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Clinical studies

Short repeats in the heme oxygenase 1 gene promoter is associated with increased levels of inflammation, ferritin and higher risk of type-2 diabetes mellitus

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ABSTRACT

Objective: We evaluated the relationship between the HO1 genotype, ferritin levels and the risk of type-2 diabetes and inflammation.
Research methods: Eight hundred thirty-five individuals were evaluated and classified according to their nutritional status and the presence of type-2 diabetes: 153 overweight (OW); 62 obese (OB); 55 type-2 diabetes mellitus (DM); 202 OWDM; 239 OBDM and 124 controls (C). We studied biochemical (glycemia, insulin, lipid profile, liver enzyme, creatinine, hsCRP), hematological (hemoglobin, free erythrocyte protoporphyrin, transferrin receptor and serum Fe and ferritin) and oxidative stress (SOD, GHS and TBARS) parameters. We determined heme oxygenase activity and the $(GT)_n$ polymorphism in its gene promoter.
Results: Individuals with diabetes, independent of nutritional status, showed high levels of ferritin and HO activity compared to control subjects. Allelic frequency was not different between the groups (Chi^2, NS) however, genotypes were different (Chi^2, $P<0.001$). The SS (short-short) genotype was higher in all DM individuals compared to controls and MM was higher in controls. SM (short-medium) genotype was an independent risk factor for DM in logistic regression analysis. We observed high risk for type-2 diabetes mellitus in the presence of SM genotype and high levels of ferritin (OR adjusted: 2.7; 1.9–3.6; $p<0.001$; compared to control group). It was also significantly related to inflammation.
Conclusion: The SM genotype in HO1 gene promoter and ferritin levels were associated with higher risk for type-2 diabetes and for having a higher marker of inflammation, which is the main risk factor for the development of chronic diseases.

1. Introduction

Type-2 diabetes mellitus is a metabolic disease characterized by increased levels of inflammation and oxidative stress, both of which could trigger the exacerbation of insulin resistance and the onset of diabetes complications [1]. Oxidative stress is closely related to inflammation, since reactive oxygen species (ROS), produced by high levels of circulating glucose, could activate inflammatory pathways such as NF-κB and JNK [2]. The increase of inflammatory cytokines in circulation induces defects in insulin secretion in the pancreas and liver, and altered muscle glucose uptake [3], but also can produce alterations in the metabolism of some micronutrients, such as iron [4]. These alterations are: decreased iron uptake, internalization of ferroportin with increased iron concentration in the enterocyte, macrophages and liver, which could induce anemia of inflammation [5]. These effects are increased when type-2 diabetes is accompanied with obesity.

Several studies have shown an association between body iron storage and the development of type-2 diabetes [6,7]. The relationship between iron and metabolic diseases is based on the oxidative capacity of iron. In physiological systems, many cytoprotective mechanisms are triggered during tissue insult in an attempt to limit injury. One of these systems is the modulation of the expression of stress proteins like heme oxygenase (HO) during cellular defense [8]. There are two genetically distinct isoform of HO: the inducible HO1 and the constitutively expressed HO2 [9]. HO1 is induced by heme, oxidative stress causing agents, including hyperthermia, oxidized lipoproteins, inflammatory cytokines, hypoxia, heavy metals and nitric oxide [10]. Heme oxygenase enzyme catalyzes the degradation of heme, generating free iron (as ferrous iron), carbon monoxide and biliverdin, which is rapidly converted to bilirubin by biliverdin reductase [11]. Considerable evidence has shown that these products are potentially anti-inflammatory, antioxidant, and anti-proliferative [12,13].

