Free Radical Research, August 2007; 41(8): 919–928

informa
healthcare

Oxidative stress in childhood type 1 diabetes: Results from a study covering the first 20 years of evolution

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Accepted by Professor F. Kelly

(Received 1 March 2007; in revised form 19 April 2007)

Abstract
This study aimed to further analyse the potential role of oxidative stress in children and adolescents with type 1 diabetes at clinical onset, during disease progression and when early microvascular complications (+ DC) appeared. Compared with age-matched controls, diabetic patients had greater oxidative damage to lipids, proteins and DNA demonstrated by analysis of plasma and erythrocyte malondialdehyde, carbonyl proteins and leukocyte 8-hydroxy-deoxyguanosine, all of which were significantly raised at onset, decreased during the first 1.5 years of evolution and rose progressively thereafter. Plasma lipid levels were significantly associated with lipid and protein oxidation products. Erythrocyte glutathione and glutathione-peroxidase activity were significantly decreased with the lowest values at onset and in + DC sub-groups. Insulin therapy in the first year improved metabolic and oxidant-antioxidant status and, consequently, hyperglycaemia-derived biomolecular oxidative damage. Diabetes-associated hyperlipidaemia is related to lipid and protein oxidation, thereby supporting the concept of glucotoxicity and lipotoxicity being inter-related. The overall increase in lipid, protein and DNA oxidative damage in diabetic patients with microangiopathy could be pathogenetically relevant in the early development of diabetes-related complications.

Keywords: *Antioxidant enzymes, glutathione, lipid peroxidation, malondialdehyde, microvascular complications, oxidative stress, protein oxidation, reactive oxygen species, type 1 diabetes mellitus*

Abbreviations: *GSH, glutathione; GPx, glutathione peroxidase; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; 8-OHdG, 8-hydroxy-2′-deoxyguanosine*

Introduction

Oxidative stress, resulting from an imbalance in redox state in which pro-oxidants overwhelm antioxidant capacity, has emerged as a potential mechanism implicated in the pathogenesis, disease progression and cell dysfunction associated with many unrelated pathologic processes including diabetes and may, therefore, contribute significantly to disease mechanisms [1]; however, increased oxidative stress may also result from pathologic processes [2]. Reactive oxygen species (ROS) are increased by hyperglycaemia which occurs during diabetes since increased glucose flux provokes oxidant production and may impair antioxidant defences by multiple interacting pathways. Several mechanisms have been proposed for the oxidative damage during chronic hyperglycaemia including mitochondrial ROS overproduction [3], direct glucose auto-oxidation, non-enzymatic glycation of proteins which can be oxidants, activation of

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ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760701435228

nitric oxide synthase, xanthine oxidase, aldose reductase and the polyol pathway which decreases NADPH/NADP ratio [4–7]. Free glucose may activate protein kinase C which, in turn, increases oxidative stress by activating mitochondrial NAD(P)H oxidase, with the subsequent decrease in regeneration of reduced glutathione, the major intracellular antioxidant [8,9]. Thus, diabetes and its complications are associated with increased oxidative stress; as a result, diabetic patients are exposed to excessive ROS production which outstrips endogenous antioxidant defence mechanisms and to consequent oxidation of biological macromolecules, such as lipids, protein, carbohydrates and DNA which may ensue and be reflected systemically, as reported in diabetes types 1 and 2 [9–14].

In type 1 diabetes (T1DM), insulin deficiency provokes high blood glucose levels and lipid metabolism alterations and as a result of these perturbations in metabolic homeostasis, substrate concentrations rise in plasma or other tissues. This excess of carbohydrate and lipids in the bloodstream could be a problem *per se*, since an overload of peroxidizable substrates in the circulation may be an underlying pathogenic mechanism related to deposition and oxidation of lipoproteins in the vascular wall [15] and contribute to the development of diabetes-related complications. Taking this metabolic situation into account, we aimed to assess whether increased triglyceride and cholesterol content in plasma of patients at diabetes onset continues to rise throughout the evolution of the disease, whether it implies greater susceptibility to oxidation and, if so, to what extent and whether it is related to antioxidant activity or the presence of additional factors.

The present study extended our previous investigation by analysing in depth the potential role of oxidative stress as a mechanism underlying diabetes-associated cellular damage in children and adolescents with T1DM from clinical onset and during progression of the disease. To this end, indicative markers of lipoperoxidation, protein oxidation, free radical-catalysed damage to DNA and changes in antioxidant defence systems were measured in blood samples and correlated with diabetes-associated metabolic abnormalities, long-term metabolic control parameters, diabetes duration and with the early appearance of complications.

