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Oxidative stress, erythrocyte ageing and plasma non-protein-bound iron in diabetic patients

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Abstract

Increased oxidative stress and decreased life span of erythrocytes (RBCs) are repeatedly reported in diabetes. In the aim to elucidate the mechanism of the latter, i.e. the events leading to erythrocyte ageing, this study determined in RBCs from diabetic patients iron release in a free desferrioxamine-chelatable form (DCI), methemoglobin (MetHb) formation, binding of autologous IgG to membrane proteins and in plasma non-protein-bound iron (NPBI), F_2-Isoprostanes (F_2-IsoPs) and advanced oxidation protein products (AOPP). DCI and MetHb were higher in diabetic RBCs than in controls and autologous IgG binding occurred in a much higher percentage of diabetic patients than controls. A significant correlation between DCI and IgG binding was found in diabetic RBCs. Plasma NPBI, esterified F_2-IsoPs and AOPP were higher in diabetic patients and a significant correlation was found between plasma NPBI and intra-erythrocyte DCI. The increased DCI and autologous IgG binding appear to be important factors in the accelerated removal of RBCs from the blood stream in diabetes and the increase in plasma NPBI could play an important role in the increased oxidative stress.

Keywords: *Redox-active iron, erythrocytes, diabetes, oxidative stress, oxidative damage, F_2-isoprostanes*

Abbreviations: *AGEs, advanced glycation end-products; AOPP, advanced oxidation protein products; NH2: aminopropyl; BSA, bovine serum albumin; BCIP-NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; BHT, butylated hydroxytoluene; DFO, desferrioxamine; DCI, desferrioxamine-chelatable iron; F2-IsoPs, F2-Isoprostanes; HbA1c, glycated haemoglobin; HPLC, high performance liquid chromatography; IgG, immunoglobulin G; LDL, low density lipoproteins; MetHb, methemoglobin; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid; C18, octadecylsilane; PBS, phosphate buffered saline; NPBI, plasma non-protein-bound iron; PGF2, prostaglandin F2; RBCs: red blood cells*

Introduction

Our previous studies [1,2] of erythrocyte ageing have shown a relation among oxidative stress, iron release in a free desferrioxamine (DFO)-chelatable form (DCI), oxidative alterations of membrane proteins and autologous IgG binding to band 3 dimers (the 170-kDa band which marks the erythrocytes (RBCs) for removal from blood stream [3]). Iron is released from haemoglobin when RBCs are challenged by an oxidative stress *in vitro* [2,4,5] and *in vivo* [6,7] and the release is accompanied by methemoglobin (MetHb) formation [1,5–7]. In a condition in which increased oxidative stress and accelerated removal of RBCs occur, the perinatal period [8], the intraerythrocyte

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DCI was higher than in adult RBCs [7,9] and the binding of autologous IgG to band 3 dimers occurred in a much higher percentage than in adults [9]. A significant correlation between intraerythrocyte DCI level and IgG binding was also found. Moreover plasma level of F_2-Isoprostanes (F_2-IsoPs), the most reliable markers of oxidative stress [10], was markedly increased [11].

Increased conditions of oxidative stress and decreased life span of RBCs are repeatedly reported in diabetic patients [12–15]. Diabetes-induced oxidative damage may be prominent in RBCs due to high iron and polyunsaturated fatty acid content [15]. RBC properties are critically affected by hyperglycaemia, with increase in glycated haemoglobin, decreased deformability, increased erythrocyte aggregation [16] and accumulation of advanced glycation end products (AGEs) in the membrane [14,17] and these alterations can lean toward early senescence with a decreased life span.

Some years ago a form of non-transferrin, non-protein-bound iron (NPBI) was described [18] in plasma of subjects with increased sensibility to oxidative stress, such as newborns, particularly premature newborns, and in subjects with disturbances in iron metabolism, such as haemochromatosis [19] and thalassemia [20]. Since such a form of iron could be redox active, it has attracted the attention of diabetologists [21,22] searching for the origin of oxidative stress in diabetes and increased levels of plasma NPBI have been recently reported in type 2 diabetes [23,24].

