Association of plasma proteins at multiple stages of glycation and antioxidant status with erythrocyte oxidative stress in patients with type 2 diabetes

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ABSTRACT

This study examines the individual stages of plasma protein glycation, antioxidant status and their association with erythrocyte oxidative stress in patients with type 2 diabetes mellitus (T2DM). Study was carried out on blood from 70 patients with T2DM and 40 healthy age- and gender-matched volunteers. Biomarkers of plasma protein glycation (fructosamine, protein carbonyls, advanced glycation end products [AGEs], amyloid), antioxidant status (thiols, total antioxidant capacity and erythrocyte oxidative parameters), osmotic fragility, lipid peroxidation (LPO), reduced glutathione (GSH) and catalase were determined. Plasma glycation markers were higher in T2DM patients than in healthy volunteers: fructosamine 578 *vs.* 525 µmol/mL; carbonyl 21.23 *vs.* 18.84 nmol/mg protein ($P \leq 0.01$); AGEs 213.94 *vs.* 178.27 AU/mg protein ($P \leq 0.05$); and amyloid 0.53 *vs.* 0.40 A530 nm ($P < 0.01$). Plasma antioxidant status was significantly reduced in patients with diabetes compared to the healthy volunteers, with lower plasma protein thiols (1.16 *vs.* 1.36 nmol/mg protein; $P < 0.01$) and total antioxidant capacity (26 *vs.* 34 µmol; $P < 0.01$). Erythrocytes from the patient group were found to show greater oxidative damage, with elevated numbers of fragile cells and increased LPO, and reduced GSH level. Among the glycation markers, positive correlations were evident between fructosamine and amyloid ($r = 0.350$, $P < 0.001$) and AGEs and amyloid ($r = 0.070$). Plasma glycation markers showed negative correlation with plasma antioxidant status while positive correlation was demonstrated between erythrocytes fragility and AGEs and amyloid. Erythrocyte LPO levels correlated positively with amyloid. These data suggest that increased levels of multiple plasma protein glycation products in T2DM patients play a key role in reduced plasma antioxidant status and amplified erythrocyte oxidative damage.

KEY WORDS: Antioxidants.
Diabetes mellitus, type 2.
Erythrocytes.
Glycation end products, advanced.

Introduction

In diabetes, endogenous glycation mainly occurs in the bloodstream[1] and collective data from various biochemical, animal and epidemiological studies suggests that the glycated plasma proteins play a critical and causative role in the pathogenesis of diabetes.[2] This complex process, which leads to the generation of various glycation intermediate products and protein modifications, involves various stages, as follows: i) reducing sugars and free amino groups react non-enzymatically to form early glycation adduct-fructosamine (Amadori rearrangement product);[3] ii) these products undergo further degradation and dehydration to form amino or carbonyl intermediates;[1] iii) the reaction of carbonyl intermediates with other amino groups and subsequent complex rearrangements form irreversible crosslinked, heterogeneous fluorescent derivatives termed advanced glycation end-products (AGEs); and iv) these changes affect the native structure of glycated protein, leading to conformational changes (favouring higher β-sheet content) and promote the formation of ordered aggregates (amyloid fibrils).[4]

Several lines of evidence from *in vitro* data suggest that amyloid aggregates are formed when albumin is exposed to glycating agents for prolonged periods of time.[4,5] These glycation-induced protein modifications cause significant alterations to intrinsic functions that have important clinical implications.[5] However, the quantitative amounts of products at different stages of glycation and of amyloid aggregates in blood of patients with diabetes remain unclear.

Glycated proteins are reported to produce reactive oxygen species (ROS) and generate oxidative stress (OS).[6] The complex interplay between glycation and OS forms an integrated cycle as ROS in diabetes accelerates the glycation; this is termed glycoxidation and contributes to the progression of vascular damage in diabetes.[7] Although plasma contains antioxidant defence mechanisms, including the SH group of albumin derived from cysteine 34 and various antioxidants (urate, ascorbate, tocopherol and vitamin E), increased OS and impaired antioxidant defences have been reported in type 2 diabetes mellitus (T2DM).[8]

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Whether or not reduced plasma antioxidant activity is a functional consequence of glycation-induced modifications has not yet been investigated.