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The promoter region of HO1 is highly polymorphic in terms of the number of GT repeats ($GT_{(n)}$), which vary from 12 to 40 [9]. The number of GT repeats can modulate the gene transcription. Interestingly, higher HO1 expression in endothelial cells is correlated with better cell survival under oxidative stress conditions. These cells carry the S allele, leading to the suggestion that $GT_{(n)}$ determines the responsiveness of the gene [14]. In an animal model with type-2 diabetes (Zucker rats), the up-regulation of the HO1 system was reported to improve glycemic control (i.e., reaching adequate levels of glycemia during fasting (<100 mg/dL) and/or post-prandial (<140 mg/dL) that could improve the pro-inflammatory and pro-thrombotic status), in order to decrease oxidative stress, and to increase oxidative status [15]. However, findings on the effects of $GT_{(n)}$ on susceptibility to type-2 diabetes are conflicting. A Chinese study reported that one or two L alleles (defined as ≥25 GT repeats) was associated with higher odds of having type-2 diabetes [16]. Previously, we found a greater susceptibility to type-2 diabetes with S allele [17]. The L/L genotype has also been associated with higher risk of coronary heart disease and other cardiovascular adverse events [9,18,19]. Data on the relationship of $GT_{(n)}$ repeats and the risk of type-2 diabetes is scarce. Alteration in glycemic control is the principal risk factor for developing type-2 diabetes and its complications, and oxidative stress and inflammation caused by hyperglycemia is believed to underlie changes in the functionality of some organs. Our aim was to determine the allelic frequencies of $GT_{(n)}$ repeats in the HO1 gene promoter in normal weight, overweight and obese subjects and type-2 diabetic patients with or without overweight or obesity, and to assess the associations between HO1 gene promoter polymorphism and the risk of type-2 diabetes.

2. Subjects and methods

2.1. Subjects

We studied 835 persons; 153 overweight (OW: BMI 25–29.9 kg/m^2); 62 obese (OB: BMI > 30 kg/m^2); 55 type-2 diabetes mellitus (DM: BMI < 25 kg/m^2); 202 OWDM; 239 OBDM and 124 normal weight non-diabetic volunteers (C). Subjects had no apparent medical or family history of diabetes, and did not have metabolic syndrome according to Adult Treatment Panel III classification [20]. Subjects that were vegetarians, that intake supplements or vitamins, 2 month before the study, or women with altered menstrual bleeding were excluded. Subjects were between 20 and 84 years old. Diabetic individuals were diagnosed and followed-up by the Diabetes Program in the Nutrition Unit of Juan de Dios Hospital (Santiago, Chile).

Clinical characteristics of study participants are shown in Table 1. A complete review of the medical history of all subjects, including arterial hypertension, body mass index, and waist circumference, was carried out before the beginning of the study. Venous blood samples (25 mL) were collected from all subjects after a 48-h low-fat diet and a 12-h overnight fast. For all non-diabetic subjects, an oral glucose tolerance test (OGTT) was performed (basal and 2 h after glucose ingestion). Subjects ingested 75 g of glucose in water (Trutol®). None of the controls had an altered OGTT. Serum aliquots were stored at −70 °C.

2.2. Ethics

Written informed consent was obtained from all subjects. The study protocol was approved by the ethics committee of the Nutrition Institute and Food Technology, University of Chile.

2.3. Biochemical and hematological characterization

Plasmatic biochemical characterization included basal and post prandial glycemia (Roche Diagnostics Mannheim, Germany) and insulin (radioimmunoassay, Siemens, LA, USA). Glycosylated hemoglobin (HbA1c) was measured with a commercial kit (N° 995025, Química Clínica Aplicada SA, Amposta, Spain). We also measured serum liver aminotransferases activities (GOT: aspartate aminotransferase or AST and GTP: alanine aminotransferase or ALT), total bilirubin, alkaline phosphatase (AP), lipid profile (total, LDL, and HDL cholesterol and triacylglycerol), creatinine and high sensitivity C-reactive protein (hsCRP: as an inflammatory marker. Cutoff: 1 mg/dl, according to The American Heart Association (Cut point for hsCRP determination of risk of cardiovascular disease as: low risk ≤ 1.0 mg/L, intermediate risk 1.0–3.0 mg/L and high risk ≥ 3.0 mg/L) [21].

For hematologic characterization we studied hemoglobin (Cell Dyn 1700 counter; Abbott Laboratories, Abbott Park, IL); free erythrocyte protoporphyrin (FEP: hematofluorimeter, model 206D; AVIV Biomed, Lakewood, NJ); total serum iron (graphite furnace atomic absorption spectrometry, Simaa 6100; Perkin-Elmer, Shelton, CT); transferrin receptors (Ramco Laboratories Inc, Houston, TX); and serum ferritin (Enzyme-linked immunosorbent assay, with an Anti-ferritin (Human Spleen; Rabbit) Antibody for ferritin H subunit; Dako Corp, Carpinteria, CA). Total body iron was calculated from the ratio of transferring receptors to serum ferritin according to the following equation [22,1]: Body iron (mg/kg) = – (log (TfR/SF ratio) – 2.8229)/0.1207.