Since the accelerated removal of newborn RBCs seems to be, as mentioned above, the result of increased oxidative stress, in the present report we investigated whether in diabetic subjects too an increased oxidative stress could be related to an accelerated removal of RBCs. To this end we evaluated in RBCs the levels of DCI, MetHb and autologous IgG bound to band 3 dimers and in plasma the levels of F_2-IsoPs, NPBI and advanced oxidation protein products (AOPP) [25].

The results indicated that, as compared to healthy controls, intraerythrocyte DCI and MetHb were higher in diabetic subjects and IgG binding occurred in a much higher percentage of cases. Also in diabetic subjects NPBI was higher and correlated to intraerythrocyte DCI. F_2-IsoPs and AOPP were similarly increased.

Materials and methods

Materials

Desferrioxamine (DFO) was supplied by Ciba-Geigy (Ciba-Geigy Ltd, Basel, Switzerland). Centrifugal filters (VIVASPIN 4) were purchased from Sartorius Stedim Biotech GmbH (Goettingen Germany) and the reservoirs for silicic acid column chromatography were purchased from Varian (Varian Inc., CA). Iron sulphate heptahydrate and HPLC grade solvents were obtained from J.T. Baker (J.T. Baker, Deventer, The Netherlands). The nitrocellulose Hybond-C extra was supplied by Amersham Life Science (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Goat anti-human IgG (Fc-specific) alkaline phosphatase conjugate, used as secondary antibody, 5-Bromo-4-Cloro-3-Indolyl Phosphate/Nitro blue tetrazolium liquid substrate system (BCIP/NBT), 2,6-Di-tert-butyl-4-methylphenol (BHT) and Chloramine T were purchased from Sigma-Aldrich (Sigma-Aldrich, St.Louis, MO). Tetradeuterated 8-epi-PGF2α were obtained from Cayman (Cayman Chemicals, Ann Arbor, MI). Sep-Pak® Vac C_{18} (500 mg) and Sep-Pak® Vac NH_2 (500 mg) cartridges were purchased from Waters (Waters Corporation, Milford, MA). Bio-Rad Protein Assay was obtained from Bio-Rad (Bio-Rad Laboratories, Hercules, CA).

Subjects

Our study included 76 diabetic subjects, types 1 and 2 (25 and 51, respectively). The subjects were randomly selected during routine examination appointments from January–June 2006. Fasting blood samples were collected from 7.30 am to 8.30 am. Diabetic patients were receiving insulin treatment or oral antidiabetics (sulphonylurea derivatives or metformin) associated to dietetic treatment [26]. Thirty-nine healthy subjects were randomly selected among blood donors and used as controls. The clinical data of diabetic patients and control subjects are reported in Table I . Diabetic patients were investigated to identify the chronic complications, both in microangiopathy (retinopathy, nephropathy-microalbuminuria and neuropathy) and in macroangiopathy (peripheral pulses, common carotid artery intimal-medial thickness (CCA-IMT) and ECG). No subjects had haematological disorders. Informed consent was obtained from all subjects. The study was approved by the Human Ethics-Deontology Committee of the Medical Faculty of the University of Siena. Blood was collected in heparinized tubes and all manipulations were carried out within 2 h after collection. The blood samples were centrifuged at $2400 \times g$ for 15 min at 4°C room temperature; the plasma was saved and the buffy coat was removed by aspiration. The erythrocytes were washed twice with physiological solution, resuspended in Ringer solution (125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 32 mM HEPES, 5 mM glucose, 1 mM $CaCl_2$), pH 7.4 as a 50% (vol/vol) suspension and then used for the determination of DCI [4], MetHb [27] and autologous IgG binding to band 3 dimers [3]. Plasma was used for F2-IsoPs, NPBI, AOPP determination and for the opsonization step in IgG binding determination. Glycated haemoglobin (HbA_{1c}) was measured by HPLC using the fully automed Glycosylated Haemoglobin Analyser System

Table I. Clinical data of diabetic patients and control subjects.