Materials and methods

Patients

This study was conducted in 176 children, adolescents and young adults with T1DM and 140 healthy control subjects matched for sex, age and body mass index. For this cross-sectional study in which diabetic patients were studied at onset and during different periods of disease evolution, they were classified according to diabetes duration in six sub-groups: diabetes onset (DO), disease duration less than 1.5 years (<1.5D), disease duration from 1.5 to 5 years (1-5D), disease duration from 5 to 10 years (5–10D), disease duration from 10 to 20 years (10–20D) and patients in the latter group recently diagnosed (<12 months) with clinical symptoms of microvascular complications (+DC); all these 34 patients had both persistent microalbuminuria (defined as an albumin excretion rate > 20 μg/min in two of three overnight urine collections) and background retinopathy. Population characteristics of the study groups are shown in Table I. The number of subjects followed during the different time points of disease evolution was: seven diabetic children were studied at four different time points with follow-ups ranging from 5 to 8 years; 12 three times with follow-ups ranging from 2 to 8 years; 67 twice and 90 once with follow-ups ranging from 1 to 8 years. The 75 DO patients were evaluated between 7–10 days after clinical onset of diabetes when hydroelectrolytic disorders and acidosis had returned to normal with therapy; this group of patients presented the highest levels of glycosylated haemoglobin (HbA1c) and fructosamine (Table I). The remaining five diabetic groups presented similar plasma values of HbA1c and fructosamine. At the time of sample extraction, all diabetic patients had plasma bicarbonate levels within the normal range of 20–23 mEq/l; serum acid-base electrolytes were also normal and none of our diabetic patients had ketone bodies in urine in any of the 5 days prior to the time blood samples were taken. The general schedule for the insulin treatment of children with T1DM in this hospital is: prepubertal stage: 0.6–0.8 U/K/day; puberty: 0.8–1.2 U/K/day; postpuberty-adulthood: 0.7–0.9 U/K/day. Insulin therapy was optimized with rapid-action insulin analogues before meals and slow-action insulin analogues in the morning and at night. In addition to this optimization, therapy was tailored to the individual needs of each patient.

Blood samples of 140 healthy controls were obtained from blood analyses prior to minor surgery (hernia, fimosis, wisdom tooth extractions, minor plastic surgery, etc). Informed consent was obtained from all the individuals after the purpose and nature of the study had been explained. All patients were diagnosed and followed up at the Paediatric Diabetes Unit of the Vall d'Hebron Children's Hospital. The study was approved by the hospital Ethics Committee for Clinical Research.

Blood sample collection and processing

Venous blood samples were drawn in fasting state into tubes containing EDTA and processed immediately as previously detailed [12–14]. A total of 288 diabetic blood samples were drawn during periodic routine control analyses. Glycated haemoglobin (HbA1c) and

Table I. Clinical and biochemical characteristics of study subjects.

	Controls	Diabetic patients				
		At onset (DO)	<1.5D	1.5–5D	5–10D	10–20D	+DC
n	140	75	30	48	61	40	34
Age (years) (range)	13.7 ± 0.9 (1–30)	9.1 ± 0.43 (1–17)	9.76 ± 0.78 (3–18)	12.2 ± 0.55 (5–22)	16.1 ± 0.55 (7–24)	20.05 ± 0.71 (13–27)	25.94 ± 0.75 (20–36)
Diabetes duration (years) (range)	–	–	0.88 ± 0.06 (0.5–1.5)	3.28 ± 0.16 (1.5–5)	7.44 ± 0.175 (5–10)	14.7 ± 0.5 (10–20)	18.96 ± 1.1 (13–32)
HbA_{1c} (%)	4.6–6.6⋆	11.6 ± 0.3	8.2 ± 0.2	9.03 ± 0.22	8.7 ± 0.24	8.39 ± 0.29	8.4 ± 0.3
Fructosamine (μM)	180–285⋆	488.6 ± 18.7	360.4 ± 18.7	371.4 ± 15.3	399.4 ± 15.3	388.5 ± 12.3	384.4 ± 15.1
Cholesterol (mM)	4.04 ± 0.05	4.8 ± 0.11[†]	4.2 ± 0.11	4.6 ± 0.11[†]	4.6 ± 0.1[†]	4.8 ± 0.14[†]	4.85 ± 0.17[†]
Triglycerides (mM)	0.6 ± 0.02	0.81 ± 0.05[‡]	0.6 ± 0.04	0.57 ± 0.03	0.69 ± 0.05	0.97 ± 0.09[†]	1.17 ± 0.14[†,¶]

Data are means ± SEM. Sub-groups of diabetic patients: diabetes onset (DO), disease duration < 1.5 years (<1.5D), disease duration from 1 to 5 years (1.5–5D), disease duration from 5 to 10 years (5–10D), disease duration from 10 to 20 years (10–20D), patients with microvascular complications (+ DC), as defined in subjects and methods section; ⋆Data in parentheses are normal ranges; [†]$p < 0.0001$ vs. control subjects; [‡]$p < 0.005$; [¶]$p < 0.05$ vs. (10–20D).

microalbuminuria were immediately tested with a DCA 2000®+ analyser with DCCT and IFCC (International Federation of Clinical Chemistry and Laboratory Medicine) tractability (Bayer Co.). For measuring HbA1c, the analyser utilizes an immunochemical technique that is linear from 2.5 to 14%. Microalbumin analysis follows an immunoturbidimetric method using an albumin-specific antibody. Creatinine was measured with a colorimetric method using Benedict–Behre reaction and the albumin/creatinine ratio was used to correct for urine concentration differences. This analyser has a high degree of reproducibility: CV < 2.6% in normal range. Aliquots of plasma and washed erythrocytes were stored at − 85°C until prompt analyses. Glutathione was analysed in erythrocytes within 6 h after blood sample collection and maintained at 4°C during this time. Erythrocyte superoxide dismutase (SOD) and whole blood glutathione peroxidase (GPx) were stored at − 85°C until analysed within 1 week since we had previously observed that the enzymatic activities of SOD and GPx did not vary in that time [12]. All the above analytical procedures were performed according to those established by our laboratory and previously reported [12–14,16].

