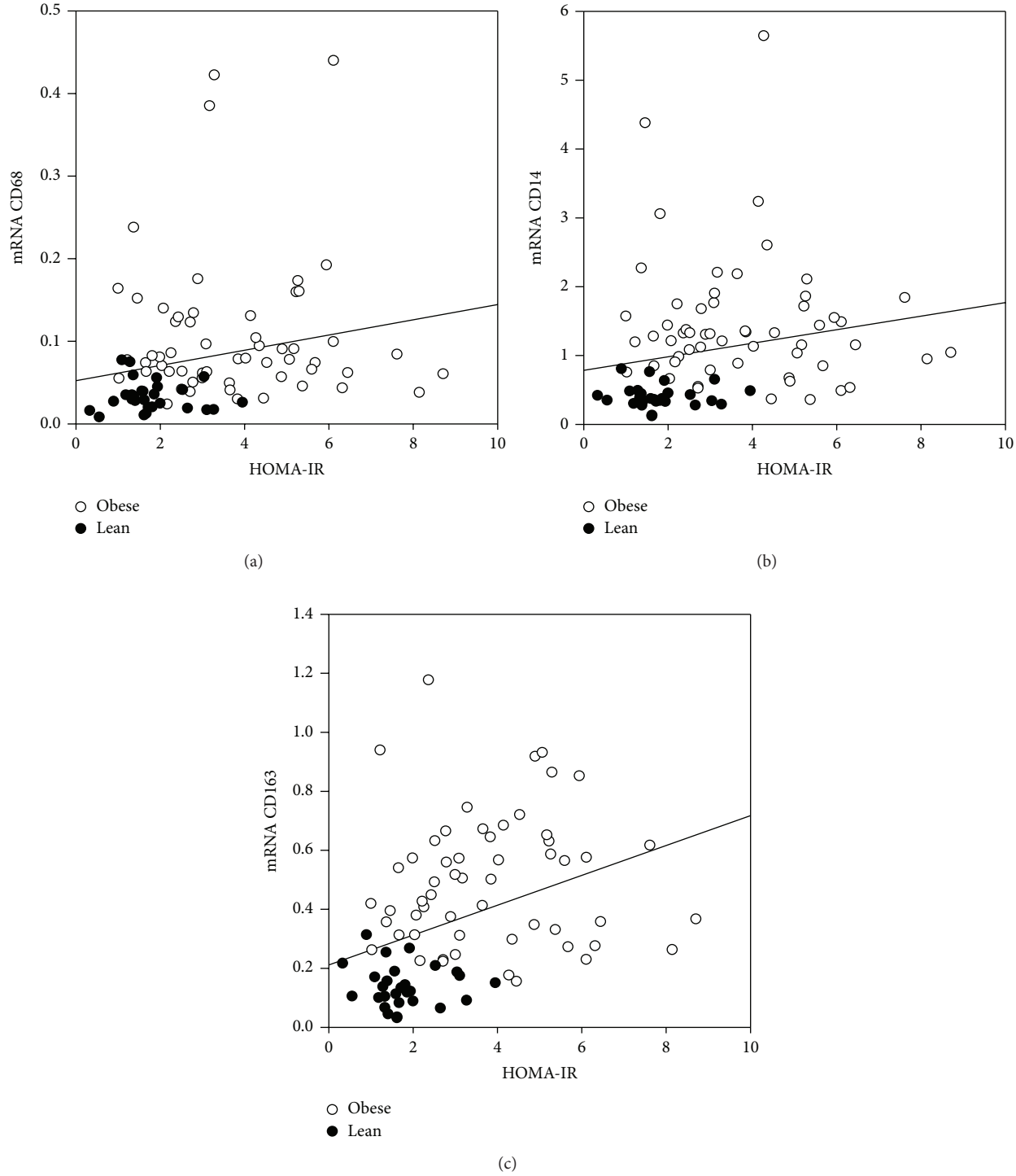

Figure 3: Association between the expression of CD68, CD14, and CD163 and HOMA-IR. Subcutaneous AT samples from lean and obese subjects ($n = 92$). Gene-expression levels of mRNA CD68, CD14, and CD163 measured by RT-PCR. HOMA-IR measured by fasting blood samples. Spearman's correlation with r = correlation coefficient. (a) Association between mRNA CD68 and HOMA-IR (r: 0.34, $P < 0.05$), (b) association between mRNA CD14 and HOMA-IR (r: 0.37, $P < 0.001$), and (c) association between mRNA CD163 and HOMA-IR (r: 0.47, $P < 0.001$).

Table 3: Association between serum protein levels and the gene-expression levels of the macrophage markers.

	sCD163	IL-6	MCP-1
mRNA			
CD14	0.33*	0.23*	0.50**
CD68	0.38**	0.30*	0.34*
CD163	0.37**	0.34*	0.43**
CD206	0.38**	0.28*	0.47**
IL-10	0.37**	0.23*	0.45**
TNF-α	0.22*	0.27*	0.33*
IL-6	0.17	0.25*	0.44*
MCP-1	0.30*	0.28*	0.46**

Subcutaneous AT and blood samples from obese and lean subjects ($n = 92$). Gene-expression levels of the general macrophage markers: CD14 and CD68, the anti-inflammatory markers: CD163, CD206, and IL-10, and the proinflammatory markers: TNF-α, IL-6 and MCP-1 relative to the housekeeping gene LRP10 measured by RT-PCR. Circulating levels of sCD163, IL-6, and MCP-1 measured by ELISA.
Statistic tests: Spearman's correlation test; r: correlations coefficient.
*$P < 0.05$; **$P < 0.001$.

Table 4: Multivariate linear regression for HOMA-IR and M1- and M2-macrophage markers.

	Model 1		Model 2	
	β	P value	β	P value
CD68	—	—	0.01	0.98
CD163	0.57	0.04*	0.57	0.05*
CD206	−0.03	0.89	0.06	0.89
IL-10	−0.47	0.07	−0.07	0.07
TNF-α	0.06	0.71	0.13	0.71
IL-6	−0.06	0.47	−0.03	0.49
MCP-1	0.13	0.79	−0.47	0.80

Subcutaneous AT samples from obese subjects ($n = 57$). Gene-expression levels of mRNA CD68, CD163, CD206, IL-10, TNF-α, MCP-1, and IL-6 measured by RT-PCR. HOMA-IR measured by fasting blood samples. Multivariate linear regression analysis on a log scale. Dependent variable: HOMA-IR. *$P < 0.05$.
Model 1 includes all pro- and anti-inflammatory macrophage markers. Model 2 includes all pro- and anti-inflammatory macrophage markers adjusted to total macrophage number by mRNA CD68.

When adjusting the total number of macrophages by CD14 there was also a positive association between HOMA-IR and CD163 though not significant (β: 0.55, P: 0.06). The other macrophage markers were not associated with HOMA-IR ($P > 0.05$).

4. Discussion

We found that the commonly used macrophage markers CD14 and CD68 were elevated in the AT from obese subjects compared with lean subjects indicating an increased number of macrophages in AT from obese subjects, which is in accordance with several other studies [2, 33]. In a study by Harman-Boehm et al. it is shown that the immunohistochemistry staining for CD68 positive cells highly correlates with the adipose tissue abundance of CD68 mRNA measured by real time PCR [34]. Thus, using gene expression of CD68 (and CD14) may be an acceptable correlate to the number of macrophages.

The gene-expression levels of both the anti- and the proinflammatory macrophage markers were elevated in the AT from the obese subjects compared with the lean subjects. Coherently, we found increased serum levels of the macrophage specific sCD163 and the proinflammatory cytokines IL-6 and MCP-1 in obese subjects compared with lean subjects. This correlates well with the idea that obesity induces a local and systemic low grade inflammation with increased level of macrophages in the AT and increased cytokine production [4]. The serum levels of sCD163, IL-6, and MCP-1 were significantly associated with the gene-expression levels of the ATM markers; however, other cells than the ATMs may also produce and release these cytokines.

To determine if obesity induces a shift in the polarization of the ATMs we measured the ratio of the pro- and anti-inflammatory markers relative to CD14. By this method we adjusted the total number of macrophages in the AT. We found a relatively higher expression of the anti-inflammatory markers, CD163 and IL-10, and a relative reduction of the proinflammatory markers, TNF-α and IL-6, in AT from obese subjects compared to lean individuals. Thus, we found that human ATMs change polarization to a more anti-inflammatory profile in obesity than towards a proinflammatory profile, which previously generally had been found both in rodent and human studies [13, 15]. Similar findings were, however, made in a study by Spencer et al. where they compared subcutaneous abdominal AT from lean and obese subjects and found that there was a shift towards a M2 phenotype in non-CLS macrophages in AT from obese subjects compared with lean ones [7]. Furthermore, they found that macrophages in AT from lean subjects expressed a mixed M1-M2 phenotype. A murine study by Shaul et al. also showed an enhanced M2 phenotype in epididymal ATMs from obese mice after 12 weeks of high fat diet compared with mice at 8 weeks [35]. The shift towards a more anti-inflammatory cell type in ATMs from obese subjects may be a protective mechanism to counteract the increased inflammation in the AT seen in obesity. It should be emphasized that both the anti- and proinflammatory markers were enhanced in association with the increased number of macrophages in AT from obese subjects and, therefore, even though there is a shift to a more anti-inflammatory profile, the AT from obese subjects is still more inflamed than the AT from lean subjects. Obesity leads to adipocyte hypertrophy, local hypoxia, and dead adipocytes. Thus, another explanation for the phenotypic switch seen in the ATMs from a proinflammatory state to a more anti-inflammatory state may be due to a need of adipose tissue repair and matrix remodeling.

In the present study we demonstrated that the anti-inflammatory macrophage marker CD163 is highly expressed in the subcutaneous AT from obese subjects and that the gene-expression level is strongly and significantly associated with both CD14 and CD68 ($P < 0.001$). An increased gene-expression level of CD163 in AT from obese subjects compared with lean subjects has been described previously [36]. However, another study found no significant change

in CD163+ counts in subcutaneous AT by immunohistochemistry when comparing lean with obese subjects [15]. Additionally, we also found a positive and significant association between the gene-expression level of CD163 and the serum level of sCD163 (r: 0.37, $P < 0.001$). Furthermore, the gene-expression level of CD163 was found to be significantly associated with HOMA-IR (r: 0.47, $P < 0.001$). A multivariate analysis emphasized this since a positive association persisted between CD163 and HOMA-IR after adjusting for the total number of macrophages by mRNA CD68 (β: 0.57, $P < 0.05$). In a study by Wentworth et al. it was found that proinflammatory macrophages were significantly associated with HOMA-IR [17]. We also found a positive association between HOMA-IR and the proinflammatory markers: TNF-α, MCP-1, and IL-6, in a univariate correlation analysis (all $P < 0.05$), but in a multivariate regression analysis CD163 was the only macrophage marker that remained significantly associated with HOMA-IR (Table 3). These findings are consistent with our recent data showing a strong association between the soluble part of CD163 (sCD163) and HOMA-IR [23] and consistent with yet another study where soluble CD163 is shown to be an independent predictor of the development of type 2 diabetes [37]. Our results indicate that CD163, both in terms of the gene expression and the soluble part of the receptor, may be of importance in regard to insulin resistance. The background for the link between CD163 and insulin resistance is yet to be understood despite a clear association.

Our study has some limitations. First, only gene expressions are investigated in the AT and gene expressions may not always reflect the protein level. Thus, it would have been interesting to reinforce our results with, for example, immunohistochemistry. Furthermore, we only examined AT from the subcutaneous depot. It would have been of great interest also to investigate visceral adipose tissue. A recent study by Michaud et al. found no depot difference in CD68 mRNA abundance between subcutaneous and visceral AT in lean, overweight, and obese subjects [33]. However, a significant difference in the mRNA CD68 expression between the visceral and subcutaneous depot with elevated levels in the visceral AT has been described [34]. These differences should be clarified in future studies. In the present study we found no age or gender specific difference in the gene-expression levels of CD68 or CD14. However, an association between the macrophage number, measured by the expression of CD68, and age has previously been observed [3, 38]. Thus, the range in age (18–49 years) may be too narrow to detect such an association in our study. Finally, another limitation is that we only analyzed AT from Caucasian subjects and not from other ethnical groups.

In conclusion we found that there is an elevated number of macrophages in the subcutaneous abdominal AT in obese compared with lean subjects. The gene-expression levels of both the anti- and proinflammatory macrophage markers were elevated in AT from obese subjects compared with lean subjects. Furthermore, the serum level of sCD163, IL-6, and MCP-1 was significantly elevated in obese subjects compared with lean subjects reflecting increased systemic low grade inflammation. Unexpectedly, we found a change in the macrophage phenotype by obesity, with a preponderance of anti-inflammatory (M2) markers and a decrement of proinflammatory (M1) markers in ATMs from obese compared with lean subjects after adjusting the total number of macrophages by CD14. A shift in the polarization towards a M2 profile may be a protective mechanism counteracting the enhanced inflammation seen in the AT in association with obesity. Finally, we demonstrated that mRNA CD163 is positively and significantly associated with HOMA-IR also after adjusting for the total number of macrophages and other macrophage markers.

Conflict of Interests

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

Authors' Contribution

Karen Fjeldborg analyzed the data, searched the literature, generated the figures, wrote the initial manuscript, and interpreted the data together with Bjørn Richelsen. Tore Christiansen, Marianne Bennetzen, and Bjørn Richelsen designed and performed the original studies. Karen Fjeldborg, Steen B. Pedersen, and Holger J. Møller measured the tissue and blood samples. All authors were involved in writing the final paper and had final approval of the submitted and published version.

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Clinical Study

Obesity and Cytokines in Childhood-Onset Systemic Lupus Erythematosus

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Background. In systemic lupus erythematosus (SLE), atherosclerosis is attributed to traditional and lupus related risk factors, including metabolic syndrome (MetS), obesity, and inflammation. *Objective*. To evaluate the association between obesity, measures of body fat content, serum tumor necrosis factor alpha (TNF-α), and interleukin (IL)-6 and -10 levels in childhood-onset SLE (cSLE). *Methods*. We screened consecutive cSLE patients followed up in the Pediatric Rheumatology Outpatient Clinic of the State University of Campinas. cSLE patients were assessed for disease and damage. Obesity was definite as body mass index (BMI) $\geq 30\,kg/m^2$. Serum TNF-α, IL-6, and IL-10 levels were measured by ELISA. Dual-energy X-ray absorptiometry was used to determine total fat mass, lean mass, and percent of body fat. *Results*. We included 52 cSLE patients and 52 controls. cSLE patients had higher serum TNF-α ($P = 0.004$), IL-6 ($P = 0.002$), and IL-10 ($P < 0.001$) levels compared to controls. We observed higher serum TNF-α ($P = 0.036$) levels in cSLE patients with obesity. An association between serum TNF-α levels and body fat percent ($P = 0.046$) and total fat mass on trunk region ($P = 0.035$) was observed. *Conclusion*. Serum TNF-α levels were associated with obesity and body fat content in cSLE. Our finding suggests that obesity may contribute to the increase of serum TNF-α levels in cSLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic systemic inflammatory disease affecting mainly women during childbearing age [1]. Although life expectancy has improved significantly, no changes in morbidity and mortality related to cardiovascular disease (CVD) have been observed in SLE patients in the past decades [2, 3]. In addition to traditional risk factors, many lupus-specific factors are linked to the increased risk of CVD observed in SLE [4–6].

Obesity-associated systemic inflammation is characterized by increased circulating proinflammatory cytokines and activation of several kinases that regulate inflammation [7–9]. Recent evidence supports that obesity-induced inflammation is mediated primarily by immune cells such as the macrophages and T lymphocytes present in metabolic tissues [9]. Adipose tissue derived cells can produce inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin (IL) 6, and IL-10 [10, 11].

