Biometals (2012) 25:725–735
DOI 10.1007/s10534-012-9540-z

Type 2 diabetic patients and their offspring show altered parameters of iron status, oxidative stress and genes related to mitochondrial activity

Solange Le Blanc · Pia Villarroel · Valeria Candia · Natalia Gavilán · Néstor Soto · Francisco Pérez-Bravo · Miguel Arredondo

Received: 14 December 2011 / Accepted: 13 March 2012 / Published online: 27 March 2012

Abstract Type 2 diabetes (T2D) is directly related to alterations in iron status, oxidative stress and decreased mitochondrial activity, but the possible interaction of these parameters among T2D patients and their offspring is unclear. The whole study included 301 subjects: 77 T2D patients and one of their offspring and 51 control subjects with one of their offspring. The offspring were older than 20 years old. We measured parameters of iron status (serum iron, ferritin and transferrin receptor), diabetes (pre and post-prandial glucose, insulin, lipids), oxidative stress (Heme oxygenase activity, TBARS, SOD, GSH, Vitamin E), as well as the expression of genes in blood leukocytes related to mitochondrial apopotosis (mitofusin and Bcl/Bax ratios). The offspring of T2D patients had increased levels of serum ferritin ($P < 0.01$) and lower transferrin receptor ($P < 0.008$); higher insulin ($P < 0.03$) and total and LDL cholesterol; higher heme oxygenase and SOD activities increased TBARS and lower GSH; decreased mitofusin and Bcl/Bax expression ratios compared to offspring of normal subjects. These results suggest that the offspring of T2D patients could have an increased metabolic risk of develop a cardiovascular disease mediated by oxidative stress and iron status.

Keywords Iron · Oxidative stress · Bcl2/Bax ratio · Mitofusin · Diabetes mellitus

Pia Villarroel, Valeria Candia participated equally in the investigation.

S. Le Blanc · P. Villarroel · V. Candia · M. Arredondo (✉)
Laboratorio de Micronutrientes, Instituto de Nutrición y Tecnología de los Alimentos (INTA), Universidad de Chile, El Líbano 5524, Macul, Santiago, Chile
e-mail: marredon@inta.uchile.cl

N. Gavilán
Consultorio Eduardo Frei Montalva, La Cisterna, Santiago, Chile

N. Soto
Unidad de Endocrinología y Diabetes, Hospital San Borja Arriarán, SSMC, Santiago, Chile

F. Pérez-Bravo
Departamento de Nutrición, Facultad de Medicina, Universidad de Chile, Santiago, Chile

Introduction

Overweight and obesity in youth is a worldwide public health problem. Obesity and body fat distribution are related to insulin resistance and could be the common pathophysiological link to metabolic syndrome (MetS) and type 2 diabetes (T2D) (Halpern et al. 2010). Also, children from families with T2D members have an increased risk of obesity; this risk increase fivefold when both parents have diabetes (Praveen et al. 2010). T2D and its principal cause/symptom, insulin resistance, are widely considered the core pathological trait defining the MetS because insulin resistance that increases both glycemia and

insulinaemia, favoring the deposition of fat, increasing the circulating lipids and induces inflammation (Alemany 2011).

Actual evidence suggests that iron overload elevated circulating ferritin concentrations and this was associated with higher risk of T2D and MetS, through metabolic disorders that affect glucose and insulin metabolism (Fernandez-Real et al. 2002). In animal models, iron excess might result in β-cell oxidative stress and decreased insulin secretory capacity (Sun et al. 2008). Indeed, it had been shown that increased iron stores contribute to insulin resistance and hyperinsulinemia by reducing hepatic insulin metabolism (Niederau et al. 1984) and by decreasing glucose uptake in muscle (Merkel et al. 1988).

The association of ferritin levels with metabolic derangements and complications in diabetes is partly dependent on association with inflammation. Iron status, estimated with the soluble transferrin receptor/ferritin index, is associated with metabolic disorders and its complications but the associations are dependent on other risk factors (Mojiminiyi et al. 2008) Also, oxidative stress and inflammatory processes have a causal role in the aetiology of T2D and MetS (Hoogeveen et al. 2007). High levels of inflammation increase the risk of developing atherosclerosis and participate as mechanism for the adverse consequences of the MetS (Dandona et al. 2005). Iron is an essential trace element that participates actively as structural or functional component of physiological systems with large reactive properties, that interacts with oxygen radicals, inducing oxidative stress (OS) (Sumida et al. 2009). Also, OS participate actively in the development of cardiovascular disease and in the diabetic complications (Bonomini et al. 2008) and it is generally defined as an excess of formation and/or insufficient removal of highly reactive molecules such as reactive oxygen species and reactive nitrogen species (Zhai et al. 2011; Johansen et al. 2005). Studies have consistently shown that both obesity and MetS are associated with increased OS (Steinberg et al. 1989; Keaney et al. 2003)

Recently, T2D was associated with a decrease in the mitochondrial activity. Mitofusin 2 (Mfn2) is a dynamin-related GTPase protein that participates in the maintenance of mitochondrial metabolism and regulation of mitochondrial fusion/fission process (Chang et al. 2010). Also, Mfn2 participate in the regulation of muscle metabolism and provide a molecular mechanism that explains the alterations in mitochondrial function in obesity or T2D. T2D patients show a reduced Mfn2 expression in skeletal muscle that is directly proportional to insulin sensitivity (Bach et al. 2005). Inhibition of Mfn2 expression in cultured cells reduces oxygen consumption, mitochondrial membrane potential and glucose oxidation; these results suggest a role of Mfn2 in both mitochondrial function and pathophysiology of obesity and/or T2D. In addition, Mfn2 was recently described as an anti-proliferative protein involved in vascular disorders (Chen et al. 2004). During the apoptotic process, Mfn2 induces the release of cytochrome C and other mitochondrial proteins that activate apoptotic pathway through caspases activation (Wang 2001).

