

Impact of diabetes mellitus on the relationships between iron-, inflammatory- and oxidative stress status

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Abstract

Background Diabetes is an inflammatory condition associated with iron abnormalities and increased oxidative damage. We aimed to investigate how diabetes affects the interrelationships between these pathogenic mechanisms.

Methods Glycaemic control, serum iron, proteins involved in iron homeostasis, global antioxidant capacity and levels of antioxidants and peroxidation products were measured in 39 type 1 and 67 type 2 diabetic patients and 100 control subjects.

Results Although serum iron was lower in diabetes, serum ferritin was elevated in type 2 diabetes ($p = 0.02$). This increase was not related to inflammation (C-reactive protein) but inversely correlated with soluble transferrin receptors ($r = -0.38$, $p = 0.002$). Haptoglobin was higher in both type 1 and type 2 diabetes ($p < 0.001$) and haemopexin was higher in type 2 diabetes ($p < 0.001$). The relation between C-reactive protein and haemopexin was lost in type 2 diabetes ($r = 0.15$, $p = 0.27$ vs $r = 0.63$, $p < 0.001$ in type 1 diabetes and $r = 0.36$, $p = 0.001$ in controls). Haemopexin levels were independently determined by triacylglycerol ($R^2 = 0.43$) and the diabetic state ($R^2 = 0.13$). Regarding oxidative stress status, lower antioxidant concentrations were found for retinol and uric acid in type 1 diabetes, α -tocopherol and ascorbate in type 2 diabetes and protein thiols in both types. These decreases were partially explained by metabolic-, inflammatory- and iron alterations. An additional independent effect of the diabetic state on the oxidative stress status could be identified ($R^2 = 0.5-0.14$).

Conclusions Circulating proteins, body iron stores, inflammation, oxidative stress and their interrelationships are abnormal in patients with diabetes and differ between type 1 and type 2 diabetes. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords diabetes; iron; inflammation; oxidative stress

Introduction

Long-term complications of diabetes mellitus have been associated with increased oxidative stress, as measured by the accumulation of oxidatively damaged macromolecules such as products of lipid peroxidation [1], glycoxidation [2] and DNA damage [3]. Biochemical pathways explaining

hyperglycaemia-induced oxidative stress include auto-oxidation of glucose, advanced glycation end-products and decreased antioxidant defences [4–7].

Another putatively important source of oxidative stress stems from the disturbances in iron metabolism that are associated with diabetes [8]. Iron in its free, redox-active form (Fe^{2+}) is one of the most reactive pro-oxidants because it catalyses the generation of the highly reactive hydroxyl radicals (Fenton reaction) that are able to initiate and propagate peroxidation cascades [9]. Diabetes-induced alterations in the availability of redox-active iron can result from excessive iron stores or from disturbances in the protective mechanisms designed to prevent its uncontrolled release. In plasma, protection is ensured by the sequestration of iron in a safe redox-inactive form (Fe^{3+}) by the combined action of iron-oxidation by caeruloplasmin and iron-binding by transferrin (Tf). In this way, plasma iron in healthy individuals is maintained at levels far below the total Tf binding capacity and it is generally accepted that only negligible amounts of free, non-Tf bound iron are present [10]. We have observed that in diabetic subjects, the levels of non-Tf bound iron are not elevated but that the capacity of plasma to bind iron in a form that prevents lipid peroxidation is impaired.

In addition to non-Tf bound iron, iron release from haem proteins has been suggested to be an important generator of hydroxyl radicals by the Fenton reaction [11]. The binding of haemoglobin to haptoglobin and haem to haemopexin, followed by clearance of these complexes by the liver and spleen, greatly decreases their pro-oxidant danger [12]. Findings of an unusual susceptibility of haem proteins to damage by glucose, which is followed by iron mobilisation, suggest a role for these proteins in the iron-mediated free radical reactions occurring in diabetic plasma [13].

With regard to the intracellular medium, it has been reported that serum ferritin concentrations, which correlate closely with body iron stores in healthy individuals [14], are elevated in diabetic patients. This has been associated with abnormal indexes of glucose homeostasis [15–17]. In view of the potential release of free iron from ferritin in the presence of reducing agents such as superoxide and ascorbic acid [18], abnormal iron stores can also be a potential source of iron-driven peroxidation reactions in diabetes.

To investigate this hypothesis, accurate identification and quantification of the various body iron compartments is essential. Moreover, the potential protein sources of redox-active iron mentioned above (Tf, ferritin, caeruloplasmin, haemopexin and haptoglobin) are also acute-phase proteins. Since diabetes is an inflammatory condition, reliable markers to pry out the role of inflammation and/or body iron stores in diabetes are still required. For example, the concentration of the soluble form of the Tf receptor in serum (sTfR) is proportional to the iron demands and decreases with iron overload [19]. Quantification of the ratio ferritin/soluble transferrin receptor (sTfR) could thus give an insight on iron stores

without being influenced by the inflammatory status in diabetes.

To address these issues, we aimed to investigate the impact of diabetes mellitus on circulating iron and body iron stores as well as the relationship to inflammatory and oxidative stress status. We collected, for the first time to our knowledge, an extensive profile of iron parameters as well as metabolic control, inflammatory and oxidative stress markers in type 1 (T1DM) and type 2 (T2DM) diabetic patients and compared them to a healthy control group.

Subjects, materials and methods

Study subjects and design

The study was approved by the ethical commission of the University Hospital of Antwerp (ID 3/25/91) and informed consent was obtained from all participants.