TNF-α and IL-6 are proinflammatory cytokines associated with an increased insulin resistance, inhibition of insulin receptor autophosphorylation, and signal transduction. These mechanisms lead to insulin resistance,

hyperglycemia, and dyslipidemia [12–18]. IL-10 is also known as an antiatherogenic cytokine. Upregulation of IL-10 locally or systemically reduces atherosclerosis development in mouse models [13–15].

The aim of this study was to evaluate the association between obesity, measures of body fat content, and serum TNF-α, IL-6, and IL-10 in cSLE.

2. Patients and Methods

2.1. Subjects. Fifty-two consecutive cSLE patients, recruited from the Pediatric Rheumatology Outpatient Clinic of the State University of Campinas were included in this study. Patients were included in the present study if they (i) fulfilled at least four criteria of the American College of Rheumatology (ACR) [19]; (ii) were below 18 years of age at disease onset; and (iii) had a follow-up duration of at least 6 months (time necessary to evaluate damage index).

Fifty-two healthy volunteers (caregivers or students) matched by age, gender, and sociodemographic characteristics were included as a control group. None of the controls had any history of chronic disease, including autoimmune diseases.

This study was approved by the ethics committee at our institution, and the informed written consent was obtained from each participant and/or legal guardian.

2.2. Clinical Features. All patients had their medical histories and clinical,and serological characteristics entered at the time of cSLE diagnosis into special computer database programs. Features included in this protocol were age at the onset of disease (defined as the age at which the first symptoms clearly attributable to SLE occurred), age at diagnosis (defined as the age when patients fulfilled four or more of the 1987 revised criteria for the classification of SLE [19]), and follow-up time (defined as the time from disease onset until December 2012).

Total doses and length of use the of corticosteroids since the onset of disease were calculated by careful review of the medical charts. Doses of oral and parenteral corticosteroids were converted to the equivalent doses of prednisone. The cumulative dose of corticosteroids used was calculated by the sum of the daily dosages versus the time (days) of treatment. We also calculated the cumulative corticosteroid dose adjusted by weight by summing up the daily corticosteroid dose per weight at each routine visit.

2.3. Disease Activity and Cumulative Damage. Disease activity was measured by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) [20]. SLEDAI scores range between 0 and 105, and the scores of ≥3 were considered as active disease [21]. Adjusted SLEDAI scores over time were calculated by careful review of the medical charts and preview exams [22]. Cumulative SLE-related damage in all patients was determined by using the Systemic Lupus International Collaborating Clinics (SLICC)/ACR Damage Index (SDI) [23].

2.4. Body Mass Index. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared (kg/m^2). Criteria used to define nutritional status were based on the World Health Organization (WHO) criteria [24]. BMI cutoff points for Brazilian children and adolescents were used for individuals between 2 and 18 years [25]. Obesity was considered when BMI was above 30 Kg/m^2.

2.5. Dual X-Ray Absorptiometry (DXA). Percentual body fat (PBF), fat mass, and lean mass were obtained by DXA scan (Hologic Discovery Wii), through Whole Body Auto Fan Beam. This scan determines total fat mass and total lean mass in kilograms in addition to total fat mass and total lean mass as a percentage of total body mass.

2.6. Blood Sampling. Blood samples were collected from peripheral veins of all individuals in dry tubes and left to clot at room temperature for 30 minutes. Blood samples were then centrifuged for 15 minutes at 3000 rpm, and the serum was then stored in aliquots at −80°C for future use. We did not collect blood samples from individuals during an episode of acute or chronic infection.

2.7. Cytokine Assay. Commercially available kits from R&D Systems (London, UK) were used for the measurement of serum TNF-α, IL-6, and IL-10 levels by enzyme-linked immunosorbent assay (ELISA), carried out in accordance with the manufacturer's instructions. The minimum detectable dose (MDD) was 0.106 pg/mL for TNF-α, 0.039 pg/mL for IL-6, and 3.9 pg/mL for IL-10.

2.8. Statistical Analysis. All the data were tested for their normal distribution (Kolmogorov-Smirnov test). Categorical variables were compared by χ^2 test. Nonnormal variables were compared by Fisher exact tests. Mann-Whitney U test was used to compare anthropometric measure and laboratory studies between patients and controls. Spearman's correlation was used to correlate continuous variables (e.g., TNF-α levels, SLEDAI, and SDI scores). For all analyses, P value ≤ 0.05 was considered to be statistically significant. Statistical analysis was carried out using IBM SPSS Statistics 16.0 software (SPSS/IBM, Chicago, IL, USA).

3. Results

3.1. Demographics. We included 52 consecutive cSLE patients. Forty-seven (90.3%) were women with mean age of 17.6 years (standard deviation (SD) ± 3.7 years). Mean disease duration was 5.14 years (SD ± 4.05). The control group consisted of 52 controls (47 women) with mean age of 18.2 years (SD ± 6.4). Patients and healthy controls were statistically comparable in terms of age and sex (Table 1).

3.2. BMI Analyses. BMI was similar between patients (median 21.74 kg/m^2; range: 16.1–31.12 kg/m^2) and controls (median 21.43 kg/m^2; range: 14.36–28.54 kg/m^2) (P = 0.101). Sixteen (31%) cSLE patients were overweight compared to 6 (11.5%) controls (P = 0.018).

Table 1: Demographics data from cSLE and controls.

	cSLE patients $N = 52$	Healthy controls $N = 52$
Age (mean ± SD)	17.6 ± 3.7	18.2 ± 6.4
Female (N; %)	47 (90.3)	47 (90.3)
Disease duration (mean ± SD)	5.14	—

Table 2: Sera cytokines levels of the individuals included in the study.

Sera levels	cSLE patients $N = 52$	Healthy controls $N = 52$
TNF-α	1.93 pg/mL* (0.8–11.17 pg/mL)	1.23 pg/mL (0.25–3.91 pg/mL)
IL-6	1.46 pg/mL* (0.34–9.74 pg/mL)	0.95 pg/mL (0.39–3.91 pg/mL)
IL-10	13.86 pg/mL* (3.93–56.92 pg/mL)	6.64 pg/mL (3.52–9.54 pg/mL)

*$P \leq 0.05$.
The data were given in median (range).
cSLE: childhood-onset systemic lupus erythemathosus; TNF-α: tumor necrosis factor alpha; IL: interleukin.

We did not observe an association between BMI and SLEDAI, SDI, and cumulative corticosteroid dose.

3.3. Body Composition Analysis. On whole body analysis, we observed a median fat mass of 22.38 kg (range: 7.67 kg–36.62 kg), a median lean mass of 35.49 kg (range: 25.31 kg–52.14 kg), and a median PBF of 34.1% (range: 12.1–54.4%) in cSLE. In the trunk region we observed a median fat mass of 8.62 kg (range 2.98 kg–17.59 kg), median lean mass of 16.80 kg (range: 11.24 kg–26.19 kg) and a PBF of 42.3% (range: 12.1–54.4%).

3.4. Cytokine Assay. Serum TNF-α ($P = 0.004$), IL-6 ($P = 0.002$), and IL-10 ($P < 0.001$) levels were significantly increased in cSLE patients when compared to healthy controls (Table 2). We observed higher serum TNF-α levels in obese cSLE patients when compared with nonobese cSLE patients ($P = 0.036$), obese controls ($P = 0.039$) and non-obese controls ($P < 0.0001$) (Table 3). No difference in serum TNF-α levels was observed between obese and non-obese healthy controls ($P > 0.05$). We observed an association between TNF-α and PBF ($P = 0.046$) and total fat mass on trunk region ($P = 0.035$) analyzed by DXA scans.

No association between serum IL-6 and IL-10 levels and SLEDAI or SDI scores was observed. In addition, no difference in these cytokine levels in cSLE patients and controls with and without obesity was observed.

4. Discussion

Adipose tissue is known to be capable of secreting cytokines such as TNF-α, IL-6, and IL-10. Therefore, the purpose of this study was to assess whether the levels of these cytokines were increased in obese cSLE when compared to nonobese cSLE and healthy controls.

The observation that obese cSLE patients had higher serum TNF-α levels when compared to nonobese cSLE and healthy controls is the major finding of our study. In addition, we observed that serum TNF-α levels correlated with PBF and total fat mass in trunk region in cSLE.

Recent studies have demonstrated that increased adipose tissue mass contributes towards an increase in chronic inflammation [26, 27]. Chronic inflammation is further enhanced by inflammatory markers produced in the liver and in other organs [28]. Recently, it has been demonstrated that obesity is associated with a low-grade inflammatory process, characterized by increased circulating levels of proinflammatory cytokines such as TNF-α, IL-6, and acute-phase proteins (CRP) [29–32]. The mechanism underlying increased inflammation in the setting of obesity remains unclear, but it is known that mononuclear cells are activated and proinflammatory cytokines are upregulated in obese individuals [33, 34].

We observed an association between serum TNF-α levels and PBF and total fat mass in trunk region. Studies analyzing the association between serum TNF-α and DXA scans have not been reported in cSLE so far, but studies on healthy women and type-2 diabetes patients showed an association between plasma levels of TNF-α and visceral adipose tissue volume measured by CT-scan [35–38]. Previous studies have shown that visceral fat accumulation is associated with increased risk of CV risk [37]. In addition, with an increase in TNF-α, a reduction in lipoprotein lipase activity in adipose tissue is observed [39]. There is also evidence that TNF-α has a local effect, regulating adipocyte size in the face of increasing energy consumption [40, 41].

Cytokines, such as TNF-α and IL-6, are primarily involved in the early stages of the inflammatory response culminating in atherosclerosis [39, 42]. Increased TNF-α levels in the endothelium promote initial atheroma plaque [39, 42]. However, so far, studies were not able to conclude whether TNF-α is a causative factor of atherosclerosis.

Both IL-6 and TNF-α are expressed and secreted by human adipose tissue [43]. In obesity, increased secretion of IL-6 may contribute to metabolic dysfunction [44, 45]. In addition, one previous study has shown that IL-6 correlated positively with BMI and with measures of insulin resistance in abdominal obese male subjects [45]. As previously described in adults SLE patients, we observed higher IL-6 and IL-10 levels in cSLE patients when compared to healthy controls [46–49]. However, no association with BMI was observed in our cSLE cohort.

IL-10 downregulates inflammatory activation of monocytes and macrophages by transcriptional and posttranscriptional inhibition of the entire range of proinflammatory cytokines [50]. IL-10 has been shown to reduce atherosclerosis and it can be found in atheromatous plaque due to local macrophages production [50]. However, IL-10 is involved in SLE pathogenesis and it is increased in SLE patients with CVD compared to SLE patients without CVD [51, 52]. In our study, we did not observe an association between sera IL-10 levels and obesity.

Table 3: Cytokines levels and therapy information from subjects subdivided into obese and nonobese.

	Obese cSLE $N = 16$	Nonobese cSLE $N = 36$	Obese controls $N = 7$	Nonobese controls $N = 45$
Cytokines levels				
TNF-α (pg/mL)	3.1 (1–11.1)*	1.8 (0.8–11.1)	1.3 (0.5–2.1)	1.2 (0.2–3.9)
IL-6 (pg/mL)	1.4 (0.3–6.9)	1.4 (0.3–9.7)	0.9 (0.4–5.9)	0.9 (0.3–3.6)
IL-10 (pg/mL)	16.7 (7.6–26.3)	13.6 (3.9–39.7)	4.9 (3.9–6)	5.6 (3.5–9.5)
Therapy				
CE dose (mean ± SD)	17.3 ± 19.8	18.3 ± 19.8		
CE/Kg (mean ± SD)	535.1 ± 339.5	444.5 ± 245.9	—	—
CE cumulative (mean ± SD)	28036.7 ± 17611.5	23057 ± 16568.7		

The cytokine data were given in median (range). * $P < 0.05$.

We also did not observe an association between sera IL-6 levels and obesity. In the literature, it has been described that plasma IL-6 levels are associated with increased CV risk and observed in SLE patients with metabolic syndrome [53] and in patients with type 2 diabetes [44, 54]. In a large healthy family population study where children were included, IL-6 levels were closely associated with traditional and nontraditional risk factors for atherosclerosis [55].

Although cSLE is rare, it is important to consider that one limitation of our study is the small number of patients and controls included.

Corticosteroids are associated with weight gain due to increased appetite and fluid retention. Corticosteroids also cause a redistribution of fat deposition, occurring predominantly in the trunk and face [56–59]. However, we did not observe an association between serum TNF-α, IL-6, and IL-10 levels and corticosteroid dose.

To the best of our knowledge, this is the first study to evaluate the association of BMI, body composition and serum TNF-α, IL-6, and IL-10 levels in cSLE patients. Although these cytokines have been shown to be associated with CVD in other populations, we only observed an association between serum TNF-α levels and obesity, and PBF and total fat mass in trunk region. Our findings suggest that total fat mass may contribute to increased levels of serum TNF-α levels in cSLE.

Conflict of Interests

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

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Review Article

Type 2 Diabetes, PUFAs, and Vitamin D: Their Relation to Inflammation

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Chronic diseases have become one of the most important public health problems, due to their high costs for treatment and prevention. Until now, researchers have considered that the etiology of Type 2 diabetes mellitus (T2DM) is multifactorial. Recently, the study of the innate immune system has offered an explanation model of the pathogenesis of T2DM. On the other hand, there is evidence about the beneficial effect of polyunsaturated fatty acids (PUFA) n-3 and n-6 in patients with chronic inflammatory diseases including diabetes. Furthermore, high vitamin D plasmatic concentrations have been associated with the best performance of pancreatic β cells and the improving of this disease. In conclusion, certain fatty acids in the adequate proportion as well as 25-hydroxivitamin D can modulate the inflammatory response in diabetic people, modifying the evolution of this disease.

1. Introduction

Changes in human behavior and lifestyles in the last century have caused a great increase in prevalence of Type 2 diabetes mellitus. WHO calculates that 171 million people around the world have diabetes and that by 2030, its prevalence will reach epidemic proportions, affecting 366 million [1]. T2DM is considered a multifactorial disease, several hypotheses have tried to explain the origin of the pathology; that is, that it is an abnormality of the anterior hypothalamus and the endocrine pancreas caused by progressive ischemia or that there is abnormal islet innervation. Recently, there is increasing evidence that the acute phase inflammatory response induced by cytokines is closely related to the generation of insulin resistance and Type 2 diabetes mellitus. Some researchers have associated these pathologies with the presence of inflammatory and immune system biomarkers, including TNF-α, IL-1, IL-6, C Reactive Protein (CRP), monocyte chemotactic protein-1 (MCP-1), sialic acid, leptin, adiponectin, resistin, and visfatin [2].

Furthermore, recent epidemiological studies have associated total fat intake (saturated, mono, and poly-unsaturated fats) with T2DM; however, the type of fat could influence insulin metabolism positively, as can be observed when saturated fat is replaced with monounsaturated fat in the diet, improving considerably insulin action. There are several studies that demonstrate the beneficial effect of polyunsaturated fatty acid supplementation (n-3) in patients with active inflammatory processes [3], and others report that physiological concentrations of certain fatty acids can modulate the inflammatory response modifying the evolution of certain diseases [4].

Some epidemiological studies have recently reported an increase in serum 25-hydroxivitamin D (25-OH Vitamin D) deficiency in various populations around the world [5–9]. Until a few years ago, this deficiency had only been observed in very specific groups, such as old-age residents of high latitudes and altitudes or in low sun exposure. However, vitamin D deficiency has increasingly been found in apparently healthy populations as well as in industrialized society older adults [10].