Bcl2 and Bax are members of the Bcl2 family, and participate as an anti-apoptotic and pro-apoptotic proteins, respectively (Wei et al. 2001). Bcl-2 family proteins are important regulators of mitochondrial morphogenesis machinery (Cleland et al. 2011) and programmed cell death (Khera et al. 2006). This family includes the pro-apoptotic protein Bax, which is essential in the mitochondrial pathway of apoptosis (Wei et al. 2001). It have been reported that a reduced Bcl2/Bax ratio is an indication of apoptosis susceptibility (Kalra et al. 2008; Metukuri et al. 2010; Jin et al. 2011). Bax dimerization in the mitochondrial membrane, induce the leakage of cytochrome C, which is essential in the mitochondrial pathway to apoptosis. By the other hand, the anchor of Bcl2 to the mitochondrial membrane prevents Bax dimerization. Therefore Mfn2, Bcl2 and Bax are closely linked to the regulation of muscle physiology, insulin sensitivity and apoptotic processes in the mitochondria of T2D subjects.

We hypothesized that offspring of diabetic subjects have increased iron stores and oxidative stress similar to their parents. The aim of the present study was to investigate if there are differences in iron status and oxidative stress parameters among the adult offspring of subjects with T2D and normal controls.

Subjects and methods

Subjects and study design

The whole study included 301 subjects: 71 families with one or both parents with T2D (38 male/39

female) with not requirement for insulin, with at least 2 years of diabetes evolution, as diagnosed and controlled by the Diabetic Program, Nutrition Unit, San Juan de Dios Hospital, and one of the offspring (35 male/36 female) older than 20 years of age. Also, we studied 51 families non-diabetic (51 male/51 female) and one of the offspring (19 male/32 female), with no apparent medical or family history of diabetes and without metabolic syndrome according to ATP III classification (NIH 2005). All of them were selected as a convenience sample, choice of the sample by random methods whose characteristics are similar to those of the target population. The study protocol was approved by the ethics committee of INTA, Universidad de Chile and Nutrition Unit of San Juan de Dios Hospital and all the individuals signed an informed consent before the study began. A complete overall revision of the medical history of all subjects, including arterial hypertension, body mass index and waist circunference was also carried out prior to the beginning of the study.

Venous blood samples (30 mL) were collected from all subjects following a 48 h low fat diet (diet that consists of little fat, especially saturated fat and cholesterol, which are thought to lead to increased blood cholesterol levels and heart attack) and a 12 h overnight fast. Plasmatic biochemical characterization included basal glycemia and insulinemia, lipid profile (total cholesterol, LDL cholesterol, HDL cholesterol, and triglyceride) and high sensitivity C-reactive protein (hsCRP). For all non diabetic persons, a oral glucose tolerance test (OGTT) was performed (two point curve was used: basal and after 2 h glucose ingestion). The subject ingested 75 g of glucose (Trutol®). None of the control had a altered OGTT. All of these parameters were measured enzimatically (DiaLab, Wiener Neudorf, Austria). hsCRP (Química Clínica Aplicada SA, Amposta, Spain) was assessed to discard chronic inflammatory events and a cutoff value over 3 mg/L was used as an upper limit. Hematological parameters included hemoglobin (Coulter counter, Cell Dyn 1700), total serum iron (Atomic absorption spectrometry with graphite furnace, Simaa 6100, Perkin Elmer), free erythrocyte protoporphyrin (FEP) (Hematofluorimeter, Aviv Model 206D); transferrin receptor (TfR; Ramco Laboratories, Texas, USA)) and serum ferritin (SF) by Elisa.

Because our hypothesis was that the offspring of diabetic patients will have elevated iron store and oxidative stress parameters, we evaluated the presence of iron overload and also the presence of anemia and iron deficiency. The following cut-offs were used: 120 and 135 g/L as the lower-normal limit for hemoglobin (Hb) in women and men, respectively (World Healths Organization 2007); 1.244 μmol/L as an upper-normal limit for free erythrocyte protoporphyrin in red blood cells and 12.5 μmol/L as a lower-normal limit for serum iron. Depleted iron stores were defined as serum ferritin concentrations below 33 pmol/L (15 μg/L). Iron deficiency without anemia was defined as having normal Hb and two other abnormal laboratory results (as serum Fe or ferritin, for instance) and iron deficiency anemia was defined as below-normal Hb and two other abnormal laboratory results (World Healths Organization 2007). Iron overload was defined as having a serum ferritin level greater than 449.4 pmol/L for women and greater than 674.1 pmol/L for men. The "cut off" for TfR (to define iron deficiency) was >8.3 mg/L (according to test kit reference value). Total body iron was calculated from the TfR/SF ratio as follows: Body iron (mg/kg) = $-(\log(\text{TfR}[\mu g/mL \times 1{,}000]/\text{SF}[\mu g/L]) - 2.8229)/0.1207$ (Cook et al. 2003).

Isolation of mononuclear leucocytes (MNCs)

MNCs were obtained on a Histopaque gradient following the protocol described by Muñoz et al. (2005). Briefly, blood was diluted in a 1:1 ratio with sterile phosphate buffered saline (PBS) (in mmol/L: 137 NaCl; 2.7 KCl; 8.1 Na_2HPO_4; 1.5 KH_2PO_4; pH 7.4), layered onto Histopaque (Histopaque 1077, density: 1.119, Sigma Diagnostic, St. Louis, MO) and centrifuged at $400\times g$ for 35 min at room temperature. The mononuclear layer (buffy coat) was then removed and washed twice in PBS at $180\times g$ for 10 min each. Immediately after, the same wash protocol was performed with RPMI 1640 medium (Invitrogen Life Technology, Carlsbad, CA). Finally, the cells were re-suspended in 500 μL of PBS.

Challenge of mononuclear leucocyte with Fe/glucose

Isolated MNCs were incubated with basal media (RPMI 1640; without glucose, Invitrogen, Cat. No. 72400-120) or with basal media supplemented with Fe 50 μmol/L ($FeCl_3$ as Fe:NTA 1:2.2, NTA was used to keep iron in soluble form) and glucose (20 mmol/L)

by 22 h. These concentrations of iron or glucose reflect excess or overload condition. Under theses condition the pH of media was maintained around 6.5 and not change in the osmolarity was observed. Then, total RNA was isolated using TRIzol according to manufacturer's instructions (Invitrogen, Cat. No. 15596-026). RNA was digested with DNase TURBO DNA-free (Ambion, Cat. No. AM1907). RNA purity was determined using 260/280 ratio and quantified using Quant-iT^{TM} RiboGreen$^{®}$ RNA Reagent (Invitrogen, Cat. No. R11491).