2.4. Heme oxygenase (HO) determination: mononuclear leukocytes isolation, HO activity and HO gene promoter polymorphism

Mononuclear leukocytes (MNCs) were obtained according to Muñoz et al. [23]. Briefly, blood was diluted in a 1:1 ratio with sterile phosphate-buffered saline (PBS, in mM) (137 NaCl, 2.7 KCl, 8.1 Na_2HPO_4, and 1.5 KH2PO4; pH 7.4), layered onto a Histopaque gradient with a density of 1.119 g/mL (Histopaque 1077; Sigma Diagnostics, St Louis, MO) and centrifuged at 400*g* for 35 min at room temperature. The mononuclear layer (buffy coat) was then removed and washed twice for 10 min in PBS at 180*g*. Immediately afterward, the same wash protocol was performed with RPMI 1640 medium (Invitrogen Life Technology, Carlsbad, CA). Finally, the cells were resuspended in 500 μL PBS.

Genomic DNA was obtained from MNCs using the Chomczinsky reagent and quantified at 260 nm (Gene Quant; Pharmacia Biotech, Cambridge, United Kingdom). HO1 gene promoter amplification was conducted by using polymerase chain reaction in a thermal cycler (model #2720; Applied Bio-Systems, Foster City, CA) using 30 cycles of 20 s at 94 °C, 20 s at 60 °C, and 20 s at 72 °C (Chen et al. [9]; Hirai et al. [32]). The primers used were HO1 s CCCAAAGCTTGCAGCTTCTCAGAT-NED and HO1a GGGAAACGAATTCTGGCCATAG-GAC. The polymerase chain reaction product (3 μL) was loaded into a 2.5% agarose gel to check amplification. Finally, capillary electrophoresis was performed to detect ($GT_{(n)}$) length. $GT_{(n)}$ repetition were classified according the $GT_{(n)}$ length: Short (S) < 27 GT; Medium (M) between 27 and 32 GT and Large (L) > 32 GT.

HO enzymatic activity was measured in MNCs. Briefly; MNCs (1×10^6) were suspended in 1 mL RPMI-1640 (Invitrogen Life Technology) and 100 μL 900 μmol H_2O_2 and incubated in 5% CO_2 for 18 h at 37 °C. Cells were centrifuged for 10 min at 1500*g* at 4 °C, and the resulting pellet was homogenized in 100 μL of non-denaturing lysis buffer (in mM: 20 KH_2PO_4, 135 KCl, and 0.1 EDTA; pH 7.4) and centrifuged for 20 min at 10,000*g* at 4 °C. The supernatant (100 μL) was incubated for 1 h in the dark at 37 °C, with 100 μL of 15 μM hemin

Table 1
Anthropometry and blood pressure parameters of the study population.

	Control	OW	OB	DM	OW DM	OB DM	*P*
M/W	61/63	73/80	27/36	28/27	113/88	130/109	–
Age (years)	40.2 ± 13.7	48.7 ± 13.3[a]	50.7 ± 13.9[a]	58.3 ± 12.7[a]	58.5 ± 11.2[a]	54.7 ± 11.5[a]	**<0.001**
Weight (kg)	62.0 ± 7.9	73.4 ± 8.6[a]	86.4 ± 14.7[a]	61.0 ± 7.7	73.7 ± 8.4[a]	88.9 ± 12.7[a]	**<0.001**
Height (mts)	164 ± 8.9	163 ± 8.8	161 ± 10.0	163 ± 9.3	163 ± 8.7	162 ± 9.8	NS
BMI (kg/mt^2)	22.9 ± 1.7	27.5 ± 1.5[a]	33.3 ± 3.2[a]	22.9 ± 1.8	27.7 ± 1.4[a]	33.7 ± 3.2[a]	**<0.001**
Waist Circ (cms)	81.3 ± 7.4	92.9 ± 7.2[a]	103 ± 8.1[a]	86.9 ± 8.9[a]	96.8 ± 8.3[a]	108 ± 9.7[a]	**<0.001**
SP (mm Hg)	119 ± 13.8	127 ± 16.6[a]	135 ± 18.5[a]	134 ± 16.7[a]	137 ± 18.3[a]	141 ± 20.7[a]	**<0.001**
DP (mm Hg)	74.8 ± 9.1	80.6 ± 11.1[a]	81.7 ± 13.9[a]	78.2 ± 12.3[a]	83.3 ± 11.1[a]	84.8 ± 11.4[a]	**<0.001**

