

Original article

## Structural modifications of human albumin in diabetes

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### Abstract

**Aim.** – Albumin, a major protein in the blood circulation, can undergo increased glycation in diabetes. From recent studies, it has become evident that glycation has important implications for albumin actions and impact on cell functioning. This study compares the structural and functional properties of albumin glycosylated by glucose and methylglyoxal (MGO) with those of albumin purified from diabetic patients.

**Methods.** – Human serum albumin (HSA) was purified from diabetic patients and control subjects using affinity chromatography, and oxidation parameters in various albumin preparations were determined. Tryptophan and 1-anilino-8-naphthalene sulphonic acid (ANSA) probe fluorescence, redox state, antioxidant and copper-binding capacities of the different preparations of albumin were also determined and compared.

**Results.** – Occurrence of oxidative modifications was enhanced in albumin whether purified from diabetic patients, or glycosylated by glucose or MGO, after determination of their fructosamine and free thiol and amino group contents, carbonyl content and antioxidant activities. Whereas more quantitative changes in oxidative and structural parameters were observed in the glucose- and MGO-modified albumins, significant impairment of albumin function (free-radical-scavenging and copper-binding capacities) were demonstrated in the HSA purified from diabetics. These findings reveal different structural and functional features of diabetic HSA compared with *in vitro* models.

**Conclusion.** – This study provides new information supporting albumin as an important biomarker for monitoring diabetic pathophysiology. In addition, it reconfirms the influence of experimental conditions in which advanced glycation end-products (AGEs) are generated in tests designed to mimic the pathological conditions of diabetes.

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**Keywords:** Albumin; AGEs; Oxidative stress; Diabetes

### Résumé

Modifications structurales de l'albumine au cours du diabète.

**But.** – Le stress oxydant et les modifications oxydatives des protéines sont directement impliqués dans les complications du diabète. Un facteur puissamment protecteur contre le stress oxydant, produit par notre organisme, n'est autre que l'albumine, protéine la plus abondante du plasma. Mais, en particulier chez les diabétiques, la structure de l'albumine se trouve modifiée par des phénomènes de glycation qui confèrent à la protéine des propriétés biologiques néfastes. Dans cette étude, nous avons cherché à comparer les modifications structurales et fonctionnelles de l'albumine glycosylée *in vitro* avec celles de l'albumine purifiée à partir de patients diabétiques.

**Méthodes.** – L'albumine humaine a été purifiée à partir de patients diabétiques ou de témoins en utilisant la chromatographie d'affinité. La glycation *in vitro* d'albumine humaine a été effectuée par des incubations en présence de méthylglyoxal (MGO) ou de glucose. Les statuts structuraux et fonctionnels (pouvoir antioxydant) des différentes préparations d'albumine ont été déterminés et comparés au moyen notamment de mesures de propriétés électrophorétiques, de fluorescence, de capacités de liaison de métaux ou de neutralisation de radicaux libres.

**Abbreviations:** AAPH, 2,2'-azobis 2-amidinopropane; AGEs, Advanced glycation end-products; HRP, Horseradish peroxidase; HSA, Human serum albumin; HSA-C, Commercial human serum albumin; HSA-ND, Human serum albumin from non-diabetic patients; HSA-D, Human serum albumin from diabetic patients; mPAGE, Phenylboronate polyacrylamide electrophoresis; PBS, Phosphate-buffered saline; -SH, Sulfhydryl group; SMBG, Self-monitoring of blood glucose; TNBS, 2,4,6-trinitrobenzene sulphonic acid.

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**Résultats.** – Une augmentation importante et significative des modifications de type oxydatif a été observée à la fois au niveau des préparations d'albumine glyquée *in vitro* par le glucose ou le MGO, mais également dans la protéine lorsqu'elle purifiée à partir de patients diabétiques. En particulier, une altération des propriétés antioxydantes de piégeage des radicaux libres et de fixation des métaux a été mise en évidence pour l'albumine provenant de diabétiques.

**Conclusion.** – Ce travail contribue à montrer l'importance de l'albumine comme un biomarqueur majeur à privilégier pour le développement de stratégies nutritionnelles ou pharmaceutiques afin de limiter les désordres inhérents à la pathologie diabétique.