Quantitative RT-PCR

300 ng of total RNA were used for reverse transcription (AffinityScript qPCR cDNA Synthesis Kit, #600559, Stratagene). 1:3 diluted cDNA (2 μL) was used for real time PCR reaction (Brilliant II Sybr$^{®}$ Green qPCR Master Mix, #600828, Stratagene) in a LightCycler system. We used the following primers: ***bax***: cgagtggcagctgacatgtttt and tgaggcaggtgaatcgcttgaa; ***bcl2***: cagttgggcaacagagaaccat and agcccttg tccccaatttggaa; ***mfn2***: aactgtctgggacctttgctca and tttc tggcatcccctgtgcttt; ***ho1***: ttcaccttccccaacattgcca and agctcctgcaactcctcaaag and ***β-actin***: tggcacccagcac aatgaaga and gaagcatttgcggtggacgat. Cycles were: 10 s to 95 °C, 10 s to 60–65 °C and 15 s to 72 °C. The results of each qPCR were analyzed with LightCycler3 Analysis Software version 3.5 (Roche). The relative abundances were normalized with actin expression.

Heme oxygenase activity

Mononuclear leucocytes (1×10^6) were suspended in 1 mL RPMI-1640 (Sigma-Aldrich, St. Louis, MO) and 100 μL of 900 μM H_2O_2 and incubated in 5 % CO_2 for 18 h at 37 °C. Cells were centrifuged for 10 min at $1{,}500 \times g$. The pellet was homogenized in 100 μL of non-denaturing lysis buffer (in mmol/L: 20 KH_2PO_4; 135 KCl; 0.1 EDTA; pH 7.4) and centrifuged for 20 min at $10{,}000 \times g$. 100 μL of the supernatant was incubated for 1 h at 37 °C in dark with 100 μL of 15 μmol/L hemin (Sigma–Aldrich, St. Louis, MO), 100 μL of 100 μg/mL biliverdin reductase isolated from rat liver (see below) and 600 μL of re-suspension buffer (100 mmol/L KH_2PO_4; pH 7,4). The reaction was started with 100 μL of 1 mmol/L NADPH (Sigma-Aldrich, St. Louis, MO). Bilirubin was extracted with 1 mL of chloroform by first stirring for 1 h and then centrifuging for 5 min at $200 \times g$. Bilirubin concentrations were measured at 530 nm (Shimadzu, model UV-1601). HO activity was expressed as nmole of bilirubin/mg protein/h (bilirubin molar extinction coefficient: $\varepsilon = 43.5\ \mathrm{mM}^{-1} \times \mathrm{cm}^{-1}$) (Arredondo et al. 2007).

Statistics

To compare the T2D and control groups we used a *t*-test. A one-way ANOVA was used to evaluate the relative gene expression and their treatment. A 2-factor repeated-measures analysis of variance was used to evaluate the effect of group (sex and diagnosis) and possible interactions between groups regarding either iron status or oxidative stress parameters. Logistic regression analysis (stepwise, forward estimation) was used to assess Fe or oxidative stress associations after adjustment for confounding variables, such as sex, age, diagnosis and family history of type-2 diabetes (STATA 11.0 software; Statacorp LP, College Station, TX). Values with a normal distribution were expressed as a means ± SDs. Because serum ferritin and HO activity had a skewed distribution, their results were transformed into logarithms and were expressed as geometric means and range (±SDs). Differences were considered significant at $P < 0.05$ (Statistica for Windows, Release 5.1, ′97 Edition, Tulsa, USA).

Results

We studied biochemical, hematological, oxidative stress parameters and gene expression associated to apoptosis and mitochondrial function in the offspring of T2D patients and control subjects. Clinical characteristics of diabetic and control parents are summarized in Table 1. As expected, diabetic parents had more conventional risk factors for cardiovascular events compared to controls. Both groups did not differ significantly in either age, weight or height. However, we found high levels and significantly different in body mass index (BMI, *T* test, $P < 0.007$), waist circumference (*T* test, $P < 0.001$) and systolic pressure (*T* test, $P < 0.001$) between diabetic and control parents, whether if analyzed as a total or by sex. Liver enzymes activities (GOT, GPT, AP), basal glycemia and insulinemia, hsCRP, cholesterol, LDL and triglycerides were

Table 1 Physical and biochemical characteristics of diabetic parents and control parents

	Controls	Diabetics	P^c
N (Female/Male)	51/51	39/38	
Age (years)	55.6 ± 7.6[a]	59.1 ± 8.9	NS
Weight (kg)	71.6 ± 10.7[a]	74.7 ± 14.4[b]	NS
Height (cm)	162.1 ± 8.1[a]	161.2 ± 1.0[b]	NS
Body mass index (kg/mt^2)	27.2 ± 3.4	28.9 ± 5.3	<0.007
Waist circumference (cm)	92.4 ± 9.3[a]	98.4 ± 14.1[b]	<0.001
Systolic pressure (mm Hg)	128.1 ± 18.6[a]	138.5 ± 20.7	<0.001
Diastolic pressure (mm Hg)	83.3 ± 14.2[a]	81.3 ± 13.5[b]	NS
GOT (UI/L)	46.0 ± 22.3	54.4 ± 36.4	<0.03
GPT (UI/L)	44.3 ± 20.2	58.3 ± 35.1	<0.001
Alkaline phosphatase (UI/L)	135.1 ± 41.3	165.0 ± 53.3	<0.001
Creatinine (μmol/L)	0.88 ± 0.32[a]	0.96 ± 0.40	NS
Total bilirubin (μmol/L)	0.56 ± 0.47[a]	0.56 ± 0.43	NS
HS-CRP (mg/L)	2.05 ± 2.69	3.70 ± 4.50	<0.001
Basal glycemia (mmol/L)	5.37 ± 0.63[a]	9.19 ± 3.80	<0.001
PP glycemia (mmol/L)	5.58 ± 1.37	–	–
Basal insulin (μUI/mL)	4.67 ± 2.99	25.8 ± 33.61	<0.001
PP insulin (μUI/mL)	38.3 ± 32.7	–	–
Total cholesterol (mmol/L)	206.8 ± 44.7[a]	192.7 ± 45.9	<0.02
HDL cholesterol (mmol/L)	33.1 ± 9.9[a]	31.5 ± 9.1[b]	NS
LDL cholesterol (mmol/L)	143.0 ± 43.4[a]	121.6 ± 36.8	<0.001
Triglycerides (mmol/L)	158.6 ± 86.5[a]	186.7 ± 94.7	<0.02

Data are expressed as means ± SD

HS-CRP high sensitivity C-reactive protein; *PP* Post prandial, after 2 h

[a] Statistical differences ($P < 0.05$) between women and men in control group

[b] Statistical differences ($P < 0.05$) between women and men in diabetic group

[c] *t* test controls versus diabetic individuals

also higher and significantly different in diabetics parents compared to control.

