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Glycated ferritin increases the *in vitro* expression of TLR2 and TLR4 in peripheral blood CD14+ cells obtained from patients with prediabetes

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Abstract: Serum ferritin is a widely-used marker of inflammation in prediabetes, diabetes and atherosclerosis. In these cases, progressive endothelial damage may involve the participation of toll-like receptors (TLR). The aim of this study was to determine the expression of TLR2 and TLR4 in peripheral blood mononuclear cell (PBMC)-derived CD14+ cells from subjects with prediabetes and with a high level of serum ferritin both at baseline and after *in vitro* cell stimulation with glycated ferritin. Blood samples were drawn from 22 subjects (13 with prediabetes and 9 with normoglycemia). Serum ferritin levels were measured by ELISA, while the expression of TLR2 and TLR4 in PBMC-derived CD14+ cells was determined by flow cytometry. IL-6 and IL-8 cytokines in PBMC-derived CD14+ supernatants were measured by ELISA. Subjects with prediabetes had a higher baseline expression of TLR4 in PBMC-derived CD14+ cells than was observed in cells from normoglycemic subjects ($p<0.05$). Glycated ferritin increased the expression of both TLR2 and TLR4 as well as IL-6 and IL-8 in PBMC-derived CD14+ cells from subjects with prediabetes when compared to normoglycemic subjects ($p<0.05$). We concluded that in prediabetes, the increased basal expression of TLR4 could be part of the low-grade inflammation, which could be increased by glycated ferritin.

Keywords: serum ferritin; glycated ferritin; prediabetes; toll-like receptors

INTRODUCTION

Several studies have shown that the low-grade inflammatory condition observed in subjects with prediabetes is characterized by increased expression of proinflammatory cytokines, as well as high levels of C-reactive protein (CRP) and serum ferritin [1-3]. Evidence shows that there is a relationship between serum ferritin and glycemia [4-5], and also a positive association between serum ferritin and the risk of coronary artery disease [6].

Serum ferritin is believed to have its primary origin in the liver, but it is unclear whether it is a reflection or the cause of inflammation, or whether it is involved in an inflammatory cycle [7]. The increase in serum ferritin correlates with the iron pool and levels of proinflammatory cytokines [7]. Probably for this reason, the presence and/or the severity of the hyperglycemic state (prediabetes and diabetes), coronary artery disease, and other conditions such as infection, trauma, chronic kidney disease, rheumatoid arthritis and cancer correlate with an increase in serum ferritin [4-7].

Transmembrane toll-like receptors (TLR) and their ligands are involved in endothelial inflammation [8]. In both healthy and sick individuals, TLR2 and TLR4, which are proteins belonging to the TLR family, sense both exogenous (bacterial and viral pathogens) and endogenous (tissue damage-associated) biochemical signals [9]. Exogenous TLR2 ligands include peptidoglycan, porins, lipoarabinomannan and zymosan [10-12]. Ligands for TLR4 are lipopolysaccharide and

glycoinositolphospholipids [9]. Both TLR2 and TLR4 also sense endogenous ligands such as hyaluronan, saturated fatty acids, heat shock proteins, glycated proteins and advanced glycation end-products (AGEs) [9,13-14].

In chronic hyperglycemic states as in prediabetes and diabetes, inflammation contributes to the development of atherosclerosis [15]. Increased expression of TLRs in monocyte-macrophages, a group of cells involved in the pathophysiology of atherosclerosis, is part of the inflammatory process that leads to accelerated atherosclerosis and vascular complications [16]. The increased activity of TLRs in atherosclerosis can be triggered by both exogenous and endogenous ligands [14]. Endogenous molecules such as AGEs interact with TLR2 and TLR4 to alter intracellular signaling, gene expression and the release of proinflammatory cytokines such as IL-1β, IL-6, IL-8 and TNF-α [13,17]. Activation of the nuclear factor-kappa B (NF-κB) is a signature downstream event for TLRs-ligand interaction [9]. In studies with AGEs, NF-κB has been shown to be activated through the MyD88-dependent pathway [18].