Considerable evidence implicates oxidative damage in various vascular cells by glycated plasma proteins and AGEs, resulting in diabetic vascular complications.[2,4] Erythrocytes are perhaps the cells mainly exposed to increased circulatory glucose and the glycated proteins in the vasculature.[9] Erythrocytes undergo glycation and the presence of glycated peptides, cytoskeletal protein and glycation motifs have been demonstrated in the erythrocyte membrane,[10] and efforts have been made to examine the associations between GLUT-1 and membrane glycation.[11] Interaction of plasma AGEs or AGE-bearing erythrocytes with their receptors for AGEs (RAGE) alters vascular function, leading to a vascular hyperpermeability/inflammatory reaction including OS and cytokine production.[9]

Epidemiological studies have demonstrated increased erythrocyte fragility in patients with T2DM.[12] Additionally, erythrocyte fragility has been examined in relation to glycosylated haemoglobin, and it is proposed that increased levels of glycated haemoglobin as well as membrane proteins have a pivotal role in increasing osmotic fragility in erythrocytes.[13] However, mechanisms linking the changes in erythrocytes from patients with diabetes and induced plasma protein glycation pathways in T2DM remain to be elucidated.

The present study aims to determine the clinical relevance of estimating plasma proteins at multiple stages of glycation (fructosamine, carbonyls, AGEs and amyloid) and antioxidant status (protein thiols, antioxidant capacity) in the plasma of patients with T2DM, and assess any correlation with erythrocyte fragility and OS parameters.

Materials and methods

Study volunteers, clinical information and sample collection

The study was undertaken at the Department of Medicine, Bharati Hospital, Pune, India, in 2011–2012. Volunteers were divided into two groups. Group 1 comprised 70 patients with T2DM (mean age: 57.87±6.9 years; mean duration of diabetes: 6.5±8.1 years; glycated haemoglobin [HbA1c]: 9±2.03%). Group 2 included 40 healthy control volunteers (mean age: 57±9.2 years; HbA1c: 5.28±0.3%).

Diagnosis of T2DM was determined using American Diabetes Association criteria. Patients with malignancy, connective tissue disease, liver dysfunction, apparent cardiovascular disease and other chronic inflammations were excluded, as were patients consuming high levels of antioxidants or hepatotoxic drugs, and those with serum creatinine >1.0 mg/dL (indicative of renal dysfunction).

Control volunteers were recruited according to the following criteria: i) absence of diabetes on oral glucose tolerance test; ii) absence of metabolic or other disease; and iii) no treatment with drugs known to affect glucose tolerance.

Ethical approval for the study was obtained from the Bharati Vidyapeeth Medical College Institutional Ethical Committee and informed written consent was obtained from each volunteer in advance.

The clinical history of each volunteer pertaining to glycaemic control markers such as fasting plasma glucose (FPG) and HbA1c, serum lipid profile, serum creatinine, blood urea, total protein and albumin (obtained from hospital records) was also incorporated as a part of the study.

For estimation of glycation and OS markers, fasting blood samples were collected in 10.8 mg EDTA and centrifuged at 3000 rpm for 20 min. Plasma and erythrocytes were then carefully collected (without disturbing the buffy layer) in separate tubes and labelled. Plasma was stored at –20°C until analysis, while erythrocytes were processed immediately for further biochemical analysis.

Plasma glycation markers

Fructosamine level was analysed using a published method[14] with modifications. Briefly, 0.8 mL nitroblue tetrazolium (0.75 mmol/L; NBT) prepared in carbonate buffer (0.1 mol/L; pH 10.35) was added to 0.04 mL plasma sample. After incubating at 37°C for 30 min. the fructosamine concentration was estimated by measuring the absorbance at 540 nm. The results were expressed as µmol/mL plasma.