Offspring of diabetic also showed higher values and differences in weight ($P < 0.003$), BMI ($P < 0.001$) and systolic blood pressure ($P < 0.001$) than controls offspring. Total bilirubin ($P < 0.004$), basal ($P < 0.03$) and post prandial ($P < 0.02$) insulinemia and total ($P < 0.004$) and LDL cholesterol ($P < 0.02$) were increased in offspring of diabetics (Table 2). Also, in offspring of diabetics we observed a positive correlation between serum ferritin and basal insulin levels ($r = 0.334$, $P < 0.01$) and a negative correlation after OGTT in controls offspring ($r = -0.386$, $P < 0.005$), suggesting that despite of ferritin values, they have a good metabolic control of the insulin levels after OGTT, measured as $HOMA_{IR}$ (Acosta et al. 2002).

Diabetic parents presented a lower hemoglobin ($P < 0.02$) and transferrin receptor ($P < 0.001$), but higher serum ferritin ($P < 0.007$), and total body iron ($P < 0.001$) than control parents. There were just 4/75 individuals with depletes iron store and only 1/75 with iron deficiency anemia in diabetic parents group. Also, 11/75 presented ferritin levels over 224.7 pmol/L and no one over 449.4 pmol/L. In control parent there were 8/102 individuals with depletes iron store and only 2/102 with iron deficiency anemia. Only 2/102 individuals had ferritin levels over 224.7 pmol/L (Table 3). In diabetic parents, only HO enzymatic activity and TBARS as an oxidative stress parameters, were higher and different from control parents ($P < 0.001$) (Table 3).

Offspring of diabetic parents showed similar behavior in relation to iron nutrition status (Table 4). There were 14/70 individuals with depletes iron store and only 1/70 with iron deficiency anemia in the offspring of diabetic group and 9/51 individuals with depleted iron store and only 2/51 with iron deficiency anemia in the offspring of control group. We found higher levels of serun ferritin ($P < 0.01$) and total body iron ($P < 0.001$) and low transferrin receptor ($P < 0.008$) than controls, indicating high levels of iron storages. Also, we observed a positive correlation only between serum ferritin and basal insulin levels levels ($R = 0.334$; $P < 0.01$). Furthermore, offspring of diabetic parents showed high levels of TBARS ($P < 0.01$), and HO ($P < 0.001$) and SOD ($P < 0.002$) activity, and lower GSH ($P < 0.002$).

Table 2 Physical and biochemical characteristics of the offspring of diabetic patients and control individuals

	Controls	Diabetics	P^c
N (Female/Male)	32/19	36/35	
Age (years)	29.9 ± 5.1[a]	33.0 ± 5.5	NS
Weight (kg)	68.8 ± 12.5[a]	76.6 ± 17.2[b]	<0.003
Height (cm)	164.5 ± 9.4[a]	164.6 ± 9.6[b]	NS
Body mass index (kg/mt^2)	25.3 ± 3.4	28.2 ± 5.1	<0.001
Waist circumference (cm)	85.3 ± 9.5[a]	103.8 ± 96.6	NS
Systolic pressure (mm Hg)	113.6 ± 11.1	120.6 ± 14.0	<0.002
Diastolic pressure (mm Hg)	75.3 ± 8.4	74.7 ± 9.8[b]	NS
GOT (UI/L)	41.1 ± 27.5	48.0 ± 31.4	NS
GPT (UI/L)	43.5 ± 53.1	62.9 ± 79.1	NS
Alkaline phosphatase (UI/L)	125.2 ± 34.1[a]	136.3 ± 40.1[b]	NS
Creatinine (μmol/L)	0.84 ± 0.22	0.90 ± 0.61[b]	NS
Total bilirubin (μmol/L)	0.55 ± 0.47	0.61 ± 0.60	<0.004
HS-CRP (mg/L)	2.50 ± 3.82	3.01 ± 4.26	NS
Basal glycemia (mmol/L)	4.99 ± 0.61[a]	4.98 ± 0.83	NS
PP glycemia (mmol/L)	5.03 ± 1.19	5.47 ± 2.05	NS
Basal insulin (μUI/mL)	5.22 ± 3.96	13.51 ± 31.52	<0.03
PP insulin (μUI/mL)	32.8 ± 26.8[a]	48.4 ± 49.8	<0.02
Total cholesterol (mmol/L)	171.6 ± 31.4	190.8 ± 43.8[b]	<0.004
HDL cholesterol (mmol/L)	34.3 ± 10.2[a]	34.5 ± 10.7[b]	NS
LDL cholesterol (mmol/L)	111.8 ± 31.6	124.8 ± 36.9	0.02
Triglycerides (mmol/L)	131.8 ± 84.0[a]	152.7 ± 78.6[b]	NS

Data are expressed as means ± SD

HS-CRP high sensitivity C-reactive protein, *PP* post prandial, after 2 h

[a] Statistical differences ($P < 0.05$) between women and men in control group

[b] Statistical differences ($P < 0.05$) between women and men in diabetic group

[c] *t* test controls versus diabetic individuals

Table 3 Hematological and oxidative stress parameters of diabetic and control parents