The etiology of this deficiency has been considered multifactorial and ethnicity is one of these factors, in this sense, hypovitaminosis D has been observed in Afro-American and Latino populations who have a higher risk of insulin resistance and T2DM compared to Caucasian subjects [11].

Additionally, some researchers [12, 13] have hypothesized that low 25-OH vitamin D concentrations may have an important role in the pathogenesis of T2DM, although the mechanisms are not yet clear.

2. Type 2 Diabetes Mellitus

2.1. Genetic Factors. Positive family history confers a risk 2 to 4 times higher for T2DM. Fifteen to twenty-five percent of first-degree relatives of patients with T2DM have glucose intolerance or diabetes. Long-life risk (at age 80) for T2DM is 38% if one parent is affected and 60% at age 60 if both parents have the disease [14]. Although genetic factors are important, it is necessary to take into account that Diabetes is a multifactorial and heterogeneous disease [15].

2.2. Physiopathology of Hyperglycemia, Insulin Resistance, and Beta Pancreatic Cell Dysfunction. Insulin is the key hormone for glucose regulation and in general normoglycemia is maintained by the balance between secretion and action of insulin (less action, higher secretion and vice versa) and normal beta pancreatic cells can adapt to changes in insulin action [16].

2.3. Glucose Homeostasis. After a night of fasting, most of the glucose uptake occurs in tissues that are independent of insulin action, mainly in the nervous system and the splanchnic organs; this balance between uptake and glucose consumption is altered after feeding, when glucose homeostasis depends on three processes that normally occur as follows:

(1) insulin secretion,

(2) glucose uptake by peripheral tissues (85% in muscle and 4-5% in adipose tissue) and by splanchnic tissues (liver and intestine),

(3) suppression of hepatic glucose production, which represents 85% of endogenous synthesis (the resting 15% is produced by the kidney) [17].

Although glucose uptake by adipose tissue is minimal, it is very important for maintaining glucose homeostasis as it coordinates fatty acid release from triacylglycerols, and produces cytokines that regulate insulin sensitivity in the liver and muscle. Alteration of any of these factors will produce glucose intolerance and open hyperglycemia. T2DM is characterized by the coexistence of two abnormalities:

(i) insufficiency in insulin secretion by pancreatic β-cells,

(ii) insulin resistance [18].

2.4. Insulin Resistance. Insulin resistance is a decrease in the ability of insulin to exert its biological action at different glucose concentrations. Unless subjects with insulin resistance produce great amounts of insulin to compensate for its effects, they will surely develop hyperglycemia and diabetes. The defects related to insulin resistance include decreased expression of insulin receptors in the surface of those cells that are sensitive to this hormone which is due to alterations in the signaling pathways that should activate after insulin binding with its receptor and abnormalities in normal pathways that usually respond to insulin action such as glucose transportation and glucagon synthesis [19].

By the time blood glucose reaches the level of diagnosis for T2DM (≥126 mg/dL) the β-cell function disorders have already taken place. The inability of these to continue hypersecreting insulin is responsible for the transition from insulin resistance and compensating hyperinsulinism with normoglycemia to insulin resistance with noncompensating hyperinsulinism and glucose intolerance; ending up in insulin resistance with hyperinsulinemia and hyperglycemia [20].

2.5. Causes of Abnormalities in Insulin Secretion

2.5.1. Alterations in β Cell Volume. β-cell volume is controlled by four independent mechanisms as follows:

(1) mitosis of existing β-cells,

(2) size of β-cells,

(3) neogenesis from pancreatic epithelial cells,

(4) β-cell apoptosis.

In normal conditions, approximately 0.5% of β-cells suffer apoptosis in adults, which is mainly compensated by mitosis and neogenesis, allowing an equilibrium between insulin production and metabolic needs [21].

There is evidence supporting the concept that β-cell apoptosis is an important factor in the decrease of islet number and the pathogenesis of T2DM [22]. Other related factors include stress of the endoplasmic reticulum, chronic hyperglycemia, oxidative stress, and the activity of some cytokines [23].

2.5.2. Glucotoxicity. Hyperglycemia alone is capable of producing alterations in insulin secretion that decrease when it is corrected, that means that any increase in glycaemia in patients with a decrease in β-cell volume can cause important abnormalities in insulin secretion in the rest of the pancreatic tissue; it also contributes to the increase of insulin resistance and the defects in insulin secretion, which are corrected when glucose levels are reduced [24, 25].

2.5.3. Lipotoxicity. Lipotoxicity is another cause of β-cell dysfunction. In normal acute conditions, β-cell exposure to physiological concentrations of free fatty acids stimulates insulin secretion; these are transformed into acyl coenzyme A (acyl-CoA) and subsequently into phosphatidic acid and diacylglycerol within β-cells [26]. These compounds activate specific isoforms of protein kinase C that stimulates insulin secretion. By comparison, chronic exposure to high concentrations of acyl-CoA inhibits insulin production through Randle's cycle. The increase in Acyl-CoA concentrations within β-cells also stimulates nitric oxide production, which increases inflammatory cytokine production, including IL-1

and TNF, that contributes to increase in the β-cell deterioration and apoptosis [25, 27].

2.5.4. Defects in Function or Synthesis of Incretins. Oral intake of glucose stimulates insulin secretion, representing 50 to 70% of the normal insulin response; it depends on the entero-insular axis represented by two intestinal hormones that stimulate insulin secretion, called incretins: glucagon-like peptide-1 (GLP-1) and insulintrophic peptide dependent on glucose (GIP). These two hormones are liberated by endocrine cells in the duodenum and jejunum in response to carbohydrates in the intestine [28].

It has been observed that the action of these incretins is deteriorated or nonexistent in Type 2 diabetic patients, and it is actually considered one of the main mechanisms affecting insulin secretion in these patients [29].

2.6. Diagnosis. For the diagnosis of T2DM, it is convenient to use the current criteria of the American Diabetes Association (ADA) which are

(i) glycosylated hemoglobin ≥ 6.5% or fasting plasma glucose ≥ 126 mg/dL (7.0 mmol/L). Fasting is defined as noncaloric intake for at least 8 hours;

(ii) plasma glucose at 2 hours ≥ 200 mg/dL (11.1 mmol/L) during a test of glucose tolerance;

(iii) in patients with classic symptoms of hyperglycemia or hyperglycemic crises, a random plasma glucose ≥200 mg/dL (11.1 mmol/L) [20].

The process that determines the appearance of the disease is slow so that early detection is desirable; therefore, detection of cases with insulin resistance is a potential strategy that can facilitate the early diagnosis of this disease. However, insulin resistance alone denotes induction of glucose uptake in most tissues, but the relationship between secretion and action is a highly complex problem. In the presence of a severe insulin resistance, a disproportionate amount is secreted with a high percentage of immature forms of insulin being released into circulation, with a different half-life and action. Therefore a method to distinguish the immature forms of insulin is required.

There are useful methods for measuring glucose utilization by insulin as "the hyperinsulinemic euglycemic clamp" or the homeostasis model (HOMA-IR) [21]. The latter estimates severity of resistance more than insulin sensitivity, since the relationship between glucose and insulin at baseline reflects the balance between hepatic glucose production and secretion of insulin which is kept by feedback between the liver and pancreatic β cells and is obtained by dividing the product of glucose and insulin between 22.5 (when SI units are used) or between 405 (when expressed in mg/dL). The simplified formula is the result of a mathematical model which fits the action of insulin to the blood glucose value [22]. The cutoff point for diagnosis of insulin resistance may vary depending on the study population but is considered from 75th percentile of the study population. However there is data in Mexican population, placing HOMA in 2.4, with an additional advantage, since, in addition to insulin resistance, it allows to value beta cell function (HOMA-B), this value is obtained by dividing the product of insulin by 20 between at least 3.5 glucose, the cutoff point is also considered from the 75 percentile of the study population [23, 24].

3. Diabetes Mellitus and the Immune System

For more than 15 years, evidence has been gathered that supports the hypothesis that chronic low grade inflammation is a risk factor for the development of T2DM [15, 30–33]; however, the mechanisms are not clear yet. Existing theories include production of proinflammatory cytokines, such as IL-1 and TNFα, and increase in central fat mass, due to chronic inflammation [34].

Additionally acute phase proteins and certain cytokines are related with and through a great number of metabolic pathways that regulate insulin, the functions of lipoproteins lipases, and adipocytes, contributing to the development of insulin resistance [35].

Dietary factors may also increase acute phase proteins; for instance, a hyperenergetic diet increases protein C reactive concentrations, while a high fat diet increases sialic acid, which is considered a marker of the acute phase response and a cardiovascular and diabetes risk factor [36, 37].

In relation to the innate cellular immune system, macrophages have been associated with Type 2 diabetes mellitus pathogenesis. Proinflammatory M1 macrophages induce an inflammatory state and insulin resistance through inhibition of insulin signaling caused by IL-6 and TNF-alpha. Eguchi et al. [38] demonstrated a direct contribution of macrophages to beta cell dysfunction. Proinflammatory M1 macrophages were recruited to islets in mice infused with ethyl palmitate, in the *db*/*db* mouse, and in the KKAy mouse. Macrophage depletion *in vivo* in all these models increased Ins and Pdx1 mRNA expression in islets and increased glucose-stimulated insulin secretion *in vivo* and in isolated islets *ex vivo*.

Other cell populations affected are dendritic cells, it has been reported that hyperglycemic states determine a decrease of the total population of these, including myeloid dendritic cells type 1 (mDC1) and plasmacytoid dendritic cells (pDC) [39].

Natural Killer (NK) lymphocytes, which are important effector cells of the innate immune system, are responsible for controlling infections, but oxidative stress and endoplasmic reticulum (ER) stress induced by high glucose levels may influence NK cell function in T2D patients [40]. Some results demonstrate defects in NK cell-activating receptors NKG2D and NKp46 in T2D patients, and implicate the Unfolded Protein Response (UPR) pathway as a potential mechanism [41].

In relation to the adaptive immune system, there have been changes in certain cell lines, including a decreased polymorphonuclear leukocyte (PMNL) function; this alteration has been associated with defective chemotaxis, bacterial killing, leukotriene (LT) release, and lysosomal-enzyme secretion [42]. It has also been shown to produce increased

levels of reactive oxygen species, possibly as a result of the effects of hyperglycemia [43]. Some studies have shown a correlation between impaired PMNL function and its improvement with adequate glycaemic control [44, 45].

T cells play an important role in the development of inflammatory processes, some experimental models suggest the association of an early T-lymphocyte occurrence in adipose tissue and the parallel initiation of insulin resistance (IR) in diet-induced obesity as a potential pathophysiological role of this cell type in the development of IR and T2DM [46, 47].

T reg cells are a subpopulation of CD4+ T cells that actively suppress physiologic and pathologic immune responses, therefore; contributing to the maintenance of immunological self-tolerance and immune homeostasis; it has been reported that T reg cells correlate with insulin resistance and glucose concentrations in T2DM patients [48].

4. Fatty Acids

Fats are organic biomolecules formed basically of carbon and hydrogen, and in a lesser extent of oxygen; they can be divided in phospholipids and triacylglycerols, both of which are made of fatty acids [49]. Fatty acids are long-chain monocarboxylic acids, with a pair number of carbon atoms, between 8 and 22. Fatty acids can be saturated, monounsaturated, or polyunsaturated according to the number of double bonds in the chain [50].

There are polyunsaturated fatty acids (PUFA) that the human organism cannot synthesize, such as linoleic acid (LA) and alpha linolenic acid (ALA) that must be obtained from the diet; they are called essential fatty acids (EFA). They belong to the n-6 or n-3 families of fatty acids, also known as ω-6 or ω-3, respectively [51]. EFAs can be converted into long chain fatty acids (LCFA) in the organism, with more double bonds such as arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic (DHA) [9].

4.1. Polyunsaturated Fatty Acids and the Immune System. Animal or tissue culture studies as well as in human beings indicate that the amount and degree of saturation of fats in the diet influence inflammatory and immunologic responses. The nature of the effect depends on the type of fatty acid, age, health status, and experimental model [52–54].

Arachidonic acid and in a lesser proportion linoleic acid are the main components of the phospholipid membrane of lymphocytes, so growth and development of lymphoid tissues as well as the structural and functional integrity of lymphoid cells (T and B) are affected by essential fatty acid deficiency, which conditions a loss of functional integrity of T-CD4+ cells, monocytes, macrophages, and neutrophils, affecting chemotaxis and eicosanoid production [54].

In vitro experiments have shown that lymphocytes incorporate into their membranes a large amount of n-6 fatty acids (AA y LA) during their development and proliferation, which could lead to believe that their requirements are very high during the normal immune response in the secondary lymph nodes [54].

It is well known that activation of mature peripheral T-cells initiates with the interaction of the *T cell receptor* (TCR) and an antigenic peptide cleaved in a cell's Major Histocompatibility Complex (MHC). This interaction requires high concentrations of AA, and any change in this may affect TCR-MHC complex affinity and TCR signal transduction, determining the nature and magnitude of the T-cell response. There are *in vitro* studies showing the regulatory effect of AA metabolites on the development and function of immune system cells including growth and differentiation of thymocytes, proliferation and migration of T-cells, Th1 and Th2 response mediated by cytokines, antigen presentation, macrophage regulation, TNFα, IL-1 and IL-2 production, as well as suppressor T-cell induction [3, 53, 55–57].

Other *in vitro* studies have evaluated the effect of supplementing lymph node cell cultures with n-6 PUFA, showing that low concentrations of these improve B and T lymphocyte proliferation, while high concentrations inhibit them [58]. There is also evidence of reduction in proinflammatory cytokines IL-1 and TNFα production with gamma linoleic (GLA) and dihomogammalinoleic (DHLA) acids [53].

On the other hand, experimental research has given evidence on the effects of different n-3 PUFA on lymphocyte cultures. Additional data indicate that low concentrations of these PUFA stimulate lymphoproliferation of B and T cells, whereas high concentrations inhibit this effect [58, 59].

There are also some studies regarding the beneficial effects of n-3 PUFA in humans. Supplementation with n-3 rich fish oil has shown a decrease in helper T-cells [60], IL-1β, IL-2, TNF, IL-6, and IL-8 production [59, 61].

Existing scientific evidence suggests that moderate intake of the main n-6 and n-3 PUFA (arachidonic and linoleic acids), as well as an adequate proportion of both may be beneficial for those diseases that are related to the inflammatory process or immunity originated [52, 53, 57, 59, 62–65].

4.2. Polyunsaturated Fatty Acids and Type 2 Diabetes Mellitus. PUFAs may have a beneficial effect on the development or control of diabetes through several mechanisms. For instance, they are able to act as activators of peroxisome proliferator activated receptor gamma (PPARγ); which stimulates the differentiation of preadipocytes to adipocytes, generating an increase in insulin receptors, thus reducing insulin resistance. Another mechanism is the protection of pancreatic beta cells from damage caused by an increase in free radicals produced in diabetes [66, 67].

Studies *in vitro* have reported that n-3 fatty acid supplementation improves the proinflammatory phenotype of macrophages, as well as insulin resistance in adipocytes [68].

Although there is strong evidence in humans and animal models that PUFAs exert a protective effect against the development of Type 2 diabetes mellitus [69–71], there are no concluding data in this respect. However, intake of diets rich in PUFA, particularly n-3 and n-6, has been shown to facilitate the action of insulin through various metabolic pathways, such as suppression of hepatic lipogenesis, reduction of the release of triacylglycerols from liver, improvement in ketogenesis, and oxidation of fatty acids in liver and skeletal muscle.