OW: Overweight; OB: obese; DM: type-2 diabetes mellitus; DM OW: diabetic with overweight; DM OB: diabetic with obesity; BMI: body mass index; Waist Circ: waist circumference; SP: systolic pressure; DP: diastolic pressure. Values are expressed as mean ± SD.
[a] One way ANOVA, post-test Dunnettís (against control).

Table 2
Biochemical parameters of the study population.

	Control	OW	OB	DM	OW DM	OB DM	*P*
Basal Glucose (mg/dl)	88.3 ± 8.8	95.3 ± 12.9	95.6 ± 17.0[a]	145 (82.9–252)[a#]	141 (91.0–219)[a#]	128 (90.0–181)[a#]	**<0.001**
PP Glucose (mg/dl)	84.0 ± 23.1	102 ± 33.7[a]	108 ± 28.4[a]	–	–	–	**<0.01**
Basal Insulin (lUI/mL)[#]	3.5 (2.1–5.8)	5.1 (2.5–10.4)[a]	8.9 (4.0–19.9)[a]	6.0 (2.0–18.1)	11.2 (4.3–29.4)[a]	13.3 (6.5–27.6)[a]	**<0.01**
PP Insulin (lUI/mL)[#]	15.2 (4.9–47.4)	26.5 (10.3–68.1)[a]	49.2 (21.9–110)[a]	–	–	–	**<0.01**
Basal Glyc Hb (%)[#]	2.60 (1.71–3.94)	3.14 (2.50–3.94)	4.06 (3.12–5.26)[a]	6.73 (4.78–9.47)[a]	6.77 (4.32–10.41)[a]	7.81 (5.42–11.26)[a]	**<0.001**
GOT (UI/L)[#]	26.0 (14.9–45.5)	31.8 (18.1–55.8)	40.7 (22.0–75.3)[a]	17.9 (8.6–37.3)[a]	19.9 (10.8–36.6)[a]	21.4 (10.6–43.2)	**<0.01**
GPT (UI/L)[#]	13.0 (3.9–42.9)	29.2 (12.8–66.7)[a]	44.6 (21.6–92.2)[a]	19.0 (9.0–39.9)	19.2 (9.0–40.9)	13.1 (4.4–39.2)	**<0.01**
Alk Phos (UI/L)	110 ± 40.0	130 ± 43.3[b]	141 ± 42.8[b]	129 ± 58.0	126 ± 45.0	134 ± 48.0[b]	**<0.01**
Bilirubin (mg/dl)[#]	0.49 (0.26–0.90)	0.48 (0.27–0.85)	0.44 (0.25–0.79)	0.47 (0.25–0.86)	0.42 (0.21–0.86)	0.39 (0.20–0.76)	NS
Creatinine (mg/dl)[#]	0.82 (0.54–1.25)	0.88 (0.64–1.22)	0.86 (0.61–1.20)	1.00 (0.70–1.43)[a]	1.03 (0.62–1.70)[a]	1.03 (0.71–1.48)[a]	**<0.01**
Total Cholesterol (mg/dl)	187 ± 45.3	203 ± 43.5[a]	206 ± 42.7	205 ± 46.7	207 ± 57.4[a]	201 ± 46.1[a]	**<0.05**
HDL Cholesterol (mg/dl)	44.3 ± 12.9	37.4 ± 12.1[b]	35.0 ± 10.9[b]	44.3 ± 10.9	39.8 ± 12.7[b]	40.9 ± 12.6	**<0.01**
LDL Cholesterol (mg/dl)	121 ± 43.2	136 ± 39.6[b]	142 ± 41.5[b]	129 ± 41.7	128 ± 45.4	121 ± 37.6	**<0.01**
Triglycerides (mg/dl)[#]	98.8 (63.7–153)	131 (79.6–215)[a]	135 (89.3–203)[a]	138 (83.4–227)[a]	163 (93.3–286)[a]	176 (99.0–313)[a]	**<0.001**
hsCRP (mg/dl)	0.97 (0.25–3.74)	1.15 (0.30–4.47)	1.61 (0.34–7.65)[a]	1.74 (0.49–6.16)	1.95 (0.51–7.45)[a]	2.54 (0.91–7.03)[a]	**<0.001**