Recently, we have been able to show that ferritin undergoes *in vitro* glycation and promotes both the expression of TLR2 and TLR4 as well as the activation of NF-κB in PBMC-derived CD14+ cells in normoglycemic subjects [19-20]. In the present study, we compared the expression of TLR2, TLR4, IL-6 and IL-8 in PBMC-derived CD14+ cells from subjects with prediabetes and with a high level of serum ferritin to that found in cells from normoglycemic subjects both at baseline and after stimulation of the cells with glycated ferritin.

MATERIALS AND METHODS

Blood samples

Blood samples were drawn from 22 subjects after informed consent, following the guidelines of the Declaration of Helsinki and the approval of the bioethics committees in research of the Department of Medicine and Health Sciences of the University of Sonora (Approval No. CBIDMCS/D-117), and of a local hospital from which the subjects were invited to participate in the study. Of the 22 participants, 13 met the criteria of the American Diabetes Association for the diagnosis of impaired fasting glucose and/or impaired glucose tolerance [21]. Fasting plasma glucose (FPG) and the oral glucose tolerance test (OGTT), with 75 g of anhydrous glucose, were conducted in every subject using the glucose oxidase assay (Hitachi Modular P800 Analyzer, Roche Diagnostics Co., IN, USA). Glycated hemoglobin A1c was performed on a DCA Vantage Analyzer (Siemens DCA Vantage Analyzer, Tarrytown, NY, USA). This last method is certified by the National Glycohemoglobin Standardization Program (NGSP).

Given that contrasting results in serum concentrations of ferritin in subjects with prediabetes and other chronic hyperglycemic conditions have been reported in relation to gender [22-24], age [25] and body mass index (BMI) [26], we took 9 blood samples from normoglycemic subjects matched by BMI, sex and age. At the time of the study, none of the 22 participants presented acute infections, usual causes of hyperferritinemia, cancer, autoimmune diseases, alcoholism or pregnancy, and none were taking antihypertensive treatment, lipid-lowering drugs, nonsteroidal antiinflammatory drugs, steroids, hormone replacement therapy, pioglitazones, thyroglobulin or fibrates.

Blood sample collection and standard biochemical assessment

All participants were asked to fast overnight for 10-12 h and to avoid strenuous exercise and any alcohol intake for 48 h before the evaluation. Venous blood samples were drawn into heparin and anticoagulant-free tubes using a standard Vacutainer method (Becton, Dickinson, NJ, USA). Samples of whole blood, plasma and serum were further split into smaller aliquots for the determination of standard biochemical parameters and isolation of peripheral blood mononuclear cells (PBMC).

Total cholesterol (TC), triglycerides (TG) and HDL-cholesterol (HDL) were measured spectrophotometrically in a Hitachi Modular P800 Analyzer (Roche Diagnostics Co., Indiana, USA) by standardized, commercially available diagnostic tests. LDL-cholesterol was calculated using the Friedewald formula [27]. Fasting serum insulin was determined using ELISA (Cat. IS130D, Calbiotech, Spring Valley, CA, USA).

Measurements of FPG and fasting serum insulin were used to determine insulin resistance (HOMA-IR) [28]. Serum CRP was measured in duplicate using the nephelometric Minineph™ highly sensitive CRP method (hsCRP, cat. ZK044LR, Binding Site Ltd, Birmingham, UK). The concentration of ferritin in sera samples was determined in triplicate using an ELISA assay (cat. ab108837, Abcam, Cambridge, UK). In this last case, the absorbances were measured employing a Bio-Rad Model 680 microplate reader (Hercules, CA, USA) at 405 nm, and data were expressed in μg/L and assessed by regression analysis, following the manufacturer's recommendations.