Protein carbonyl concentration was estimated by the method of Uchida *et al*.[15] with some modification. Briefly, 0.1 mL plasma sample was mixed with 0.5 mL 2,4-dinitrophenylhydrazine (10 mmol/L) prepared in 2.5 mol/L HCl and incubated at 37°C for 60 min. After incubation, the protein was precipitated using 1 mL 10% trichloroacetic acid (TCA) and centrifuged at 7000 rpm for 10 min. The supernatant was discarded and the pellet was washed (x3) with 1 mL ethanol:ethyl acetate (1:1; v/v) mixture and dissolved in 1 mL urea (6 mol/L), followed by centrifugation at 5000 rpm for 5 min. Finally, the resulting supernatant was diluted with 0.5 mL urea (6 mol/L) and absorbance was measured at 365 nm and 280 nm. Protein carbonyl concentration was calculated (extinction coefficient 21 mM-1 cm-1) and the results were expressed as nmol/mg protein.

Plasma AGE concentration was estimated spectro-fluorometrically according to the method described by Grillo *et al*.[3] Fluorescence intensity was expressed in arbitrary units (AU)/mg protein.

Plasma amyloid groups were determined using the amyloid-specific dye Congo red (CR),[1] and results were expressed as absorbance ($A_{530\ nm}$) values.

Plasma antioxidant status

Protein thiol concentration was estimated using the method of Ellman.[17] Free thiol concentration was calculated using a standard curve (Microlowry)[32] obtained with various native BSA concentrations (0.8–4 mg/mL, corresponding to 19–96 nmol/L total thiols). The results were expressed as nmol/mg protein.

Antioxidant capacity of plasma was estimated by the ferric reducing antioxidant power (FRAP) assay and the method of Benzie and Strain.[18] The results were expressed as µmol/L reduced ferrous by comparing with standard.

Erythrocyte oxidative stress

Osmotic fragility was estimated using the method of Raghuramulu *et al*.[19] Mean erythrocyte fragility (MEF), defined as the concentration at which 50% of erythrocytes lysed, was also calculated.

Estimation of erythrocyte lipid peroxidation (LPO) was performed using the method of Placer *et al*.,[20] and assessed by measuring malondialdehyde (MDA), the end-product of fatty acid peroxidation. The results were expressed as nmol MDA/mL haemolysate.

Table 1. Clinical characteristics of participants in the control and diabetic groups.

	Control group	Diabetic group
n	40	70
Gender (Male/Female)	29/11	45/25
Age (Years)	54.3±11.5	56.68±8.60
Duration of diabetes (Years)	–	5.39±6.83
FPG (mg/dL)	113.33±35.30	228.6±98.82*
Haemoglobin (g/dL)	11.34±2.29	11.51±2.31
HbA1c (%)	5.28±0.309	9.5±2.77*
Blood urea (mg/dL)	28.1±17.52	32.7±21.28
Serum creatinine (mg/dL)	1±0.21	0.10±0.19
Total protein (g/%)	6.58±0.83	6.29±0.58
Albumin (g/%)	3.93±0.52	3.54±0.48*
Total cholesterol (mg/dL)	172.41±47.97	151.83±47.80
Triglycerides (mg/dL)	126.67±91.02	125.42±65.66
S-HDL cholesterol (mg/dL)	42.92±11.41	40.49±13.90
S-VLDL cholesterol (mg/dL)	25.33±18.20	27 .05±16.68
S-LDL cholesterol (mg/dL)	104.16±40.01	85.45±39.23
Cholesterol–total/ HDL (mg/dL)	4.14±1.15	3.84±1.06
Cholesterol–LDL/ HDL (mg/dL)	2.47±0.74	2.17±0.90

Data are presented as mean±SD. *P<0.001.

FPG: fasting plasma glucose; HbA1c: glycated haemoglobin; S-HDL: serum high-density lipoprotein; S-LDL: serum low density lipoprotein; S-VLDL: serum very low density lipoprotein.

Reduced glutathione (GSH) estimation was performed using the method of Ellman.[17] The results were expressed as nmol/mL haemolysate.

Catalase activity was assayed by the method of Chance, as modified by Aebi.[21] The results were expressed as Katal units (KU)/g haemoglobin.