Isolation, preparation and enzymatic digestion of leukocyte DNA samples

Total DNA was extracted from white blood cells and purified using a DNA purification kit (MasterPure™ Epicentre, Madison, WI, USA) and hydrolysed for nucleoside preparation. Extracted DNA was dissolved and stored at − 80°C under nitrogen until hydrolysis with DNase I, P1 nuclease and alkaline phosphatase [17].

Determination of 8-hydroxy-2′-deoxyguanosine (8-OHdG)

The specific content of 8-OHdG in DNA hydrolysate samples was measured in duplicate using a competitive ELISA kit (8-OHdG Check; JAICA, Shizuoka, Japan) following the supplier's instructions. The amount of hydrolysed DNA used for assay in the wells was 2–10.3 μg/50 μl as determined by ultraviolet analysis and lay in the range suitable for analysis of 8-OHdG in DNA samples. Precision assessed by eight repeated measurements of the same 8-OHdG standard showed a coefficient of variation of 4.8% and 3–5% with the duplicates of hydrolysed DNA samples.

Determination of lipid and protein oxidation parameters

Plasma MDA concentration was determined as its diethylthio-barbituric acid adduct (TBA-MDA), after reverse-phase isocratic HPLC separation of the MDA-TBA complex, as previously described in detail [13,14,18]. Erythrocyte MDA content was also

determined by reversed-phase HPLC with fluorescence detection, as we detailed previously [14,16]. Plasma protein carbonyl group levels were evaluated following the 2,4-dinitrophenylhydrazine assay [19] with slight modifications.

Statistical analysis

Data were analysed with the StatView 4.5 statistical program (Abacus, Berkeley, CA). Statistical comparisons among groups were performed by analysis of variance. Linear regression analysis was used to study associations between variables. Data were expressed as means ± SEM. *P*-values ≤0.05 were considered significant.

Results

A description of the clinical characteristics and biochemical and metabolic control parameters of this wide age-range group of diabetic patients from disease onset up to 20 years of disease evolution and of the control group can be found in Table I. Glycated haemoglobin (HbA_{1c}) and fructosamine levels were raised in all sub-groups of diabetic patients, with the highest values being found at disease onset; insulin therapy produced a clear improvement in metabolic control as reflected by a marked reduction in HbA_{1c} and fructosamine. Plasma cholesterol and triglyceride levels were significantly increased at diabetes onset; however, while triglyceride remained in normal range during the first 10 years of evolution and rose significantly thereafter, a slight decrease in cholesterol was observed in the first 1.5 years followed by a gradual, significant increase over the study period.

Plasma levels of MDA, the most widely used marker of lipid peroxidation, were significantly elevated at diabetes onset and decreased during the first 1.5 years of evolution, after which, a significant trend towards greater and progressive increases was clearly observed (Figure 1A). Plasma protein carbonyl content, a marker of protein oxidative damage, was patent and significantly increased in all diabetic patient groups throughout the study period, with levels at onset (+66%) and in + DC (+90%) being the most marked (Figure 1B) and were positively correlated with the sum of plasma triglyceride and cholesterol values ($r = 0.244$; $p < 0.001$). Single linear regression analyses revealed a significant correlation between plasma levels of oxidizable lipids and MDA ($r = 0.254$; $p < 0.0001$).

Erythrocyte MDA concentrations were significantly higher only at diabetic onset, remaining similar to those of controls thereafter; er-MDA values in (+ DC) subgroup were 35% higher than in controls (Figure 2A). The drop in er-MDA was associated with the improvement in metabolic control since significant correlations were found between er-MDA and HbA_{1c} ($p < 0.01$) and er-MDA and fructosamine ($p < 0.001$). At diabetes onset, an increase in oxidative DNA damage was observed, as determined through leukocyte 8-OHdG content, which decreased up to 5 years, rising progressively thereafter and reaching significance in the group of diabetic patients with complications (Figure 2B).