GOT, glutamate oxaloacetic acid transaminase; GPT, glutamate pyruvate transaminase; hsCRP, high-sensitivity C-reactive protein; PP: Post prandial, after 2 h; Glyc Hb: glycosylated hemoglobin (HbA1c). Values are expressed as: mean ± SD or [#]geometric means (range ± 1 SD).
[a] Kruskal-Wallis, post hoc Dunnís.
[b] One way ANOVA, post hoc Dunnettís (against control).

Table 3
Hematological and oxidative stress parameters of the study population.

	Control	OW	OB	DM	OW DM	OB DM	*P*
Hemoglobin (g/dl)	14.5 ± 1.7	14.5 ± 1.4	14.3 ± 1.7	14.3 ± 1.6	14.5 ± 1.7	15.2 ± 1.8[b]	**<0.01**
FEP	63.9 ± 21.1	68.4 ± 20.7[b]	73.4 ± 29.0	63.6 ± 16.0	65.1 ± 19.5	61.6 ± 19.0	**<0.01**
Fe (μd/dl)	110 ± 43.5	100 ± 34.4	96.9 ± 32.0	119 ± 44.1	121 ± 49.6	129 ± 54.8[b]	**<0.01**
Transferrin Sat (%)	31.3 ± 12.3	29.1 ± 10.4	28.8 ± 11.0	36.5 ± 15.3	30.4 ± 10.5	29.9 ± 8.0	NS
Ferritin (μd/l)[#]	34.6 (15.0–79.8)	39.1 (17.1–89.3)	43.5 (18.7–101)	56.7 (30.3–106)[a]	60.4 (35.4–103)[a]	65.5 (38.4–118)[a]	**<0.001**
TfR (μg/ml)[#]	4.5 (2.5–7.9)	3.8 (1.5–9.5)	3.3 (0.8–14.0)	3.7 (2.0–6.9)	4.7 (2.7–8.1)	5.0 (3.2–7.9)	NS
TBI (mg/kg)[#]	5.0 (2.3–11.2)	5.0 (2.3–11.2)	6.1 (2.3–16.0)	6.9 (3.4–14.4)[a]	7.2 (4.8–10.7)[a]	7.4 (5.2–10.5)[a]	**<0.01**
HO[#]	1.56 (0.41–5.94)	2.30 (0.49–10.8)[a]	2.68 (0.41–17.5)[a]	3.15 (0.87–11.3)[a]	2.48 (0.62–9.86)[a]	2.16 (0.59–7.92)[a]	**<0.001**
SOD (pg/mL)	3.7 ± 0.8	3.4 ± 0.4	4.0 ± 0.7	3.7 ± 0.5	3.5 ± 0.6	3.7 ± 0.9	NS
GSH (μmol/L)	53.2 ± 16.7	52.5 ± 8.6	35.9 ± 13.6	46.9 ± 4.9	45.9 ± 14.1	46.6 ± 18.8	NS
Vit E (μmol/L)[#]	0.12 (0.02–0.59)	0.11 (0.02–0.74)	0.17 (0.04–0.67)	0.17 (0.06–0.44)	0.14 (0.02–0.90)	0.19 (0.04–0.92)	NS
TBARS (nmoles/ml)[#]	0.97 (0.36–2.59)	1.04 (0.35–3.11)	1.23 (0.44–3.41)	1.23 (0.60–2.53)	1.79 (0.94–3.40)[a]	1.66 (0.86–3.21)[a]	**<0.01**

FEP: free erythrocyte protoporphyrin (μg red blood cells/dL); Transferrin Sat: transferrin saturation; TfR: transferrin receptor; TBI: total body iron; HO: heme oxygenase (enzymatic activity: nmol bilirubin/mg protein/h). Values are expressed as mean ± SD or [#]geometric means (range ± 1 SD).
[a] Kruskal-Wallis, post hoc Dunnís.
[b] One way ANOVA, post hoc Dunnettís (against control).