Materials for the *in vitro* assays

PerCP/Cy5.5 anti-human CD14 antibody (clone HCD14, cat. 325622), FITC anti-human CD282 antibody (TLR2, clone TL2.1, cat. 309706), PE anti-human CD284 antibody (TLR4, clone HTA125, cat. 312806) and fixation buffer (Cat. 420801) were obtained from BioLegend (San Diego, CA, USA). Ferritin type I, from horse spleen (Cat. F4503), zymosan A from *Saccharomyces cerevisiae* (Cat. Z4250) and D-(+)-glucose (Cat. 5767) were obtained from Sigma Aldrich (Saint Louis, MO, USA). The human IL-6 ELISA Kit (cat. 900-K16) and the human IL-8 ELISA Kit (cat. 900-K18) were acquired from Peprotech (Rocky Hill, NJ, USA). All other reagents used were of the highest grade available.

Ferritin glycation

To obtain glycated ferritin, 1 mg of native ferritin was dissolved in 1 mL of 20 mmol/L phosphate buffered saline (PBS), pH 7.4, and was then incubated in a solution of 500 mL of 20 mmol/L PBS, pH 7.4 with 0.4 M D-(+)-glucose, containing 1 mmol/L EDTA, 0.1 mg/mL chloramphenicol and 0.02% NaN_3, pH 7.4 at 37°C for 7 days under a nitrogen atmosphere. Glycated ferritin was incubated with 1.3 g/500 mL $NaBH_4$ for 1 h at 4°C and dialyzed against 20 mmol/L PBS, pH 7.4 containing 0.1 mmol/L EDTA for 24 h to remove free glucose [19-20]. Finally, the concentration of glycated ferritin was estimated with the Bradford technique [29] and the protein modification was evaluated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) [30].

Stimulation of PBMC-derived CD14+ cells and estimation of TLR2 and TLR4 expression (mean fluorescence intensity units (MFI)) by flow cytometry

Previously, we established that glycated ferritin induces both TLR2 and TLR4 in CD14+ PBMC from normoglycemic subjects in a dose-dependent manner, reaching a maximum effect at 50 ng/mL in 2 h [19]. Accordingly, for the present work the same concentration of glycated ferritin (50 ng/mL) was used to stimulate for 2 h the PBMC-derived CD14+ cells obtained from the subjects. For this, 1×10^5 PBMC in 100 μL of 20 mmol/L PBS, pH 7.4 was used. PBS has no significant effect on cell viability, the expression of TLRs or on cytokine secretion in experiments of 2 h or less, and it prevents reaction or costimulation of glycated ferritin with other proteins [19,31-33]. Native ferritin and zymosan (50 ng/mL each) were used as controls to stimulate the expression of TLR in PBMC-derived CD14+ cells (1×10^5 cells in 100 μL of 20 mmol/L PBS, pH 7.4) for 2 h. Zymosan, a positive control for both TLR2 and TLR4 activation, is known for its ability to induce activation of the MyD88 signaling pathway in CD14+ PBMC in order to increase the expression of TLR4 mediated by NF-κB [19,34-35].

Cells were then incubated with a pool of antibodies of PerCP/Cy5.5 anti-CD14, FITC anti-TLR2 and PE anti-TLR4 (all used at the concentration recommended by the manufacturer) for 30 min at room temperature in the dark. Flow cytometry analysis was conducted, evaluating 20,000 events per assay using a FACSCalibur cytometer from Becton Dickinson (NJ, USA) and Summit Software Informer (Beckman coulter, CA, USA).

Quantitation of IL-6 and IL-8

The quantitation of the proinflammatory interleukins IL-6 and IL-8 in supernatants of stimulated PBMC-derived CD14+ cells was performed by ELISA. Briefly, the microplates were prepared by coupling the capture antibodies (anti-IL-6 or anti-IL-8). Supernatants were then added and incubated for 2 h at 25°C with constant agitation. After washing and using the corresponding antibody for detection, plates were incubated for 2 h at 25°C with constant agitation. Detection was performed using the substrate 2,2'-azino-bis

Table 1. Baseline characteristics of the donor subjects of the blood samples.