All these mechanisms promote glucose uptake and decrease insulin resistance due to hypoactivity Δ5D desaturase and elevated activity of Δ6D and Δ9D desaturases [72].

Several investigations have been developed to evaluate the effect of fatty acid supplementation in the evolution of Type 2 diabetes mellitus [70, 71, 73–81]. Beneficial effects have been described on triacylglycerols, lipoproteins, haemostasia, atherogenic plaque stability, blood pressure, leukocyte function, glucose metabolism, insulin resistance, and even diabetic neuropathy; however, the results depend on dose and duration of intervention.

5. Vitamin D

Vitamin D or calciferol is an unsaponifiable heterolipid of the steroid group; it has two basic forms, D_2 (ergocalciferol) found in plants as a product from ultraviolet B radiation on ergosterol and D_3 which originates as dehydrocholesterol produced by ultraviolet B radiation, after becoming previtamin D_3. Vitamin D_3 can be synthesized in the human epidermis or ingested through fish oil, egg yolk, fortified foods, or supplements [82].

Vitamin D is converted into 25-hydroxivitamin D (25(OH)D) in the liver, which is the main circulating metabolite. Its measurement reflects intake and endogenous production; the active form is 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) or dihydroxycholecalciferol, which is hormone produced mainly in the kidney and regulated by parathyroid hormone, calcium, and phosphorous concentrations [83].

Vitamin D receptors are present in most tissues, including the endothelium, vascular smooth muscle, and myocardium; the first two are able to convert 25(OH)D into $1,25(OH)_2D$. Directly or indirectly $1,25(OH)_2D$ has a role in the regulation of many genes, such as those involved in insulin production and development of vascular smooth muscle cells, which is the reason it is thought to be an important contributing factor to cardiovascular diseases [84].

5.1. Vitamin D and Type 2 Diabetes Mellitus. Epidemiologic data suggest that 9 out of 10 cases of T2DM can be attributed to modifiable lifestyles [85, 86]; however, changes in lifestyle are hard to accomplish and maintain in the long term. There is recent evidence in humans and animal models suggesting that vitamin D may play an important role in modifying the risk of diabetes [87].

Vitamin D receptors are present in pancreatic β and in immune system cells. Additionally, its role in the regulation of calcium absorption is well known; vitamin D participates in the activity of β-cell endopeptidases dependent on calcium and can act through two main pathways:

(1) directly inducing β-cells to secrete insulin through an increase in intracellular calcium concentration through Ca channels,

(2) by mediating β-cell calcium-dependent activation to facilitate conversion of proinsulin to insulin [88].

The role of vitamin D in the function of pancreatic cells can be mediated by the union of 1,25-dihydroxyvitamin D to its receptors in the beta cell. Alternatively, vitamin D can work through the activation of 25 hydroxyvitamin D (25(OH)D) by 1-alpha-hydroxylase expressed in pancreatic beta cells, directly improving insulin sensitivity by stimulating insulin receptor expression and the activation of PPAR-δ (peroxisome proliferator activated receptor delta), which has been associated with the regulation of fatty acid metabolism in skeletal muscle and adipose [89].

The expression of calbindin-D28K (vitamin D dependent on the union of proteins and calcium) has demonstrated a protective effect on beta cells from cytokine mediated cell death, reducing the risk of T2DM [88]. There are few studies in humans associating vitamin D and chronic inflammatory status of T2DM patients; however, the evidence suggests that vitamin D can improve insulin sensitivity and promote pancreatic β-cell survival by modulating the effects of cytokines and nuclear transcription factors such as NF-κB [90].

Some cohort studies in the US and Finland have reported an association between vitamin D status and the risk of T2DM [91–93]. Some other studies [94, 95] have found associations between serum vitamin D levels, insulin resistance, and β-cell dysfunction.

Additionally, clinical studies have examined the effect of vitamin D supplementation and related indicators in different T2DM populations. These studies were carried out from 2 months to 7 years, while vitamin D doses were between 400 and 100,000 IU/day; some have reported improvements in central glycaemia [96], insulin sensitivity, and even lipid profile and endothelial function [97]. Other studies [98–102] of vitamin D supplementation without calcium showed no effect on glycaemia neither reduction in diabetes incidence after years of follow-up [103].

On the other hand, it has been demonstrated that vitamin D is a predictive factor for death by cardiovascular disease in T2DM patients [104, 105].

6. Adipokines

Adipose tissue has its own innervation and vascularization, with two morphological and functional distinct types of cells. White adipose tissue is dedicated to energy storage, while brown adipose tissue dissipates it [106]. Triacylglycerol and fatty acid storage in white adipose tissue are produced through the ability of insulin to stimulate glucose uptake and lipogenesis [107, 108].

It is well known that the distribution of energy within adipocytes due to an increase in adipose tissue mass or the amount of free circulating fatty acids, leads to obesity, dyslipidaemia, insulin resistance, and T2DM [109]; on the other hand, there is evidence indicating that the loss of adipose tissue in lipodystrophic syndromes results in insulin resistance and T2DM. These functions that are apparently opposite, can be explained by the functional complexity of adipose tissue and the great number of signaling molecules that it secretes, which are called "adipocytokines" or "adipokines;" however, not all of these peptides have cytokine family characteristics; therefore, the term "adipokine" is used for a protein produced and secreted only by adipocytes and not by other cells

present in adipose tissue [110]. These polypeptides have been associated with various physiological processes such as food intake, energy balance, insulin action, glucose metabolism, vascular remodeling, and blood pressure regulation and coagulation [111, 112]; additionally, high levels of acute phase proteins and inflammatory cytokines in obese individuals have demonstrated that they suffer from a chronic state of low grade inflammation, which has been associated with the development of insulin resistance, metabolic syndrome, and T2DM [113, 114].

7. Leptin

Leptin is a 16 kDa hormone produced mainly by adipose tissue, and at a lesser extent by other tissues like muscle, stomach, and placenta [115, 116]; it also acts as a cytokine. Adipocytes secrete leptin in direct proportion to adipose tissue mass and nutritional status, being higher in subcutaneous in relation to visceral adipose tissue [117].

As a hormone, leptin helps monitor body weight (mainly by body fat content) to adjust the metabolic level, while as a cytokine, it can exert an effect on the innate and adaptive immune systems; leptin receptors have been found in neutrophils, monocytes, and lymphocytes. Leptin is able to activate proinflammatory cells, promoting a Th1 response and mediating TNFα, IL-2, or IL-6 production [118].

The effect of leptin on the immune system is explained by the fact that there are leptin receptors not only at the hypothalamus and adipose tissue, but also on cells of the immune system such as lymphocytes and monocytes [119]. Structurally, leptin receptors (Ob-R) belong to the class I cytokine family of receptors which include receptors for IL-2, IL-3, IL-4, IL-6, IL-7, and granulocyte-monocyte colony stimulating factor (GM-CSF) [118].

It is well known that leptin can affect T lymphocytes as they express ObR and high concentrations of leptin which seem to be associated with the production of proinflammatory cytokines by these cells [120, 121]. Additionally, leptin also acts on other immune cells, as has been demonstrated in human blood mononuclear cell cultures in presence of various amounts of leptin. The results of this study showed that leptin is able to induce dose-dependent mononuclear cell proliferation; to increase the expression of monocyte activation markers such as CD38, CD25, and CD71; and to increase TNF-α and IL-6 by cultured monocytes. The authors concluded that leptin could amplify monocyte activation and increase the proinflammatory response through cytokine production [122, 123].

Human macrophages and neutrophils also express a great amount of leptin receptors, causing chemotactic and apoptosis retardation. Additionally, dendritic cells may be playing a role on their development and function [118].

It has also been postulated that leptin may activate endothelial cells and stimulate macrophage activation in white adipose tissue (WAT). An increase in WAT and the consequent expression of inflammatory adipokines and decrease in adiponectin contribute to the chronic inflammatory state associated with obesity and the metabolic syndrome. Leptin has also been found to be involved in inflammation associated with atherosclerosis, acting as a signal for insulin sensitivity regulation in the organism. Leptin resistance has been identified as a causal factor of cardiovascular complications in obesity [124].

8. Adiponectin

Adiponectin (AMP1) is a protein hormone of 247 aminoacids, which is largely produced by WAT, it circulates in plasma at higher concentrations than the majority of hormones, its concentration is 5 to 30 μg/mL, representing approximately 0.1% of all plasma proteins and in different amounts between genders, probably due to its regulation by sex hormones [125].

Adiponectin is an anti-inflammatory adipokine, showing improvement in hepatic insulin sensitivity, exerting a synergic effect with leptin, decreasing free nonesterified fatty acid flow, increasing fat oxidation, and reducing hepatic glucose release, plus stimulating glucose use by muscle [114, 126].

The effect of adiponectin on insulin sensitivity is mediated by an increase in oxidation of fatty acids through activated adenosine-monophosphate protein-kinase (AMPK) in skeletal muscle and liver, decreasing glucose synthesis.

As opposed to the majority of adipokines, adiponectin expression and its circulating concentrations are decreased in pathologies with insulin resistance and obesity. It has been demonstrated that Pima Indians have an inverse correlation between adiponectin concentrations and BMI, and that individuals with high adiponectin concentrations are less prone to develop T2DM in comparison to those with low concentrations [110, 114].

TNF-α and IL-6 are potent inhibitors of adiponectin expression and secretion in WAT biopsies and culture cells, suggesting that insulin resistance induction by TNF-α and IL-6 may also be caused by an inhibition of the autocrine-paracrine liberation of adiponectin [127]. It has been demonstrated that the administration of recombinant adiponectin in its complete or isolated form, exerts hypoglycemic effects, decreasing insulin resistance in murine models for obesity and diabetes [128].

In contrast, in lipoatrophic mice, insulin resistance was totally reversed with a combination of leptin and adiponectin at physiological doses, whereas the reversion was only partial when they were administered separately, showing that their joint effect can produce insulin sensitization in peripheral tissues [129].

9. Resistin

Resistin is a dimeric protein named for its apparent effect on the induction of insulin resistance in mice. It belongs to a family of cysteine-rich proteins called FIZZ (*found in inflammatory zone*), which were initially called Resistin-Like Molecules (RELM), and it has been found in adipocytes, macrophages, and other types of cells [130].

High plasma resistin concentrations have been found in experimental obesity models [131]; however, visceral adipose tissue has been associated with low concentrations of this

adipokine [132]. In humans, resistin comes mainly from cells of the immune system and not from adipocytes. The possible effect of resistin on the development of insulin resistance has been evaluated; however, the results are not clear yet [133].

Experimental mice models have shown that recombinant resistin promotes insulin resistance and decreases insulin-stimulated glucose transporters in adipose tissue, while antiresistin antibodies produce the opposite effect [110, 128]. Furthermore, in humans, resistin may be implicated in inflammatory processes as mononuclear cells secrete it in important amounts. Resistin, IL-6, and TNF seem to influence each others action in monocytes through NF-kappa B specialized signaling routes [134].

10. Visfatin

Visfatin is another adipokine, initially thought to mimic insulin, although this is still in debate. It was first found in liver, skeletal muscle, and bone marrow, as a B-lymphocyte precursor growth factor, and was originally called pre-B cell colony stimulator. Its circulating values are related to WAT accumulation [135]. It is also produced by neutrophils preventing apoptosis by a mechanism that is mediated by caspases 3 and 8. It has been found to be elevated in patients with gastrointestinal inflammatory diseases [136]. There is also evidence that it induces chemotaxis, IL-1β, TNF, and IL-6 production, as well as synthesis of CD14+ monocyte costimulator molecules, increasing their ability to induce alloproliferative responses; it may also be involved in the regulation of the inflammatory response and other compensatory mechanisms [137].

The effects of visfatin are similar to insulin's, as it stimulates glucose transportation in muscle and adipocytes, and inhibits hepatic glucose production. Visfatin is forced to activate insulin receptors causing their phosphorylation and the activation of their signaling molecules; however, insulin and visfatin do not compete for the union to the insulin receptor, indicating that they are recognized by different regions [138].

In a KKAy mouse model for obesity and diabetes mellitus, the expression of visfatin in visceral fat tissue and its serum concentrations was found to be directly associated with the increase in obesity in animals fed a high fat diet compared to controls [139].

11. Cytokines

Cytokines are a large group of low molecular weight proteins that mainly regulate the immune response; however, they may have other functions such as embryogenesis, cellular differentiation, and migration, amongst others. They are mainly produced by leukocytes, but some of them can also be secreted by other cells. They were originally called "lymphokines" as they were considered biological products of lymphocytes in response to antigens [140].

In general, these molecules are not constitutively produced, cell activation is necessary for them to be produced in sufficient quantities to exert a biological effect. Most cytokines are secreted in a glycosylated form which increases their stability and solubility [141]. Cytokines have a very short half-life and act at very low concentrations through the union with high affinity receptors. They exert an autocrine effect when anchored to receptors in the producing cell; they also have paracrine effects on other adjoining cells, while in some cases they can be released into the blood or lymphatic circulation, exerting their effect on other organs and tissues acting as hormones [142].

Regarding the inflammatory response, some cytokines promote its development (proinflammatory), while others suppress it (anti-inflammatory) [143].

12. Tumor Necrosis Factor Alpha (TNF-α)

TNF-α was the first cytokine associated with insulin resistance in animals; it is overexpressed in adipose tissue in obesity and decreases with weight loss, being also considered as an important insulin resistance regulator [144]. It has been found to affect insulin signaling *in vivo* and *in vitro*, decreasing the expression of adiponectin in adipose tissue [145]. It stimulates lipolysis, inhibiting the expression of lipoprotein lipase (LPL) and glucose transporter 4 (GLUT 4), which are two key elements for fat accumulation thus, it could be considered a mechanism for the reduction of large fatty acids. However, high concentrations of TNF-α could also be implied in the development of metabolic abnormalities such as insulin resistance [127].

TNF-α seems to play a role in the physiopathology of blood hypertension (BHP) associated with obesity and insulin resistance, as it inhibits insulin dependent glucose uptake by interfering with its signaling [30, 127].

13. Interleukin 1 (IL-1)

IL-1 is an obesity and insulin resistance associated cytokine, produced by several types of cells, but mainly by activated macrophages. It is a key mediator of the inflammatory response, with stimulator and inhibitor actions on some cells, even promoting apoptosis in others. It is able to promote the expression of the same genes that produce it, as well as synthesis of prostaglandins, leukotrienes, interleukin-8, and certain protooncogenes like *c-fos* y *c-jun* [146].

Insulin signaling is directly affected by IL-1 through the induction of cytokine-3 signaling suppressor (SOCS-3); it is mainly stimulated by TNF-α and catecholamines and inhibited by glucocorticoids. It has multiple effects on various tissues, acting together with IL-6 as an endogenous pyrogens, stimulating thermogenesis [147]. It is also a regulator of Protein C Reactive (PCR) hepatic production, and its increase in adipose tissue may stimulate PCR synthesis, which is another inflammatory response modulator [126, 148, 149].

