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Oxidative modifications impair albumin quantification

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ABSTRACT

Background: Hypoalbuminemia is a measure of malnutrition, inflammation and a predictor of mortality in uremia. It is controversial whether albumin levels *per se* are associated with the clinical outcomes in uremic patients. The co-occurrence of hypoalbuminemia and oxidative stress in hemodialysis (HD) patients led us to hypothesize that oxidative modifications of albumin decrease its detection and influence albumin quantification.

Methods: Albumin levels are determined in clinical laboratories mainly by the bromocresol green (BCG) spectrophotometric assay. The detection of serum albumin was investigated in HD patients and in healthy controls using an "albumin-detection index", defined as the ratio between BCG read-out (albumin-specific) to total albumin. The detection efficacy of albumin was also investigated *in vitro*, after glycoxidation, HOCl-mediated-oxidation, and metal-catalyzed-oxidation. Oncotic pressure was measured to assess albumin function.

Results: The albumin-detection index of patients was significantly lower compared with controls, correlating negatively with oxidative stress markers (serum advanced oxidation protein products-AOPP and glycoxidized serum albumin) and positively with serum albumin levels. The albumin-detection index was also decreased after *in vitro* oxidation.

Conclusions: The study shows, both *in vivo* and *in vitro*, decreased detection of oxidized albumin by a commonly-used clinical assay, thus providing the molecular link between oxidative stress and hypoalbuminemia. Oxidative stress as reflected by hypoalbuminemia, rather than actual albumin levels, may be related to cardiovascular morbidity outcomes in HD patient.

1. Introduction

Hypoalbuminemia is considered a measure of malnutrition, inflammation and a predictor of mortality in various clinical states [1]. In end-stage renal disease patients treated by hemodialysis (HD), hypoalbuminemia is a predictor of vascular morbidity [2] and mortality [3].

Along with hypoalbuminemia, increased oxidative stress is prevalent in HD patients, contributing to cardiovascular complications [4]. Oxidative stress causes molecular modifications of human serum albumin (HSA), such as carbonylation [5,6], formation of advanced glycoxidation end products (AGEs) [7] and advanced oxidation protein products (AOPP) [8]. HSA oxidation disturbs its biological functions due to conformational alterations (such as increased negative charge and exposure of hydrophobic regions), ending in impaired site II-ligand binding capabilities, decreased drug binding and decreased antioxidant activity [6,8–10]. In HD patients hypoalbuminemia, oxidative stress, and inflammation are linked [11,12]. It is likely the cause of hypoalbuminemia, rather than albumin levels *per se*, that is related to morbidity in uremic patients [12].

This study assessed the hypothesis that oxidative modifications alter albumin, resulting in decreased detection by a common biochemical assay and may result in "apparent" hypoalbuminemia. The molecular link between oxidative stress and hypoalbuminemia was explored, supporting the *in vivo* experiments with verification of this hypothesis *in vitro*.

2. Materials and methods

All chemicals and antibodies were obtained from SIGMA (St. Louis, MO, USA), unless specified otherwise.

Abbreviations: AGE, advanced glycoxidation end products; AGE-HSA, glycoxidized human serum albumin; AOPP, advanced oxidation protein products; BCG, bromocresol green; CML, carboxy-methyllysine; CO-HSA, carbonylated human serum albumin; DNPH, 2,4-dinitrophenylhydrazine; HC, healthy control subjects; HD, hemodialysis; HSA, human serum albumin; IMA, ischemia modified albumin; MIA, malnutrition-inflammation-atherosclerosis syndrome; MS, mass spectroscopy; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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2.1. Subjects

Blood was drawn from 12 healthy controls (HC, 7 males/5 females; age 45.5 ± 3.2 yr, non-smokers), and from 12 patients (4 males/8 females; age 62.9 ± 3.9 yr) who underwent 4 h of HD treatment thrice weekly for over a year, with normal liver function and without evidence of infection, malignancy or severe hyperparathyroidism. Blood was drawn before dialysis, from the arterial line. Informed consent was obtained from all participants and the institutional Helsinki committee approved the protocol. Sera were used for determination of oxidative stress markers (AOPP levels, oxidized albumin), serum albumin levels (by bromocresol green – BCG) and albumin-detection index.