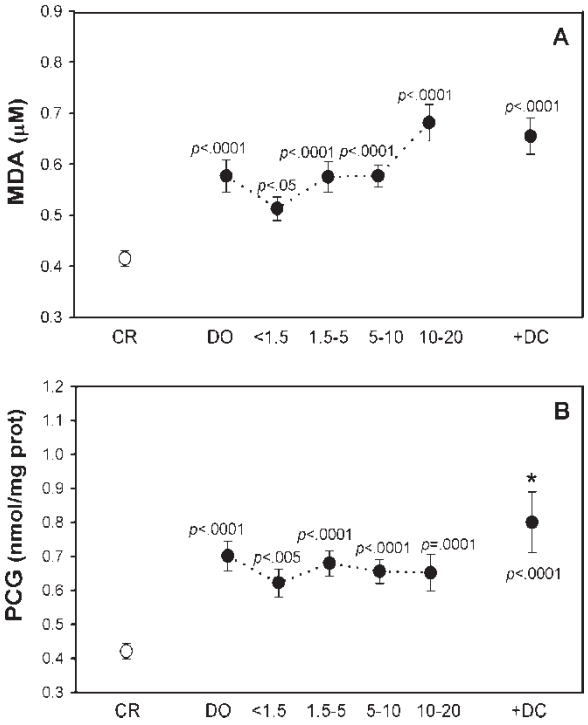

Figure 1. Plasma levels of malondialdehyde (A) and plasma protein carbonyl groups (PCG) (B) in diabetic patients at DO, during the first 20 years of disease evolution (four sub-groups: < 1.5, 1.5–5, 5–10, 10–20), in diabetic patients with complications (+DC) (filled black circles) and in controls (CR) (open circles). Values are expressed as mean ± SEM vs. controls; *p*-values are shown on the graphs. *$p < 0.05$ diabetic patients with complications (+ DC) vs. [10–20] sub-group of patients.

Both GPx activity and erythrocyte glutathione content in all diabetic patient groups, except < 1.5D, were significantly lower than in controls and followed a similar pattern, with the DO sub-group presenting the lowest glutathione values (Figure 3A and B). Antioxidant enzyme SOD activity was significantly elevated in red blood cells of diabetic patients at onset and from 5 years of evolution on (Figure 3C). Correlation coefficients and rank of significance with age, disease duration and sex of diabetic patients for every measured parameter are shown in Tables II and III. We found no relationship between sex and any of the metabolic or oxidative stress parameters studied (Table III).

The subdivision of the control group into four subgroups with ages comparable to the respective diabetic groups, i.e. 1–5 years; 6–9 years; 10–15 years and > 15 years is shown in Table IV, where it can be observed that concentrations of the different metabolites, parameters of molecular oxidative

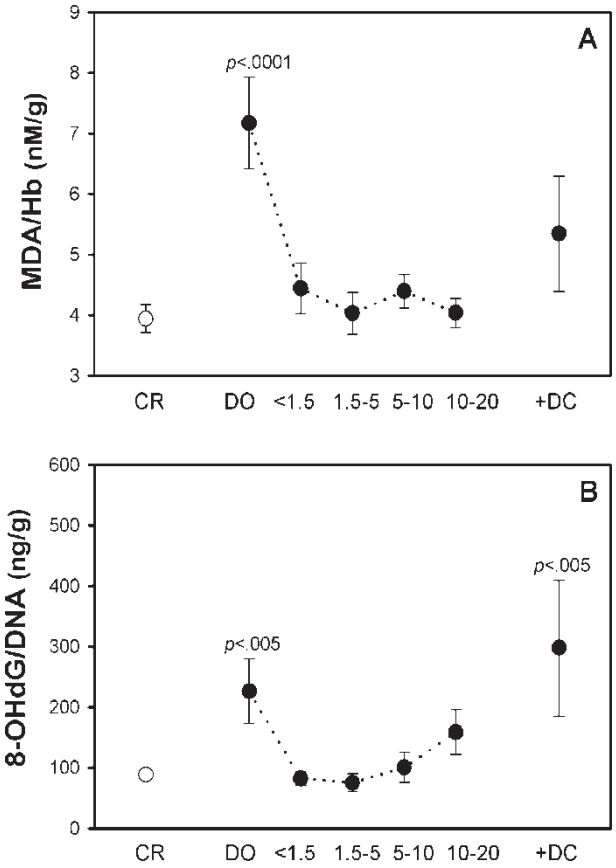

Figure 2. Erythrocyte malondialdehyde levels (A) and leukocyte 8-hydroxy-2′-deoxyguanosine levels (B) in diabetic patients at DO, during the first 20 years of disease evolution (four sub-groups: < 1.5, 1.5–5, 5–10, 10–20), in diabetic patients with complications (+DC) (filled black circles) and in controls (CR) (open circles). Data are means ± SEM vs. controls; *p*-values are shown on the graphs.