In the past years, it has been demonstrated that high concentrations of glucose stimulate IL-1β production by β-cells [31], implying an effect on the development of T2DM. The evidence suggests the presence of an inflammatory process that leads to failure of the β-cell to secrete sufficient amounts of insulin in diabetic patients. Insulitis is associated with the

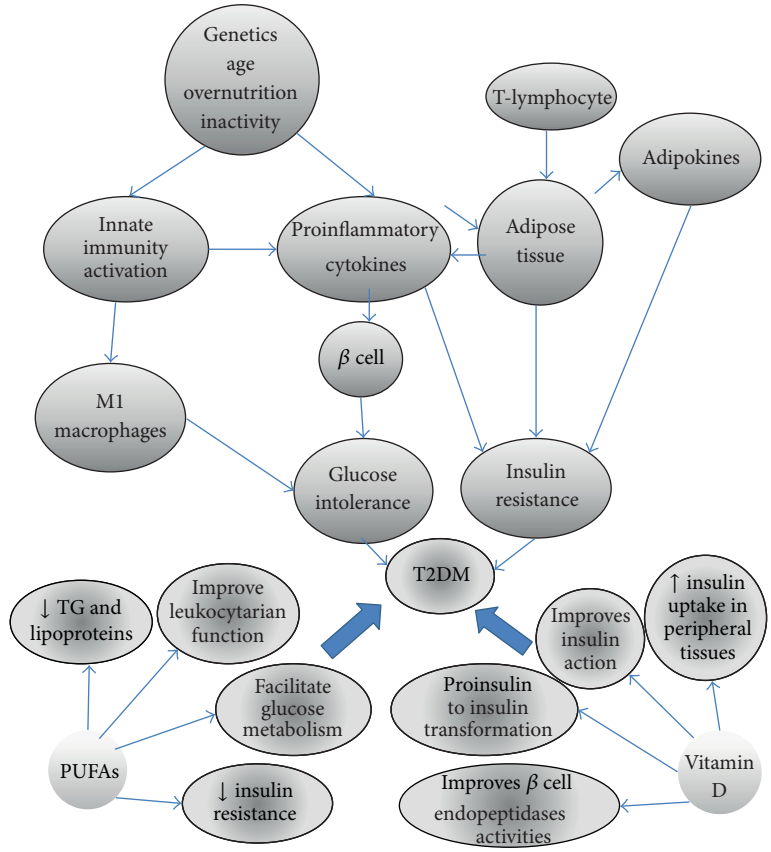

Figure 1: Diverse factors such as overnutrition, physical inactivity, age, and genetics can activate the innate immune system and consequently the production of cytokines, which by themselves lead to insulin resistance and diabetes, but there is evidence that some of these alterations can be improved through the consumption of PUFAs and vitamin D.

pathologic activation of the innate inflammatory system by metabolic stress, which is mediated by IL-1 signaling causing lesions in the pancreatic parenchyma [32].

Overnutrition is the main cause of Type 2 diabetes. Exposure of pancreatic islets to glucose or free fatty acids induces production and release of IL-1β [33, 146, 150], which may also be induced by leptin [151]. IL-1β gene expression has been found to be 100 times increased in beta cells of islets from T2DM patients compared to nondiabetic controls [33].

Fatty acids from adipocytes, which are also a source of IL-1β [152], could amplify these signals by self-activation through their own IL-1 [33, 153].

14. Conclusions

Recent epidemiological studies have associated total fat intake (saturated, mono, and poly-unsaturated fats) with T2DM; however, the type of fat could influence insulin metabolism positively, as can be observed when saturated fat is replaced with monounsaturated fat in the diet, improving considerably insulin action. There are several studies that demonstrate the beneficial effect of polyunsaturated fatty acid supplementation (n-3) in patients with active inflammatory processes and others that report that physiologic concentrations of certain fatty acids can modulate the inflammatory response modifying the evolution of some diseases including Type 2 diabetes. On the other hand, some epidemiological studies have recently reported an increase in serum 25-hydroxivitamin D (25-OH vitamin D) deficiency in diabetic populations. The etiology of this deficiency has been considered multifactorial and ethnicity is one of these factors; low 25-OH vitamin D concentrations may have an important role in the pathogenesis of T2DM, although the mechanisms are not yet clear. Some of these alterations are resumed in Figure 1.

Conflict of Interests

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

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Review Article

Anti-VEGF for the Management of Diabetic Macular Edema

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Diabetic retinopathy (DR) is an important cause of vision loss around the world, being the leading cause in the population between 20 and 60 years old. Among patients with DR, diabetic macular edema (DME) is the most frequent cause of vision impairment and represents a significant public health issue. Macular photocoagulation has been the standard treatment for this condition reducing the risk of moderate visual loss by approximately 50%. The role of vascular endothelial growth factor (VEGF) in DR and DME pathogenesis has been demonstrated in recent studies. This review addresses and summarizes data from the clinical trials that investigated anti-VEGF for the management of DME and evaluates their impact on clinical practice. The literature searches were conducted between August and October 2013 in PubMed and Cochrane Library with no date restrictions and went through the most relevant studies on *pegaptanib*, *ranibizumab*, *bevacizumab*, and *aflibercept* for the management of DME. The efficacy and safety of intravitreal anti-VEGF as therapy for DME have recently been proved by various clinical trials providing significantly positive visual and anatomical results. Regarding clinical practice, those outcomes have placed intravitreal injection of anti-VEGF as an option that must be considered for the treatment of DME.

1. Introduction

Obesity is a major risk factor for type 2 diabetes and has increased in prevalence in the last decades [1, 2]. Diabetic retinopathy (DR) is a leading cause of vision loss in working-age patients around the world. One percent of all cases of blindness worldwide can be attributed to DR [3, 4]. Diabetic macular edema (DME) is primarily responsible for vision impairment in diabetic patients [5–7] (Figure 1). A large epidemiological study indicated that 26% of patients with diabetic retinopathy presented with DME [8]. According to another study, the prevalence of macular edema in patients with recently diagnosed diabetes is 0 to 3%, increasing to 29% in diabetic patients with over 20 years of disease [9]. Therefore, ophthalmic complications of the diabetes, especially DME, represent a significant public health issue (Figure 2).

Both proliferative and nonproliferative DR may show DME, which is classified as either focal, if edema is caused by a focal leakage from microaneurysms, or diffuse, if generalized leakage from retinal capillaries with abnormal permeability is observed throughout the posterior pole [10–12]. Besides the abnormal permeability, edema may also occur due to occlusion of the capillary bed that leads to dilation of the patent capillaries and leakage [13].

Controlling DME risk factors such as systemic hypertension, hyperlipidemia, and poor blood glucose control may decrease the development of edema and lower progression of DR [14]. Other risk factors are adult-onset diabetes mellitus, cardiovascular disease, impaired renal function, advanced DR, increased number of retinal microaneurysms, and vitreomacular traction [13, 15].

The Early Treatment Diabetic Retinopathy Study (ETDRS) showed the benefit of focal/grid laser for the

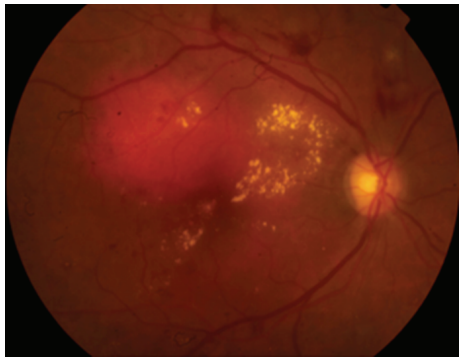


Figure 1: Diabetic retinopathy showing intraretinal hemorrhages, hard exudates, and microaneurysms in the posterior pole associated with diabetic macular edema.

management of DME, reducing the risk of moderate visual loss by approximately 50%, and since then, macular photocoagulation (MPC) has been the gold standard treatment [16]. Recently, data from the Diabetic Retinopathy Clinical Research Network (DRCR.net) studies demonstrated best-corrected visual acuity (BCVA) improvement of more than 5 letters of vision in 51, 47, and 62% of eyes treated with monthly 0.5mg of intravitreal ranibizumab after 1, 2, and 3 years of follow-up, respectively [7, 17–19].

Vascular endothelial growth factor (VEGF) is an important mediator of blood-retinal barrier breakdown, which leads to fluid leakage and the development of macular edema (Figure 3) [20]. Observing that VEGF intraocular levels are increased in DME, it was hypothesized that alternative or adjunct therapies using VEGF inhibitors (anti-VEGF) could be beneficial in reversing vision loss from macular edema [21].

The aim of this review was to address and compare, where possible, data from the clinical trials that assessed anti-VEGF for the management of DME and to evaluate their impact on clinical practice.

2. Methods

The literature searches were conducted between August and October 2013 in PubMed and Cochrane Library with no date restrictions. Relevant unpublished data regarding the topic "anti-VEGF for the management of diabetic macular edema" presented at official retina conferences during this period were also considered in this review. The search strategy used the following words: *diabetic retinopathy*, *diabetic macular edema*, *vascular endothelial growth factor*, *anti-VEGF*, *pegaptanib*, *Macugen*, *bevacizumab*, *Avastin*, *ranibizumab*, *Lucentis*, *aflibercept*, *VEGF Trap Eye*, and *Eylea*.

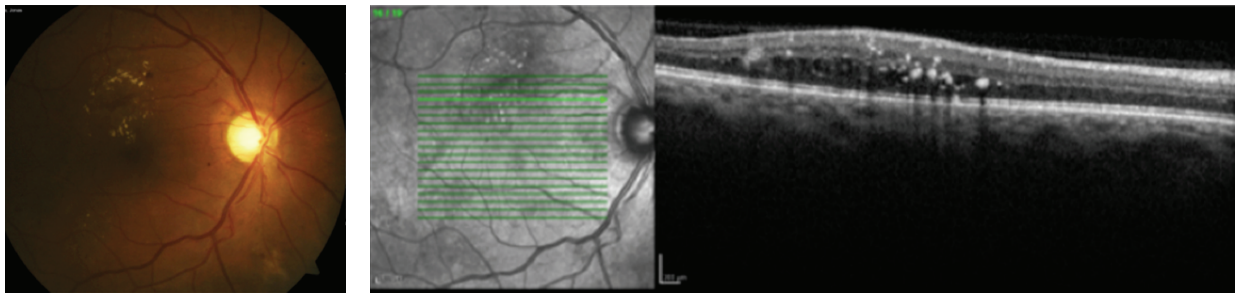
3. Results and Discussion

On the basis of evidence that VEGF expression and signaling are deregulated in diabetic retinopathy, anti-VEGF compounds have been studied as a pharmacological alternative treatment for DME. Considering agents originally used to treat neovascular age-related macular disease (AMD), recent trials have addressed the efficacy and safety of different types of anti-VEGF in the treatment of DME, including pegaptanib (Macugen, OSI/Eyetech, USA), ranibizumab (Lucentis, Genentech, Inc., USA), bevacizumab (Avastin, Genentech, Inc., USA), and aflibercept (EYLEA, Regeneron Pharmaceuticals, Inc., USA).

Pegaptanib sodium is a selective VEGF antagonist that binds with the 165 isoform of VEGF and was approved by the US Food and Drug Administration (FDA) for the treatment of neovascular AMD [22]. Ranibizumab is a recombinant humanized immunoglobulin G1 kappa antibody fragment that binds with and inhibits the biologic activity of all isoforms of human VEGF-A. It was approved by the FDA for the treatment of neovascular AMD, macular edema associated with retinal vein occlusion, and since 2012, it is approved for the treatment of DME [23]. Bevacizumab is a full-size, humanized, recombinant monoclonal IgG antibody that inactivates all VEGF-A isoforms and is approved for systemic use in the treatment of certain metastatic cancers, but its use for ocular diseases is off-label. Aflibercept, or VEGF Trap-Eye, is a new, fully human, 115 kDa recombinant fusion protein that binds with and inhibits all isoforms of 4 VEGF-A and B as well as binds placental growth factors 1 and 2. It has the advantages of a longer half-life in the eye and a higher binding affinity for VEGF-A [24]. VEGF Trap-Eye was approved for the treatment of AMD in 2011 (Table 1) [25].

3.1. Pegaptanib. A phase II randomized double-masked multicenter controlled trial investigated different doses of intravitreal pegaptanib (0.3, 1, and 3 mg) and sham injections in patients with diabetic macular edema. Data published in 2005 showed that 172 individuals with DME involving the center of the macula were included with BCVA at baseline between 20/50 and 20/320. Injections were given at study entry, week 6, and week 12. Additional injections and/or laser therapy could be performed as needed, after week 12 until the end of week 36. Subjects receiving pegaptanib had better BCVA outcomes compared to sham at week 36, with a larger proportion of those receiving 0.3 mg of the drug having a visual acuity gain of 2 lines or more (34 versus 10% $P = 0.003$). The same positive results in favor of 0.3 mg pegaptanib were observed with regard to reduction of central retinal thickness. Subjects assigned to pegaptanib were less likely to need additional laser therapy [26].

In 2011, a phase-2/3, multicenter, randomized, double-blinded trial conducted in the United States included 260 subjects with DME involving the center of the macula and BCVA at baseline between 20/50 and 20/200. They received 0.3 mg of either intravitreal pegaptanib or sham injection every 6 weeks and were followed for 102 weeks. At week 18, macular grid/focal laser was performed as needed, based on ETDRS criteria. The primary efficacy endpoint was the proportion gain of 10 letters or more of visual acuity (VA) from baseline to year 1. No safety issues were identified throughout the study. Again pegaptanib was superior to sham injection regarding visual acuity gain at the end of the first year (37 versus 20%; $P = 0.0047$). The group treated with



(a) (b)

Figure 2: (a) Fundus photograph of the right eye of a patient with diabetic retinopathy with hard exudates and focal edema temporal superior to the macula. (b) Optical coherence tomography of the patient showing intraretinal edema and hard exudates.

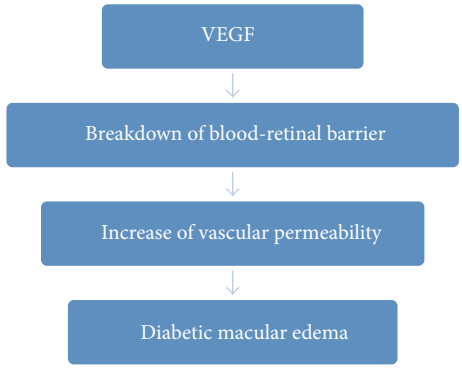


Figure 3: VEGF and pathophysiology of diabetic macular edema.

pegaptanib gained 6.1 letters in mean BCVA at week 102, while the sham injection group gained 1.3 letters ($P < 0.01$). Significantly fewer macular laser indications were observed in the pegaptanib group compared to sham injection [27].

3.2. Ranibizumab. A small pilot study, in 2006, provided early data proving that intravitreal ranibizumab was effective and improved vision acuity in patients with DME. Ten diabetic patients with chronic macular edema were included and received intravitreal ranibizumab (IVR) at study entry and at months 1, 2, 4, and 6. At month 7, the study showed an improvement in mean visual acuity and reduction in mean foveal thickness, demonstrating the importance of VEGF in the pathophysiology of DME [28].

A multicenter, phase II trial, READ-2, was conducted randomizing 126 subjects with DME evenly into 3 groups: group 1 received 0.5 mg of ranibizumab at baseline and months 1, 3, and 5; group 2 received focal/grid laser at baseline and at month 3 if needed; and group 3 received a combination of focal/grid laser and 0.5 mg ranibizumab at baseline and at month 3. The primary outcome was mean change in BCVA at 6 months. Group 1 (+7.24 letters) was superior to group 2 (−0.43 letters, $P = 0.01$) regarding ETDRS BCVA, while improvement in group 3 (+3.80 letters) was not significant compared to the other two groups. A visual gain of 3 lines or more was observed in 22% in group 1, 0% in group 2, and 8% in group 3 ($P = 0.002$) [29].

Table 1: Anti-VEGF agents.