Variable	All	Control	Prediabetes	*p*-value*
n	22	9	13	
Sex (M/F)	10/12	4/5	6/7	
Age (years)	45.68±10.47	42.88±9.50	47.61±11.04	0.31
BMI (kg/m^2)	31.62±5.08	32.60±6.54	30.94±3.93	0.466
FPG (mmol/L)	5.13±0.59	4.72±0.40	5.41±0.54	0.004
OGTT (mmol/mol)	6.57±1.83	5.19±1.05	7.53±1.66	0.001
A1c (%), (mmol/mol)	5.7±0.37, 39.09±4.06	5.4±0.32, 35.55±3.67	5.96±0.20, 41.53±2.02	<0.001
TG (mmol/L)	1.32 (1.10, 1.93)	1.23 (1.00, 1.54)	1.87 (1.23, 2.36)	0.043
TC (mmol/L)	4.99±0.80	4.97±0.61	5.00±0.94	0.930
LDL-C (mmol/L)	3.04±0.82	3.17±0.71	2.94±0.90	0.547
HDL-C (mmol/L)	1.24±0.31	1.22±0.34	1.25±0.30	0.810
FSI (μIU/mL)	4.22±1.68	3.65±1.34	4.63±1.82	0.186
HOMA-IR	0.85 (0.64, 1.37)	0.75 (0.61, 0.82)	1.05 (0.79, 1.62)	0.036
hsCRP (mg/L)	1.55 (0.86, 4.06)	1.28 (0.70, 1.81)	2.25 (1.08, 4.36)	0.186
SF (μg/L)	120.54±91.56	68.73±68.71	156.41±90.08	0.023

Data are presented as mean ± standard deviation, medians (25th percentile, 75th percentile). *P*-values were determined by the t-test for parametric data or the Mann-Whitney U test for nonparametric data. Two-tailed statistical significance level: *$p \leq 0.05$. BMI – body mass index; FPG – fasting plasma glucose; OGTT – oral glucose tolerance test; A1c – glycated hemoglobin A1c; TG – triglycerides; TC – total cholesterol; LDL – low density lipoprotein; HDL – high density lipoprotein; FSI – fasting serum insulin; HOMA-IR – homeostatic model assessment of insulin resistance; hsCRP – highly sensitive C-reactive protein; SF – serum ferritin.

(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) at 405 nm using a microplate reader (Bio-Rad model 680, Hercules, CA, USA).

Statistical analysis

Data were expressed as means and standard deviations, or medians and interquartile ranges. A t-test or Mann-Whitney U test was applied to determine differences between parameters in peripheral blood. Pearson's coefficient adjusting for triglycerides was calculated to assess correlation between variables. For the expression of TLRs, Wilcoxon's test was applied to evaluate differences between glycated ferritin and basal expression, and the Mann-Whitney U test was applied to calculate differences between groups. Statistical analysis was performed using SPSS 21 (SPSS Inc., IL, USA). Results were regarded as significant at $p<0.05$.

RESULTS

The profiles of tested parameters in peripheral blood of the 22 participants at the start of the study (13 with prediabetes and 9 normoglycemic subjects) are shown in Table 1. The concentration of serum ferritin as well as that of TG were significantly higher in subjects with prediabetes when compared to those normoglycemic. A significant correlation was found between the concentrations of serum ferritin, FPG and glycated hemoglobin A1c (Table 2).

Table 2. Correlation between serum ferritin (SF) and glycemic variables.

Variable	r	*p*-value
FPG	0.542	0.02*
A1c	0.503	0.02*
HOMA-IR	0.224	0.329

A *p*-value<0.05 was regarded as statistically significant. All correlations were adjusted for triglycerides. FPG – fasting plasma glucose; A1c – glycated hemoglobin; HOMA-IR – homeostatic model assessment of insulin resistance.

TLR2 and TLR4 expression in PBMC-derived CD14+ cells in the resting state

Baseline expression of TLR4 in PBMC-derived CD14+ cells was significantly higher in subjects with prediabetes than in normoglycemic subjects (Fig. 1F). The expression of TLR2 did not differ between normoglycemic controls and prediabetic patients.