Blood was collected from 39 T1DM, 67 T2DM patients and 100 healthy volunteers. The diabetic patients were consecutively recruited in the period from August 2003 to May 2005 when they attended the outpatient diabetes clinic at the Antwerp University Hospital for their annual check-up. The healthy controls were recruited through advertisement from among the hospital and faculty staff and external company employees undergoing their compulsory medical check-up. Patients were divided into subgroups (0, 1, or 2) according to their complication profile, which was evaluated by specialised medical doctors. Nephropathy was scored as 1 when microalbuminuria (20–200 $\mu\text{g}/\text{min}$ albumin excretion rate) was present and 2 for macroalbuminuria (>200 $\mu\text{g}/\text{min}$). Ophthalmologists diagnosed pre-proliferative 1 and proliferative 2 retinopathy according to the Early Treatment Diabetic Retinopathy Study [20]. Neuropathy was evaluated by electromyography: the presence of abnormal motoric or sensoric nerve conduction velocity in the extremities was scored as 1 and when aggravated by denervation as 2. The presence of cardiovascular complications was assessed by cardiologists and scored as 1 in case of abnormal electrocardiogram and 2 with established antecedents of myocardial infarction, coronary heart disease, angina and/or stroke.

Measurements of glycaemic control, inflammation and iron status

HbA_{1c} was measured by ion-exchange HPLC (ADAMS HA 8160 Menarini, Firenze-Italy) (CV 0.7%). Glucose (CV 0.6%), serum iron (CV 3.0%) and total iron-binding capacity (TIBC) (CV 2.8%) were measured colorimetrically (Vitros 950 AT Ortho Clinical Diagnostic Inc., Rochester-USA). Tf saturation was calculated by the formula: serum iron/TIBC. Levels of Tf (CV 2.8%), sTfR (CV 4.6%), ferritin (CV 3.2%), haemopexin (CV 6.0%), caeruloplasmin (CV 3.2%), haptoglobin (CV 3.6%) and

high-sensitive C-reactive protein (CRP) (CV 3.9%) were assayed nephelometrically (BNII Nephelometer, Dade Behring, Marburg-Germany). Fibrinogen (CV 5.0%) and Von Willebrand antigen (CV 3.5%) were determined using an electromagnetic method (STA Diagnostica Stago, Asnières-France). Copper was analysed by electrothermal Zeeman atomic absorption spectrometry (Perkin-Elmer Spectrometer model 3030; Graphite furnace HGA600).

Total serum protein glycation and Tf glycation were measured as described in detail earlier. By measuring fructosamine concentration using the nitroblue tetrazolium assay [21] adapted for 96-well plates, glycation was determined in the total serum proteins (CV 3.2%) and in the Tf isolated from serum by immunocomplexation (CV 3.7%). Results are expressed as fructosamine per g albumin and per g Tf, respectively.

Oxidative stress status

Oxidative stress status was evaluated by measuring global plasma antioxidant capacity, concentrations of individual antioxidants and products of peroxidation.

The global plasma antioxidant capacity was evaluated by measuring the inhibition of chemiluminescence after addition of plasma to a reaction mixture containing 75 $\mu\text{mol/L}$ luminol and peroxy radicals liberated by the thermal decomposition of 20 mmol/L 2,2-azo-bis(2-amidinopropane) hydrochloride [22]. Two antioxidant effects were distinguished: first, the duration of the lag-time before appearance of the chemiluminescence signal was compared to a calibration with Trolox and expressed as Trolox equivalents (total antioxidant capacity-trolox equivalents (TAC-TE)); second, the percent inhibition of the maximum chemiluminescence peak (total antioxidant capacity-percent inhibition (TAC-PI)) (CV < 9%).

Plasma α -tocopherol and retinol were measured by HPLC (Shimadzu, Kyoto-Japan) with a reversed-phase C18 column LiChrospher RP C18 (Alltech, Deerfield-IL-USA) with 100% methanol as mobile phase and detection at 292 and 325 nm, respectively [23], (CVs 4.8% and 4.1%). Ascorbate in plasma was measured by reversed-phase HPLC with 2 mmol/L KCl mobile phase and electrochemical detection at 1000 mV [24] (CV 6%).

Reduced glutathione in whole blood and protein thiols in plasma were measured by a colorimetric method using Ellman's reagent and expressed relative to haemoglobin (Drabkin reagent) and proteins (Biuret reagent), respectively [25,26], (CVs 7 and 6%).

Plasma determinable reactive oxygen metabolites (d-ROM) were measured using a commercial kit (Pharmalab d-ROMs, Parma-Italy) and calibrated against known concentrations of *tert*-butyl hydroperoxide (CV 7%).

Plasma malondialdehyde (MDA) was analysed by reverse-phase HPLC with 10 mmol/L methanol/ KH_2PO_4 (40/60 v/v) as mobile phase and detection at 532 nm [27] (CV 9%).

Statistical analysis

Results were expressed as mean \pm SD or as geometric mean (95% CI) for data not compatible with a Gaussian distribution. The statistical package SPSS Version 11.0, Chicago-IL was used. Differences between groups were calculated using two-way ANOVA to test for the independent effect of the subgroup (Control, T1 and T2DM) and of the sex, as well as for the interaction between these two factors. The Tukey and Bonferroni post-hoc tests were applied to identify the differences between the three subgroups. Non-Gaussian data were log-transformed (Ln) before application of the parametric tests. The χ^2 or Fisher's exact test was performed to compare frequency distributions. Multiple linear regression (stepwise) was applied to identify the relationship between the various parameters. Two tailed *p*-values ≤ 0.05 (or adjusted for multiple comparisons according to Bonferroni) were considered as statistically significant.

Results

Clinical characteristics

The main subject characteristics are shown in Table 1. Non-diabetic controls and T1DM subjects were similar with regard to age and BMI while T2DM patients were older and had higher BMI. Glycaemic indices such as fasting blood glucose, HbA_{1c} and fructosamine content in total serum proteins and in serum Tf were significantly higher in diabetic subjects, this difference being more pronounced in T1DM ($p < 0.0005$). Diabetic complications were present in less than half of the patients with a significantly higher proportion of cardiovascular complications in the T2DM group.