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**Mots clés :** Albumine ; Produits de glycation avancée ; Stress oxydant ; Diabète

## 1. Introduction

Non-enzymatic glycation is one of the underlying modification factors that contribute to various alterations of intrinsic protein functions. It is the result of covalent binding of glucose to amino groups of circulating proteins, such as haemoglobin (HbA<sub>1c</sub>) and albumin, or proteins present in the extracellular matrix (such as collagen and laminin) [1]. Because of its long half-life (about 21 days) compared with other proteins and its high concentrations in the circulatory system, serum albumin is a plasmatic protein that is highly sensitive to glycation. Elevated levels of glycated albumin (two- to threefold increases) in cases of diabetes mellitus can lead to irreversible damage associated with metabolic disorders such as retinopathy, nephropathy, neuropathy and coronary artery disease [2,3]. The development of these diabetic complications is attributed to the formation of deleterious and irreversible conjugates called “advanced glycation end-products” (AGEs) during the glycation process [3].

The two main clinical parameters used for chronic glycaemic control are glycated HbA<sub>1c</sub> levels and SMBG [4]. Measurement of glucose in blood, a short-term indicator, reflects the diabetic status over a 24-hour period, while the HbA<sub>1c</sub> value represents a long-term glycaemic indicator. However, the HbA<sub>1c</sub> is not always a relevant indicator of glycaemic control in patients with diabetes-associated pathologies that affect erythrocyte lifespan, such as haemolytic or renal anaemia and liver cirrhosis [5,6]. In such cases, glycated albumin appears to be an alternative marker for glycaemic control: the glycated albumin level is thought to indicate blood glucose status over a short period (2 to 4 weeks), while HbA<sub>1c</sub> reflects the glycaemic state over a longer period (2 months). Indeed, numerous studies support the use of glycated albumin levels in the detection of short-term changes in glycaemic control [7–9].

Albumin is known to have a set of diverse beneficial functions, including oncotic pressure regulation, and binding and transport capacities for a wide variety of metabolites, including those of therapeutic drugs [10,11]. For this reason, albumin can play an important role in drug disposition and efficacy [12]. However, the most prominent property of albumin is its major antioxidant activity in a circulatory system that is constantly subjected to powerful oxidative stress [13]. Previous reports from our group have demonstrated impairment of these properties with the glycation process [14,15]. In these studies, modified albumin *in vitro* exhibited different structural and functional properties attributed to the heterogeneous products formed by glycation, depending on the nature of albumin used (bovine or

human), the nature and concentration of carbohydrates (glucose, methylglyoxal [MGO]) and the duration of incubation (3 weeks or more) [16]. These heterogeneous glycation products derived from albumin are often used as glycation models in studies dealing with the involvement of AGEs in cellular pathophysiology in the context of diabetes. However, the question remains as to whether these glycated albumins *in vitro* constitute relevant models in diabetes-associated cellular disorders. For this reason, it was important to compare the structural and functional differences between *in vitro* glycated albumins and albumin purified from diabetic patients.

The present study examined the differential structural and functional aspects of albumin purified from diabetic and non-diabetic subjects. The study was also extended to compare commercial albumin glycated by glucose and MGO incubation.

## 2. Methods

### 2.1. Materials

The biconchonic acid, albumin from human serum (96 to 99%), MGO solutions (40% aqueous), anti-DNPH (dinitrophenyl hydrazine) antibody, 2,2'-azobis-(2-methylpropionamide) dihydrochloride (AAPH) and 3,3',5,5'-tetramethylbenzidine liquid substrate system for the enzyme-linked immunosorbent assay (Elisa) tests were all obtained from Sigma-Aldrich (St Louis, MO, USA). ECL<sup>TM</sup> anti-rabbit immunoglobulin G (IgG) and HRP-linked whole antibody (from donkey) were purchased from GE Healthcare Ltd (Little Chalfont, Buckinghamshire, UK), and the monoclonal anti-AGE antibody was from Cosmo Bio Co., Ltd (Tokyo, Japan).

### 2.2. Purification of albumin

Blood was obtained from a pool of diabetic patients and non-diabetic subjects, and anticoagulated in EDTA tubes (BD Vacutainer<sup>®</sup>) in the biochemistry laboratory of our institution in Saint-Denis, Réunion. Albumin purification was conducted using pooled serum from 15 diabetic patients (HbA<sub>1c</sub>: 12.0 ± 1.8%) and 38 non-diabetic subjects (HbA<sub>1c</sub>: 5.3 ± 0.3%). Included in the diabetic pool were only those patients with high albuminaemia levels (>40 g/L) but no acute inflammatory syndrome (C-reactive protein [CRP] levels <2 mg/L), acute coronary syndrome (troponin Ic <0.03 µg/L) and high hypertriglyceridaemia (>3.5 g/L) in the 2 months prior to the analysis (Table S1, Supplementary data). Patients with HbA<sub>1c</sub>

abnormalities and/or hyperleukocytosis were excluded from the plasma pool.