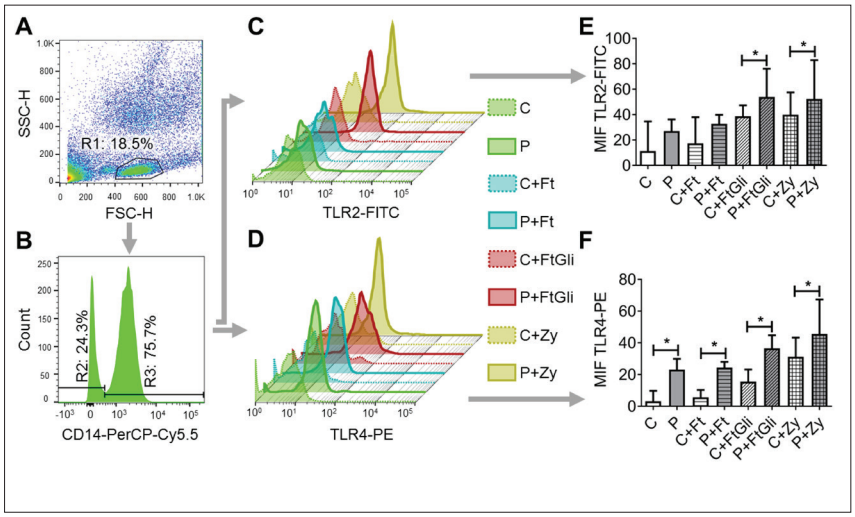

Fig. 1. TLR2 and TLR4 protein expression. Flow cytometry measurements of TLR2 (**E**) and TLR4 (**F**) expression were performed on CD14+ PBMC from normoglycemic controls (C, n=9) and prediabetic (P, n=13) subjects, after challenge of the cells with 50 ng/mL of native ferritin (Ft) or glycated ferritin (FtGli) or zymosan (Zy), as described in Section 2.5. **A** – Representative dot plot of the selected PBMC region (R1). **B** – Representative histogram for selected CD14+ PBMC region (R3). **C**, **D** – representative histograms of median fluorescence intensity units (MIF) of TLR2 and TLR4 on CD14+ PBMC after stimuli. Values are expressed as MFI/10^5 cells with interquartile range. Mann-Whitney U test, *p<0.05.

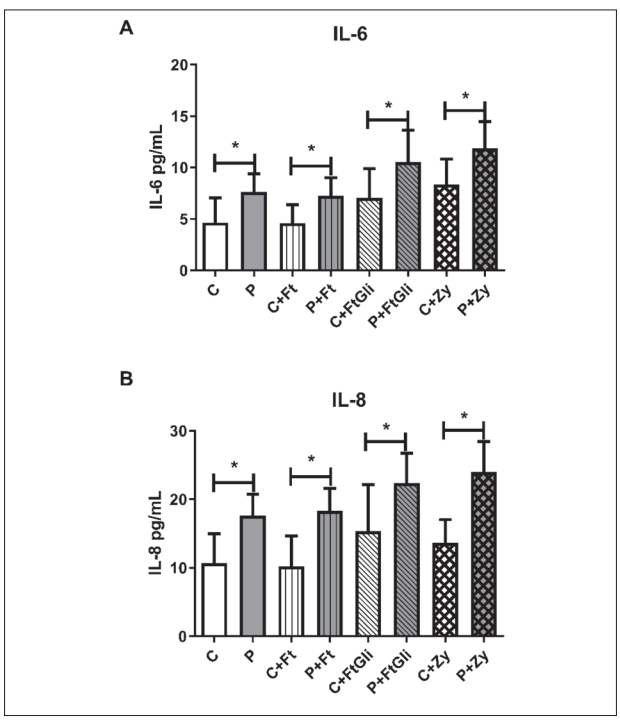

Fig. 2. Release of cytokines in resting and activated PBMC supernatants. IL-6 (**A**) and IL-8 (**B**) were measured in supernatants of PBMC from normoglycemic control (C) and prediabetic (P) subjects at baseline (rest) and after 50 ng/mL of native ferritin (Ft) or glycated ferritin (FtGli) or zymosan (Zy) stimulation using an ELISA assay, as described in the materials and methods section. Values are expressed as mean±standard deviation in pg/mL; *p<0.05.