(Sigma-Aldrich), 100 μL of 100*g* biliverdin reductase/mL isolated from rat liver, and 600 μL suspension buffer (100 mM KH_2PO_4; pH 7.4). The reaction was started with 100 μL of 1 mM NADPH (Sigma-Aldrich). We extracted bilirubin with 1 mL chloroform by stirring it for 1 h and then centrifuging it for 5 min at 200*g* and 4 °C. Bilirubin concentrations were measured at 530 nm (Spectrophotometer model UV-1601; Shimadzu, Tokyo, Japan). HO activity was expressed as nmol bilirubin/mg protein/h (bilirubin molar extinction coefficient: $\varepsilon = 43.5\ \text{mmol/L}^{-1} \times \text{cm}^{-1}$) [24,25].

2.5. SOD, GSH and TBARS determination

GSH and Zn/Cu-SOD activity were determined in erythrocytes using an ELISA kit (Cayman Chemical Com, Michigan, USA) and Bioxytech SOD-525 assay kit (OXIS International Inc., Portland OR), respectively. Thiobarbituric acid reactive substances (TBARS) were measured according to Jo and Ahn [26]. Briefly, 0.5 mL of each serum was incubated with both 1 mL 0.67% thiobarbituric acid and 0.3 mL 50% trichloroacetic acid for 30 min at 90 °C and centrifuged at 1620*g* for 15 min. The supernatant was used to measure TBARS at 530 nm in a Metertech SP830 spectrophotometer (Metertech, Inc. Taiwan).

Table 4
Allele frequencies at the polymorphic locus (n/%).

Allele	Control	OW	OB	DM	OW DM	OB DM
S	81 (33)	118 (39)	55 (44)	48 (44)	174 (43)	205 (43)
M	129 (52)	159 (52)	58 (47)	49 (45)	168 (42)	188 (39)
L	38 (15)	29 (9)	11 (9)	13 (12)	62 (15)	85 (18)

S: short; M: medium; L: large. Chi-square test: all $p > 0.05$.

Table 5
Distribution of Genotypes (n/%).

Genotype*	Control	OW	OB	DM	OW DM	OB DM
SS	12 (10)	15 (10)	11 (18)	3 (5)	16 (8)	16 (7)
SM	45 (36)	81 (53)	30 (48)	36 (65)	129 (64)	152 (64)
SL	12 (10)	7 (5)	3 (5)	6 (11)	13 (6)	21 (9)
MM	38 (31)	35 (23)	12 (19)	6 (11)	11 (5)	7 (3)
ML	8 (6)	8 (5)	4 (6)	1 (2)	17 (8)	22 (9)
LL	9 (7)	7 (5)	2 (3)	3 (5)	16 (8)	21 (9)

*Chi-square test: $P < 0.001$.

2.6. Statistics

Values were expressed as mean ± SD. Variables with a skewed distribution were transformed to recover the original units and were expressed as geometric means ± SDs. To determine differences between the control group and the others groups we use a One way ANOVA (post hoc: Dunnettís test) for results with a normal distribution or Kruskal-Wallis (post hoc: Dunnís test) for results not normally distributed. Logistic regression techniques (stepwise, forward estimation) were used to assess gene-disease associations after adjustment for confounding variables (STATA software; version 15; Stata Corp LP, College Station, TX). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Anthropometric parameters in the study population

Using BMI, 153 subjects were classified overweight; 62 obese; 55 DM; 202 overweight DM; 239 obese DM and 124 controls without any of the ATP III criterion. In the C, OW, OB groups (61/124; 49.2%); (73/153; 47.7%) and (27/63; 42.9%) were males, respectively; and in DM; OWDM and OBDM groups (28/55; 50.9%); (113/201; 56.2%) and (130/239; 54.4%) were males, respectively (Table 1). The age range in the studied group was between 19.7 and 84.2 years old and 18.0 and 73.9 years old in controls. There were no age differences between men and women compared by group, however age (years) was different in all groups (OW: 48.7 ± 13.3; OB: 50.7 ± 13.9; DM: 58.3 ± 12.7; OW DM: 58.5 ± 11.2 and OB DM: 54.7 ± 11.5) compared to controls (40.2 ± 13.7) (One way ANOVA, $P < 0.001$, Table 1). Weight and BMI was different in all groups against controls (One way ANOVA, $P < 0.001$) except for DM group. Waist circumference and systolic and diastolic blood pressure were also different and higher in all groups compared to controls (One way ANOVA, $P < 0.001$).