Anti-VEGF agents	Mechanism of action	Molecular weight	FDA approval
Pegaptanib sodium (Macugen)	Selective VEGF antagonist (165 isoform).	50 kDa	AMD (2004)
Ranibizumab (Lucentis)	Recombinant humanized IgG1 kappa antibody fragment. Inhibits all isoforms of human VEGF-A.	48 kDa	AMD (2006) RVO edema (2010) DME (2012)
Bevacizumab (Avastin)	Full-size, humanized, recombinant monoclonal IgG antibody. Inhibits all isoforms of human VEGF-A.	149 kDa	Off-label use in ophthalmology
Aflibercept (Eylea)	Fully human recombinant fusion protein. Inhibits all isoforms of human VEGF-A and B as well as binds placental growth factors 1 and 2.	115 kDa	AMD (2011) RVO edema (2012)

RVO: retinal vein occlusion.

Another phase II clinical trial, RESOLVE, randomized 151 patients with DME to receive either 0.3 or 0.5 mg of ranibizumab as monotherapy versus sham injection, monthly for 3 months. After one month, patients were allowed to have their doses doubled to 0.6 mg or 1 mg (or double sham) if indicated by specific study criteria. Both groups were eligible for rescue laser on the basis of foveal thickness and visual acuity. Patients in the sham group had their doses doubled more often (91.8 versus 68.6%), as well as rescue laser being more often performed in the sham group (34.7 versus 4.9%). The ranibizumab group had BCVA improvement averaging +10.3 letters at 1 year, while the sham group had +1.4 letters ($P < 0.001$); the same superiority was

observed in central retinal thickness improvement, −194.2 versus −48.4 μm in the ranibizumab and sham groups, respectively ($P < 0.001$). Visual gain of 10 letters or more was observed in 60.8% of the ranibizumab-treated patients, compared with 18.4% of the sham-treated patients ($P < 0.001$) [30].

The RESTORE phase III clinical trial conducted in Europe randomized 345 subjects into 3 different groups: (A) receiving 0.5 mg of ranibizumab and sham laser, (B) receiving 0.5 mg of ranibizumab and active laser, and (C) receiving laser and sham injection. Monthly treatment was given for 3 months followed by "as needed" treatment. In the 12-month report, visual acuity improvement was 6.1 letters in group A, 5.9 letters in group B, and 0.8 letters with laser alone in group C. There was a statistically significant difference between both ranibizumab groups and the laser group ($P < 0.0001$), but no differences were seen between the ranibizumab groups. Mean central retinal thickness also decreased significantly in both ranibizumab groups compared with laser alone. The mean number of injections was 7 in the ranibizumab group A and 6.8 in the ranibizumab plus laser group B. No safety issue was observed in this study [31].

Two methodologically identical phase III trials, RIDE and RISE, were intended to support FDA approval of ranibizumab for treatment of DME and were sponsored by Genentech (Genentech Inc., South San Francisco, USA). The parallel, multicenter, double-masked, sham injection-controlled, randomized studies were conducted in the United States and South America. RIDE enrolled 377 patients with DME and RISE enrolled 382. They were evenly assigned to 3 different groups to receive either 0.3 or 0.5 mg of ranibizumab or to receive sham injections, monthly treatment for 24 months. At 3 months, rescue laser was allowed for all patients. After 24 months, the protocol was changed and all patients previously assigned to sham injections became eligible to receive 0.5 mg ranibizumab injection [32].

At 2 years, RISE and RIDE outcomes showed significant superiority of both ranibizumab groups over the sham injection groups regarding improvement of visual acuity and reduction of central retinal thickness. The primary efficacy point was improvement of 15 letters or more, and considering the sham injection, 0.3 mg ranibizumab, and 0.5 mg ranibizumab groups, the achievement rate was 18.1, 44.8, and 39.2% of patients in RISE and 12.3, 33.6, and 33.3% of patients in RIDE. It is worth noting that in the RIDE and RISE studies there was no direct comparison between ranibizumab and laser, due to a 3-month delay in laser treatment, even in the sham groups. Similarly to other ranibizumab trials, safety findings were acceptable. Endophthalmitis occurred at a rate of 0.8%. The incidence rates of nonfatal myocardial infarction, cerebrovascular accident, and death from vascular or unknown causes were 4.9–5.5% in the sham groups and 2.2–8.8% in the ranibizumab groups. Based on these trials, FDA approved ranibizumab as the first anti-VEGF for the treatment of DME.

The same primary endpoint was evaluated at 36 months and the visual effects were maintained. Improvement of 15 letters or more, in the sham injection, 0.3 mg ranibizumab, and 0.5 mg ranibizumab groups was, respectively, 22.0, 41.6, and 51.2% in RISE patients and 19.2, 36.8, and 40.2% in RIDE patients [33].

The Diabetic Retinopathy Clinical Research Network (DRCR.net) conducted a study with a more complicated design. Although other trials had shown the benefits of anti-VEGF as a treatment for DME, monthly injections or monthly evaluations were not feasible in clinical practice. DRCR.net protocol I tried to give more flexibility to the treatment and to differentiate between the effect of ranibizumab and laser. A total of 854 patients with DME were randomized into 4 groups: sham injection plus prompt laser; 0.5 mg ranibizumab plus prompt laser, 0.5 mg ranibizumab plus deferred laser (at or after 24 weeks), and 4 mg triamcinolone plus prompt laser. Treatment was given according to the "4 : 2 : 7 rule": four monthly injections; additional injections if required at the next 2 study visits, and 7 subsequent study visits during which injection could be indicated at the investigator's discretion if the study eye was considered to show "no improvement" [17].

The primary outcome was mean change in BCVA at 1 year and the findings showed that both ranibizumab groups, with prompt or deferred laser, gained 9 letters, superior to the triamcinolone plus laser and sham plus laser groups, which gained 4 and 3 letters, respectively. At 2 years, improvements in mean change in BCVA were maintained and fewer injections were performed in the ranibizumab groups throughout the second year: from 8 in the prompt laser and 9 in the deferred to 2 and 3, respectively. It is noteworthy that the number of injections was similar between prompt laser group and deferred laser group [17].

Data from the third year of protocol I suggest that early initiation of focal/grid laser treatment not only lacks benefit but also may be detrimental to visual outcomes, since the deferred laser group showed 57% of patients gaining 10 letters or more and 5% losing 10 letters or more, while the prompt laser group showed 42% gaining 10 or more letters and 10% losing 10 or more letters [34].

An exploratory analysis of protocol I was performed to evaluate the effect of intravitreal ranibizumab and triamcinolone on worsening diabetic retinopathy. Despite acknowledging the limitations of exploratory analysis, the results indicated that ranibizumab, as well as triamcinolone, appears to reduce the risk of worsening diabetic retinopathy [35].

3.3. Bevacizumab. Intravitreal bevacizumab (IVB) has been widely used off-label for the treatment of AMD, especially because of its significantly lower cost compared to ranibizumab, in addition to positive clinical effects demonstrated in early studies [36]. The widespread use of IVB for the management of DME led to the need of a formal evaluation of its safety and efficacy [37–39].

DRCR.net conducted a phase II exploratory trial including 121 eyes with DME over a 12-week period to assess the short-term effect of IVB [39]. The eyes were randomized into five groups: (I) focal laser, (II) two intravitreal injections of 1.25 mg of bevacizumab at 0 and 6 weeks, (III) two intravitreal injections of 2.5 mg of bevacizumab at 0 and 6 weeks, (IV) 1.25 mg of bevacizumab at week 0 followed by a sham

injection at week 6, and (V) 1.25 mg of bevacizumab at 0 and 6 weeks plus focal laser at 3 weeks. Eyes assigned to groups II and III had a significant BCVA improvement over the laser-only group I, and this difference was seen throughout the 12 weeks. These two groups also had a greater improvement in central subfield thickness (CST) at the 3-week visit. No differences were seen between groups 1.25 mg and 2.5 mg bevacizumab. The single injection group was not superior to the laser group. Bevacizumab plus laser showed results comparable to laser-only treatment. This study suggested that bevacizumab was an effective drug for treating DME as a primary treatment and also for refractory eyes, since 69% of included eyes were refractory to previous treatment. However, eyes that received primary treatment had greater improvement than the refractory ones ($P = 0.04$). No safety concerns were detected in 24 weeks. Similar outcomes showing no difference between 1.25 mg and 2.5 mg of bevacizumab have been previously reported in other studies [40, 41].

A randomized clinical trial compared IVB injection alone or in combination with intravitreal triamcinolone acetonide (IVT) versus macular laser photocoagulation as a primary treatment for DME. A total of 150 eyes were randomly assigned to the following groups: (I) 1.25 mg IVB, (II) IVB/IVT, with 1.25 mg IVB and 2 mg IVT, and (III) macular laser. The IVB group showed significant superiority in visual acuity improvement after six months, but this was not sustained after 24 months. The mean BCVA was significantly better in the IVB-only group compared to baseline, after 24 weeks [7, 42].

The study conducted by the Pan-American Collaborative Retina Study Group (PACORES) examined IVB as the primary treatment for diffuse DME at 11 centers in 8 countries [38]. This retrospective, multicenter, interventional, comparative case series reviewed clinical data of 139 eyes with diffuse DME treated with at least 1 off-label intravitreal injection of either 1.25 or 2.5 mg of bevacizumab. The dose received at baseline was the same dose delivered throughout the study. Follow-up considered BCVA measurement with ETDRS charts and OCT at baseline and 1, 3, 6, 12, and 24 months after the initial injection. The reinjection criterion was recurrence of diffuse DME [43].

No significant differences between the 1.25 mg and 2.5 mg dose groups were detected. Mean BCVA and central macular thickness (CMT) improved at 1 month after the first IVB and such significant outcomes were sustainable all along the 24 months; when the results demonstrated that 72 (51.8%) eyes improved by 2 or more ETDRS lines, 62 (44.6%) eyes remained stable, and 5 (3.6%) eyes decreased by 2 or more ETDRS lines of BCVA. At 24 months, OCT analysis showed that CMT decreased from 446.4 ± 154.4 to $279.7 \pm 80\,\mu$m. The mean number of injections per eye was 5.8 (range of 1–15 injections) at a mean interval of 12.2 ± 10.4 weeks [43].

The bevacizumab or laser therapy (BOLT) study is a prospective, randomized, blinded, single-center study that compared IVB to macular laser photocoagulation in patients with persistent DME after at least one macular laser treatment [44]. Eighty eyes were randomized into either the bevacizumab group, receiving injections every 6 weeks, with a minimum of 3 and a maximum of 9 injections, or the laser group, receiving treatment every 4 months, with a minimum of 1 and a maximum of 4 treatments. Mean BCVA after 1 year increased in the bevacizumab group and declined in the laser group. The CMT results were superior in the bevacizumab group as well. The mean number of interventions was 9 injections and 3 laser treatments during the first year.

The 2-year outcome report from the BOLT study was published in 2012 and presented similar results to those obtained in the first year report [45]. The mean BCVA was 20/50 in the group treated with bevacizumab and 20/80 in the laser group ($P = 0.005$), with a mean gain of 8.6 letters for bevacizumab versus a mean gain of 0.5 letters for the laser group. Regarding improvement of 15 letters or more, 32% of the eyes treated with bevacizumab achieved this target versus 4% for the laser-treated eyes ($P = 0.004$). On the other hand, the proportion of subjects that lost fewer than 15 letters in the laser group was 86% versus 100% for the bevacizumab group ($P = 0.03$). CMT decreased significantly in both groups at 2-year follow-up and the mean number of treatments was 13 injections and 4 macular laser interventions. These outcomes provided by the BOLT study support the longer term use of IVB for the treatment of DME.

3.4. Aflibercept. Encouraged by positive results from a phase I study [46], a phase II, multicenter, randomized clinical trial was conducted to investigate different dosing regimens of intravitreal VEGF Trap-Eye for the treatment of DME compared to standard macular laser [24]. The DA VINCI (DME A and VEGF Trap-Eye: Investigation of Clinical Impact) study enrolled 221 subjects with center-involved DME and BCVA between 20/40 and 20/320 randomized into 5 groups: 0.5 mg VEGF Trap-Eye every 4 weeks (0.5q4), 2.0 mg VEGF Trap Eye every 4 weeks (2q4), 2.0 mg VEGF Trap Eye monthly for 3 months and then every 8 weeks (2q8), 2.0 mg VEGF Trap Eye monthly for 3 months and then as needed (2 PRN), and macular laser treatment. All VEGF Trap Eye groups received sham laser and all laser patients received sham injection. The primary endpoint was mean change in BCVA. The change from baseline in central retinal thickness and proportion of patients gaining at least 15 letters at week 24 were among secondary outcomes [24].

Improvement in mean change of BCVA was observed ranging from 8.5 to 11.4 letters in groups receiving aflibercept versus 2.5 letters in the laser group, at week 24. Central retinal thickness significantly decreased more in the groups treated with VEGF Trap-Eye compared to the laser group. No significant differences were seen between the aflibercept groups, supporting a treatment regimen of every 8 weeks instead of every 4 weeks [24]. At 52 weeks, the change in mean BCVA ranged from 9.7 to 13.1 letters in the aflibercept groups versus a loss of 1.3 letters in the laser group [47].

Two phase III trials are ongoing and have recently divulged early outcomes. VIVID-DME (VEGF Trap-Eye in Vision Impairment due to DME), in Europe, Japan, and Australia, and VISTA-DME (Study of Intravitreal Administration of VEGF Trap-Eye in Patients with Diabetic Macular Edema), in USA, are randomized, double-masked, active controlled trials that investigate the efficacy and safety of

repeated doses of intravitreal VEGF Trap-Eye in subjects with DME [48]. The trials are both sponsored by Bayer (Bayer AG, Leverkusen, Germany) and Regeneron Pharmaceuticals (Regeneron Pharmaceuticals, Inc., Tarrytown, USA) and may support the FDA approval for the use of aflibercept in DME [49, 50].

VIVID-DME enrolled 404 patients and VISTA-DME 461, randomized (1 : 1 : 1) to receive intravitreally either 2.0 mg aflibercept every 4 weeks (2q4) or 2.0 mg aflibercept every 8 weeks after 5 initial monthly doses (2q8) or laser photocoagulation. Primary endpoint was mean change in BCVA at week 52. The patients were scheduled for continued treatment for 3 years [48]. After the first year, the primary results showed the superiority of aflibercept groups over the laser treatment group. Mean change in BCVA in the VIVID-DME study was plus 10.7 letters in the 2q8 group and plus 10.5 in the 2q4 versus plus 1.2 in the laser group. The VISTA-DME showed a similar mean change in BCVA of plus 10.7 letters in the 2q8, plus 12.5 in the 2q4, and plus 0.2 in the laser group. The mean change in central retinal thickness, proportion of patients gaining at least 15 letters, and improvement of Diabetic Retinopathy Severity Score (DRSS), all secondary outcomes, showed a significant superiority of aflibercept over laser treatment. On average, the aflibercept 2q8 group performed similarly as the aflibercept 2q4 group. No systemic safety signal was detected in either aflibercept treatment group through week 52 [48].

4. Further Study and Concerns

The efficacy of intravitreal anti-VEGF for the treatment of DME has recently been proved by various studies. Safety issues concerning intravitreal injection of anti-VEGF are well known from AMD studies, although none of those trials or DME trials had enough power to detect significant differences between the study groups regarding adverse events. Serious ocular adverse events are of low frequency and include endophthalmitis, uveitis, and retinal detachment; likewise the risk of occurrence does not seem to be greater in patients with DME than in AMD. Serious systemic adverse events could be death, myocardial infarction, and stroke. Most safety studies, however, have failed to identify issues regarding such systemic events related to intravitreal anti-VEGF injection [32, 36, 47, 48].