Statistical analysis

Data were expressed as mean±standard deviation (SD). Statistical analysis was carried out using Graphpad Prism (version 5.01). Significance of difference between means was assessed by Student's *t*-test. The relationships between different parameters were expressed as the correlation coefficient (*r*).

Results

Clinical characteristics of the study population are given in Table 1. Fasting blood glucose and HbA1c were significantly higher in the patient group (P<0.001). Total protein levels were lower in the patients, accompanied by a significantly reduced albumin level (P<0.001). Lipid profile levels did not differ significantly between the groups.

Protein glycation markers

Changes in plasma protein glycation markers and antioxidant status are presented in Table 2.

No significant differences were observed in fructosamine.

Table 2. Plasma protein glycation markers and antioxidant status in the control and diabetic groups.

	Control group	Diabetic group
Glycation markers		
Fructosamine (µmol/mL)	526.0±166.8	578.85±199.7^{NS}
Protein carbonyl (nmol/mg protein)	1.88±0.29	2.12±0.44†
AGEs (AU/mg protein)	1783±442.7	2139±740.9*
Amyloid (A530)	0.40±0.12	0.53±0.26†
Antioxidant status		
Protein thiols (nmol/mg protein)	1.36±0.26	1.16±0.27†
Total antioxidant capacity (µmol)	32.88±14.61	24.64±9.21†

Data are presented as mean±SD.

*P<0.05; †P<0.01; ^{NS}: not significant.

AGEs: advanced glycation end products.

Protein carbonyl content was significantly higher in the patient group (P<0.01), and AGEs were approximately 1.2-fold higher than in the control group (P<0.05). Plasma amyloid aggregation was found to be associated with diabetes as absorbance was significantly higher (P<0.01) in the patient group (0.53±0.26 $A_{530\ nm}$) compared to control group (0.40±0.12 $A_{530\ nm}$).

Protein antioxidant status

Protein thiol groups were significantly reduced in patients with diabetes (P<0.01; Table 2). The total antioxidant capacity as assayed by the FRAP method was 24.63±9.2 µmol/L in the patient group, which is significantly lower (P<0.01) than in the control group (32.87±14.6 µmol/L).

Erythrocyte oxidative stress

Erythrocyte levels of OS markers are listed in Table 3. Erythrocyte osmotic fragility (% haemolysis) was higher in patients with diabetes (Fig. 1). The MEF, calculated from % haemolysis, was found to be elevated in the patient group

Table 3. Changes in erythrocyte oxidative stress markers in the control and diabetic groups.

	Control group	Diabetic group
MEF (50% haemolysis)	0.609±0.096	0.666±0.074^{NS}
LPO (nmol MDA/mL haemolysate)	0.074±0.034	0.11±0.049*
GSH (nmol/mL haemolysate)	132.7±39.21	101.8±36.07†
Catalase activity (KU/g haemoglobin)	0.068±0.043	0.076±0.061^{NS}

Data presented as mean±SD.

*P<0.01; †P<0.001; ^{NS}: not significant.

MEF: mean erythrocyte fragility; LPO: lipid peroxidation; GSH: reduced glutathione; MDA: malondialdehyde; KU: Katal unit.

compared to controls. The extent of erythrocyte LPO, measured as TBARS, was found to be higher in patients with diabetes (0.11 nmol MDA/mL haemolysate) compared to the control group (0.074 nmol MDA/mL haemolysate) ($P<0.01$). Erythrocyte GSH and catalase were lower in the patient group ($P<0.001$).

Overall correlations

Positive correlations were observed between fructosamine and amyloid ($r=0.350$, $P<0.001$), AGEs and amyloid ($r=0.070$), erythrocyte fragility (MEF) correlated positively with AGEs ($r=0.113$) and amyloid ($r=0.130$), erythrocyte LPO level with plasma amyloid ($r=0.151$) (Fig. 2A–E).

Negative correlations were observed for fructosamine with protein carbonyl ($r=–0.274$, $P<0.01$) and protein thiols ($r=–0.072$), plasma total antioxidant capacity with AGEs ($r=–0.099$) and amyloid ($r=–0.036$), and within erythrocytes between GSH and LPO (Fig. 2F–J).