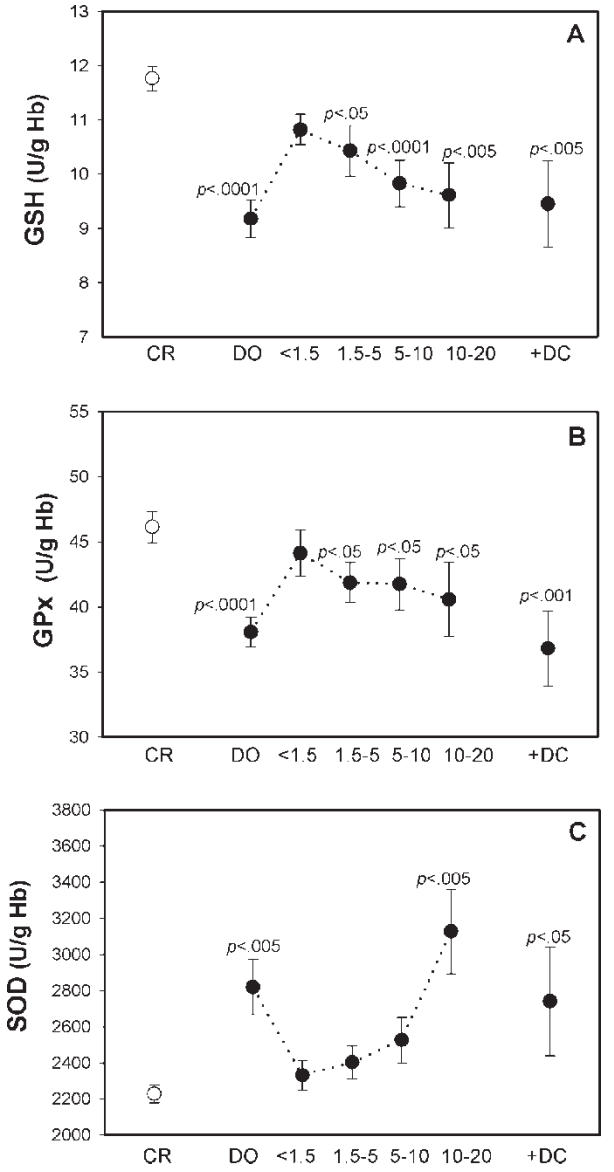

Figure 3. Erythrocyte glutathione content (A), erythrocyte glutathione peroxidase activity (B) and erythrocyte superoxide dismutase activity (C) in young diabetic patients at disease onset (DO), during the first 20 years of disease evolution (four sub-groups: < 1.5, 1.5–5, 5–10, 10–20), in diabetic patients with complications (+ DC) (filled black circles) and in controls (CR) (open circles). Values represented are mean ± SEM vs. controls; *p*-values are shown on the graphs.

damage or enzyme activities measured are not age-related or affected by sex or pubertal stage.

Discussion

This comprehensive study closely followed, for the first time, the evolution of oxidative stress from onset and for up to 20 years in relation to parameters of metabolic control of the disease and lipid abnormalities in children with T1DM. The results of this work provide evidence that hyperglycaemia-derived oxidative stress was already present at diabetes onset and almost disappeared during the first year of insulin treatment. Thus, the metabolic efficacy of insulin is not limited to its direct action on glucose homeostasis maintenance; indeed, insulin exerts other beneficial effects, probably by reducing levels of free fatty acids and triglycerides, substrates which are susceptible to lipid peroxidation. As a consequence of this beneficial impact of insulin, an improvement in levels of cytotoxic products of oxidative damage was observed in this 'honeymoon' period. In the present study in diabetic children and adolescents, we observed that triglycerides remained unaltered during the first 10 years of disease evolution but began to rise thereafter, with the highest values being observed in patients in whom complications had already appeared. In parallel, plasma MDA levels rose from < 1.5 years and continued to rise thereafter and, again, the highest values were found in patients with diabetic complications, i.e. changes in plasma lipid levels were in line with those observed in MDA formation with similar graphic patterns and, again, the nadir was in the 'honeymoon' period. These results are consistent

Table II. List of measured parameters and statistical analysis of their correlations with age and disease duration in the diabetic patient group.

	Age		Disease duration	
	r	*p*	*r*	*p*
HbA_{1c} (%)	−0.28	<0.0001	−0.302	<0.0001
Fructosamine (μM)	−0.19	0.01	−0.2	0.002
Cholesterol (mM)	0.06	NS	0.08	NS
Triglycerides (mM)	0.28	<0.0001	0.33	<0.0001
MDA (μM)	0.19	0.001	0.17	0.004
er-MDA (nmol/g Hb)	−0.28	0.001	−0.2	0.02
PCG (nmol/mg prot)	0.13	0.04	0.13	NS
8-OHdG/DNA (ng/g)	0.14	NS	0.07	NS
GSH (μmol/g Hb)	−0.16	0.01	−0.13	0.02
GPx (U/g Hb)	−0.04	NS	−0.02	NS
SOD (U/g Hb)	0.08	NS	0.11	NS

Note: NS, no significant values ($p > 0.05$); HbA_{1c}, glycosylated haemoglobin; MDA, malondialdehyde; er-MDA, erythrocyte malondialdehyde; PCG, protein carbonyl groups; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; GSH, glutathione; GPx, glutathione peroxidase; SOD, superoxide dismutase; Hb, haemoglobin.

with the hypothesis that diabetes-associated dyslipidemia could, together with hyperketonaemia, contribute to lipid peroxidation, as has been observed in diabetic patients [20,21]; however, none of our diabetic children had presented episodes of hyperketonaemia and no traces of ketones were found in urine at the time of extraction. However, puberty, with the changes in sex hormone levels, did not appear to be a factor affecting oxidant–antioxidant status in either diabetic patients or their corresponding controls in this study. Neither were differences in oxidative damage biomarkers or mechanisms of antioxidant activity observed between diabetic girls and boys; thus, the possible protective effect of oestrogens in normalizing blood lipids in post-pubertal girls does not appear to withstand the augmented oxidative stress provoked by diabetes.