Inflammatory status

Inflammation measured as CRP, fibrinogen and Von Willebrand antigen was higher in the diabetic population, being most pronounced in T2DM ($p < 0.0005$) (Table 2). BMI was related to CRP in diabetes ($r = 0.53$, $p = 0.001$ in T1DM and $r = 0.36$, $p = 0.010$ in T2DM) but not in control subjects. Inflammation parameters were not related to lipid status. After subdivision of the study groups based on the median CRP of the whole population ($<$ or ≥ 0.12 mg/dL), the T2DM patients with high CRP levels tended to have a higher frequency of cardiovascular complications ($p = 0.075$). In T1DM, duration of diabetes was positively associated with the Von Willebrand antigen ($r = 0.49$, $p = 0.002$) and fibrinogen ($r = 0.40$, $p = 0.014$).

Iron status

With regard to the circulating iron status (Table 2), serum iron was lower in T2DM female patients while

Table 1. Clinical characteristics and metabolic control of diabetic versus healthy subjects

	Control		T1DM		T2DM		P-value ANOVA – χ^2
	Men (n = 56)	Women (n = 44)	Men (n = 21)	Women (n = 18)	Men (n = 33)	Women (n = 34)	
Age (years) ^a	47 [45–50]	40 [38–43]	47 [43–52]	41 [37–45]	62 [58–57]	63[59–68]	<0.0005 ^{dfgij}
Body mass index (kg/m ²)	25 ± 2.9	22.8 ± 3.1	26.6 ± 4.8	24.1 ± 3.7	28.6 ± 4	31.2 ± 5.8	<0.0005 ^{dgiij}
Duration of diabetes (years)	Not relevant		24 ± 13	21 ± 13	10 ± 7	13 ± 11	<0.0005 ^{dhiij}
Systolic blood pressure (mmHg) ^a	120 [113–127]	110 [102–118]	130 [119–143]	117 [106–129]	137 [127–148]	141 [121–142]	<0.0005 ^{dei}
Diastolic blood pressure (mmHg) ^a	70 [66–75]	67 [62–73]	76 [68–84]	69 [68–77]	76 [70–83]	73 [67–79]	0.239
Serum total cholesterol (mmol/L)	5.44 ± 0.97	5.21 ± 0.77	4.97 ± 0.56	5.24 ± 0.98	5.29 ± 1.01	5.49 ± 0.86	0.284
Serum HDL cholesterol (mmol/L)	1.45 ± 0.45	1.94 ± 0.43	1.61 ± 0.46	1.93 ± 0.45	1.20 ± 0.34	1.47 ± 0.52	<0.0005 ^{dij}
Serum triacylglycerol (mmol/L)	1.30 ± 0.71	0.96 ± 0.32	1.10 ± 0.79	1.41 ± 2.13	1.73 ± 0.83	1.69 ± 1.63	0.022 ^{di}
Treatment (insulin/OAD) ^b	Not relevant		21/3	19/3	16/20	20/22	<0.0005
Insulin dose (units/kg/day)	Not relevant		0.72 ± 0.18	0.61 ± 0.28	0.44 ± 0.26	0.48 ± 0.24	0.021 ^c
Anaemia (yes/no) ^b	1/55	0/44	3/18	1/17	3/30	2/32	0.070
Smoker (no/yes/ex/unknown) ^b	35/13/2/6	29/8/4/3	5/5/2/9	5/2/1/10	9/3/3/18	11/3/1/19	<0.0005
Nephropathy (0/1/2) ^b	–	–	15/4/1	12/2/0	22/6/2	23/7/3	0.55
Retinopathy (0/1/2) ^b	–	–	8/8/1	9/9/0	17/10/3	18/11/0	0.44
Neuropathy (0/1/2) ^b	–	–	11/6/1	13/5/0	17/11/3	18/8/2	0.51
Cardiovascular disease (0/1/2) ^b	–	–	13/3/3	15/3/0	15/6/10	13/9/8	0.010 ^c
Glucose (mmol/L)	4.9 ± 0.5	4.6 ± 0.4	11.6 ± 3.8	9.9 ± 4.5	8.1 ± 2.8	7.4 ± 3.1	<0.0005 ^{dehij}
HbA _{1c} (%)	5.4 ± 0.3	5.3 ± 0.2	7.8 ± 1.0	7.8 ± 0.7	7.2 ± 1.2	7.3 ± 1.3	<0.0005 ^{dhiij}
Protein glycation (μmol/g albumin)	5.36 ± 0.68	5.72 ± 0.73	8.09 ± 1.41	8.32 ± 1.43	6.44 ± 1.32	6.79 ± 1.30	<0.0005 ^{dhiij}
Tf glycation (μmol/g transferrin)	0.78 ± 1.10	0.80 ± 1.08	1.94 ± 0.84	1.97 ± 1.22	1.02 ± 1.06	1.09 ± 1.04	<0.0005 ^{dhiij}

OAD, oral antidiabetic medication. The scoring of the complications is explained under 'Methods'. Values are expressed as mean ± SD, as ^ageometric mean (95% confidence interval) obtained after log transformation for non-normally distributed data or as ^bnumber of observations. ^cand ^ddenotes $p < 0.05$ and $p < 0.005$ when comparing according to diabetic state within each gender group. ^eand ^fdenotes $p < 0.05$ and $p < 0.005$ when comparing males to females within the different groups. ^gdenotes $p < 0.05$ for the interaction between sex and diabetes. Post-hoc analysis revealed significant differences ($p < 0.05$) between the control and T1DM (^h), control and T2DM (ⁱ) or between T1DM and T2DM (^j).