	Controls	Diabetics	*T*-test
Hemoglobin (g/L)	145.1 ± 12.3[a]	140.3 ± 16.1[b]	<0.02
Serum iron (μmol/L)	17.50 ± 5.99[a]	17.11 ± 6.01[b]	NS
Serum Ferritin (SF) (pmol/L)*	97.7 (46.1–208.1)[a]	122.5 (64.3–233.5)[b]	<0.007
Transferrin Receptor (μg/ml)	4.93 ± 2.36	3.44 ± 2.95	<0.001
Total body iron (mg/kg)	6.91 ± 3.69[a]	9.56 ± 3.65[b]	<0.001
Zn PP (μmol/L red blood cells)	1.27 ± 0.34[a]	1.28 ± 0.31	NS
HO (nmole bilirubin/mg protein/hr)*	2.5 (0.8–8.1)	9.4 (2.5–34.8)	<0.001
SOD (pg/mL)	3.62 ± 0.56[a]	3.67 ± 0.70	NS
GSH (μmol/L)	51.7 ± 12.6	47.9 ± 14.3	NS
TBARS (nmoles/ml)	1.33 ± 1.08	1.75 ± 1.14	<0.001
E Vitamin (μmol/L)	6.96 ± 4.17	7.89 ± 3.95	NS

Data are expressed as means ± SD. ANOVA for repeated measures: $P < 0.001$ for Fe and HO activity with diagnosis, $P < 0.001$ for FS, TfR and TBI with groups (sex and diagnosis). There were not interactions between groups and parameters

Zn-PP Zn protoporphyrin, *HO* Heme oxygenase (enzymatic activity)

* Geometric means and ±1 SE range

[a] Statistical differences ($P < 0.05$) between women and men in control group

[b] Statistical differences ($P < 0.05$) between women and men in diabetic group

Table 4 Hematological and oxidative stress parameters of the offspring of diabetic and control parents

	Controls	Diabetics	T-test
Hemoglobin (g/L)	143.2 ± 17.3[a]	142.5 ± 16.7[b]	NS
Serum iron (μmol/L)	16.11 ± 6.41	17.77 ± 6.91[b]	NS
Serum Ferritin (SF) (pmol/L)*	52.6 (19.9–139.1)[a]	70.1 (25.8–190.5)[b]	<0.01
Transferrin Receptor (μg/mL)	4.92 ± 2.16	3.52 ± 2.96	<0.008
Total Body Iron (mg/kg)	4.11 ± 3.86[a]	8.01 ± 5.39[b]	<0.001
Zn PP (μmol/L red blood cells)	1.30 ± 0.37	1.30 ± 0.45[b]	NS
HO (nmole bilirubin/mg protein/h)*	2.27 (0.69–7.46)	5.69 (1.77–18.27)	<0.001
SOD (pg/mL)	3.29 ± 0.42	3.85 ± 0.64	<0.002
GSH (μmol/L)	56.5 ± 12.5	42.0 ± 15.5	<0.002
TBARS (nmoles/mL)	1.11 ± 0.80[a]	1.51 ± 1.18	<0.01
E Vitamin (μmol/L)	6.50 ± 3.25	6.04 ± 3.48	NS

Data are expressed as means ± SD. ANOVA for repeated measures: $P < 0.001$ for Fe and HO activity with diagnosis, $P < 0.001$ for FS, TfR and TBI with groups (sex and diagnosis). There were not interactions between groups and parameters

* Geometric means and ± 1 SE range

[a] Statistical differences ($P < 0.05$) between women and men in control group

[b] Statistical differences ($P < 0.05$) between women and men in diabetic group

Table 5 Logistic regression for diabetes diagnosis, familiar history, age and sex as outcome and serum ferritin, heme oxygenase (HO) enzymatic activity, hsCRP and BMI as contributing variables

	Odds ratio	95% CI	*P*
Familiar History	47.565	6.335–357.088	0.001
Age	1.070	1.042–1.097	0.001
Sex	1.912	0.880–4.153	0.102
Serum Ferritin	1.014	1.001–1.026	0.030
HO activity	1.055	1.022–1.089	0.001
hsCRP	1.059	0.974–1.152	0.181
BMI	0.980	0.915–1.049	0.564

HO Heme oxygenase (enzymatic activity), *hsCRP* high sensitivity C Reactive Proteins

Logistic regression analysis (adjusted by familiar history, sex, age and diagnosis) to determine the association between diabetic offspring conditions and possible contributing variables, such as iron status or oxidative stress parameters, showed a significant association with serum ferritin; HO activity, TfR and TBARS (Table 5).

As an indication of apoptosis suceptibility and mitochondrial activity, we studied the Bcl2/Bax ratio and Mfn2 relative expression. Both, diabetic parents and their offspring had lower Bcl2/Bax ratio compared to controls (One way ANOVA, $P < 0.001$) (Fig. 1).

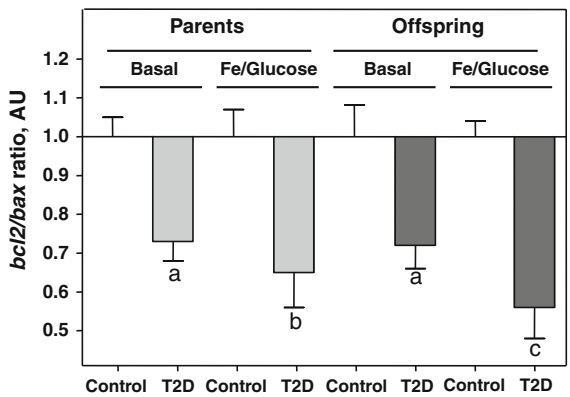

Fig. 1 *bcl2/bax* ratio in mononuclear cells. Bcl2 and Bax relative expressions were measured in mononuclear cells isolated from T2D, controls and their offspring. Mononuclear cells were incubated in basal conditions or with Fe/glucose media (50 μmol/L/20 mmol/L, respectively). Bcl2/Bax ratio mRNA relative abundance, using RT PCR was normalized to mRNA actin relative abundance. *Different letters* mean significantly different (One way ANOVA, **a**: $P < 0.01$; **b**: $P < 0.005$; **c**: $P < 0.001$)

Mfn2 expression was lower in diabetic parents and their offspring than controls (One way ANOVA, $P < 0.001$) (Fig. 2). We also incubated the MNCs with Fe (50 μmol/L) or glucose (20 mmol/L) alone and we observed a similar effects (data no showed).