Hyperlipidaemia in diabetes is considered to be a significant risk factor for the development of micro-vascular complications [8,15] and an association between lipid peroxidation parameters and diabetic angiopathy has been reported in both young adults with early nephropathy [22] and in diabetic patients with long-term complications [10,11]. A direct effect of triacylglycerols causing increased mitochondrial ROS production and resulting in cellular necrosis in cultured macrophages has recently been described as a further mechanism underlying oxidative stress-mediated lipotoxicity and may be relevant to the development of atherosclerosis [23]. Should an excess of lipids, the substrate under greatest attack by free radicals, be a major source of increased oxidative stress in diabetes, this pathogenic pathway could be a therapeutic target for attenuating molecular oxidative damage, thereby preventing the acceleration of atherosclerosis from the early stages of diabetes. Therefore, it is worthwhile considering the use of statins, the beneficial effects of which are mainly attributed to their cholesterol-lowering properties but which also include other possible pleiotropic effects related to their anti-inflammatory and antioxidant activities [24,25]. Notably, recent reports have revealed that statins may inhibit superoxide production in vascular cells [26] and that they attenuate high glucose-induced and diabetes-associated oxidative stress *in vitro* and *in vivo* [27], thereby providing new insights into antioxidative therapy in diabetes.

Oxidative damage to proteins was also clearly shown to be continuous from onset and through the first 20 years of evolution in our diabetic patients and was significantly greater in those with early complications; interestingly, blood levels of these abnormal products correlated positively with those of total lipids in the whole study cohort. This noteworthy finding points to the deleterious role of oxidative injury to proteins in the early development of diabetic microvascular complications, thereby linking protein oxidation to disease progression, which is plausible since cells are unable to repair oxidatively-modified proteins and the

Table III. Plasma levels of cholesterol, triglycerides, malondialdehyde and protein carbonyl groups, leukocyte 8-hydroxy-2′-deoxyguanosine levels (8-OHdG), erythrocyte malondialdehyde content, glutathione content, glutathione peroxidase activity and superoxide dismutase activity in erythrocytes of diabetic patients according to sex.

	Male ($n = 137$)	Female ($n = 151$)	*p*
HbA_{1c} (%)	9.02 ± 0.2	9.28 ± 0.2	NS
Fructosamine (μM)	415.5 ± 16.1	420.2 ± 10.05	NS
Cholesterol (mm)	4.6 ± 0.08	4.7 ± 0.07	NS
Triglycerides (mM)	0.72 ± 0.03	0.81 ± 0.05	NS
MDA (μM)	0.58 ± 0.02	0.59 ± 0.021	NS
er-MDA/Hb (nmol/g)	4.9 ± 0.4	5.8 ± 0.4	NS
PCG (nmol/mg prot)	0.7 ± 0.03	0.67 ± 0.03	NS
8-OHdG/DNA (ng/g)	129.2 ± 20.3	149.3 ± 27.6	NS
GSH (μmol/g Hb)	9.4 ± 0.2	9.8 ± 0.2	NS
GPX (U/g Hb)	38.8 ± 1.1	41.7 ± 1.03	NS
SOD (U/g Hb)	2727.8 ± 110.1	2618.2 ± 95.1	NS

Data are means ± SEM. NS, No significant values ($p > 0.05$).

Table IV. Plasma levels of cholesterol, triglycerides, malondialdehyde and protein carbonyl groups, leukocyte 8-hydroxy-2′-deoxyguanosine levels (8-OHdG), erythrocyte malondialdehyde content, glutathione content, glutathione peroxidase activity and superoxide dismutase activity in erythrocytes of control subjects.

	n	Cholesterol (mM)	Triglycerides (mM)	MDA (μM)	MDA/Hb (nM/g)	PCG (nmol/mg protein)	8-OHdG/DNA (ng/g)	GSH (μmol/g Hb)	GPx (U/g Hb)	SOD (U/g Hb)
Age (years)										
1–5	17	3.8 ± 0.2	0.56 ± 0.05	0.43 ± 0.04	3.3 ± 0.5	0.46 ± 0.04	73.4 ± 4.7	11.7 ± 0.9	47.4 ± 1.6	2295.8 ± 260.8
6–9	23	3.8 ± 0.13	0.57 ± 0.02	0.37 ± 0.03	4.1 ± 0.4	0.39 ± 0.06	89.7 ± 34.02	10.6 ± 0.6	46.6 ± 1.8	2458.5 ± 100.5
10–15	53	4.09 ± 0.07	0.62 ± 0.03	0.42 ± 0.02	3.8 ± 0.3	0.43 ± 0.03	91.89 ± 15.7	11.3 ± 0.3	44.9 ± 1.6	2349.8 ± 86.8
>15	47	4.1 ± 0.1	0.63 ± 0.03	0.42 ± 0.02	4.2 ± 0.5	0.37 ± 0.05	88.1 ± 9.7	11.8 ± 0.4	47.8 ± 3	2109.3 ± 70.5
Sex										
Male	77	4.03 ± 0.07	0.61 ± 0.02	0.43 ± 0.02	3.8 ± 0.34	0.4 ± 0.03	89.5 ± 11.2	11.3 ± 0.33	46.2 ± 1.7	2309.3 ± 87
Female	63	4.06 ± 0.08	0.6 ± 0.03	0.39 ± 0.02	4.0 ± 0.31	0.41 ± 0.04	87.6 ± 8.1	11.9 ± 0.42	46 ± 1.6	2157.9 ± 69