2.2. Determination of "albumin-detection index" in sera

An albumin-detection index was established as the ratio between HSA concentration determined by BCG (ABBOTT laboratories, USA, performed according to the instructions for the AEROSET chemical analyzer, ABBOTT), to total HSA concentration. Total concentration of albumin was determined by its absorbance at 280 nm (OD_{280}) or by ELISA. According to the hypothesis, if oxidized albumin is detected equally accurately by the BCG assay as by other methods, the ratio between the read-out results should equal 1. In case of decreased BCG read-outs resulting from oxidative modifications, this ratio will be <1.

Since all proteins and peptides absorb at 280 nm, serum albumin had to be purified. Each serum sample was subjected to gel-filtration chromatography as described [13]. After identification of albumin-containing fractions (by silver-staining with ProteoSilver Plus kit and by Western analysis with anti-human albumin), i.e., fractions containing the highest proportion of the 67 kD albumin and merely traces of other proteins, albumin concentrations were determined by OD_{280} and BCG. To subtract the contribution of contaminating proteins (trace amounts) to the OD_{280} of the albumin-fraction, proteins were separated by SDS–PAGE and silver-stained. Bands corresponding to immunoglobulins, transferrin and albumin were identified, quantified by densitometry and their percentages calculated, assuming that contamination by less abundant proteins was negligible. Total protein concentration was then calculated in each fraction using the fraction's OD_{280} value and the appropriate extinction coefficients (1.4, 1.12 and 0.531 for 1 mg/ml of immunoglobulins, transferrin and albumin, respectively; data obtained from SIGMA website). Finally, the OD_{280} of immunoglobulins and transferrin were subtracted from the total OD_{280} of the fraction, giving the net OD_{280} of albumin. Albumin-detection index was then calculated by the ratio between HSA concentrations determined by BCG to total HSA.

In part of the samples, the index was calculated using total HSA concentration determined by ELISA.

2.2.1. Determination of AOPP

AOPP levels, a marker of protein oxidation, were measured in sera as described previously [8].

2.3. Oxidative modifications of albumin in sera

2.3.1. AGE-albumin determination

Albumin-enriched fractions were simultaneously separated on two SDS–PAGE gels: One gel was silver-stained and the other was used for Western analysis with anti-AGE (clone 6D12, COSMO-BIO, Japan) and anti mouse-HRP-conjugate as secondary antibody. The 6D12 clone detects Nε-carboxy-methyllysine (CML)-protein adducts and not early products (Schiff base and Amadori products). Western analysis signals were quantified by densitometry and the ratio of AGE signal to silver-stained albumin band was considered as a measure of AGE-albumin.

2.3.2. Carbonylated-albumin determination

The levels of carbonylated-albumin were determined as described previously [14]. Briefly, albumin-enriched fractions were reacted with 2,4-dinitrophenylhydrazine (DNPH) and separated on two parallel SDS–PAGE gels. One gel was silver-stained and the other was used for Western blotting with anti-DNP. The signals were quantified by densitometry. The degree of albumin-carbonylation was determined by the ratio of carbonyl signal to silver-stained albumin band.

2.4. Densitometry

The densities of Western analysis signals and silver-stained protein bands were quantified using the BioCapt and Bio-Profil (Bio-1D) softwares.

2.5. Direct ELISA for albumin

Albumin-enriched fractions were absorbed onto a Maxisorp plate (NUNC) by overnight incubation at 4 °C. The following steps were performed at room temperature. After washing, wells were blocked with 0.25% fish gelatine solution in phosphate-buffered saline (PBS) for 1 h. Wells were incubated with anti-human albumin in a 1/50,000 dilution in blocking solution (prepared in PBS with 0.05% Tween-20, PBST) for 1 h. After washing in PBST, wells were incubated with Goat-anti-Rabbit-horseradish peroxidase conjugate (1/20,000 dilution in blocking solution) for 1 h. Peroxidase activity was determined by addition of tetramethylbenzidine substrate (TMB, eBIOSCIENCE, USA) for 5 min, stopped with 0.1 M sulfuric acid, and the O.D. results were immediately read at 450 nm in an automated plate reader.

2.6. Albumin oxidations in vitro

Commercial human serum albumin (HSA, powder, cat#A3782) was dissolved in PBS to give physiological concentrations and oxidized in different oxidation systems. Specific oxidants concentrations were employed as described [8,15,16]. Three oxidation systems were used.