	Type 1 Diabetic patients	Type 2 Diabetic patients	Control subjects
Number	25	51	39
Age (years)	50.6±3.1	68.8±1.5	46.2±0.8
Sex (M/F)	9/16	29/22	21/18
Diabetes duration (years)	17.8±4.2	12.8±1.3	—
Body Mass Index (kg/m^2)	25.2±0.8*	27.4±0.6*	22.5±0.2
Glycaemia (mmol/l)	7.93±0.54*	7.48±0.33*	4.73±0.11
HbA1c (%)	7.6±0.2*	7.5±0.2*	5.0±0.2
Total cholesterol (mmol/l)	4.92±0.17*	5.0±0.12*	4.44±0.09
HDL-cholesterol (mmol/l)	1.57±0.05	1.34±0.05*	1.70±0.04
LDL-cholesterol (mmol/l)	2.80±0.14*	2.99±0.12*	2.35±0.05
Triglycerides (mmol/l)	1.28±1.17	1.61±0.16	1.35±0.04
Creatinine (μmol/l)	74.0±7.6	69.4±3.0	61.0±7.6
Sideremia (μmol/l)	16.3±1.1	15.6±0.3	18.4±2.9
Transferrin (g/l)	2.50±0.08	2.37±0.21	2.38±0.17
Ferritin (μg/l)	87.8±27.2	91.5±5.1	115.0±32.4
Transferrin saturation (%)	26.1±1.7	26.5±1.6	26.6±0.7
Hypertension (%)	33.3	56.0	—
Macrovascular complications (%)	25.0**	57.1	—
Microvascular complications (%)	38.0	40.0	—

Data are expressed as means±SEM or as percentage.
*p <0.001 vs control subjects; **p <0.01 vs Type 2 diabetic patients.

(Bio-Rad Laboratories, Richmond, CA). The other clinical determinations were performed using routine clinical chemistry methods.

Binding of autologous IgG to band 3 dimers

The method of Turrini et al. [3] was used to evaluate the binding of autologous IgG to oxidatively modified band 3 (dimers). The advantage of the isolation of autologous IgG bound to band 3 dimers (as described in Turrini et al. [3]) is that, of all the autologous IgG antibodies, only the small fraction which specifically binds to the band 3 dimers is used and therefore the method is much more sensitive and reliable.

Briefly, RBCs were centrifuged, washed twice with HEPES buffered saline (130 mM NaCl, 10 mM HEPES, 10 mM glucose pH 7.4) and suspended at 10% hematocrit with the same buffer containing 30% (vol/vol) autologous plasma and incubated for 30 min at 37°C, to allow the binding of autologous IgG (opsonization step). The RBCs were then washed twice in HEPES and the erythrocyte membranes were prepared according to Dodge et al. [28]. Autologous IgG were then eluted from erythrocyte membrane and used as the primary antibody. At this end an aliquot of membranes (90 μl) was incubated for 5 min at 4°C with 50 mM glycine (pH 3.0) and then centrifuged at 17500 × g in an Beckman centrifuge (Beckman Coulter, Fullerton, CA); the supernatant containing IgG was neutralized with 1 M Tris-HCl, pH 7.5. Membrane proteins were quantified according to Lowry et al. [29]. Membrane proteins (10 μg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE: 10% acrylamide) under non-reducing conditions according to Laemmli [30] and then transferred to nitrocellulose according to Towbin et al. [31]. After blocking for 1 h in PBS containing 1% (wt/vol) BSA, the primary antibody (diluted 1:100) was added to PBS and the nitrocellulose was incubated for 1 h at room temperature. After washing, the nitrocellulose was incubated for 30 min at room temperature with the second antibody diluted 1:1000 in PBS. The second antibody was anti-human IgG conjugated to alkaline phosphatase. The blot was developed with BCIP-NBT for alkaline phosphatase and was quantified using scanning densitometry.

The positive control was obtained by preparing samples in which 10% control erythrocyte suspension was incubated with H_2O_2 (5 mM final concentration) for 30 min at 4°C. Afterwards the cells were washed and incubated with autologous plasma as reported above. Negative controls (not shown) were the samples not exposed to autologous plasma. The membrane proteins were blotted and then incubated with the second antibody (anti-human IgG).