Data are means ± SEM.

introduction of carbonyl derivatives may determine the partial or total inactivation of proteins [28]. Furthermore, oxidative damage to proteins is one mechanism by which oxidants promote inflammatory damage, enhance the tissue-destructive effects of proteolytic enzymes [29] and provoke other modifications, all of which may lead to severe failure of biological functions and cell death. In addition, a recent study described a novel reaction pathway for glucose-mediated protein oxidation which requires both polyunsaturated fatty acids and reactive carbonyl groups and the complicity of this pathway in diabetic microvascular disease [30].

Diabetes induced significant changes in erythrocyte antioxidant enzyme activities in our children and adolescents as early as at onset, which stabilized in ensuing stages and became significant again after 1.5 years, and to a greater extent in patients with microvascular complications. Interestingly, the two enzymes which form the first line of intracellular antioxidant defence behaved differently: SOD activity was significantly elevated particularly at onset and from 10 years of evolution onwards while GPx was markedly decreased at onset, improved with insulin but worsened after 1.5 years. At DO, an early upregulation of endogenous CuZnSOD was produced, which resulted in clearly increased antioxidant activity coinciding with the periods of greater oxidant stress, consistent with a concerted adaptive response to elevation of oxidizing species that involves a specific compensatory increase in the activity of this important scavenger protein. Studies on the expression of antioxidant enzymes in blood mononuclear cells from diabetic patients exposed to hyperglycaemia *in vitro* found that CuZnSOD was increased two-fold in patients without nephropathy, while the response of antioxidant genes was abnormal in those with diabetic nephropathy [31]. Our results indicate that CuZnSOD is induced in erythrocytes of young diabetic patients with or without complications, which suggests that the antioxidant response to hyperglycaemia *in vivo* appears to differ from that observed *in vitro* or when cells from diabetic patients are exposed to hyperglycaemia *ex vivo*.

In contrast, activation of endogenous GPx did not appear to be produced, likely due to decreased erythrocyte content of glutathione, the most abundant intracellular antioxidant. In fact, we observed a parallel decrease in both the total content of tripeptide glutathione and GPx activity in diabetic patients throughout the study period. Antioxidant functions of glutathione include scavenging singlet oxygen and hydroxyl radicals and acting as a cosubstrate of GPx in the elimination of organic peroxides. Interestingly, a beneficial rise in antioxidant activity of cytosolic GPx was produced with insulin treatment over the first 1.5-year honeymoon stage and permitted acceptable levels to be maintained for up to 10 years of disease

progression. However, in patients with diabetic microangiopathy, GPx activity was markedly low, which could lead to unfavourable sequelae, since GPx is the only antioxidant enzyme known to directly reduce phospholipid hydroperoxides within membranes and lipoproteins. Although glutathione and enzymes included in the glutathione redox cycle play an essential role in protecting cells against free radical-mediated damage, they may themselves be susceptible to oxidation and, in fact, GPx may be inactivated in high oxidative stress conditions. It has been reported that GPx presents higher susceptibility to oxidation in diabetic patients than that of controls and even greater in those from diabetics with poor metabolic control [32]. In our group of young diabetic patients, we found no significant positive correlation between HbA1c levels and GPx activity throughout the period of study; however, it was at disease onset when the highest HbA1c levels and lowest Gpx activity were observed, which coincided with the lowest glutathione concentrations, indicating that poor metabolic control is associated with blood glutathione depletion. Insulin administration boosted recovery of redox state which can be observed via the replenishing of levels of the major intracellular non-protein thiol. The decrease in glutathione content does not appear to be related to the decrease in synthesis, as recently described in a group of diabetic adolescents [33]; rather, their findings suggest that glutathione depletion arises from an increased utilization rate. Chronic hyperglycaemia, through the polyol pathway, increases consumption of NADPH, the cofactor also required by glutathione reductase, thus impairing oxidized glutathione regeneration [9,34]. Glutathione depletion may have deleterious long-term consequences in diabetic adolescents since their endogenous antioxidant defences, mainly GPx activity, become weakened. Indeed, erythrocyte GPx activity has recently been shown to be independently associated with an increased risk of cardiovascular events and may therefore have prognostic value [35].