Tf concentration and associated TIBC were lower only in the T1DM group, especially in the male population ($p < 0.0005$ for the effect of diabetes and $p = 0.002$ for the effect of gender). Although Tf saturation was not significantly different between the groups, saturations lower than the median (25%) tended to occur more frequently in T2DM patients ($p = 0.069$). Caeruloplasmin and associated copper ($r = 0.87$) were higher in females ($p < 0.0005$) but did not differ in patients with diabetes. Haptoglobin was significantly higher in both types of diabetes ($p < 0.001$) while haemopexin ($p < 0.001$), ferritin ($p = 0.022$) and sTfR ($p = 0.009$) were only significantly elevated in T2DM. Women had higher levels of haemopexin ($p = 0.04$) but lower ferritin ($p < 0.0005$) in all groups. When taking the ratio sTfR/ferritin the gender difference remained, but the difference between the control and diabetic groups disappeared ($p = 0.15$). The inverse relation between ferritin and sTfR in the whole group was only significantly maintained in T2DM ($r = -0.38$, $p = 0.002$) (Figure 1(A)). Ferritin was positively and sTfR was negatively associated with Tf saturation in the control as well as in the diabetic subjects. The presence of diabetic complications did not affect

ferritin levels but sTfR concentration was significantly higher in patients with macroalbuminuria ($p = 0.046$) or severe (score 2) cardiovascular complications ($p = 0.019$).

Relationship between the inflammatory, metabolic and iron status

Multiple regression analysis explaining the variance of ferritin in the whole study population identified HDL cholesterol, serum iron and associated proteins (Tf, albumin, haemopexin, and sTfR) (total $R^2 = 0.32$, $p < 0.0005$) as independent determinants but not haptoglobin, caeruloplasmin or the diabetic state. In other models, glycaemic-, inflammatory status, body mass index and triacylglycerol levels were excluded as determinants for ferritin. Moreover, the positive correlation between ferritin and body mass index was lost in the diabetic groups (Figure 1(B)).

There was no significant correlation between the inflammation parameter CRP and ferritin (Figure 2(A)) or

Table 2. Inflammatory- and iron status in diabetic versus control subjects

	Control		T1DM		T2DM		P-value ANOVA - χ^2
	Men (n = 56)	Women (n = 44)	Men (n = 21)	Women (n = 18)	Men (n = 33)	Women (n = 34)	
CRP (mg/dL) ^a	0.06 [0.05–0.09]	0.11 [0.08–0.15]	0.14 [0.08–0.22]	0.21 [0.12–0.36]	0.25 [0.17–0.37]	0.40 [0.27–0.61]	<0.0005 ^{dehij}
Fibrinogen (g/L)	2.93 ± 0.45	3.34 ± 0.69	3.88 ± 1.40	3.59 ± 0.73	4.16 ± 1.05	4.43 ± 0.70	<0.0005 ^{dij}
Von Willebrand antigen (%)	101 ± 31	113 ± 36	154 ± 70	154 ± 39	173 ± 74	159 ± 56	0.030 ^{dhi}
Serum iron (μmol/L)	18 ± 7	18 ± 6	16 ± 5	16 ± 6	17 ± 6	14 ± 5	0.025 ^{ci}
TIBC (μmol/L)	66 ± 7	73 ± 10	60 ± 9	67 ± 11	70 ± 13	69 ± 11	<0.0005 ^{dfghj}
Tf saturation (%)	28 ± 12	26 ± 8	27 ± 9	24 ± 9	27 ± 21	21 ± 9	0.268 ^e
Tf concentration (g/L)	2.52 ± 0.36	2.83 ± 0.47	2.27 ± 0.31	2.52 ± 0.54	2.66 ± 0.39	2.68 ± 0.38	<0.0005 ^{dfhj}
Ferritin (μg/L) ^a	147 [119–181]	43 [34–54]	117 [84–163]	48 [34–68]	158 [121–206]	73 [56–95]	<0.0005 ^{cf}
sTfR (mg/L) ^a	1.32 [1.25–1.38]	1.32 [1.24–1.40]	1.32 [1.20–1.44]	1.48 [1.34–1.62]	1.46 [1.36–1.57]	1.47 [1.37–1.58]	0.030 ^{ci}
sTfR/ferritin (μg/μg) ^a	9.0 [7.2–11.4]	30.9 [23.9–40.1]	11.2 [7.8–16.2]	30.9 [21.0–45.5]	9.2 [6.9–12.4]	20.2 [15.1–27.0]	<0.0005 ^f
Haptoglobin (g/L)	1.04 ± 0.39	1.03 ± 0.34	1.31 ± 0.62	1.22 ± 0.63	1.42 ± 0.41	1.63 ± 0.45	<0.0005 ^{dhi}
Caeruloplasmin (g/L)	0.23 ± 0.04	0.34 ± 0.10	0.25 ± 0.04	0.34 ± 0.12	0.25 ± 0.04	0.30 ± 0.05	<0.0005 ^{fg}
Copper (mg/L)	0.80 ± 0.17	1.15 ± 0.38	0.86 ± 0.26	1.34 ± 0.51	0.93 ± 0.20	1.13 ± 0.26	<0.0005 ^f
Haemopexin (g/L)	0.87 ± 0.12	0.96 ± 0.15	0.93 ± 0.20	0.95 ± 0.16	1.15 ± 0.15	1.20 ± 0.27	<0.0005 ^{deij}
Albumin (g/L)	43 ± 3	44 ± 3	42 ± 2	41 ± 3	43 ± 3	42 ± 3	<0.0005 ^{deghij}

Values are expressed as mean ± SD, as ^ageometric mean (95% confidence interval) obtained after log transformation for non-normally distributed data or as ^cand ^ddenotes $p < 0.05$ and $p < 0.005$ when comparing according to the diabetic state within each gender group. ^eand ^fdenotes $p < 0.05$ and $p < 0.005$ when comparing males to females within the different groups. ^gdenotes $p < 0.05$ for the interaction between sex and diabetes. Post-hoc analysis revealed significant differences ($p < 0.05$) between the control and T1DM (^h), control and T2DM (ⁱ) or between T1DM and T2DM (^j).

sTfR in any of the subgroups. In contrast, higher CRP was associated with elevated caeruloplasmin, haemopexin and haptoglobin concentrations (Figure 2(B), (C) and (D)). This association was maintained after subdivision according to the diabetic state except for haemopexin in T2DM. The haemopexin values were consistently high in these patients even at low levels of CRP. Multiple regression explaining the variance in haemopexin identified as the independent determinants, triacylglycerol ($R^2 = 0.43$), the diabetic state ($R^2 = 0.13$), CRP ($R^2 = 0.05$) and Tf ($R^2 = 0.03$).