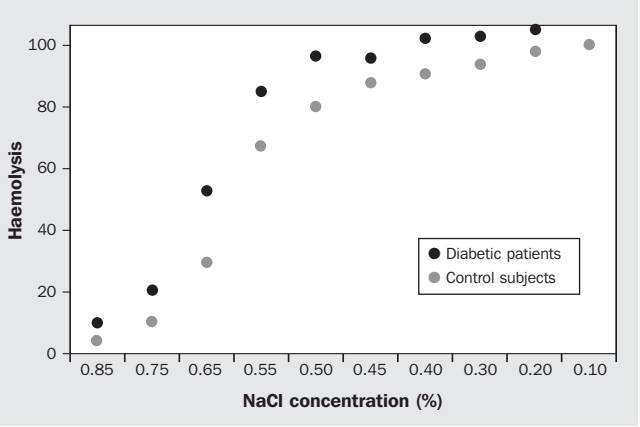

Fig. 1. Erythrocytes osmotic fragility curves as % haemolysis of patients with diabetes and controls. The degree of haemolysis was calculated by comparison with 0.1% NaCl solution which represented 100% lysis.

Discussion

Type 2 diabetes is often associated with chronic diabetic complications, and increase in protein glycation has been correlated to OS.[6,7] The functional attributes of proteins are determined by the structure, which is often described in terms of alterations in structural characteristics such as functional groups and conformation, and glycation or oxidative changes culminate in changes and functions generally associated with pathological conditions. Erythrocyte dynamics are determined by the structural integrity of the membrane and alterations in terms of membrane characteristics (e.g., osmotic fragility and antioxidant status) complicate their flow through the microcirculation. Therefore, the present study aimed to investigate plasma protein glycation-induced multistage modifications and the implications for erythrocytes.

Results in the diabetic group demonstrated higher fructosamine levels in plasma, which suggests enhanced glycation due to poor glycaemic control represented by enhanced FPG and HbA1c levels. This initial adduct in the glycation reaction is reported to increase due to persistent hyperglycaemia,[22] where fructosamine reflects mean blood glucose concentration over a short period of three to four weeks.[22] Through a series of complex reactions, fructosamine forms carbonyls and AGEs, high levels of which were evident in the present study in patients with diabetes.

Protein carbonyls provide an index of protein oxidation generated either through direct oxidation of amino acid side chains or by glycation that induces formation of protein carbonyls (e.g., ketoamine derivatives), thus generating reactive radicals and perpetuating a vicious cycle.[23] A significant negative correlation is seen between protein carbonyl and fructosamine, illustrating that glycated plasma proteins subsequently undergo oxidative degradation and heightened carbonyl stress.[24] Recently, Bansal *et al*.[25] found 1.4-fold and 1.2-fold increases in protein carbonyls and AGEs in patients with diabetes compared to healthy individuals.

Glycation-induced modification of protein has been reported to result in protein aggregation, protein crosslinking and amyloid fibril formation.[4,5] Congo red has been used widely to identify tissue amyloid deposits and to follow amyloid fibril formation from purified proteins and peptides *in vitro*.[26] Most of these studies focused on the structural and functional changes of commercial BSA-induced glycation *in vitro*.[4,27,28] Thus, it is conceivable that the glycation of plasma proteins may contribute to the formation of globular amyloid-like structures *in vivo*.

In the present study, plasma protein amyloid aggregation was assessed using Congo red. Similarly, Griffin *et al*.[29] measured amyloid fibrils and amyloid-like structures in serum using another amyloid-specific dye, thioflavin T. In the present study, plasma amyloid content was found to be significantly higher in patients with T2DM, which may be caused by an amplified rate of modification as a result of hyperglycaemia, as well as a decreased rate of elimination. The positive correlation of plasma amyloid with AGEs reported in this study is consistent with the results of other *in vitro* studies.[26] Interestingly, the present results also indicate a significant association of plasma amyloid with fructosamine.