Oxidative stress, due to the above-mentioned consequences of hyperglycaemia, altered energy metabolism and changes in antioxidant defence systems among the other suggested causes, contributes to macromolecular oxidative damage of lipids and proteins, and an important aim of this study was to ascertain whether it causes oxidative alterations on DNA. The lesions induced by ROS on DNA are typically breakage of a single filament or oxidative alteration of purinic and/or pyrimidinic bases such as 8-OHdG, an oxidized form of guanine, the major DNA-damage product [36]. Our results showed that 8-OHdG accumulation in DNA of circulating leukocytes was markedly raised at DO, became normal once insulin therapy had been started and during evolution of the disease, and clearly reappeared in patients with microvascular complications. Hence, DNA oxidative damage appears to occur in the early stages of disease, probably as a consequence of both hyperglycaemia and autoimmune inflammation, and at later stages only in leukocytes from patients with early development of complications, thereby pointing to these periods as the most sensitive to oxidative damage. Differential sensitivity to DNA oxidation, alterations in the antioxidant defence mechanisms or in repair capacity are among the causes suggested to account for the extent of DNA oxidative damage [37]. As DNA damage is efficiently repaired by cellular enzymes, its measurement gives a snapshot view of the level of oxidative stress, in contrast to measurement of oxidation of other biomolecules which are not repaired or have a slow turnover, such as lipids or proteins [38]. Moreover, the possibility that individual defects in enzymes that reduce oxidative DNA damage contribute to an increase in damaged DNA cannot be ruled out, since 8-OHdG levels in DNA reflect the balance between oxidative damage and repair rate.

To our knowledge, this is the first study to assess leukocyte 8-OHdG levels in young diabetic patients at disease onset, over 10 years of diabetes evolution and up to the early appearance of complications. Our findings, therefore, are not fully comparable to those previously reported in adult type 1 or 2 diabetic patients [38–40], although a recent study conducted in diabetic children found no substantial changes in oxidative DNA damage probably due to a significantly increased DNA repair capacity [37]. It is interesting to note that the increased 8-OHdG content in nuclear DNA may result from a generalized increase in cellular oxidative damage and/or a decrease in antioxidant defences such as glutathione and GPx activity, which are inversely correlated with leukocyte 8-OHdG. The low GPx activity observed in our diabetic patients, particularly those with microangiopathy, may have been insufficient to reduce ROS generated by overexpressed SOD activity, leading to a disequilibrium between the two antioxidant enzymes that could account for ROS overproduction and contribute to enhancing DNA oxidation. In this regard, it has been observed that, under hyperglycaemic conditions, endogenous aldehydes have great potential to oxidatively damage DNA [41].

While we consider this work contributes to understanding of the mechanisms underlying oxidative stress generation in T1DM after close observation of clinical and metabolic control parameters together with the main molecular indicators of oxidative stress in a young patient cohort over a long period of time, the study has some limitations. First, different numbers of patients were studied in each group of children and adolescents with T1DM at clinical onset, during disease progression and when microvascular complications appeared; although the majority of patients were consecutively studied in at least two stages, this was mainly a cross-sectional study with

follow-up over a long period of time rather than a strictly longitudinal study of the same patients. Second, the most patients who developed complications could have had increased oxidative stress, potentially induced by their dyslipidaemia or weakened antioxidant defences, as noted earlier, the individual detection of which should be a major therapeutic goal. In conclusion, the results of this novel, comprehensive study, which closely followed oxidative stress evolution in children and adolescents with T1DM, provide further solid evidence linking oxidative processes to diabetes. The link is supported by the finding that increased hyperglycaemia-derived molecular oxidative damage was already present at DO, almost disappeared during the first 1.5 years of insulin treatment, thereby improving both glycometabolic control and oxidant status, and rose progressively thereafter, particularly in young diabetic patients with complications. The overall increase in lipid, protein and DNA oxidative damage in diabetic patients with microangiopathy points to molecular oxidative damage as a potential mechanism that precedes or accompanies the early stages of diabetes-related complications, which further supports the hypothesis that increased oxidative stress may be implicated in the high incidence of vascular disease found in diabetes. This cumulative increase in cellular and molecular oxidant injury markers in young diabetic patients and their association with the early development of microvascular complications merit further study to pinpoint the pathogenetic disease mechanisms and enable the development of possible therapeutic strategies. Moreover, we showed that hyperlipidaemia caused by diabetes-associated loss of optimal regulation of lipid metabolism implies greater susceptibility to oxidation that would lead and significantly contribute to the extent of lipid and protein oxidation, thereby supporting the concept of glucose toxicity and lipotoxicity being inter-related. Therefore, concerted efforts should be made to reduce oxidative lipaemia and oxidative stress in an attempt to potentially delay the development of premature microvascular complications in diabetes.

Acknowledgements

This work was supported by grants from FIS (99/1103, 02/0852), Centre Network RCMN (C03/08) and Group Network RGDM (G03/212) financed by the Carlos III Institute of Health, Spain. We are grateful to Miss C. O'Hara for her help in the English version of this manuscript.

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