Purification of serum albumin from fresh human plasma was based on extensive dialysis against 50 mM Tris/HCl (pH 7.4) followed by affinity chromatography using Cibacron Blue 3G linked to agarose (Amersham catalogue number 17-0948-01) as a ligand for albumin. A 1.5-M NaCl (pH 7.4) buffer was used for the desorption of bound albumin from Cibacron Blue–agarose, following the elution of other plasma proteins using a 50-mM Tris/HCl (pH 7.4) buffer. Each fraction of eluate was examined by gel electrophoresis, and the most purified and concentrated extracts were pooled before extensive dialysis against phosphate buffered saline (PBS), then stored at  $-80^{\circ}\text{C}$ .

### 2.3. Preparation of advanced glycation end-products (AGEs)

AGEs were prepared, as described elsewhere [15], by incubating 0.37 mM of HSA-C and purified HSA from a HSA-ND pool without and with glucose (100 mM) in PBS (pH 7.4) under sterile conditions at  $37^{\circ}\text{C}$  for 3 weeks or with MGO (10 mM) for 2 days. The proteins were dialysed against PBS, sterile-filtered with a 0.2- $\mu\text{m}$  Millipore filter and stored at  $-80^{\circ}\text{C}$ .

### 2.4. Fructosamine assay and albuminaemia

Albumin concentrations were determined using an albumin reagent kit (ALB2, COBAS<sup>®</sup>) based on a colorimetric assay at 570 nm of albumin mixed with bromocresol green (BCG, 3',3'',5',5''-tetrabromo-m-cresolsulphonephthalein) [17]. Fructosamine was determined by the method developed by Johnson et al. [18] using a commercial reagent kit (FRA, COBAS<sup>®</sup>). The results were expressed as mmol/L of 1-deoxy-1-morpholino-d-fructose (DMF), a synthetic ketoamine used as a primary standard.

### 2.5. Quenching of tryptophan and 1-anilino-8-naphthalene sulphonic acid (ANSA) fluorescences

Fluorescence spectra were carried out on a Perkin-Elmer LS55 spectrometer with protein samples at a concentration of 10  $\mu\text{M}$  in PBS. Tryptophan emission spectra were obtained in the range of 250 to 600 nm under excitation at 270 nm. All fluorescence spectra were corrected for their different respective absorptions.

1-anilino-8-naphthalene sulphonic acid (ANSA) was dissolved in 5- $\mu\text{M}$  albumin samples at an ANSA/albumin ratio of approximately 0.4 (mol/mol) to ensure that the dye was linked to the hydrophobic sites of albumin. ANSA–protein complex emission fluorescence spectra were performed in the range of 350 to 700 nm under an excitation wavelength of 470 nm.

### 2.6. Oxidative modification of –SH

Thiol groups in native and modified albumin were measured by Ellman's assay using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) [19], as has been well described elsewhere [14].

Various concentrations of L-cysteine (10 to 100 nmol) were used to perform the standard curve. Thiol group content was measured by reading the absorbance at 412 nm. Results were expressed as the number of free–SH groups per mol of bovine serum albumin (BSA).

### 2.7. 2,4,6-trinitrobenzene sulphonic acid (TNBS) assay

The TNBS assay is a sensitive method for determining the primary free amino groups in proteins [20]. Again, this method has been described in detail elsewhere [16].

### 2.8. Methacrylamido phenylboronic acid (MPBA) polyacrylamide gel

Discriminating analysis between glycosylated and non-glycosylated albumin has previously been performed using MPBA polyacrylamide electrophoresis [21]. MPBA-resolving acrylamide gels were prepared by adding 1% (w/v) of MPBA (provided by J. van den Elsen, Department of Biology and Biochemistry, University of Bath, UK) to 8% acrylamide solution. The 4% stacking acrylamide gel was prepared without boronic acid. Albumin samples (20  $\mu\text{g}$ ) were applied to the gel in denaturing sodium dodecyl sulphate (SDS) and reductive dithiothreitol (DTT) buffers.

### 2.9. Copper-binding affinity

The capacity of albumin to bind copper ions can be measured spectrophotometrically using bathocuproinedisulphonic acid (BCS, Fluka #11870) [22,23]. AGE preparations (40 nmol in 0.15 M of NaCl) were incubated in triplicate for 2 h with 50 nmol  $\text{CuSO}_4$  in a final volume of 500  $\mu\text{L}$ . The unbound ligand fractions were separated from the albumin-linked ligand fractions by ultrafiltration, using the Amicon<sup>®</sup> Ultra system (Millipore, Billerica, MA, USA), with centrifugation at 3000 g for 20 min. The concentration of copper bound to albumin was determined by adding BCS (1.2 mM) diluted in 1 mM of sodium ascorbate solution, followed by an absorbance reading at 480 nm after 5 min of incubation at room temperature. The concentrations of copper bound to modified albumins were calculated using a calibration standard curve by increasing the amount of  $\text{CuSO}_4$  up to 100 nmol.