Erythrocyte DFO—chelatable iron (DCI)

DCI was determined as a DFO–iron complex (ferrioxamine) as previously reported [4]. Briefly, 25 μM DFO was added to the samples, the erythrocytes were then lysed by adding water (1 vol) and by freeze (−70°C)-thawing. The hemolysate was ultrafiltered in centrifugal filters with a 30 kDa molecular weight cut-off and stored at −20°C until analysis. The DFO excess was removed by silica column chromatography. The DFO–iron complex was determined by HPLC and the detection wavelength was 229 nm.

Plasma non-protein-bound iron (NPBI)

NPBI was determined as a DFO–iron complex (ferrioxamine) as reported for erythrocyte DFO–chelatable iron (DCI). In particular 25 μM DFO was added to plasma (1 ml), the samples were diluted with Ringer solution (1 ml) and then ultrafiltered, as above. The excess of DFO was removed by silicic acid column chromatography and the DFO-iron complex was determined by HPLC. Since in these studies the level of DFO-chelatable iron to be determined in plasma was much lower (0.1–1.0 μM) than that usually measured (1.0–30.0 μM) in erythrocytes, the linearity of the method for these low levels of iron was demonstrated ($y = 78951x + 17689$) and the standard curve was obtained with increasing amounts (0.1–1.0 μM) of iron sulphate heptahydrate in the presence of 25 μM DFO.

Plasma F_2-isoprostanes (F_2-IsoPs)

BHT (90 μM) was added to plasma and plasma was stored at -70°C. The purification and the quantification of the plasma F_2-IsoPs were carried out according to Nourooz-Zadeh et al. [32] and Signorini et al. [33], respectively.

In particular, for total (sum of esterified and free) F_2-IsoPs, immediately after thawing, KOH (1 M, 500 μl) was added to plasma (1 ml). After incubation at 45°C for 45 min, the sample was acidified to pH 3 with HCl 1N (500 μl). The plasma was then spiked with tetradeuterated prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (500 pg in 50 μl of ethanol) as an internal standard and applied on an octadecylsilane (C_{18}) cartridge. Lipids were eluted with a solvent mixture, applied on an aminopropyl (NH_2) cartridge and then derivatized and examined by gas chromatography/negative-ion chemical ionization tandem mass spectrometry analysis [33]. The isomer considered for F_2-IsoPs determination was 8-epi-$PGF_{2\alpha}$ (or 8-iso-$PGF_{2\alpha}$), also referred as 15-F_{2t}IsoP [11,33].

For free F_2-IsoPs, immediately after thawing, the plasma (1 ml) was acidified with water (pH 3), spiked with tetradeuterated $PGF_{2\alpha}$ (500 pg in 50 μl of ethanol) as an internal standard and applied on an C_{18} cartridge. Subsequently the procedure used was the same as that of total F_2-IsoPs.

Advanced oxidation protein products (AOPP)

After centrifugation, the plasma was stored at -70°C until use. AOPP were determined according to Witko-Sarsat et al. [25]. Briefly, AOPP were measured by spectrophotometry on a microplate reader (Benchmark, Bio-Rad Laboratories, Hercules, CA) and calibrated with chloramine T solutions that absorb at 340 nm in the presence of potassium iodide. In standard wells, 10 μl of 1.16 M potassium iodide was added to 200 μl of chloramine T solutions (0–100 μmol/L) followed by 20 μl of acetic acid. In test wells, 200 μl of plasma diluted 1:5 in PBS was placed on a 96-well microtiter plate and 20 μl of acetic acid was added. The absorbance of the reaction mixture was immediately read at 340 nm on the microplate reader against a blank containing 200 μl of PBS, 10 μl of potassium iodide and 20 μl of acetic acid. The chloramine T absorbance at 340 nm was linear within the range of 0–100 μmol/L. AOPP concentrations were expressed as μmol/g protein. Plasma protein concentration was evaluated using the Bradford [34] method with Coomassie Brilliant Blu G-250.

Statistical analysis

Results are reported as means ± SEM. Comparisons between groups were carried out using the Student's *t*-test for independent samples. Pearson's coefficient was used for correlations. The χ^2-test was used to examine bivariate associations between percentages. All tests were two-tailed. The value of $p < 0.05$ was considered statistically significant.