Soluble TfR correlated positively with the levels of Von Willebrand antigen in both types of diabetic patients ($r = 0.53$, $p = 0.001$ in T1DM and $r = 0.37$, $p = 0.002$

in T2DM) but not in control subjects ($r = -0.36$, $p = 0.170$).

Oxidative stress status

Oxidative stress status is shown in Table 3 and reveals diabetes-related differences in both antioxidant status and in levels of peroxidation products. As regards antioxidants, both T1DM and T2DM had lower thiol content in the plasma proteins ($p < 0.0005$). Levels of uric acid, retinol and total antioxidant capacity in plasma, expressed as Trolox equivalents (TAC-TE), were significantly lower in T1DM ($p < 0.0005$) and female

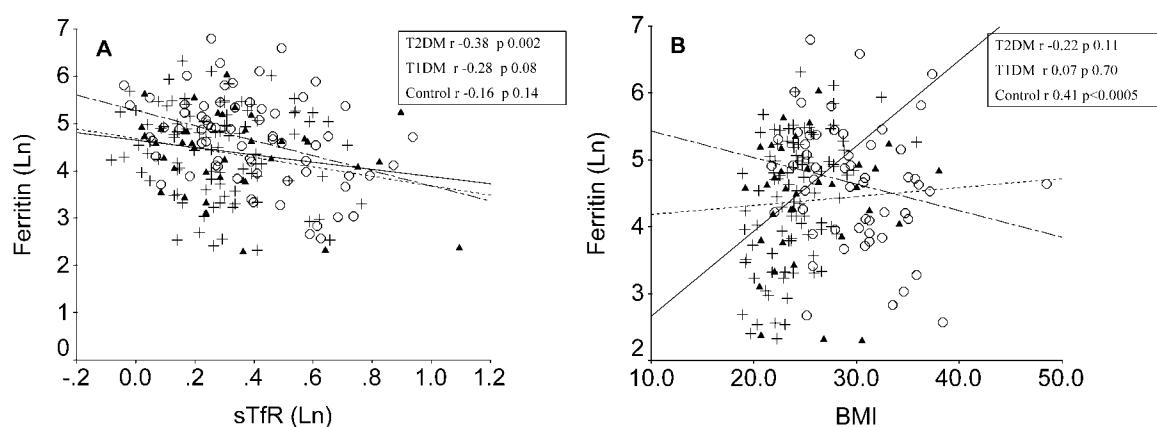


Figure 1. Correlation of sTfR (A) and body mass index (B) with ferritin in type 1 (closed triangles and dotted line) and type 2 (open circles and dashed line) diabetic patients and control subjects (crosses and full line)

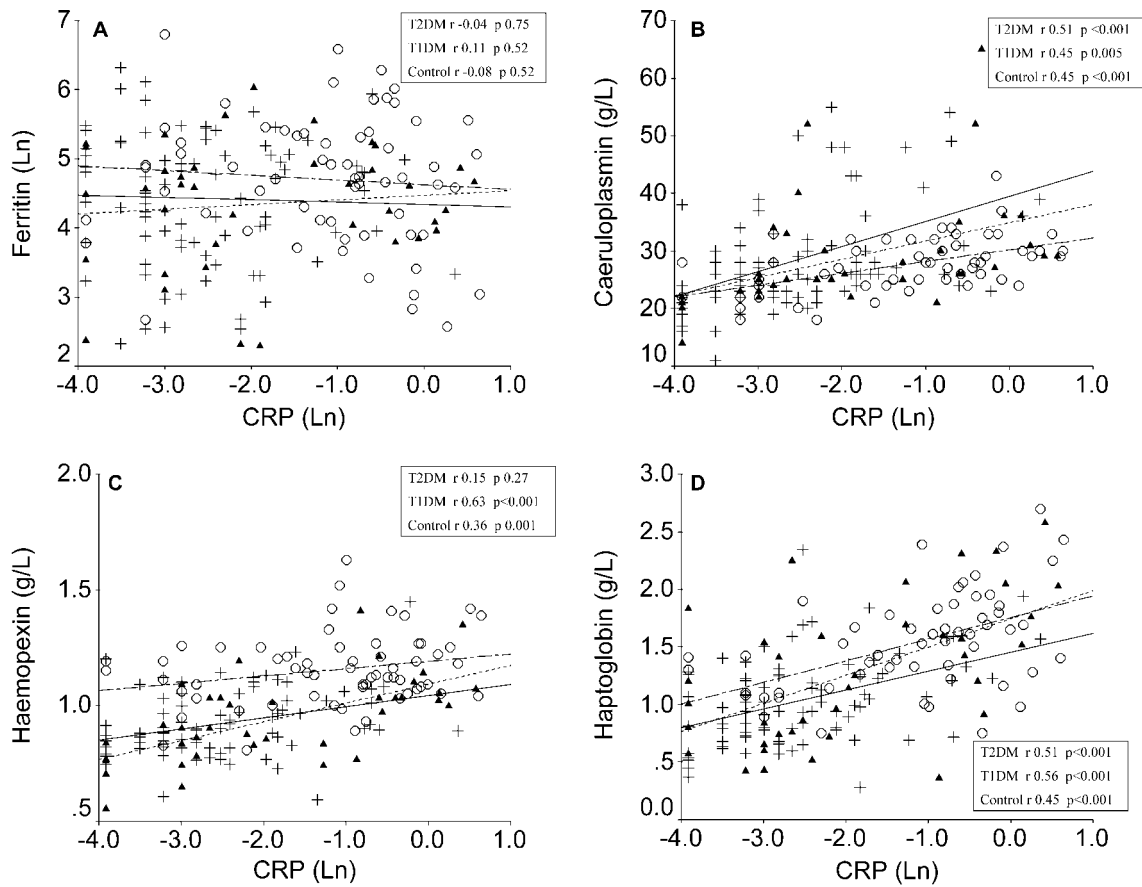


Figure 2. The relationship between inflammation (CRP) and the levels of ferritin (A), caeruloplasmin (B), haemopexin (C) and haptoglobin (D) in type 1 (closed triangles and dotted line) and type 2 (open circles and dashed line) diabetic patients and control subjects (crosses and full line)