### 2.10. Enzyme-linked immunosorbent assay (Elisa) quantification of carbonylated proteins

The degree of carbonylation of glycosylated albumin was determined by carbonyl Elisa assay based on recognition of protein-bound DNPH in carbonylated proteins with an anti-DNP antibody. This method has been described in detail in previous published studies from our group [16,24].

### 2.11. Haemolysis test

Each well of a 96-well plate was filled with 100  $\mu\text{L}$  (about  $1.10^8$  erythrocytes; final concentration: 400,000 cells/ $\mu\text{L}$ ) of a

diluted solution of red blood (1/10 in 0.15 M of NaCl). Different albumin samples (final concentration: 10  $\mu$ M) were added in triplicate. Haemolysis was initiated by adding 100  $\mu$ L of 0.45 M of AAPH solution to each well. Turbidimetry at 450 nm was recorded at 10-min intervals using a 37 °C thermostated microplate reader. Results were expressed as 50% of maximum haemolysis time (HT<sub>50</sub>) in min.

### 2.12. Statistical analysis

Data were expressed as the means  $\pm$  standard deviation (SD) of a minimum of three experiments. Statistical significances

were determined using one-way analysis of variance (Anova) followed by Tukey's test for multiple comparisons, with a *P* value < 0.05 required for significance.

## 3. Results

### 3.1. Biochemical characterizations of human serum albumin (HSA) samples

Clinical and biochemical characteristics of the diabetic plasma samples are presented in [Table S1, Supplementary data](#). All purified albumin samples exhibited approximately