Results

Figure 1A shows the intraerythrocyte DCI level in diabetic patients and the respective controls. The level was significantly higher in diabetic patients and no difference between type 1 (2.19 ± 0.47 nmol/ml erythrocyte suspension) and type 2 (1.57 ± 0.24 nmol/ml erythrocyte suspension) was observed. Also, the MetHb content was significantly higher in diabetic than in control RBCs (Figure 1B) and it was somewhat correlated with glycated haemoglobin (HbA_{1C} values $7.51 \pm 0.15\%$) ($r = 0.322$, $p < 0.01$, $n = 67$). No difference was again found between type 1 and type 2 diabetes (66 ± 6.4 and 65 ± 5.0 nmol/ml erythrocyte suspension, respectively).

The percentages of diabetic and control subjects showing the binding of autologous IgG to band 3 dimers in RBCs are reported in Figure 2A. The binding was present in a much higher percentage of diabetic subjects than of controls and again no difference was found between type 1 (52.2%) and type 2 (46.8%). Typical Western blots of erythrocyte membrane proteins of a diabetic and a control subject showing IgG binding (although to a much lesser extent as compared to the diabetic subject) are presented in Figure 2B. The 170 kDa band, indicating autologous IgG binding to band 3 dimers, was quantified using scanning densitometry and the values of optical density of all the samples are reported in Figure 2C. As can be seen, the values were markedly higher in diabetic patients. This means that diabetic erythrocytes present with 170 kDa band, which marks the aged cells for removal, with a markedly higher frequency and that band 3

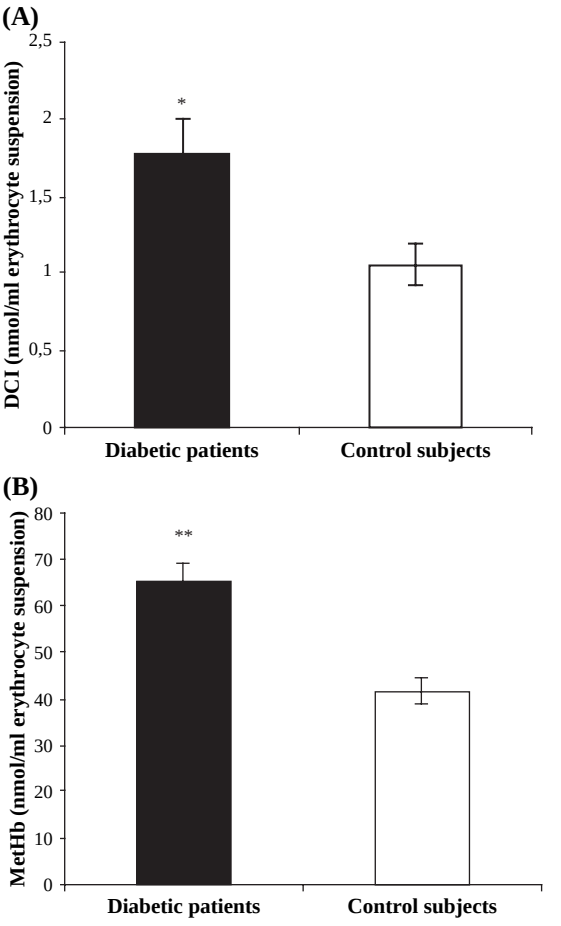

Figure 1. (A) DFO-chelatable iron (DCI) level in diabetic and in control erythrocytes. The results, expressed as nmol/ml erythrocyte suspension, are the means ± SEM of determinations carried out in 76 diabetic patients and in 39 control subjects. $^{\star}p < 0.05$ vs control subjects. (B) Methemoglobin (MetHb) values in diabetic and control erythrocytes. The results, expressed as nmol/ml erythrocyte suspension, are the means ± SEM of determinations carried out in 68 diabetics and in 29 controls. $^{\star\star}p < 0.001$ vs controls.