TLR2 and TLR4 expression in stimulated PBMC-derived CD14+ cells

The challenge with glycated ferritin caused increased expression of TLR2 and TLR4 (p=0.028 and p=0.011, respectively) in PBMC-derived CD14+ cells from subjects with prediabetes, and caused increased expression of TLR2 and TLR4 (p=0.001 and p=0.001, respectively) in PBMC-derived CD14+ cells from normoglycemic subjects. This effect was greater in cells of prediabetic individuals (Fig. 1). The challenge with native ferritin did not induce changes in the expression of TLRs while zymosan upregulated both TLR2 and TLR4, with a response significantly higher in cells from subjects with prediabetes (Fig. 1). The expression of TLR2 and TLR4 showed a correlation with FPG (r=0.602, p=0.03 and r=0.616, p=0.025, respectively), but the serum ferritin concentration showed a significant correlation only with TLR4 expression of baseline PBMC-derived CD14+ cells (r=0.891, p=0.043) (not shown in the figures).

IL-6 and IL-8 measurement

Measurement of the downstream functional products of TLRs, IL-6 and IL-8 showed significant upregulation in both baseline and activated PBMC-derived CD14+ cells from prediabetic subjects (Fig. 2).

DISCUSSION

Serum ferritin is a widely-used marker of inflammation [36]. It is also a risk marker of prediabetes [2,23] and coronary artery disease [6]. In such conditions, progressive endothelial damage can involve the participation of TLRs [8-9,37]. TLRs are a family of transmembrane receptors that sense both exogenous and endogenous damage signals that trigger innate and adaptive immune responses through the activation of cellular TLR-dependent signaling [8-9].

Studies have demonstrated that various glycated molecules can activate, through TLR, a range of inflammatory responses, including chemotaxis and synthesis of proinflammatory cytokines [37-38]. To our knowledge, there is no method to measure glycated ferritin in the sera of patients, but glycation can occur under conditions of subclinical hyperglycemia, with at least two factors favoring this process: (i) the increase in the concentration of serum ferritin in conditions of hyperglycemia [5], and (ii) the half-life of glycosylated ferritin, which is up to 10-fold greater than the non-glycosylated isoform [39] and is the predominant isoform of ferritin in serum [40].

Our recent studies have shown that glycated ferritin serves as a ligand for TLR and that it promotes its *in vitro* expression in PBMC-derived CD14+ cells from normoglycemic subjects [19-20]. Results from the present study support the finding that glycated ferritin promotes the increased expression of TLR2 and TLR4 in CD14+ PBMC cells from subjects with prediabetes to a significantly greater extent than in cells from normoglycemic subjects. It is important to highlight that the activation of TLR2 and TLR4 was triggered by glycated but not by native ferritin.

The observed effect of glycated ferritin on the expression and activation of TLR in PBMC CD14+ cells is consistent with that established in experimental studies that show the activation of TLR by glycated molecules; for example, AGE-LDL activates a TLR4-mediated signaling pathway, thus inducing proinflammatory cytokine production in mice [37], while glycated serum albumin can promote the release of IL-8 in vascular smooth muscle cells via the TLR4-dependent pathway [38].

It is worth noting that results from *in vitro* assays as well as *in vivo* observations have demonstrated that hyperglycemia on its own activates the expression of TLR (both TLR2 and TLR4) in human monocytes [16], and increases the expression and functional activation of TLR in recently diagnosed diabetic subjects [41]. At the experimental level, the expression of TLR2 and TLR4 also increases in rats with prediabetes [42] and the knockdown of both receptors results in the decrease of the high glucose-induced NF-κB response [16].