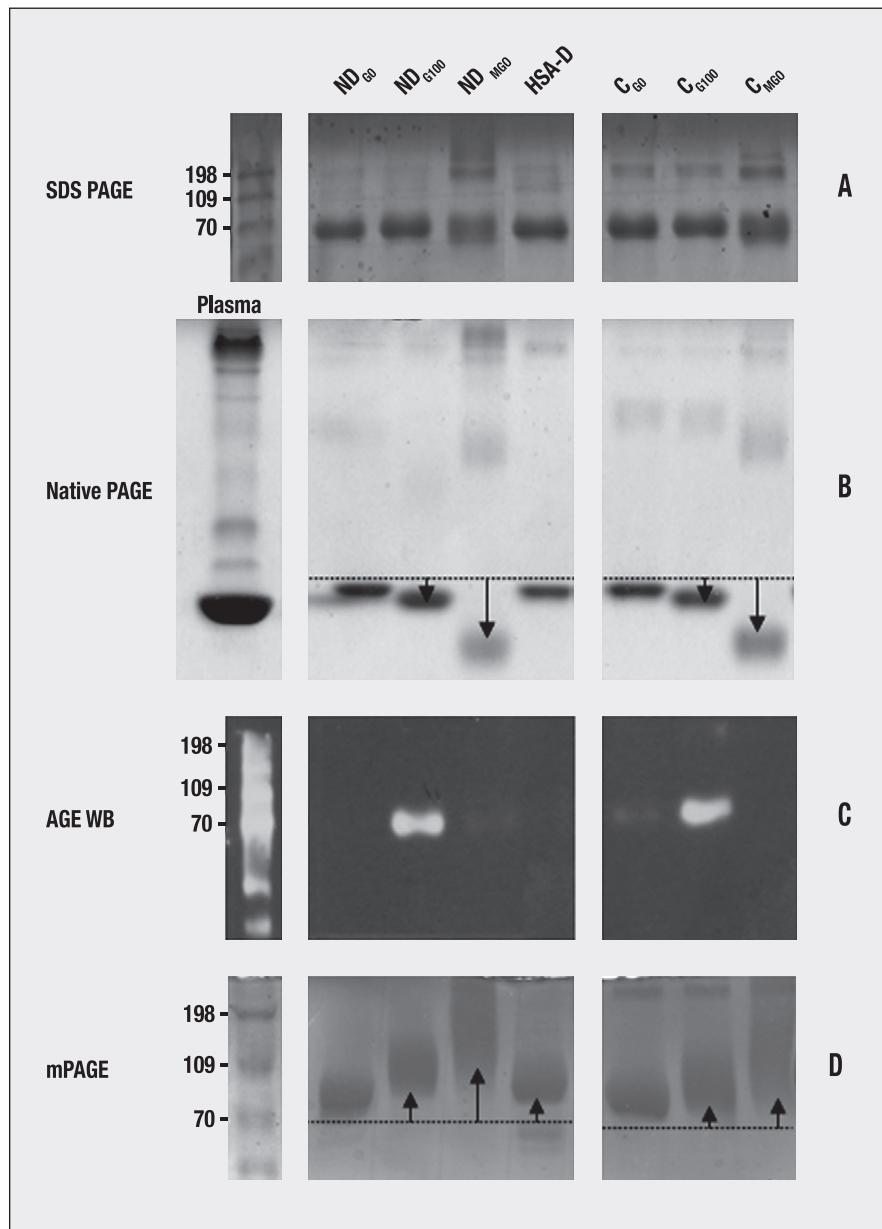


Fig. 1. Characterization of purified human serum albumin from non-diabetic patients (HSA-ND) and human serum albumin from diabetic patients (HSA-D) and commercial human serum albumins (HSA-C) modified by glycation; a: coomassie staining of human serum albumin (HSA) samples separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) (12%); b: electrophoretic migration profile of native polyacrylamide gel electrophoresis (12%); c: advanced glycation end-product (AGE) content of human serum albumin samples as assessed by Western blotting (WB); d: separation of human serum albumin samples using phenylboronate acrylamide gel electrophoresis (mPAGE).



identical molecular weights (about 66 kDa), with a slight variation of a few Daltons for modified HSA corresponding to attachment to the protein of one or several glucose (162 Da) or MGO (54 Da) units (Fig. 1a). In contrast, these albumins differed in their net charge, as confirmed by native polyacrylamide gel electrophoresis (PAGE, Fig. 1b). In this situation, the protein charge constituted the main factor affecting its migration. A higher migration was observed for albumins (HSA-ND and HSA-C) modified by glucose and MGO compared with native HSA. Indeed, electrophoretic migration was dramatically enhanced for albumin modified by MGO. Such enhanced migrations for HSA-ND and HSA-C modified by glucose and MGO vs native HSA indicate impairment of the isoelectric point of albumin following glycation. Also, there was a decrease in cationic charges in albumin after glycation that could be attributed to the involvement of positively charged residues (arginine and lysine) in condensation with carbohydrate [14].

The end stage of the glycation process gives rise to irreversible conjugates known as AGEs [3]. Of these AGEs, N<sup>ε</sup>-carboxymethyl-lysine (CML) and N<sup>ε</sup>-carboxyethyl-lysine (CEL) were formed during the incubation of albumin with glucose and MGO, respectively. Western blotting (Fig. 1c) revealed that using antibodies directed against AGEs showed enhanced signals only for HSA modified by 100 mM of glucose, whereas no signal was observed for HSA purified from the plasma of diabetic patients. The lack of signal for MGO-modified albumin suggests that our AGE antibody was directed against CML rather than CEL.

Several oxidative parameters in our albumin preparations are shown in Table 1. As expected, levels of free thiol and amino groups decreased significantly in albumin after glycation by glucose and MGO. For both parameters, the decrease was more evident with MGO HSA-ND (HSA-C) modification than with glucose, although such severe decreases with MGO have been previously reported [25]. Similarly, the increase in carbonyl rate, attributed to enhanced oxidation of albumin HSA-ND (HSA-C) with *in vitro* modification, was more marked with MGO than

with glucose. Significant differences in the levels of free thiol and amino groups and carbonyl rate were also found between albumin from non-diabetic subjects (HSA-ND<sub>G0</sub>) and diabetic patients (HSA-D). Their free amino group modification level and carbonyl rate values were similar to the values obtained for HSA-ND<sub>G100</sub>. In contrast, free thiol content was more affected in diabetic albumin than in MGO-modified albumin *in vitro* compared with non-modified and non-diabetic albumins.

Fructosamine levels (μg/g of HSA) were significantly enhanced in albumin from diabetic patients compared with the controls. In comparison to diabetic albumin, fructosamine levels increased markedly with modification by glucose *in vitro* and, to a lesser extent, by MGO of both albumins (HSA-ND and HSA-C). The lower fructosamine level for HSA<sub>MGO</sub> can be explained by the higher reactivity of MGO compared with glucose, thereby giving rise more quickly to AGE formation.

In numerous studies, boronate affinity chromatography has proved its efficacy in separating Amadori products (ketoamine) from non-modified albumin [14,27]. According to Morais et al. [21], Amadori products such as fructosamine can also be directly characterized by using phenylboronate incorporated into acrylamide gel electrophoresis. Phenylboronic acid has also shown its capacity to function as a saccharide receptor in the aqueous solution of many sensory systems [26,27].