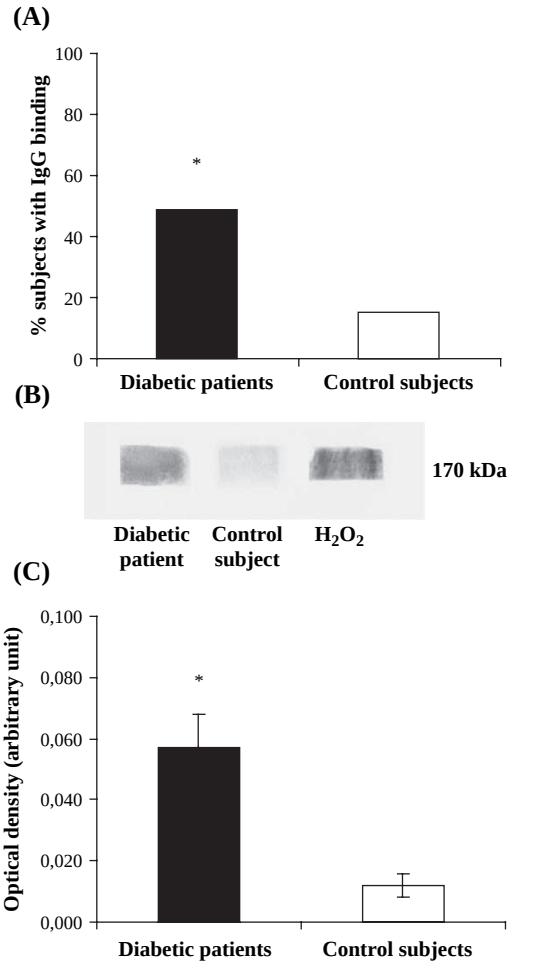

Figure 2. (A) Percentages of diabetic patients and control subjects showing the binding of autologous IgG to band 3 dimers in the erythrocytes. The results derive from determinations carried out in 70 diabetic patients and in 39 control subjects. $^{\star}p < 0.001$ vs controls (χ^2-test). (B) Typical western blots of membrane proteins isolated from diabetic and control erythrocytes showing the IgG binding to band 3 dimers (170 kDa band). Control erythrocytes incubated with H_2O_2 are also shown. (C) Scanning densitometry data of 170 kDa band of 70 diabetic patients and 39 control subjects. The results are expressed as means ± SEM. $^{\star}p < 0.001$ vs controls.

dimers are much more expressed than in controls. That is to say that a great part of erythrocytes from diabetic subjects are aged and ready to be eliminated.

When in diabetic RBCs the values of optical densities for autologous IgG binding to band 3 dimers were plotted against the corresponding values of DCI, a significant correlation was found (Figure 3), suggesting that, as previously proposed, the oxidant stress-induced iron release is strictly related to band 3 dimer formation. The strict association between DCI and IgG binding was also demonstrated by the fact that DCI level was significantly higher ($p < 0.05$) in diabetic RBCs showing IgG binding (2.32 ± 0.44 nmol/ml erythrocyte suspension) than in diabetic RBCs showing no binding (1.07 ± 0.21 nmol/ml erythrocyte suspension).

Table II shows the plasma levels of F_2-IsoPs, NPBI and AOPP in diabetic patients and respective controls. Plasma levels of total and esterified F_2-IsoPs were significantly higher in diabetic than in control subjects. No difference was observed in plasma free F_2-IsoPs levels between diabetics and controls. Plasma level of NPBI was significantly higher in diabetics than in controls and it was present in almost all (87.5%) diabetic subjects and in 20.8% only of controls (χ^2-test, $p < 0.001$). A correlation ($r = 0.475$; $p < 0.05$) was found, in diabetic subjects, between plasma level of NPBI and erythrocyte DCI. Plasma AOPP were significantly higher in diabetics than in controls. Again no difference was found between type 1 and type 2 diabetes.