3.2. Biochemical and iron status parameters

Basal glycemia and glycosylated hemoglobin were high in all DM groups and obese individuals (Table 2: One way ANOVA, $P < 0.001$). Basal insulin was higher in all the groups except in DM group compared to control subjects. Postprandial glucose and insulin were higher in both OW and DOB groups (One way ANOVA, $P < 0.01$). Liver enzyme activities (i.e., GOT, GPT, and AP) and creatinine were above normal levels in some of the groups (Table 2). Lipid profile was also altered, mainly in OW and OB groups. hsCRP was higher in OB; OWDM and OBDM, compared with controls (One way ANOVA, $P < 0.001$). Among controls, 25.7% had hsCRP values higher than 3 mg/L compared to 21.9% (OE); 37.8 (OB); 39.6% (DM); 29.5 (OWDM) and 50.7% (OBDM), respectively.

DM; OWDM and OBDM showed the highest levels of iron stores measured by serum ferritin and total body iron (Table 3: ANOVA, $P < 0.01$). Only OBDM showed significantly different levels of Fe compared to controls. HO enzymatic activity was elevated in DM; OWDM and OBDM (ANOVA, $P < 0.001$). TBARS showed differences only in OWDM and OBDM groups (ANOVA, $P < 0.01$).

Table 6
Logistic regression model of the association between the SM genotype and diabetes.

	Adjusted OR	95% CI	p
SM and hsCRP	1.5	1.1–2.1	**<0.008**
SM, age and sex	2.1	1.5–2.9	**<0.001**
SM, BMI and waist circumference	2.2	1.5–2.8	**<0.001**
SM and ferritin	2.7	1.9–3.6	**<0.001**
Final model (including all variables)	1.9	1.3–2.8	**<0.001**

3.3. Microsatellite polymorphism in promoter of heme oxygenase-1 gene

As we previously reported [17], the distribution of allelic frequency of micro-polymorphism in the gene promoter of hem oxygenase, did not differ significantly between controls and the other groups (Table 4: Chi^2, NS). However, genotypes differed significantly between groups (Table 5: Chi^2, $P < 0.001$). The SS genotype was significantly higher in DM groups compared to controls. On the other hand, MM genotype was lower in DM groups.

The SM genotype was independently associated with diabetes (OR = 1.9; 95% CI: 1.5–2.6) and was related to odds ratios for type-2 diabetes, in a model controlling for hsCRP, age, sex, BMI, waist circumference and serum ferritin. The OR was higher when the model was adjusted by serum ferritin only (Table 6: Logistic regression, $p < 0.001$). Using the MM genotype as a protector genotype, the analysis for the presence of inflammation by logistic regression showed that genotype SM had an increased risk for inflammatory profile (Table 7: Logistic regression, $p < 0.0001$) in both an unadjusted model (OR: 2.6 IC: 1.69–4.11) and one adjusted for age, BMI, abdominal circumference and serum ferritin (OR: 2.5, IC: 1.56–3.93).

Table 7
Risk of inflammation according to genotype.

Genotype	Unadjusted OR	95% CI	p	Adjusted OR[a]	95% CI	p
MM	1.0			1.0		
SL	1.4	0.75–2.92	0.258	1.4	0.70–2.94	0.322
LM	0.9	0.50–1.79	0.867	0.8	0.40–1.57	0.517
SM	2.6	1.69–4.11	**<0.0001**	2.5	1.56–3.93	**<0.0001**
SS	1.5	0.81–2.86	0.194	1.4	0.73–2.73	0.301
LL	1.1	0.60–2.18	0.680	1.2	0.61–2.47	0.574

[a]OR adjusted by age, sex, BMI, waist circumference, and serum ferritin.