The SH group of albumin derived from cysteine 34 represents an important antioxidant reserve to scavenge hydroxyl and peroxinitrite radicals, and is oxidised in glycoxidation.[27] In the current study, the plasma thiol concentration was found to be reduced significantly in patients with diabetes, and may be due to: i) decreased plasma albumin level, as albumin is the major contributor of circulating plasma protein thiols,[30] or ii) glycoxidation of protein carbonyls which react primarily with thiol groups (strong nucleophiles) on plasma protein surfaces, leading to their reduction.[31] Similarly, other reports indicate a 1.4- to 1.5-fold decrease in SH groups in T2DM.[23,25,27] In addition to thiols, there are antioxidant defences in plasma that provide an index of ability to resist glycoxidative damage,[32,33] which was assessed predominantly as FRAP level.[34] The present results support the general observations of higher oxidative damage in patients with diabetes, as plasma FRAP level was significantly lower. In addition, AGEs and amyloid were found to significantly and negatively correlate with antioxidant capacity (FRAP level), emphasising the possible involvement of AGEs and amyloids in inducing the OS. These results demonstrate the complex interplay between glycation and OS as in glycoxidation in T2DM.

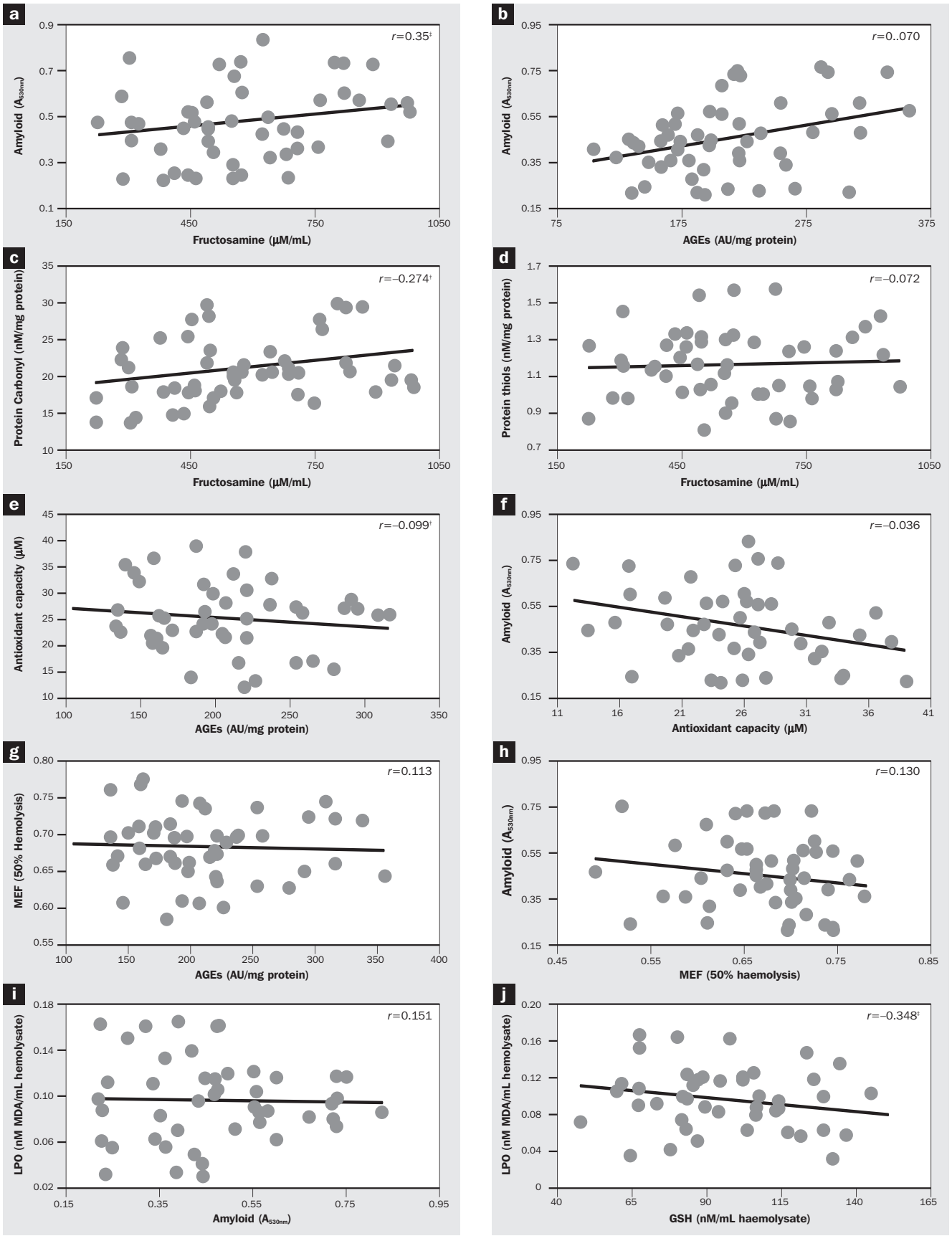

Fig. 2. Correlation of various biochemical parameters in patients with diabetes. **a)** fructosamine with amyloid; **b)** AGEs with amyloid; **c)** AGEs with MEF; **d)** MEF with amyloid; **e)** LPO with amyloid; **f)** fructosamine with protein carbonyls; **g)** fructosamine with plasma protein thiols; **h)** AGEs with antioxidant capacity; **i)** antioxidant capacity with amyloid; **j)** between erythrocyte status – LPO and GSH levels. Data are presented as correlation coefficients (*r*). *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.

Accumulating evidence suggests that the structural and functional alterations of glycated proteins and subsequent OS have important clinical implications for various cell physiologies.[4] In patients with T2DM, erythrocytes show increased tendency towards *in vivo* haemolysis, but reports on erythrocyte status with glycation are limited.[35] Another potential factor contributing to the modification of erythrocyte dynamic properties is increased glycosylation-derived internal viscosity, which is common in diabetes.[35] The present study found a significant positive correlation for erythrocyte fragility with plasma AGEs and amyloid, indicating the deleterious effects of these extracellular glycated products on erythrocytes.