The small number of relevant trials and variation in their characteristics limit comparisons between different anti-VEGF drugs. A relevant ongoing study conducted by the DRCR.net, the protocol T, proposes as its primary objective to compare the efficacy and safety of intravitreal aflibercept, bevacizumab, and ranibizumab when used to treat central-involved DME. To date, 660 subjects with DME and BCVA between 20/32 and 20/320 were enrolled and will be randomized to receive either 1.25 mg bevacizumab, or 0.3 mg ranibizumab, or 2.0 mg aflibercept [51]. Considering that ranibizumab is the only approved anti-VEGF for DME, the markedly lower cost of bevacizumab, and the potential of aflibercept to decrease treatment burden and associated cost, the outcomes from this study should have an extensive impact on clinical practice regarding the management of DME.

5. Conclusions

Diabetic macular edema is an important cause of vision impairment and macular photocoagulation has been the standard treatment for this condition. Recent studies have presented significantly positive visual and anatomical results regarding the use of anti-VEGF for the treatment of DME, both as primary intervention and in refractory cases. Although the protocols are all consistently different, undoubtedly anti-VEGF therapy has assumed an important role in the management of DME, either as a first choice or as adjuvant to photocoagulation.

This review was conducted to better understand the impact of the outcomes of recent trials on clinical practice. Further studies are necessary, especially to investigate long-term efficacy and safety, to compare drugs, and establish guidelines. However, confronted with a diagnosis of center-involved diabetic macular edema, it has become mandatory to consider treatment with intravitreal anti-VEGF injections.

Conflict of Interests

The authors have no conflict of interests regarding the present study.

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Research Article

The Role of Cytokines in the Functional Activity of Phagocytes in Blood and Colostrum of Diabetic Mothers

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Immune response changes induced by diabetes are a risk factor for infections during pregnancy and may modify the development of the newborn's immune system. The present study analyzed colostrum and maternal and cord blood of diabetic women to determine (1) the levels of the cytokines IFN-γ and TGF-β and (2) phagocytic activity after incubation with cytokines. *Methods.* Colostrum and maternal and cord blood samples were classified into normoglycemic ($N = 20$) and diabetic ($N = 19$) groups. Cytokine levels, superoxide release, rate of phagocytosis, bactericidal activity, and intracellular Ca^{2+} release by phagocytes were analyzed in the samples. Irrespective of glycemic status, IFN-γ and TGF-β levels were not changed in colostrum and maternal and cord blood. In maternal blood and colostrum, superoxide release by cytokine-stimulated phagocytes was similar between the groups. Compared to spontaneous release, superoxide release was stimulated by IFN-γ and TGF-β in normoglycemic and diabetic groups. In the diabetic group, cord blood phagocytes incubated with IFN-γ exhibited higher phagocytic activity in response to EPEC, and maternal blood exhibited lower microbicidal activity. These data suggest that diabetes interferes in maternal immunological parameters and that IFN-γ and TGF-β modulate the functional activity of phagocytes in the colostrum, maternal blood, and cord blood of pregnant diabetic women.

1. Introduction

Maternal interaction with the fetus is bidirectional. Fetal and placental tissues require suitable environment, under homeostasis, whereas the maternal body is affected by factors related to metabolic adjustments. In this relationship, the fetus receives passive immunity from the mother, which is crucial for newborn adaptation to the extrauterine environment because it provides protection against infectious agents during the first months of life [1, 2].

Cells with phagocytic and microbicidal activity are among the multiple immune components of blood and human milk that play an important role in child protection [3, 4]. A number of studies report that diabetic patients have low phagocytic and microbicidal activity and reactive oxygen species production due to changes in their antioxidant systems. Moreover, the reduction in phagocytic and microbicidal activity of leukocytes is likely related to an increase in blood glucose levels [5–7].

In diabetic individuals, the balance between proinflammatory and anti-inflammatory cytokines is not fully understood. Some studies show that they prioritize the production of proinflammatory cytokines [8], whereas others relate that the production of both cytokine types is increased by diabetes [9].

The cytokine gamma interferon (IFN-γ) and transforming growth factor β (TGF-β) seem to act in the early stages of pregnancy [10, 11] and participate in cellular functions. It

is believed that IFN-γ promotes the inflammatory reaction that enables trophoblast implantation, whereas TGF-β acts on maternal immunological response, embryo implantation, and placental and fetal development [10, 12].

IFN-γ and TGF-β play a role in the activation and regulation of immune cells. IFN-γ promotes the microbicidal response of phagocytes, increasing the expression of surface receptors and rates of phagocytosis [13]. TGF-β, in turn, likely induces and maintains the immune tolerance to the fetus [12] and the production of regulatory cells [14]. Nevertheless, the action of these cytokines on the microbicidal activity of blood and colostrum phagocytes of diabetic mothers has yet to be investigated.

The present study analyzed the colostrum and maternal and cord blood of diabetic women in order to determine (1) the levels of the cytokines IFN-γ and TGF-β and (2) phagocytic activity after incubation with cytokines.

2. Materials and Methods

The functional activity of colostrum and maternal and cord blood phagocytes in diabetic women was evaluated in a cross-sectional study. The subjects attended the Diabetes and Pregnancy Facility, School of Medicine Obstetrics Course, UNESP, Botucatu, SP, Brazil. This study was approved by the institutional Research Ethics Committee, and all the subjects gave written informed consent before entering the experimental protocol.

2.1. Subjects. Blood and colostrum samples from pregnant women (18–45 years old) were analyzed by maternal glycemic status. According to the results of the 75 g oral glucose tolerance test (OGTT 75 g) [15] and glucose profile (GP) test [16], 39 pregnant women were classified into the following groups: normoglycemic group (normal 75 g OGTT and normal GP; $n = 20$) and diabetes mellitus group (altered GTT 75 g, prior to or during the pregnancy and abnormal GP; $n = 19$). The subjects continued attending the facility, irrespective of diagnosis, and the diabetic patients followed a specific treatment for glycemic control [16].

2.2. Blood Sampling and Separation of Blood Cells. Samples of 8 mL of maternal blood were collected prior to the beginning of labor and cord blood at birth in tubes with anticoagulant. We centrifuged them at 160 G for 15 min to separate plasma from the cells. Cells were separated by a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden), producing preparations with 95% of pure mononuclear cells, analyzed by light microscopy. Purified macrophages were resuspended independently in serum-free medium 199 at a final concentration of 2×10^6 cells/mL. The cells were used immediately for assays of superoxide release, phagocytosis, microbicidal activity, and calcium release. The plasma was stored at −80°C for later glucose and cytokines analysis.

2.3. Colostrum Sampling and Separation of Colostral Cells. About 8 mL of colostrum from each woman was collected in sterile plastic tubes between 48 and 72 hours postpartum. The samples were centrifuged (160 G, 4°C) for 10 min, which separated colostrum into three different phases: cell pellet, an intermediate aqueous phase, and a lipid-containing supernatant. The upper fat layer was discarded and the aqueous supernatant was stored at −80°C for later analyses. Cells were separated by a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden), producing preparations with 98% of pure mononuclear cells, analyzed by light microscopy. Purified macrophages were resuspended independently in serum-free medium 199 at a final concentration of 2×10^6 cells/mL. The cells were used for assays of superoxide release, phagocytosis, microbicidal activity, and calcium release. The colostrum supernatant was stored at −80°C for later glucose and cytokines analysis.

2.4. Glucose Determination. Glucose levels were determined by the enzymatic system. Samples of 20 μL colostrum/maternal or cord blood, standard of 100 mg/dL (Doles), were placed in 2.0 mL phosphate buffer solution (0.05 M, pH7.45, with aminoantipyrine 0.03 mM, 15 mM sodium p-hydroxybenzoate, 12 kU/L glucose oxidase, and 0.8 kU/L peroxidase). The suspensions were mixed and incubated for 5 min at 37°C. The reactions were read on a spectrophotometer at 510 nm.

2.5. Cytokine Dosage By ELISA (Enzyme-Linked Immunosorbent Assay). IFN-γ concentrations in the colostrum and milk supernatants were determined by an ELISA kit from BioLegend Legend Max (San Diego, USA), and TGF-β concentrations were analyzed using an ELISA kit from Enzo Life Sciences (UK). The reaction rates were measured by absorbance in a spectrophotometer with a 450 nm filter. The results were calculated using the standard curve and shown in pg/dL.

2.6. Escherichia Coli Strain. The enteropathogenic *Escherichia coli* (EPEC) used was isolated from stools of an infant with acute diarrhea (serotype 0111: $H^- AL^-$, eae^+, eaf^+, bfp^+). This material was prepared and adjusted to 10^7 bacteria/mL, as previously described by Honorio-França [17].

2.7. Treatment of Blood and Colostral Phagocytes with Cytokines. To assess the effect of cytokines (IFN-γ and TGF-β) on superoxide anion release, phagocytic, microbicidal activity, and intracellular Ca^{2+} release, MN phagocytes (2×10^6 cells/mL) were incubated with 5 μL of cytokines (Sigma St. Louis, USA, final concentration 100 ng/mL) for 1 h at 37°C. The phagocytes were then washed once with 199 medium at 4°C and immediately used in the assays. A control was performed with only 199 medium.

2.8. Release of Superoxide Anion. Superoxide release was determined by cytochrome C (Sigma, St. Louis, USA) reduction [17, 18]. Briefly, mononuclear phagocytes (blood and colostrum) and bacteria were mixed and incubated for 30 min for phagocytosis. Cells were then resuspended in PBS

Table 1: Mean (±SD) glucose level, leukocyte count, viability, IFN-γ, and TGF-β concentrations in colostrum, maternal blood, and cord blood from normoglycemic and diabetic women.

Parameter	Sample	Normoglycemic	Diabetic
Glucose level (mg/dL)	Colostrum	66.0 ± 7.4	114.2 ± 9.6*
	Maternal blood	90.5 ± 8.6	122.1 ± 8.9*
	Cord blood	69.0 ± 7.3	87.0 ± 8.5*
Mononuclear phagocytes count ($\times 10^6$ cell/mL)	Colostrum	2.8 ± 0.5	2.4 ± 0.8
	Maternal blood	4.8 ± 0.7	5.1 ± 0.5
	Cord blood	2.9 ± 0.6	3.2 ± 0.9
Mononuclear phagocytes viability (%)	Colostrum	92.0 ± 3.5	90.0 ± 2.2
	Maternal blood	91.0 ± 2.4	90.0 ± 3.3
	Cord blood	90.0 ± 2.6	91.0 ± 2.7
IFN-γ (pg/mL)	Colostrum	9.2 ± 1.5	7.9 ± 3.1
	Maternal blood	8.2 ± 1.7	8.4 ± 0.5
	Cord blood	8.9 ± 1.8	7.5 ± 2.6
TGF-β (pg/mL)	Colostrum	22.1 ± 1.6	24.0 ± 4.3
	Maternal blood	26.1 ± 4.7	26.6 ± 3.7
	Cord blood	26.0 ± 4.6	27.6 ± 4.4

*Statistical differences in glucose levels between normoglycemic and diabetic groups, considering the same kind of samples.

containing 2.6 mM $CaCl_2$, 2 mM $MgCl_2$, and cytochrome C (Sigma, St. Louis, USA; 2 mg/mL). The suspensions (100 μL) were incubated for 60 min at 37°C on culture plates. The reaction rates were measured by absorbance at 550 nm and the results were expressed as nmol/O_2^-. All the experiments were performed in duplicate.

2.9. Bactericidal Assay. Phagocytosis and microbicidal activity were evaluated by the acridine orange method [19]. Equal volumes of bacteria and cell suspensions were mixed and incubated at 37°C for 30 min under continuous shaking. Phagocytosis was stopped by incubation in ice. To eliminate extracellular bacteria, the suspensions were centrifuged twice (160 ×g, 10 min, 4°C). Cells were resuspended in serum-free 199 medium and centrifuged. The supernatant was discarded and the sediment was dyed with 200 μL of acridine orange (Sigma, St. Louis, USA; 14.4 g/L) for 1 min. The sediment was resuspended in cold 199 medium, washed twice, and observed under immunofluorescence microscope at 400x and 1000x magnification.

The phagocytosis index was calculated by counting the number of cells ingesting at least 3 bacteria in a pool of 100 cells. To determine the bactericidal index, we stained the slides with acridine orange and counted 100 cells with phagocytized bacteria. The bactericidal index is calculated as the ratio between orange-stained (dead) and green-stained (alive) bacteria x100 [4]. All the experiments were performed in duplicate.

2.10. Intracellular Ca^{2+} Release Determined by Fluorescence and Flow Cytometry. We performed fluorescence staining at the FACS Calibur (BD, San Jose, USA) to assess intracellular Ca^{2+} release in phagocytes [20]. Cells were loaded with the fluorescent radiometric calcium indicator Fluo3-acetoxymethyl (Fluo3-AM-Sigma, St. Louis, USA). Cell suspensions, pretreated or not with 50 μL of cytokines (Sigma, final concentration of 100 ng/mL), were mixed and incubated at 37°C for 30 min under continuous stirring. Suspensions were centrifuged twice (160 ×g, 10 min, 4°C) and resuspended in PBS containing BSA (5 mg/mL). This suspension was incubated with 5 μL of Fluo-3 (1 μg/mL) for 30 min at 37°C. After incubation, cells were washed twice in PBS containing BSA (5 mg/mL; 160 ×g, 10 min, 4°C) and then analyzed by flow cytometry (FACS Calibur system, BD, San Jose, USA). Calibration and sensitivity were routinely checked using CaliBRITE 3 Beads (BD, Cat. no 340486, USA). Fluo-3 was detected at 530/30 nm filter for intracellular Ca^{2+}. The rate of intracellular Ca^{2+} release was expressed in geometric mean fluorescence intensity of Fluo-3. Data shown in the figures correspond to one of several trials performed.

2.11. Statistical Analysis. Data were expressed as the mean ± standard deviation (SD). The statistically significant difference was evaluated using the analysis of variance (ANOVA) for superoxide release anion, phagocytosis, bactericidal index, and intracellular Ca^{2+} release in the presence or absence of cytokines and the difference was compared at the statistical significance was considered for a P-value less than 0.05.

3. Results

Glucose levels in colostrum, maternal blood, and cord blood were higher in hyper- than in normoglycemic women. However, leukocyte retrieval and viability and IFN-γ and TGF-β levels in maternal blood, cord blood, and colostrum samples were similar between the groups (Table 1).

Diabetic and normoglycemic groups had similar spontaneous superoxide release by mononuclear (MN) phagocytes in colostrum. When exposed to EPEC and cytokines, the phagocytes of both groups increased superoxide release ($P < 0.05$). Irrespective of the presence of cytokines, phagocytes

Table 2: Superoxide release by colostrum and mononuclear phagocytes of blood (mean ± SD, $N = 10$ in each treatment).

Phagocytes	Incubated with	Superoxide release (nmol)	
		Normoglycemic	Diabetic
Colostrum	PBS	1.7 ± 0.4	2.0 ± 0.5
	Bacteria	4.4 ± 0.8*	4.1 ± 0.6*
	Bacteria plus IFN-γ	4.6 ± 0.5*	3.5 ± 0.4*+
	Bacteria plus TGF-β	4.1 ± 0.5*	3.6 ± 0.1*+
Maternal blood	PBS	1.8 ± 0.2	1.7 ± 0.2
	Bacteria	3.5 ± 0.8*	3.8 ± 0.4*
	Bacteria plus IFN-γ	4.1 ± 0.8*	3.7 ± 0.4*
	Bacteria plus TGF-β	4.3 ± 0.5*	4.5 ± 0.1*#
Cord blood	PBS	3.2 ± 0.5#	4.9 ± 1.1+#
	Bacteria	4.8 ± 0.8*	4.6 ± 1.0
	Bacteria plus IFN-γ	5.4 ± 0.7*	5.1 ± 0.5#
	Bacteria plus TGF-β	4.6 ± 0.3*	5.6 ± 0.1#+

Colostrum and blood mononuclear cells were treated or not with cytokines, in the presence or absence of EPEC. *Indicates differences between phagocytes treated or not with cytokines and incubated with bacteria and the control (without bacteria) within each group and sample (colostrum, maternal blood, or cord blood); #indicates differences between sample (colostrum, maternal blood, and cord blood) within each treatment (cytokines or PBS) and group; and +indicates intergroup differences within each treatment (cytokines or PBS) and sample (colostrum, maternal blood, or cord blood).

in maternal blood showed the highest superoxide release when exposed to bacteria. In the diabetic group, cord blood phagocytes displayed higher spontaneous superoxide release than those in the normoglycemic group. In the normoglycemic group, cord blood phagocytes exhibited the highest superoxide release when exposed to bacteria and cytokines. In the diabetic group, cytokines did not affect superoxide release by cord blood phagocytes (Table 2).