In mPAGE gel profiles (Fig. 1d), a shift in the main protein band to a higher molecular weight (> 90 kDa) was observed for modified commercial and purified HSA, and accompanied by broadening for MGO-modified HSA. A slight shift in the protein band (≈ 80 kDa) was observed for diabetic HSA.

### 3.2. Fluorescence studies

Tryptophan fluorescence emission (at 350 nm) decreased in both modified HSA (HSA-ND<sub>G100</sub> and HSA-ND<sub>MGO</sub>) and diabetic HSA (HSA-D). The quenching of fluorescence was stronger for HSA-ND<sub>MGO</sub> than for HSA-ND<sub>G100</sub> in comparison

Table 1  
Oxidation parameters for various albumin preparations from three independent experiments.

Methods	Fructosamine/human serum albumin (HSA) (μg/g)	Thiols/human serum albumin (HSA) (mol/mol)	Free amino groups (% /control)	Carbonyl rate increase (% /control)
<i>Purified HSA-ND<sup>a</sup></i>				
HSA-ND <sub>G0</sub>	1.96 ± 0.08	0.483 ± 0.023	100.00 ± 4.70	100.00 ± 5.72
HSA-ND <sub>G100</sub>	45.77 ± 1.83***	0.371 ± 0.019*	60.39 ± 6.72***	87.12 ± 11.25
HSA-ND <sub>MGO</sub>	31.02 ± 1.24***	0.307 ± 0.016**	29.43 ± 1.39***	593.76 ± 16.39***
<i>Purified HSA-D<sup>a</sup></i>				
HSA-D <sub>G0</sub>	5.65 ± 0.10***	0.263 ± 0.003***	79.07 ± 3.20**	195.66 ± 19.39***
<i>Commercial HSA<sup>b</sup></i>				
HSA-C <sub>G0</sub>	2.62 ± 0.23	0.372 ± 0.004	100.00 ± 5.05	100.00 ± 7.96
HSA-C <sub>G100</sub>	47.54 ± 1.90***	0.325 ± 0.006**	61.45 ± 2.35***	86.55 ± 8.13
HSA-C <sub>MGO</sub>	29.76 ± 1.19***	0.329 ± 0.008*	21.99 ± 3.39***	348.13 ± 22.33***

Fructosamine levels were obtained by nitroblue tetrazolium (NBT assay); free thiol group content was assessed by Ellman's method; unmodified primary amino group content in proteins was by trinitrobenzene sulphonic acid (TNBS) assay; carbonyl rate (%) was assessed by Elisa carbonyl assay; all data are expressed as means ± SD; \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$ , \*  $P < 0.05$ .

<sup>a</sup> Effect of glycation or diabetes in purified human serum albumin (HSA) (vs HSA-ND<sub>G0</sub>).

<sup>b</sup> Effect of glycation in commercial human serum albumin (vs HSA-C<sub>G0</sub>).