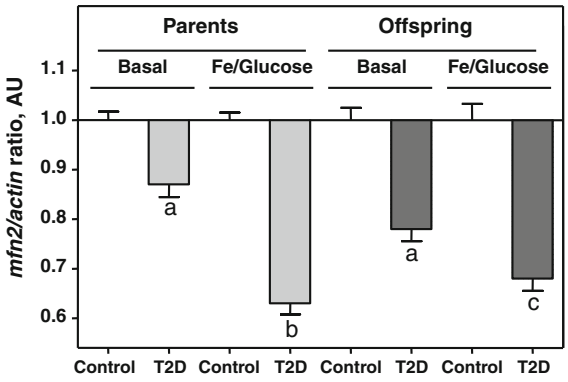

Fig. 2 Mitofusin 2 relative expression. Mitofusin 2 relative expression was measured in mononuclear cells mRNA isolated from T2D, controls and their offspring. Mononuclear cells were incubated in basal conditions or with Fe/glucose media (50 μmol/L/20 mmol/L, respectively). Mfn2 mRNA relative abundance, using RT PCR was normalized respect to mRNA actin relative abundance. *Different letters* mean significantly different (One way ANOVA, **a**: $P < 0.05$; **b**: $P < 0.001$; **c**: $P < 0.005$)

Discussion

In the present study we hypothesized that the offspring of T2D patients will have higher level of iron nutrition status and altered oxidative stress parameters compared with control individuals of similar age. Probably by the interaction of genetic, epigenetic and environmental factors, this may explain the higher propensity to develop obesity and diabetes in later life (Imamura and Maeda 2011; Reusens et al. 2011; Celis-Morales et al. 2011). We found that despite a normal glycemia, the offspring of T2D patients showed differences in insulin level and cholesterol profile. Also, they had increased levels of iron nutrition parameters reflected as high ferritin and total body iron and low transferrin receptor and an indication of altered of oxidative stress parameters (high heme oxygenase activity, SOD and TBARS and low GSH). Also, diabetic patients as their offspring had lower Bcl2/Bax ratio and Mfn2 mRNA relative abundance, a signal of increased apoptosis activity and mitochondrial disfunction.

In our study, we showed that offspring of T2D patients had elevated glycemia, insulin and altered cholesterol profile, at least an indication of the presence of metabolic syndrome. The positive correlation between serum ferritin and basal insulin levels might suggest that the alteration in iron metabolism observed in the offspring could be a result of their prediabetic state and thus give an indication on the causal relationship between alterations in iron metabolism and diabetes.

Obesity and iron-deficiency anemia are a major global health concern and they appear to be linked because individuals with overweight have a higher risk of develop iron deficiency than normal-weight individuals. To explain this association several hypothesis have been suggested: dilutional hypoferremia, poor dietary iron intake, increased iron requirements, and/or impaired iron absorption in obese individuals. In our study, no differences in iron intake were observed (data not shown) and after adjustment for familiar history, age and gender, we observed an association mainly with ferritin levels and HO activity in the studied groups (control parents, diabetics parents, control offspring, diabetics offspring). Recent evidence suggests that obesity-related inflammation may play a central role through hepcidin regulation. Hepcidin levels are higher in obese and are linked to subclinical inflammation. Thus, may reduce iron absorption and a decreased iron status in overweight individuals may result from a combination of a reduced absorption or increased iron sequestration (Cepeda-Lopez et al. 2010). We support the hypothesis that obesity-related inflammation may modified the bioavailability, metabolism or distribution of iron (Bekri et al. 2006).

T2D is being diagnosed more frequently in children and adolescents (Callahan and Mansfield 2000). The prevalence of T2D in children is mostly linked to some risk factors: obesity and sedentary lifestyle, puberty, membership of ethnic minorities, features of insulin resistance, family history of T2D, female gender and perinatal factors (Marcovecchio et al. 2005). Clearly in this study, young adults present a high probability of develop cardiovascular disease or diabetes, according their biochemical parameters and gene expression.

We showed that altered iron nutrition status was associated with adult T2D patients. They showed elevated serum ferritin and TBI (Arredondo et al. 2007). In this study, we found similar behavior in both T2D parents and their offspring, with elevated serum ferritin, TBI and low transferrin receptor. It has been shown that systemic and body iron increase with age, being similar between men and post-menopause women (Crist et al. 2009). Then, these also indicate the possibility of a higher risk of developing or exacerbating the cardiovascular disease.

HO1, a heat shock protein that participates in the degradation of heme, has been also recognized as playing a protector role in cellular defense against stressful conditions (Loboda et al. 2008). A high HO enzymatic activity, mRNA relative abundance and protein expression had been observed in mononuclear cells isolated from T2D patients incubated with high levels of heme iron than in normal controls of heme iron (Mendiburo et al. 2011). Also, a high HO activity in mononuclear cells isolated from T2D patients (Arredondo et al. 2007). Because of the high HO activity and a high levels of mRNA and protein expression, cells have two consequences, a decreasing of intracellular heme, which is toxic for the cell, and an increase in total intracellular inorganic iron, raising the possibility of generating oxidative stress (Sheftel et al. 2007).

The association between serum ferritin and diabetes was described previously (Zhai et al. 2011; Bacha et al. 2010; Jiamsripong et al. 2008). Men with high stores of iron were 2.4 times as likely to develop diabetes than were men with lower stores of iron, as assessed by using a logistic regression model (Bacha et al. 2010). Similarly, the results of the present study confirm that even a modest elevation in the ferritin concentration predicts incident diabetes independently of known risk factors and confounders (Rocha et al. 2010).