Table 3. Oxidative stress status in diabetic patients versus control subjects

	Control		T1DM		T2DM		P-value ANOVA
	Men (n = 56)	Women (n = 44)	Men (n = 21)	Women (n = 18)	Men (n = 33)	Women (n = 34)	
Plasma TAC-PI (% inhibition)	73 ± 7	79 ± 6	79 ± 5	77 ± 5	79 ± 6	79 ± 7	<0.0005 ^{aeg}
Plasma TAC-TE (μmol/L)	208 ± 33	169 ± 32	196 ± 49	136 ± 46	215 ± 53	188 ± 42	<0.0005 ^{bdfh}
Plasma thiols (μmol/g protein)	4.85 ± 1.17	5.00 ± 1.32	3.24 ± 1.33	3.31 ± 1.04	3.88 ± 1.33	3.68 ± 1.36	<0.0005 ^{bfg}
Blood glutathione (μmol/g Hb)	5.37 ± 0.78	5.92 ± 0.99	5.57 ± 0.75	6.14 ± 1.29	5.43 ± 1.02	5.75 ± 0.96	0.009 ^d
Plasma uric acid (μmol/L)	341 ± 71	230 ± 65	303 ± 95	194 ± 75	363 ± 129	311 ± 85	<0.0005 ^{bdfgh}
Serum bilirubin (μmol/L)	11.29 ± 9.92	10.17 ± 5.78	10.17 ± 4.28	10.26 ± 8.11	9.85 ± 4.63	7.54 ± 3.18	0.271
Plasma retinol (μmol/L)	6.21 ± 3.04	5.24 ± 2.65	4.54 ± 1.57	3.49 ± 1.15	6.11 ± 2.20	4.61 ± 1.50	<0.0005 ^{bdfh}
Plasma α-tocopherol (μmol/L)	35.1 ± 12.1	36.2 ± 13.5	33.9 ± 10.9	31.8 ± 9.8	36.2 ± 9.3	33.0 ± 12.5	0.6
(μmol/mmol total lipid)	5.5 ± 1.8	5.8 ± 2.2	5.7 ± 2.3	4.9 ± 1.1	5.2 ± 1.1	4.5 ± 1.0	0.026 ^{ag}
Plasma ascorbate (μmol/L)	55 ± 20	62 ± 17	58 ± 30	51 ± 24	42 ± 21	49 ± 21	0.003 ^{bg}
Plasma d-ROM (mmol/L TBOOH)	2.13 ± 0.54	3.26 ± 1.45	2.56 ± 0.77	3.95 ± 1.39	2.72 ± 0.75	3.40 ± 0.81	<0.0005 ^{bdf}
Plasma MDA (μmol/L)	0.67 ± 0.21	0.50 ± 0.22	0.52 ± 0.14	0.62 ± 0.35	0.61 ± 0.16	0.62 ± 0.25	0.008 ^e
(μmol/mmol total lipid)	0.106 ± 0.041	0.083 ± 0.040	0.088 ± 0.027	0.098 ± 0.060	0.089 ± 0.020	0.090 ± 0.037	0.196

TBOOH, *tert*-butyl hydroperoxide equivalents; Hb, haemoglobin. Values are expressed as mean ± SD. ^aand ^bdenotes $p < 0.05$ and $p < 0.005$ when comparing according to the diabetic state within each gender group. ^ddenotes $p < 0.005$ when comparing males to females within the different groups. ^edenotes $p < 0.05$ for the interaction between gender and diabetes. Post-hoc analysis revealed significant differences ($p < 0.05$) between the control and T1DM (^f), control and T2DM (^g) or between T1DM and T2DM (^h).

subjects ($p < 0.0005$). In the regression model containing all antioxidants, the variance in TAC-TE was mainly explained by the presence of uric acid (76%, $p < 0.005$). In contrast, the second aspect of total antioxidant capacity, expressed as TAC-PI, was lower in non-diabetic subjects, this effect being seen only in men ($p = 0.003$ for the interaction between diabetic state and gender). T2DM patients displayed lower plasma ascorbate levels ($p = 0.001$). Plasma α -tocopherol was also lower in T2DM but only when expressed relative to serum lipids ($p = 0.019$).

Concerning peroxidation products, diabetic subjects and females in all subgroups had higher levels of peroxides measured as d-ROM ($p < 0.0005$). MDA, even when corrected for serum cholesterol plus triglycerides, did not differ in the three subgroups.

Oxidative stress status did not differ in the patients with complications or in those with CRP levels higher than 0.12 mg/dL (median of the whole study population) and, except for the affect of uric acid, was not affected by age or body mass index.

Interrelation of glycaemic control, inflammation and iron homeostasis with oxidative stress

To analyse the effect of diabetes on these interrelationships, multiple regression was conducted in models that included the subgroup (control, T1 and T2DM), age and sex together with each of the following sets of parameters (Table 4):

Metabolic control

Although significant differences in oxidative stress parameters could be found according to the diabetic state, no relation with glycaemic control was seen within each diabetic subgroup. In addition to the small independent effect of HDL-, LDL cholesterol and triacylglycerol, oxidative stress parameters were also independently determined by the diabetic state (7–10% of the variance).

Inflammation

Inflammatory markers that were identified as independent determinants of oxidative stress were CRP for α -tocopherol as well as d-ROM and fibrinogen for plasma protein thiols.