Discussion

The present study shows that RBCs from diabetic patients present with a higher content of DCI and

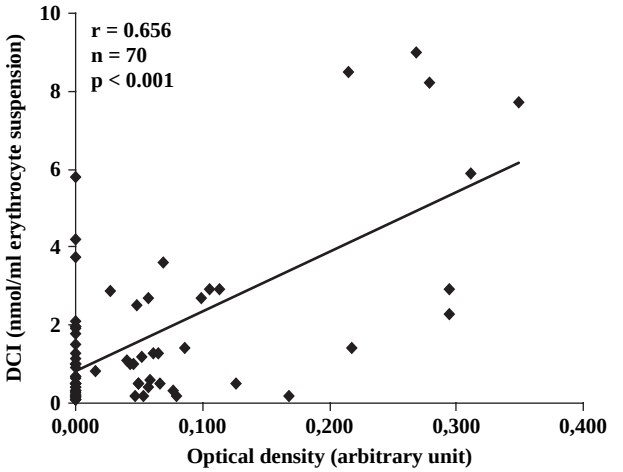

Figure 3. Correlation between autologous IgG binding (optical density) and DFO-chelatable iron (DCI) in erythrocytes from 70 diabetic subjects ($r = 0.656$, $p < 0.001$).

MetHb (the most direct effect of erythrocyte oxidative stress) than control RBCs. Also, in diabetic subjects autologous IgG are bound to erythrocyte band 3 dimers in a much greater percentage of cases as compared to healthy control subjects. DCI is strictly associated with IgG binding, as demonstrated by the significant correlation found (Figure 3) and also by the fact that its level is significantly higher in diabetic RBCs showing IgG binding than in diabetic RBCs showing no binding. These results suggest that a large part of the erythrocytes from diabetic subjects is ready to be eliminated by the phagocytic system and reflects an accelerated turnover of RBCs.

A number of studies [3,35–37] have demonstrated that the phagocytic removal of senescent, oxidatively damaged RBCs appears to be mediated by the binding of autologous antibodies to a modified band 3. Native band 3 in non-senescent RBCs is physiologically present as a mixture of dimers and tetramers [3]. Since non-senescent RBCs do not bind anti-band 3 antibodies and are not recognized by phagocytes, putative band 3 epitopes are not available for bivalent antibody binding in native dimers. Turrini et al. [3] have demonstrated that band 3 is recognized by specific autologous antibodies if the quaternary structure is modified by oxidative cross-linking of cytoplasmic domains, with ensuing reorientation of band 3 within the dimers, and that autologous IgG do not recognize non-oxidatively generated, covalently linked band 3 dimers. It has been concluded that the disulphide-cross-linked band 3 dimers are the minimal band 3 aggregates with enhanced affinity for anti-band 3 antibodies [3]. Band 3 modifications are mostly due to oxidative insults that gradually accumulate during the RBC lifespan or that impact massively in a shorter time period in pathological conditions such as in a series of genetic RBC defects [38].

Table II. F_2-Isoprostanes, NPBI and AOPP in plasma from diabetic patients and control subjects.

	Diabetic patients	Control subjects
Total F_2-IsoPs (pg/ml plasma)	390.8±33.6 (27)*	165.9±11.3 (12)
Free F_2-IsoPs (pg/ml plasma)	40.8±2.6 (12)	36.5±4.4 (12)
Esterified F_2-IsoPs (pg/ml plasma)	347.1±37.4 (12)*	129.4±13.4 (12)
NPBI (nmol/ml plasma)	0.53±0.09 (24)*	0.10±0.03 (24)
AOPP (μmol/g protein)	1.03±0.05 (40)**	0.82±0.05 (12)

The results are the means±SEM. Plasma esterified F_2-IsoPs were calculated by subtracting free F_2-IsoPs values from total F_2-IsoPs ones. The number of cases is in brackets.
*$p < 0.001$; **$p < 0.05$ vs controls.