T2D patients show reduced Mfn2 expression in skeletal muscle compared with control subjects (Zorzano et al. 2009). Also, emerging evidence indicates that impaired expression of mitochondrial fusion proteins or their dysfunction participates in oxidative stress-induced and cardiovascular injury supported by hyperglycaemia-induced mitochondrial oxidative stress causes alterations in mitochondrial function, which can induce cellular injury and dysfunction, therefore oxidative stress is a mediator of mitochondrial fragmentation, which was confirmed by the fact that oxidative stress induce further mitochondrial fragmentation in coronary endothelial cells (Ong and Hausenloy 2010). On the other hand, downregulation or dysregulation of Mfn2 leads to vascular proliferative disorders (Zheng and Xiao 2010) antiproliferative activity implicated in vascular disorders (Chen et al. 2004) and vascular smooth muscle cell apoptosis (Guo et al. 2007) may be crucial to explain the metabolic changes and insulin resistance that characterize T2D and cardiovascular disease.

Here we also showed that T2D individuals and their offspring have a lower Bcl2/Bax ratio. Also, facing an oxidative stress stimuli (Fe/glucose), the cell response from these patiens and offspring increase toward the apoptosis pathway. Taking both results together, Bcl2/Bax ratio and Mfn2 expression, we can suggest that the offspring of T2D parents will have a high risk of decreased muscle response to insulin. This may induce an apoptotic and inflammatory condition, that alter the metabolic control, a condition can be reversed with a weight loss (Bach et al. 2005).

In summary, offspring of parents with T2D have a more risk factors for developing both diabetes and/or a cardiovascular disease. These results are an indication of how important are the prevention polices in susceptible population including individuals belonging to families with a T2D and obese members.

Acknowledgments We thank the staff from the Diabetic Program, Nutrition Unit, of San Juan de Dios Hospital, for their teamwork and persistent efforts in this project. This work was supported by Grant 1085173 from Fondo Nacional de Ciencia y Tecnología (FONDECYT), Chile to M. Arredondo.

References

Acosta AM, Escalona M, Maiz A, Pollak F, Leighton F (2002) Determinación del índice de resistencia insulínica mediante homa en una población de la región metropolitana de Chile. Rev Med Chile 130:1227–1231

Alemany M (2011) Utilization of dietary glucose in the metabolic syndrome. Nutr Metab 8:74

Arredondo M, Jorquera D, Carrasco E, Albala C, Hertrampf E (2007) Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with iron status in persons with type 2 diabetes mellitus. Am J Clin Nutr 86:1347–1353

Bach D, Naon D, Pich S, Soriano FX, Vega N, Rieusset J, Laville M, Guillet C, Boirie Y, Wallberg-Henriksson H, Manco M, Calvani M, Castagneto M, Palacin M, Mingrone G, Zierath JR, Vidal H, Zorzano A (2005) Expression of Mfn2, the Charcot-Marie-Tooth neuropathy type 2A gene, in human skeletal muscle: effects of type 2 diabetes, obesity, weight loss, and the regulatory role of tumor necrosis factor alpha and interleukin-6. Diabetes 4:2685–2693

Bacha F, Lee S, Gungor N, Arslanian SA (2010) From prediabetes to type 2 diabetes in obese youth: pathophysiological characteristics along the spectrum of glucose dysregulation. Diabetes Care 33:2225–2231

Bekri S, Gual P, Anty R, Luciani N, Dahman M, Ramesh B, Iannelli A, Staccini-Myx A, Casanoca D, Ben Amor I, Saint-Paul MC, Huet PM, Sadoul JL, Gugenheim J, Kaila S, Tran A, Marchand-Brustel YL (2006) Increased adipose tissue expression of hepcidin in severe obesity is