When analysing the subgroups separately, several differences were observed. In T1DM, CRP was negatively associated with the α -tocopherol content per lipids ($r = -0.33$, $p = 0.039$) and glutathione concentrations ($r = -0.34$, $p = 0.033$). In T2DM, an inverse relation could be demonstrated between fibrinogen and retinol ($r = -0.33$, $p = 0.008$) and plasma protein thiols ($r = -0.30$, $p = 0.018$).

Iron

Taking into account all iron-related serum proteins, the diabetic state was identified as a significant independent

determinant of oxidative stress (3–13%). In the whole study population as well as in the control, T1DM and T2DM groups, a positive association was found between Tf saturation and bilirubin ($r = 0.37$, $p < 0.0005$). Tf saturations higher than median ($\geq 25\%$) were associated with significantly lower TAC-TE (183 ± 43 vs 197 ± 49 $\mu\text{mol/L}$ trolox equivalents in Tf saturation $< 25\%$, $p = 0.035$) and with diminished uric acid concentrations (283 ± 95 vs 317 ± 109 $\mu\text{mol/L}$ in Tf saturation $< 25\%$, $p = 0.024$). In contrast, increased iron body status defined by higher than median concentrations of serum ferritin (≥ 102 $\mu\text{g/dL}$), the ratio sTfr/ferritin (≥ 13.06 $\mu\text{g}/\mu\text{g}$) and Tf saturation were not associated with the levels of plasma protein thiols, glutathione, MDA per lipids, retinol, ascorbate or the presence of complications.

Other findings include positive correlations in the control population of TAC-PI with Tf ($r = 0.36$, $p < 0.001$), caeruloplasmin ($r = 0.33$, $p = 0.001$) and albumin ($r = 0.31$, $p = 0.003$), which were lost in both types of diabetic patients. In the control population, MDA was higher in the case of lower caeruloplasmin concentration ($r = 0.27$, $p = 0.014$) but this relation was lost in diabetic subjects ($p > 0.35$). In T2DM, copper was related with lower ascorbate ($r = -0.33$, $p = 0.007$) and α -tocopherol ($r = -0.27$, $p = 0.03$) contents. For haptoglobin and haemopexin, no significant relations with oxidative stress could be retrieved.

Discussion

Emerging scientific evidence describes the bidirectional relationship between glucose- and iron metabolism. These relations are influenced by oxidative stress as well as by inflammation and can in this way amplify and potentiate the pathogenic processes leading to diabetic complications [8]. To gain more insight into these interrelations, we investigated an extensive profile of glycaemic control, inflammation, iron parameters and oxidative stress status in T1DM- as well as T2DM patients in comparison to a healthy control group.

Acute-phase serum proteins as markers of low-grade chronic inflammation are increased in diabetes [28] in accordance with the higher CRP levels in our diabetic patients. Fibrinogen and Von Willebrand antigen were higher with longer duration of the disease in the T1DM group. By respectively elevating blood viscosity and reflecting endothelial impairment, these molecules are a risk factor of micro- and macroangiopathy and have already been implicated in peripheral arterial diseases [29]. In this study we could not find differences in these parameters according to the complication profile of the patients, and the relatively small numbers of participants did not allow for sufficient statistical power.

Similarly, iron proteins such as caeruloplasmin, haptoglobin, haemopexin and ferritin belong to the family of acute-phase proteins, which are up-regulated in pathologies with an inflammatory component. We detected higher levels of haptoglobin in both types of

Table 4. Factors influencing oxidative stress status in the whole study population

Independent	Dependent				
	α -tocopherol	Ascorbate	Protein thiol	Glutathione	d-ROM
Glycaemic control	Age (5%)	Diabetic state (5%)	Diabetic state HbA _{1c} (12%) Total R ² (14%)	Sex (7%)	Sex Protein glycation (25%) Total R ² (33%)
Lipid status	Body mass index (4.4%)	Diabetic state HDL cholesterol (9%) Total R ² (12%)	Diabetic state HDL cholesterol (2%) Total R ² (12%)	Sex Triacylglycerol (8%) Total R ² (11%)	Sex LDLcholesterol (2.5%) Sex Triacylglycerol (4.5%) Total R ² (13%)
Inflammation	CRP Ln (9.5%)	Fibrinogen (5%)	Fibrinogen (5%)	Sex CRP Ln (5%) Total R ² (9%)	Sex CRP Ln (25%) Age Fibrinogen (12%) Total R ² (61%)
Iron proteins	Age Serum iron (5%) Total R ² (8%)	Diabetic state (4%)	Diabetic state (13%)	Sex Haemopexin (6%) Total R ² (10%)	Caeruloplasmin (80%) Diabetic state (3%) Haptoglobin (1%) Total R ² (84%)

Multiple regression analysis was conducted in models containing as independent variables glycaemic- (fasting glucose, HbA_{1c}, protein- and Tf glycation) and lipid control (body mass index, triacylglycerol, HDL- and LDL cholesterol), inflammation (CRP, fibrinogen and Von Willebrand antigen) and iron proteins (serum iron, Tf, caeruloplasmin, haptoglobin, haemopexin, ferritin, sTfR, albumin). The parameters diabetic state (control vs T1DM vs T2DM), age and sex were always included. Shown are the independent determinants explaining the variance (% R² change) of antioxidants and oxidative damage. Tocopherol and MDA were expressed relative to serum total lipids (total cholesterol and triglycerides).