Evidence shows that both TLR2 and TLR4 are also overexpressed in PBMC CD14+ cells in obese subjects, which correlated with the increased expression of TNF-α and IL-6 [43]. This association could be related to the insulin resistance observed in most obese subjects. In the present work, the elevated baseline expression of TLR4 we observed in PBMC CD14+ cells from patients with prediabetes correlated with FPG after adjusting for triglycerides (TG) and the homeostatic model assessment of insulin resistance (HOMA-IR), which suggests an independent relationship between hyperglycemia and the expression of TLR.

In studies of inflammation and atherosclerosis, it has been observed that the expression/activation of TLR4 is more prominent than that of TLR2 in mouse mesangial cells, vascular smooth muscle cells and renal tubular epithelial cells [18,38,44]. Our results agree with those observations, since we only found differences in the baseline expression of TLR4 (not TLR2) in PBMC CD14+ cells between subjects with prediabetes and normoglycemic subjects.

Activation of NF-κB is the canonical downstream event of the TLR-ligand interaction [8]. In a previous report, we showed that glycated ferritin induces the expression of TLR2, TLR4 and cytokines in PBMC CD14+ cells through differentiation of the myeloid primary response 88 (MyD88)/NF-κB pathway [20]. Although we did not study the independent signaling of MyD88, studies with glycated proteins have shown that NF-κB is activated through the MyD88-dependent pathway [18,37,38]. Moreover, another study showed that silencing MyD88 almost completely inhibited the activation of NF-κB triggered by AGE-LDL, but that silencing the adapter containing the toll/IL-1 receptor (TIR) domain that induces the TIR-domain-containing adaptor inducing IFN-β (TRIF) had no effect [18].

AGEs interact with both TLR2 and TLR4 to alter intracellular signaling, gene expression and the release of the proinflammatory cytokines IL-6 and IL-8 through the activation of NF-κB [8-9]. In a recent study, diabetic patients exhibited significantly higher serum IL-8 levels than non-diabetic subjects. The increase in IL-8 concentration also correlated with higher IL-6 and TNF-α concentrations and suggested that IL-8 may be a marker of a worse inflammatory and metabolic profile in diabetic patients [45]. The present study also showed a significant positive regulation of IL-6 and IL-8 induced by glycated ferritin, both at baseline and in activated PBMC CD14+ cells, from subjects with prediabetes. This means that if the glycation of ferritin occurs under conditions of subclinical hyperglycemia,

then it could contribute to the increase in proinflammatory cytokines in subjects with prediabetes.

CONCLUSIONS

This study provides information on increased basal expression of TLR4 in PBMC CD14+ cells, which could be part of the low-grade inflammation associated with subclinical hyperglycemia in prediabetes. The results also showed that TLR2 may not be part of the inflammatory process in prediabetes, although its expression increases in PBMC CD14+ cells when they are exposed to glycated ferritin. Glycated ferritin increased the expression of both TLR2 and TLR4 as well as IL-6 and IL-8 in PBMC-derived CD14+ cells from subjects with prediabetes. Therefore, we conclude that in prediabetes, the increased basal expression of TLR4 could be part of the low-grade inflammation, which could be increased by glycated ferritin. Glycated ferritin could also play a role in the inflammatory process in subjects with prediabetes, since it proved to be a strong enough stimulus to increase the basal inflammatory response in PBMC CD14+ cells in those subjects. Further studies would help to corroborate our findings, as the group of subjects that participated as donors of the CD14+ PBMC cells was small. Nonetheless, we believe that the results we present here serve as a basis for future research into the role of high serum ferritin in subjects with prediabetes.

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Author contributions: Both GM-JM and MS-JM contributed equally to the development of the work, helping design and carry out the experimental work. LS-LF, SG-JA and CV-AY were experimental advisors and contributed to the research report; AH-G performed the statistical analysis and reviewed the research report; MP-AL performed the biochemical assessment of patients and reviewed the research report; CP-MC: designed the study, conducted the clinical assessment of the participating subjects and oversaw the conduct of the study.

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