In agreement with the previous studies [1,2,9,39] these results suggest that iron released from its physiological complexes (Hb, heme) into the cytoplasm of erythrocyte plays an important role in band 3 oxidative modifications (dimers formation) and therefore in removal of erythrocytes from the blood stream. Nagababu et al. [40] have shown that iron release occurs as a consequence of haemoglobin autooxidation, formation of reactive oxygen species and loss of normal tetragonal symmetry around heme iron. It has also been shown [41,42] that iron can be found associated with the cytoplasmic side of the membrane in which several discrete iron compartments (denaturated haemoglobin, free heme, molecular iron, etc.) can be demonstrated. In particular, molecular iron [42] could be able to cycle between ferric and ferrous states and therefore participate in several redox reactions with consequent oxidative damage to membrane structure. Kar and Chakraborti [43] have shown that in purified haemoglobin isolated from diabetic patients free iron release occurs and this is proportionally increased with the level of blood glucose. Compared to Hb_{Ao}, HbA_{1c} is in fact more rapidly auto-oxidized [44]. Glycation can induce structural modifications in haemoglobin with an unfolding of the tetrameric structure and weaker heme-globin linkage, leading to heme degradation and iron release [44]. Cussimanio et al. [45] have demonstrated that haemoglobin and myoglobin are extremely susceptible to damage by glucose *in vitro* through a process that leads to the complete destruction of the heme group and to iron release.

In addition to the erythrocyte membrane damage and to the appearance of the oxidative modifications of band 3, the present paper also shows that in diabetes iron is increased in plasma (NPBI) outside the erythrocyte. It has been reported [46] that NPBI is not detectable in adult healthy subjects. In agreement with Sulieman et al. [23] and Lee et al. [24] we found very low NPBI levels in five out of 24 controls and no detectable levels in the others. Therefore no substantial discrepancy comes out from such results. It must also be noted that different assays were used for such determinations. The occurrence of redox

active iron in plasma could help to explain the oxidative stress frequently reported in diabetes and particularly in its long-term complications [12,20,22,23,47]. The possible implications of plasma redox active iron in endothelial damage and development of atherosclerosis have been in fact suggested [22,47]. The correlation found between plasma NPBI and erythrocyte DCI makes it tempting to suggest that part of the released iron can cross the erythrocyte membrane and appear in plasma. Indeed we have previously reported [39] that in the neonate this seems to be the case.

The relatively low concentrations of NPBI does not imply that such concentrations are not relevant for biological effects since it is likely that additional iron can be released from macromolecular complexes under conditions in which an iron-induced oxidation occurs. Indeed, it has been reported [23] that such concentrations of NPBI are correlated with enhanced risk of mortality after myocardial infarction in diabetes.

Many studies have evaluated the plasma levels of F_2-IsoPs, the most reliable and specific markers of oxidative stress. They were found to be increased in both type 1 and type 2 diabetes [13]. Also, it has been reported [48] that the levels of plasma esterified F_2-IsoPs in type 2 diabetes were three times higher than in healthy individuals, while no measurable levels of free IsoPs were detected. Our results too show that plasma levels of esterified F_2-IsoPs are significantly higher in diabetics than in controls, whereas no difference is found in free F_2-IsoPs. This suggests that some form of lipid peroxidation is active in diabetes. The fact that only esterified but not free isoprostanes are increased may suggest that the source of lipid peroxidation are plasma lipids (lipoproteins), from which esterified F_2-IsoPs are likely to be directly derived. Plasma lipids are likely more exposed to NPBI as compared to lipids in peripheral tissues, as suggested by Salahudeen et al. [49]. We have recently observed that [50] in newborns there is a significant correlation between plasma esterified F_2-IsoPs and plasma NPBI. The increase of NPBI together with oxidized (or very susceptible to oxidation) LDL [51] make possible to suggest that in diabetes the increase of NPBI is responsible for LDL oxidation resulting in increased esterified F_2-IsoPs. An alternative explanation could be that in diabetics an increased elimination of free F_2-IsoPs occurs, as reported by Feillet-Coudray et al. [52].

AOPP, originated from free radical attack of proteins, particularly albumin [25], and often used as biomarker of oxidative stress [53], were increased in diabetic patients as compared to controls (Table II), and this is in agreement with another report [54], suggesting that plasma proteins too are involved in oxidative damage.

In conclusion, iron release, MetHb formation and binding of autologous IgG to band 3 dimers appear to be strictly related in diabetic RBCs which are likely exposed to an increased oxidative stress as demonstrated by an increased level of esterified F_2-IsoPs, NPBI and AOPP in plasma.

Also plasma NPBI appears to be correlated with intraerythrocyte DCI. Thus, iron and oxidative stress appear to be involved in the early senescence of diabetic RBCs.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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