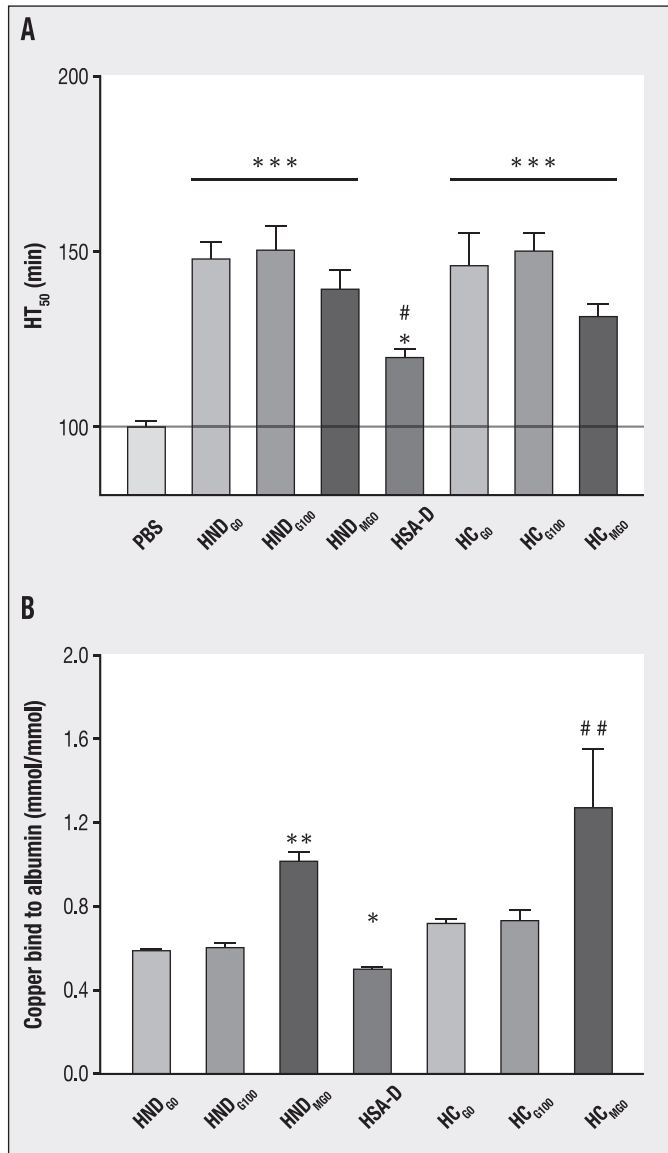


Fig. 2. Impairment of functional albumin properties in diabetes; a: antioxidant activities as assessed by free-radical-mediated blood haemolysis test (HT): histograms represent haemolysis test half-time (HT<sub>50</sub> in min) expressed as the means  $\pm$  SD of three separate experiments. The thin line allows comparison with controls (no albumin). Statistical analyses were performed using one-way Anova for multiple comparisons. \* Effect of HSA-ND or HSA-C (vs. control): \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ ; effect of glycation in commercial human serum albumin (vs HSA-CG0); b: copper-binding capacity of human serum albumin (HSA) samples as assessed by the bathocuproinedisulphonic (BCS) method: histograms represent copper-binding capacity of modified albumin (nmol/ $\mu$ mol albumin). Data are expressed as means  $\pm$  SD ( $n = 3$ ); statistical analyses were performed using one-way Anova for multiple comparisons. Effect of glycation or diabetes in purified human serum albumin (vs HSA-ND<sub>G0</sub>): \*\* $P < 0.01$ , \* $P < 0.05$ . Effect of glycation in commercial human serum albumin (vs HSA-CG0): ### $P < 0.01$ .

to native HSA-ND, while HSA-D samples had an intermediate fluorescence emission that was in between those of both glycosylated HSA-ND samples (Supplementary data, Fig. S1a). While the emission fluorescence spectra (Supplementary data, Fig. S1b) showed extrinsic fluorescence quenching at 300 nm for MGO-modified HSA and diabetic HSA, glycation by glucose

enhanced ANSA fluorescence in comparison to non-modified albumin. These data show the differential conformational changes induced by glucose- and MGO-induced glycation.

### 3.3. Functional properties

To assess the effect of glycation on the antioxidant properties of albumin, their free-radical-scavenging properties were investigated in a free-radical-induced haemolysis test. In the test, the intrinsic capacity of albumin to protect erythrocytes from haemolysis induced by free radicals was evidenced by a higher haemolysis half-time (HT<sub>50</sub>) vs a PBS control (Fig. 2a). While *in vitro* glycoxidation of both albumins by glucose and MGO did not significantly affect their anti-radical activity, this protective effect was partially lost in the case of diabetic HSA. Compared with HSA-ND<sub>G0</sub>, a reduction of about 20% ( $P < 0.05$ ) in HT<sub>50</sub> for HSA-D was observed.

The antioxidant capacity of albumin is also related to its affinity to several metals. Copper deserves special consideration as most mammalian albumins bind to this cation more tightly and more specifically than in other animals [28]. Copper absorbed through the intestines is transported by albumin in the portal circulation before being incorporated in ceruloplasmin in the liver [29]. While glucose-mediated glycation did not affect albumin affinity for copper (0.5 mol of copper per mol of albumin), modification by MGO drastically increased albumin capacity to bind to the metal (1.1 mol of copper per mol of albumin; Fig. 2b). Albumin affinity for copper was also significantly affected in cases of diabetes. HSA-D showed a significant decrease (–16%,  $P < 0.05$ ) in its copper affinity compared with non-diabetic HSA.

## 4. Conclusion

Glycation-induced albumin structural and functional changes are of particular interest as numerous studies *in vivo* have reported the strong involvement of glycosylated albumin in the development and progression of chronic diabetes complications. Most of these studies were focused on the structural and functional changes of commercial BSA-induced glycoxidation *in vitro*. However, only a few studies compared the structural and functional changes between models of glycoxidation of human albumin *in vitro* with albumin in hyperglycaemia *in vivo*. For this reason, the present study was undertaken to evaluate the suitability of *in vitro* models of glycation mimicking diabetic pathology.