4. Discussion

Development of type-2 diabetes results in the alteration of a series of metabolic processes such as, response to oxidative stress, increased inflammation, and gluco-lipotoxicity events. It has been previously described that the activity or stress responsiveness of heme oxygenase could be altered depending of the number of GT repeats in the highly polymorphic promoter region of the heme oxygenase gene. There are several reports indicating increased risk of various diseases, including type-2 diabetes, renal disease, and coronary heart disease [9,27,28]. In this study, we explored the relationship between GT repeats in the promoter of the HO1 gene and risk of type 2 diabetes among subjects of different nutritional statuses. We also investigated inflammation risk associated with the HO1 genotype, as this is the most frequent condition among patients with high risk for developing type-2 diabetes.

HO1 is a gene located on chromosome 22q13.1, and its expression is induced by heme iron. HO1 enzymatic activity is essential for heme degradation in a reaction that produces biliverdin, carbon monoxide and iron [29]. Chronic diseases are strongly associated with oxidative stress and inflammation. Also, HO1 is up-regulated in response to cellular stress, and participates in the degradation of ROS levels, which are necessary to decrease inflammation and apoptosis [14,30]. Indeed, we found the highest HO1 activity in those subjects that had obesity and/or type-2 diabetes, indicating that these groups are under major stress since they also had higher levels of plasmatic glucose, iron and TBARS. Additionally, in these groups, we found the highest frequency of the SM genotype, especially in those subjects with diabetes; however individuals with type-2 diabetes without overweight or obesity had a low frequency, showing that the SM genotype and the dysregulations that occur in obesity may have a synergistic effect in the development of type-2 diabetes.

Data related to the HO1 promoter gene polymorphism and its relationship with type-2 diabetes are conflicting. In addition, the association of L alleles with a higher risk for developing of type-2 diabetes [16], the L alleles have been also associated with coronary artery disease [31]. However, Choi et al. [27], showed that the S allele did not have any beneficial effect over glycemic control, oxidative stress, or outcome in type- 2 diabetes. Previously, we showed that the S allele has the greatest susceptibility for type-2 diabetes [17]. *In vitro* studies also show conflicting findings. Taha et al. [14] showed that the presence of the S allele in endothelial cells was associated with lower production of proinflammatory mediators and with cytoprotective functions. Hirai et al. [32] found that lymphoblastic cell lines with S/S genotype were more resistant to oxidant-induced apoptosis than those with LL.

In concordance with our previous findings, we found that the SM genotype had the greatest risk for type-2 diabetes in a model with or without confounding variables. However, when the model was only adjusted by serum ferritin levels, the risk was higher, suggesting that the risk is dependent on iron stores. In fact, the subjects with the highest body iron had the worst glucose management. Risk was increased when models were adjusted by BMI and waist circumference, suggesting that type-2 diabetes together with SM genotype are dependent on subject adiposity.

Because inflammation is a common condition in chronic diseases, we also investigated the relationship between genotype and inflammation. Subjects with SM genotype, compared with MM genotype, had a higher risk for developing inflammation in a model with or without confounding variables. Increased adiposity, especially central body fat accumulation, is related to type-2 diabetes, because adipose tissue produces inflammatory mediators that could induce insulin resistance. Increased levels of circulating glucose and fatty acids also induce oxidative stress and inflammation in obese subjects [33]. The inflammatory state observed during obesity may trigger several conditions, such as cardiovascular disease, kidney disease and liver dysfunction [1]. ROS, glycated products and species from lipoperoxidation activate inflammatory pathways inducing the pro-inflammatory cytokines release; both cytokines and inflammatory mediators could cause insulin resistance [33]. Finally, this inflammatory state induces apoptosis in beta cells, decreasing insulin secretion from the pancreas and causes decreased insulin receptor activity and receptor ubiquitination into the proteasome, producing insulin resistance and glucose intolerance in muscle and adipose tissue. Furthermore, inflammation provokes nonalcoholic fatty liver disease, which induces hepatic insulin resistance [34]. Our results suggest that the SM genotype not only represents increased risk for type-2 diabetes, but also is one of the risk factor in order to develop the disease which is causing inflammation.

5. Conclusions

Our data demonstrated that the S allele alone or together with ferritin levels is a risk, not only for type-2 diabetes, but also for inflammation, at least in this particular population.

Conflict of interest

The authors declare no conflicts of interest.

Author contributions

Conceived and designed the experiments: MA; EL; MAO. Performed the experiments: MA; MAO. Analyzed the data: MA; EL; MAO. Contributed to the writing of the manuscript: MA; EL; MAO.

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