MN phagocytes from colostrum and maternal blood, treated or not with cytokines, exhibited similar phagocytic activity against EPEC, irrespective of glycemic status (Figure 1). Cord blood phagocytes from the diabetic group displayed a higher phagocytic index when exposed to bacteria and IFN-γ ($P < 0.05$—Figure 1).

In general, maternal blood phagocytes in the diabetic group showed low bactericidal activity against EPEC. Both groups exhibited equivalent rates of EPEC elimination by mononuclear phagocytes when treated with cytokines. Maternal blood phagocytes, in both the groups studied, treated with IFN-γ exhibited a higher bactericidal index. The bactericidal index of colostrum and cord blood phagocytes did not vary between the groups (Figure 2).

In the diabetic group, maternal blood phagocytes had low intracellular Ca^{2+} release, irrespective of cytokine treatment. In the normoglycemic group, cord blood phagocytes showed lower intracellular Ca^{2+} release than those in maternal blood. In the presence of TGF-β, intracellular Ca^{2+} release was higher in cord cells from the normoglycemic group. In the diabetic group, colostrum phagocytes not incubated with cytokines showed lower intracellular Ca^{2+} release when compared with cells from normoglycemic group (Table 3 and Figure 3).

4. Discussion

The present study describes IFN-γ and TGF-β levels in colostrum and maternal and cord blood of diabetic women and how these cytokines affect phagocytic activity in the maternal body and the gestation products evaluated.

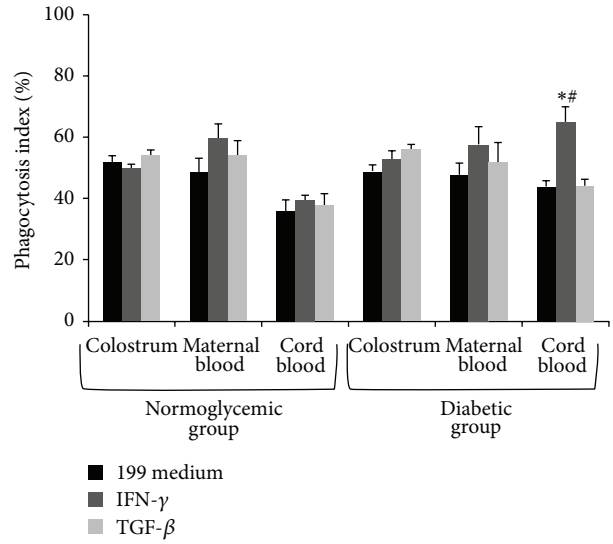

Figure 1: Bacterial phagocytosis by colostrum and maternal and cord blood phagocytes (mean ± SD, $N = 10$ in each treatment and sample), determined by the acridine orange method. Phagocytes were incubated with enteropathogenic *Escherichia coli* (EPEC) in the presence of gamma interferon (IFN-γ) and transforming growth factor β (TGF-β). *indicates differences from the 199 medium and cytokines use within each sample and group; #indicates differences between normoglycemic and diabetic groups within each treatment (cytokines or 199 medium) and sample (colostrum, maternal blood, or cord blood).

Several factors affect cytokine production and action during pregnancy [21]. One of these cytokines, IFN-γ, is implicated in the network of mediators of diabetes [22]. In the present study, however, hyperglycemia did not affect the levels of IFN-γ and TGF-β in colostrum and maternal and cord blood.

Table 3: Intracellular Ca^{2+} release by mononuclear (MN) colostrum phagocytes of diabetic mothers indicated by fluorescence intensity.

Phagocytes	Incubated with	Fluorescence intensity (%)	
		Normoglycemic	Diabetic
Colostrum	PBS	20.2 ± 0.3	17.8 ± 1.5^{+}
	IFN-γ	22.0 ± 2.4	19.6 ± 5.2
	TGF-β	20.4 ± 0.56	18.1 ± 3.28
Maternal blood	PBS	14.2 ± 2.3$^{\#}$	9.6 ± 2.3$^{\#+}$
	IFN-γ	16.6 ± 2.6$^{\#}$	10.2 ± 2.2$^{\#+}$
	TGF-β	15.5 ± 3.8$^{\#}$	9.1 ± 2.6$^{\#+}$
Cord blood	PBS	10.2 ± 2.1$^{\#}$	10.8 ± 1.4$^{\#}$
	IFN-γ	10.3 ± 2.5$^{\#}$	10.9 ± 1.4$^{\#}$
	TGF-β	19.6 ± 5.2*	10.3 ± 1.6$^{\#+}$

Colostrum and blood mononuclear cells were preincubated or not with cytokines. *Indicates differences between phagocytes incubated with cytokines and the control (PBS) within each group and sample, $^{\#}$indicates differences between samples (colostrum, maternal blood, and cord blood) within each treatment (cytokines or PBS) and group; and $^{+}$indicates differences between normo- and diabetic groups within each treatment (cytokines or PBS) and sample (colostrum, maternal blood, or cord blood).

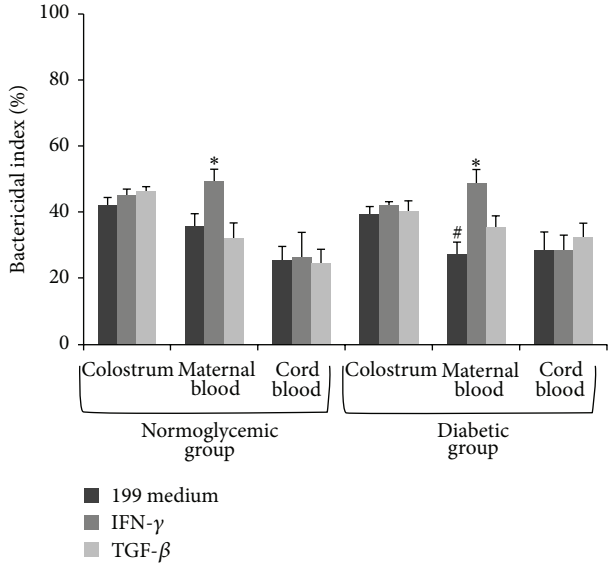

Figure 2: Bactericidal index (mean ± SD, $N = 10$ in each treatment and sample), determined by the acridine orange method. Phagocytes were incubated with enteropathogenic *Escherichia coli* (EPEC) in the presence of gamma interferon (IFN-γ) and transforming growth factor β (TGF-β). *Indicates differences from the 199 medium and cytokines use within each sample and group; $^{\#}$indicates differences between normoglycemic and diabetic groups within each treatment (cytokines or 199 medium) and sample (colostrum, maternal blood, or cord blood).

A number of studies show that part of the clinical picture of diabetic patients is associated with excessive release of proinflammatory cytokines. Cytokines exert profound effects on the biological signaling and regulation of important physiological processes that are compromised by diabetes [22]. Cytokines may also be related to phagocyte activation and production of reactive oxygen species [23].

In the present study, the cytokines tested modulated superoxide release. Colostrum phagocytes and maternal blood phagocytes increased superoxide release in both groups studied. On the other hand, in the diabetic group, cord blood phagocytes exhibited the highest spontaneous superoxide release, suggesting phagocyte activation irrespective of cytokine presence. A number of mechanisms possibly contribute to the formation of these reactive oxygen-free radicals. Glucose oxidation is believed to be the main source of free radicals [24]. Hyperglycemia also promotes lipid peroxidation by a superoxide-dependent pathway, producing free radicals [25, 26]. Another important source of free radicals in diabetic individuals is the products of glucose-protein interaction [27].

The functional activity of phagocytes was assessed in the colostrum and blood samples of diabetic patients [1, 3, 7, 28] and animals with induced diabetes [29, 30]. Phagocytes play an important role in host defense. Here, we showed that, in the presence of cytokines, phagocytes from colostrum and maternal blood exhibit similar phagocytic activity against EPEC, irrespective of the women's glycemic status. In the diabetic group, cord blood phagocytes increase phagocytosis rate in the presence of IFN-γ.

Phagocytosis and microbicidal activity of phagocytes in colostrum and blood, with production of active oxygen metabolites such as free radicals, consist of an important defense mechanism against a number of bacterial [4, 31], fungal [32], and protozoal infections [33]. In the present study, we found that phagocytes in the blood of diabetic mothers exhibited low bactericidal activity against EPEC. However, after incubation with cytokines, bacterial elimination by MN phagocytes of diabetic women increased to rates similar to those obtained in the normoglycemic group.

Earlier studies have reported that diabetic patients have low phagocytic and microbicidal activity due to derangements in prooxidative systems. An increase in blood glucose levels is indeed related to a decrease in the phagocytic activity of leukocytes [5–7]. On the other hand, cytokines such as IFN-γ act primarily on macrophages by activating their phagocytic and microbicidal abilities [13, 34]. The present study is the first to report the effects of IFN-γ and TGF-β on the functional activity of colostrum and maternal and cord blood phagocytes of diabetic mothers.

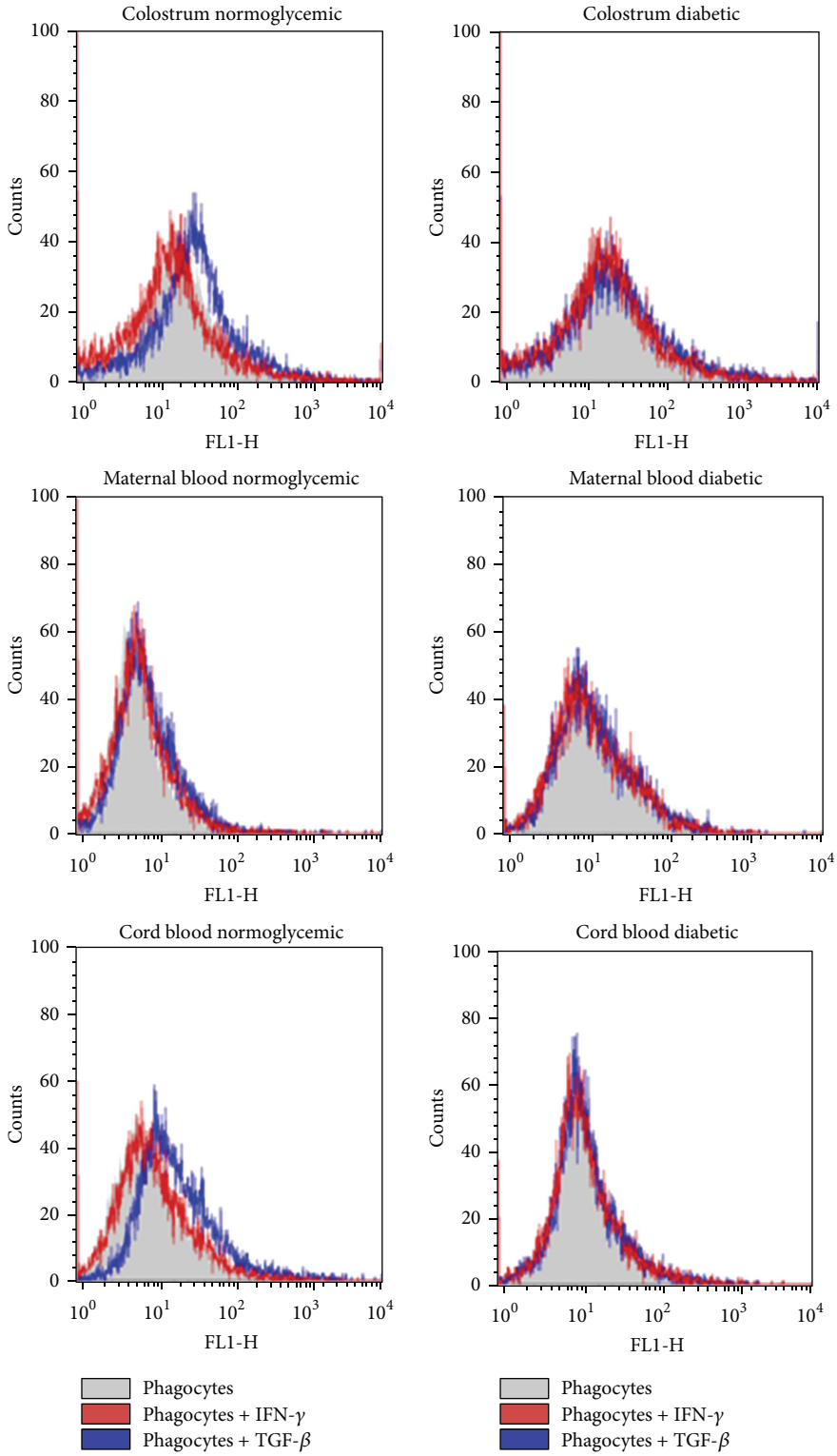

Figure 3: Intracellular Ca^{2+} release by colostral, maternal blood and cord blood phagocytes of diabetic mothers stimulated or not with cytokines. Cells were stained with Fluo-3, and immunofluorescence analyses were carried out by flow cytometry (FACScalibur, Becton Dickinson, USA).

Interestingly, cord blood phagocytes exposed to EPEC and stimulated by IFN-γ showed an increase in phagocytosis but not in their bactericidal activity. A number of studies report that these cells display low bactericidal activity because they lack nonspecific surface receptors, while others argue that they are still too immature to exhibit this response [1].

In diabetic patients, cytokine production may be associated with a number of processes such as alterations in intracellular Ca^{+2} by phagocytes, which is triggered by advanced glycation and products [35] and hyperglycemia [36]. In the present study, cytokines did not stimulate intracellular Ca^{2+} release by maternal blood phagocytes in the diabetic group, and in the normoglycemic group, TGF-β increased intracellular Ca^{2+} release in cord cells.

Transforming growth factor β (TGF-β) is involved in the balance of maternal immune response and deployment process and produced early in the maternal-fetal interface by the embryo and decidua [10, 14].

Although colostral phagocytes of diabetic women decrease intracellular Ca^{2+} release in relation to normoglycemic women [7], we show here that colostrum phagocytes, irrespective of cytokine incubation, increase intracellular Ca^{2+} release in relation to maternal and cord blood phagocytes.

The maternal transfer of immune components during pregnancy and breastfeeding represents a remarkable immunologic interaction between the mother and newborn. Breast milk is an excellent source of immunological components, and it decreases the high rates of maternal and infant complications. Adequate maternal glycemic control during pregnancy and breastfeeding duration in diabetic mothers is crucial to ensure that the immunity components act against the infections.

5. Conclusion

Our findings support the hypothesis that diabetes interferes in maternal immunological parameters due to alterations in glucose metabolism and that IFN-γ and TGF-β can modulate the functional activity of colostrum, maternal blood, and cord blood phagocytes of diabetic women and these cytokines can be alternative for use in future clinical applications in diabetic patients.

Conflict of Interests

The authors declare no conflict of interests and nonfinancial competing interests regarding the publication of this paper.

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