Using different techniques, modifications in intrinsic properties were studied in HSA induced by glycation *in vitro* with glucose (100 mM) and with MGO (10 mM). Comparison of these modifications with those observed in diabetic albumin could constitute a key factor in helping investigators to choose the most suitable glycation model for cellular physiology studies. Of albumin properties, the redox state, scavenging capacity and binding affinity of the protein were also investigated. As expected and consistent with previous studies, the incubation of human albumin with glucose and MGO *in vitro* contributed

to the increase in fructosamine and carbonyl rates, indicating the formation of Amadori products accompanied by an oxidation process. This increase in oxidative state was confirmed by the impaired redox states of the single thiol group in albumin (Cys 34). The glycation of albumin also induced a decrease in free amino group levels, indicating the involvement of several exposed negatively charged lysine and arginine residues that were neutralized by glucose and MGO during glycation. The increase in the net negative electrical charge has already been described for oxidized lipoproteins increasingly involved in atherosclerosis [30]. The tertiary structure is also partially affected by *in vitro* glycation by glucose or a derivative, as confirmed by tryptophan and ANSA fluorescence results. Overall modification of the three-dimensional structure was also induced by glycation with, in particular, a conformation change in hydrophobic pockets. Consequently, these structural changes associated with modification of the redox state have a direct bearing on the intrinsic biological functionality of albumin, including its free-radical-scavenging properties and copper-binding affinity. In albumin, the single reduced cysteine unit constitutes an important redox regulator in extracellular compartments [31,32]. Under our experimental conditions, glucose-mediated glycation did not affect albumin antioxidant activity, as assessed by the haemolysis test, as also observed elsewhere [16]. On the other hand, in MGO-modified albumins, alteration of the protective effect of albumin was consistent with the redox state of cysteine. These results could be attributed to the different tertiary structures of HSA<sub>G100</sub> and HSA<sub>MGO</sub>, as revealed by the fluorescence results of the ANSA probe. The partial unfolding of hydrophobic pockets of albumin induced by incubation with glucose revealed specific amino acids, previously buried in the native conformation of HSA, to be potential free-radical scavengers.

The antioxidant capacity of albumin also depends on the protein's capacity to bind copper ions. HSA contains one high-affinity site for copper, the N-terminal tripeptide Asp-Ala-His [33]. Protein sequestration of copper ions has been shown to prevent reactive oxygen species (ROS)-generating reactions [34]. Given that albumin conformation plays a key role in its transport capacity, the significant increase in affinity for copper observed in MGO-modified albumin can be attributed to the specific tertiary structure induced by glycation.

Regarding the data for diabetic HSA, the fundamental question to emerge was: are the structural and functional properties of models *in vitro* relevant to diabetic HSA characteristics? While most quantitative changes in oxidative and structural parameters were observed in glucose- or MGO-modified albumins, significant impairment of albumin function (free-radical-scavenging and copper-binding capacities) was demonstrated in HSA purified from diabetics. However, these findings showed different structural and functional features in diabetic HSA compared with *in vitro* models.

As for structural and biological results, glycated albumin *in vivo* appears to be the most relevant model for mimicking hyperglycaemia or diabetes. The diabetic model of HSA *in vivo* may be more suitable than *in vitro* cellular physiology studies examining the relationship between albumin status and diabetes-associated

cardiovascular complications. Also, activated macrophage cells lines, key elements of atherosclerosis development, may be the most suitable cell model for evaluating the physiological impact of the *in vivo* diabetic albumin model. While considering glycated HbA<sub>1c</sub> levels from SMBG as the best indicator of diabetic status, glycated albumin would appear to be a suitable marker for the monitoring of diabetes-associated complications.

Albumin plays an important role in the systemic distribution of many therapeutic drugs [35,36]. A reduced affinity of albumin for drugs with a narrow therapeutic index can result in their toxic effect because of an increase in concentrations in their free forms and the impact of their pharmacological properties. It would be of great interest to extend the present study to an analysis of albumin affinity for specific therapeutic drugs relevant to diabetes to obtain a better understanding of the impact of diabetes on the pharmacokinetic and pharmacodynamic properties of these drugs.

### Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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*Author contributions:* A. Guerin-Dubourg researched data, contributed discussion, reviewed the manuscript. A. Catan researched data. E. Bourdon contributed discussion, reviewed the manuscript. P. Rondeau researched data, wrote, reviewed and edited the manuscript.

### Appendix A. Supplementary data

Supplementary material (Table S1 and Fig. S1) associated with this article can be found at <http://www.sciencedirect.com>, at doi:10.1016/j.diabet.2011.11.002.

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