independent from diabetes and NASH. Gastroenterology 131:788–796
Bonomini F, Tengattini S, Fabiano A, Bianchi R, Rezzani R (2008) Atherosclerosis and oxidative stress. Histol Histopathol 23:381–390
Callahan ST, Mansfield MJ (2000) Type 2 diabetes mellitus in adolescents. Curr Opin Pediatr 12:310–315
Celis-Morales CA, Perez-Bravo F, Ibañes L, Sanzana R, Hormazabal E, Ulloa N, Calvo C, Bailey ME, Gill JM (2011) Insulin resistance in Chileans of European and indigenous descent: evidence for an ethnicity × environment interaction. PLoS ONE 6:e24690
Cepeda-Lopez AC, Aeberli I, Zimmermann MB (2010) Does obesity increase risk for iron deficiency? A review of the literature and the potential mechanisms. Int J Vitam Nutr Res 80:263–270
Chang JC, Kou SJ, Lin WT, Liu CS (2010) Regulatory role of mitochondria in oxidative stress and atherosclerosis. World J Cardiol 2:150–159
Chen KH, Guo X, Ma D, Guo Y, Li Q, Yang D, Li P, Qiu X, Wen S, Xiao RP, Tang J (2004) Dysregulation of HSG triggers vascular proliferative disorders. Nat Cell Biol 6:872–883
Cleland MM, Norris KL, Karbowski M, Wang C, Suen DF, Jiao S, George NM, Luo X, Li Z, Youle RJ (2011) Bcl-2 family interaction with the mitochondrial morphogenesis machinery. Cell Death Differ 18:235–247
Cook J, Flowers CH, Skikne BS (2003) The quantitative assessment of body iron. Blood 101:3359–3363
Crist BL, Alekel DL, Ritland LM, Hanson LN, Genschel U, Reddy MB (2009) Association of oxidative stress, iron, and centralized fat mass in healthy postmenopausal women. J Womens Health (Larchmt) 18:795–801
Dandona P, Aljada A, Chaudhuri A, Mohanty P, Garg R (2005) Metabolic syndrome: a comprehensive perspective based on interactions between obesity, diabetes, and inflammation. Circulation 111:1448–1454
Fernandez-Real JM, Lopez-Bermejo A, Ricart W (2002) Cross-talk between iron metabolism and diabetes. Diabetes 51:2348–2354
Guo X, Chen KH, Guo Y, Liao H, Tang J, Xiao RP (2007) Mitofusin 2 triggers vascular smooth muscle cell apoptosis via mitochondrial death pathway. Circ Res 101:1113–1122
Halpern A, Mancini MC, Magalhães ME, Fisberg M, Radominski R, Bertolami MC, Bertolami A, de Melo ME, Zanella MT, Queiroz MS, Nery M (2010) Metabolic syndrome, dyslipidemia, hypertension and type 2 diabetes in youth: from diagnosis to treatment. Diabetol Metab Syndr 2:55–75
Hoogeveen RC, Ballantyne CM, Bang H, Heiss G, Duncan BB, Folsom AR, Pankow JS (2007) Circulating oxidised low-density lipoprotein and intercellular adhesion molecule-1 and risk of type 2 diabetes mellitus: the atherosclerosis risk in Communities Study. Diabetologia 50:36–42
Imamura M, Maeda S (2011) Genetics of type 2 diabetes: the GWAS era and future perspectives [Review]. Endocr J 58:723–739
Jiamsripong P, Mookadam M, Honda T, Khandheria BK, Mookadam F (2008) The metabolic syndrome and cardiovascular disease: part I. Prev Cardiol 11:155–161
Jin Y, Zheng S, Pu Y, Shu L, Sun L, Liu W, Fu Z (2011) Cypermethrin has the potential to induce hepatic oxidative stress, DNA damage and apoptosis in adult zebrafish (Danio rerio). Chemosphere 82:398–404
Johansen JS, Harris AK, Rychly DJ, Ergul A (2005) Oxidative stress and the use of antioxidants in diabetes: linking basic science to clinical practice [Review]. Cardiovasc Diabetol 4:5
Kalra N, Roy P, Prasad S, Shukla Y (2008) Resveratrol induces apoptosis involving mitochondrial pathways in mouse skin tumorigenesis. Life Sci 82:348–358
Keaney JF Jr, Larson MG, Vasan RS, Wilson PW, Lipinska I, Corey D, Massaro JM, Sutherland P, Vita JA, Benjamin EJ (2003) Obesity and systemic oxidative stress: clinical correlates of oxidative stress in the Framingham Study. Arterioscler Thromb Vasc Biol 23:434–439
Khera T, Martin J, Riley S, Steadman R, Phillips AO (2006) Glucose enhances mesangial cell apoptosis. Lab Invest 86:566–577
Loboda A, Jazwa A, Grochot A, Rutkowski AJ, Cisowski J, Agarwal A, Jozkowicz A, Dulak J (2008) Heme oxygenase-1 and the vascular bed: from molecular mechanisms to therapeutic opportunities. Antioxid Redox Signal 10: 1767–1812
Marcovecchio M, Mohn A, Chiarelli F (2005) Type 2 diabetes mellitus in children and adolescents. J Endocrinol Invest 28:853–863
Mendiburo MJ, Le Blanc S, Espinoza A, Pizarro F, Arredondo M (2011) Transepithelial heme-iron transport: effect of heme oxygenase overexpression. Eur J Nutr 50(5):363–371
Merkel PA, Simonson DC, Amiel SA, Plewe G, Sherwin RS, Pearson HA et al (1988) Insulin resistance and hyperinsulinemia in patients with thalassemia major treated by hypertransfusion. N Engl J Med 318:809–814
Metukuri MR, Reddy CM, Reddy PR, Reddanna P (2010) Bacterial LPS-mediated acute inflammation-induced spermatogenic failure in rats: role of stress response proteins and mitochondrial dysfunction. Inflammation 33: 235–243
Mojiminiyi OA, Marouf R, Abdella NA (2008) Body iron stores in relation to the metabolic syndrome, glycemic control and complications in female patients with type 2 diabetes. Nutr Metab Cardiovasc Dis 18:559–566
Muñoz C, López M, Olivares M, Pizarro F, Arredondo M, Araya M (2005) Differential response of interleukin-2 production to chronic copper supplementation in healthy humans. Eur Cytokine Netw 16:261–265
Niederau C, Berger M, Stremmel W, Starke A, Strohmeyer G, Ebert R et al (1984) Hyperinsulinaemia in non-cirrhotic haemochromatosis: impaired hepatic insulin degradation? Diabetologia 26:441–444
NIH Publications (2005) National Cholesterol education Program. Detection, evaluation and treatment of high blood colesterol in adults (adult treatment panel III). No 02-5215
Ong SB, Hausenloy DJ (2010) Mitochondrial morphology and cardiovascular disease. Cardiovasc Res 88:16–29
Praveen EP, Kulshreshtha B, Khurana ML, Sahoo JP, Gupta N, Kumar G, Dwivedi SN, Ammini AC (2010) Obesity and metabolic abnormalities in offspring of subjects with diabetes mellitus. Diabetes Technol Ther 12:723–730
Reusens B, Theys N, Remacle C (2011) Alteration of mitochondrial function in adult rat offspring of malnourished dams. World J Diabetes 2:149–157

Rocha M, Apostolova N, Hernandez-Mijares A, Herance R, Victor VM (2010) Oxidative stress and endothelial dysfunction in cardiovascular disease: mitochondria-targeted therapeutics. Curr Med Chem 17:3827–3841

Sheftel AD, Kim SF, Ponka P (2007) Non-heme induction of heme oxygenase-1 does not alter cellular iron metabolism. J Biol Chem 282:10480–10486

Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL (1989) Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. N Engl J Med 320:915–924

Sumida Y, Yoshikawa T, Okanoue T (2009) Role of hepatic iron in non-alcoholic steatohepatitis. Hepatol Res 39:213–222

Sun L, Franco OH, Hu FB, Cai L, Yu Z, Li H, Ye X, Qi Q, Wang J, Pan A, Liu Y, Lin X (2008) Ferritin concentrations, metabolic syndrome, and type 2 diabetes in middle-aged and elderly Chinese. J Clin Endocrinol Metab 93:4690–4696

Wang X (2001) The expanding role of mitochondria in apoptosis. Genes Dev 15:2922–2933

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 292:727–730

World Healths Organization (WHO) and Centers for Disease Control and Prevention (2007) Assessing the iron status of populations, 2nd edn. Geneva, 6–8 April 2004. ISBN 9789241596107 (electronic version)

Zhai L, Ballinger SW, Messina JL (2011) Role of reactive oxygen species in injury-induced insulin resistance. Mol Endocrinol 25:492–502

Zheng M, Xiao RP (2010) Role of mitofusin 2 in cardiovascular oxidative injury. J Mol Med 88:987–991

Zorzano A, Liesa M, Palacín M (2009) Role of mitochondrial dynamics proteins in the pathophysiology of obesity and type 2 diabetes. Int J Biochem Cell Biol 41:1846–1854