diabetes, higher levels of haemopexin and ferritin in T2DM, but no differences in caeruloplasmin in contrast to other studies [30]. Since iron metabolism is also known to be disturbed in diabetes, these parameters are probably the result of the cumulative effect of iron abnormalities as well as inflammation. However, little is known on the relative contribution of these two factors and the implications for oxidative stress and complications. With regard to inflammation, we observed that caeruloplasmin and haptoglobin correlated strongly with CRP in all the subgroups, whereas haemopexin only in the control subjects and T1DM patients. In contrast, ferritin did not correlate with CRP in any of the subgroups. In this regard, the role of ferritin as either a marker of iron storage or reflecting other processes such as systemic inflammation, has recently gained attention. Increased ferritin concentration as an indicator of elevated iron stores has been reported extensively as a risk factor for the development of diabetes, impaired insulin sensitivity and cardiovascular disease [31–33]. However, other studies do not support the hypothesis of iron involvement in the disease [34]. Because sTfR levels are not influenced by the acute-phase response, their measurement can aid in investigating the sources of ferritin increase. In this study, sTfR was not decreased in diabetic patients and was even higher in patients with T2DM. This observation argues against elevated body iron stores and is in accordance with the lower serum iron levels. Indeed, Tf saturation was not increased in our group of T2DM patients, which even had a higher proportion of patients with Tf saturations below 25% and with anaemia. However, the reciprocal relation between ferritin and sTfR found in T2DM patients as well as the absence of a relation between ferritin and CRP argues in favour of elevated iron stores rather than a pro-inflammatory state as the source of higher serum ferritin in this group of T2DM patients. This observation is in contrast with a recent study in which the inverse relation between sTfR and ferritin was neglected in diabetic patients and the role of inflammation as major contributor to high ferritin in diabetes was suggested [35]. These apparently contradictory observations might in part be due to interference by insulin. Since insulin increases both the translocation of TfR to the membrane [36] and the synthesis of ferritin [37], it can be postulated that the higher TfR and ferritin observed in our T2DM patients might be caused by hyperinsulinism modulating iron distribution. Indeed, several reports demonstrate independent associations between ferritin and markers of adiposity such as body mass index, body fat and waist-to-hip ratio [38,39]. In our study group this relationship was only maintained in the non-diabetic group, again emphasizing the importance of a strict control for insulin levels, body fat and degree of insulin resistance when testing this hypothesis.

In this study, we also aimed to gain more insight on the effects of inflammation and iron disturbances on diabetic oxidative damage. Our results confirm that diabetes is associated with lower antioxidant defences, depending on the type of diabetes. For example, ascorbate [7]

and α -tocopherol [40] was lower only in T2DM while retinol and plasma total antioxidant capacity, which is predominantly determined by uric acid, were lower only in T1DM [41,42]. These diabetes-related differences were significant even after correcting for age, sex, body mass index, serum lipids and smoking, all factors that are well known to affect the delicate oxidant-antioxidant balance.

When analysing the effect of high iron indices on oxidative stress parameters, we observed that the antioxidant capacity expressed as TAC-TE and its main contributing factor (uric acid), were diminished in case of higher Tf saturation in diabetic but not in control subjects. Urate exerts an important antioxidant function not only by radical scavenging but also by stabilising ascorbate through inhibition of its iron-catalysed oxidation by iron chelation [43]. On the other hand, high serum uric acid is associated with cardiovascular disease and death [44] and was elevated in our group of T2DM patients. Higher production results from increased metabolism of purines (via the xanthine oxidase pathway) as a consequence of cellular damage and ischemia-reperfusion. It has been postulated that at these high levels, uric acid can even become a pro-oxidant and thus accelerate the atherosclerotic process [45].

With regard to the protective effects of the serum proteins involved in iron transport and metabolism, Tf, caeruloplasmin and albumin determined the TAC-PI of control subjects but not of diabetic patients. We have also found that the iron-binding antioxidant capacity of diabetic plasma to prevent lipid peroxidation was impaired as a result of the loss of the protective action of Tf, caeruloplasmin and albumin and that this was caused by so far unidentified diabetic factors. These alterations in antioxidant capacity were not mediated by Tf glycation and were not accompanied by higher concentrations of non-transferrin bound iron, which is considered to be an important source of redox-active iron [46]. Other possible sources of redox-active iron that should thus merit consideration include the haem proteins. Haem proteins are extremely susceptible to damage by glucose and glycation through a process that leads to complete destruction of the haem groups and thus the possible liberation of redox-active free iron [13]. Physiologically, the ability of haem proteins to catalyse iron-induced oxidative stress is hindered by the binding of haemoglobin to haptoglobin and haem to haemopexin followed by clearance of the resulting complexes by the monocytes/macrophages and hepatocytes [12,47]. It has been observed that individuals with the haptoglobin 2 allele are more susceptible to diabetic vascular disease [48]. This is associated with impaired clearance of the glycohaemoglobin-haptoglobin-2-2 complexes, which is accompanied by an increase in redox-active iron [49]. In our study, haptoglobin concentration was found to be increased in all diabetic groups and was influenced by inflammation as illustrated by the strong positive correlation with CRP in all subgroups. Haemopexin correlated with CRP only in T1DM and control subjects but not in T2DM where the association

with inflammatory status was lost and higher levels were found independently of the CRP concentration. This difference was explained by the higher serum triacylglycerol in these patients (responsible for 43% of the variance in haemopexin). The positive relation between haemopexin and triacylglycerol has also been reported earlier in type 1 diabetic children [50]. An additional diabetes-related effect on haemopexin levels (13%) still remains to be identified. In this regard, glycation of haemopexin (Amadori products) has been detected in T2DM, where the levels decreased significantly after intensive insulin treatment [51]. However, neither haemopexin nor haptoglobin were significantly associated with oxidative stress parameters in our study group, and after correcting for the influence of CRP there remained a significant independent influence of the diabetic state and of the type of diabetes on the serum levels of these proteins. This independent effect was also observed when analysing the impact of metabolic, inflammatory and iron status on each antioxidant and oxidation product. Taken together, these observations indicate that the differences between controls, type 1 and type 2 diabetic patients with regard to metabolic, inflammatory and iron status could not totally explain the different oxidative stress status in the three groups. The pathogenic mechanisms underlying these differences merit further investigation.

In conclusion, our results indicate that the increased oxidative stress in diabetes mellitus is associated with disturbances in iron and inflammatory status, which differ in type 1 and type 2 diabetes.

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