Indirect *in vitro* evidence by Guerin-Dubourg *et al*.[27] demonstrates the protective antioxidant effect of non-diabetic albumin on free-radical-induced erythrocyte haemolysis, an effect partially lost from diabetic glycated albumin. The present study found elevated levels of various OS markers in erythrocytes (e.g., high LPO, low GSH and catalase) in patients with diabetes compared to healthy individuals. Furthermore, Wautier *et al*.[36] reported that the interaction of AGEs with their receptors (RAGE) on the surface of diabetic erythrocytes induces oxidant stress, resulting in membrane lipid peroxidation and accumulation of peroxidation product such as MDA. Interestingly, the present study shows that increased erythrocyte LPO is positively associated with plasma amyloid adducts, suggesting the possible detrimental interaction of plasma amyloid with erythrocyte peroxidation. The observed decreased GSH activity and significant negative correlation between GSH and LPO is in line with similar studies.[37] Riquelme *et al*.[38] observed that glycation of cell surface proteins results in decreased erythrocyte dynamic elasticity and aggregation due to glycated albumin, which may complicate the flow of cells through the microcirculation.[39] The present results indicate that erythrocytes suffer increased oxidative damage due to glycated plasma proteins, and this may amplify the severity of diabetic vascular complications.

In conclusion, this study supports the hypothesis that protein glycation is common among patients with T2DM, but the pathogenesis is complex and multifaceted. Compared with matched non-diabetic controls, T2DM patients had significantly increased products at multiple stages of plasma protein glycation, which correlated with increased OS and erythrocyte mechanical fragility. It is suggested that erythrocytes from patients with diabetes are highly sensitive and as a result are more fragile to subsequent damage, and this is related directly to plasma amyloid content. Predisposing conditions, compounded by increased glycation, reduced antioxidant potential in plasma and higher mechanical fragility of diabetic erythrocytes with reduced cellular antioxidants may promote development and progression of vascular complications in type 2 diabetes. □

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