2.6.1. HOCl-mediated oxidation (mainly formation of AOPP-HSA)

HSA in physiological concentrations (40 mg/ml) was exposed to 50 mM HOCl (ALDRICH) as described [8]. A control HSA sample was incubated likewise without HOCl. Samples were then dialyzed against PBS (cutoff 12,000 Da).

2.6.2. Metal-catalyzed oxidation (resulting in carbonylated-HSA, CO-HSA)

HSA was incubated for 20 h at 37 °C with $FeCl_3$ and ascorbate as described [15]. Oxidizing agents were then removed by overnight dialysis. An untreated HSA sample was analyzed as control.

2.6.3. Glycoxidation (formation of AGE-HSA)

HSA was incubated with 1 M glucose and antibiotics under sterile conditions for 60 days at 37 °C, as described [16]. Control samples were treated identically, but without glucose.

2.7. Determinations of albumin concentration and detection index after in vitro oxidations

HSA concentration was measured by OD_{280}, (extinction coefficient: 0.531 for 1 mg/ml, SIGMA website) and the BCG assay. In part of the experiments the absorbance spectrum was measured

in the range of 240–320 nm to rule out any possible changes in OD_{280} due to the oxidation.

2.8. Assessment of oncotic pressure

Colloid osmotic pressure (oncotic pressure) was measured using a colloid osmometer (Wescor, Logan, USA) in samples of glycoxidation experiments ($n = 4$).

2.9. Statistical analysis

Data parameters were analyzed by Wilcoxon rank sum test and Wilcoxon signed rank test as appropriate (according to the results of Spearman correlation coefficient test) and by linear regression analysis. $p < 0.05$ was considered significant. The mean ± SD of albumin levels and albumin-detection index was calculated.

3. Results

3.1. Determination of albumin-detection index in subjects

After gel-filtration chromatography of sera, the albumin-detection index was calculated in the fractions comprising the 67 kD albumin. In these fractions, albumin concentrations determined by OD_{280} were similar in HD and HC ($p = 0.74$), while the levels determined by the BCG assay were decreased by 38% in the HD vs HC ($p = 0.058$). The calculated albumin-detection index in the HD group was 0.62, significantly ($p = 0.0004$) lower by 35% compared to HC (0.96, Fig. 1A).

In an attempt to measure total albumin concentration by a different method (other than OD_{280}) we have determined total HSA concentration by ELISA and re-calculated the detection index. This BCG/ELISA index was 0.64 in HD, significantly ($p = 0.048$) lower by 30% compared to HC (0.92, Fig. 1B). The indices calculated by the two methods correlated significantly ($p = 0.004$, Fig. 1C).

Albumin levels were measured by BCG in sera of HD patients and controls. Patients' albumin levels were 3.8 ± 0.3 g/dl, significantly lower than 4.4 ± 0.2 g/dl, measured in healthy subjects. The albumin-detection index, (determined in fractionated sera by BCG/OD_{280}), correlated with the albumin levels in serum ($r = 0.65$, $p = 0.0007$, Fig. 1D). When this correlation was studied in each group separately, only in patients was the correlation significant ($r = 0.63$, $p = 0.029$), while in the HC group $p = 0.39$ was found.

3.2. Oxidative stress in subjects

Since the ability of the BCG reagent to bind albumin was decreased in patients, we assumed that the surface structure of albumin in these patients was altered. Oxidative stress can, potentially, modify the interaction of BCG with albumin and therefore oxidative stress markers (AOPP, AGE-HSA, CO-HSA) were evaluated in patients' sera.

Serum AOPP level was 183 ± 14 μM in HD, significantly higher than in HC (111 ± 7 μM; $p = 0.0004$). AGE-HSA and CO-HSA were studied in albumin-enriched fractions by Western analysis and densitometry quantification of the Western analysis signals. A clear and significant increase in AGE-HSA ($p < 0.01$, Fig. 2A) and CO-HSA ($p = 0.026$, Fig. 2B) signal intensities were observed in HD samples compared to HC.

AGE-HSA signals correlated significantly with serum AOPP levels ($r = 0.55$, $p = 0.021$) but a correlation between CO-HSA and serum levels of AOPP could not be unequivocally demonstrated ($p = 0.062$), probably due to the limited subject number.

The albumin-detection index was found to correlate significantly with serum levels of AOPP (Fig. 2C) and of AGE-albumin

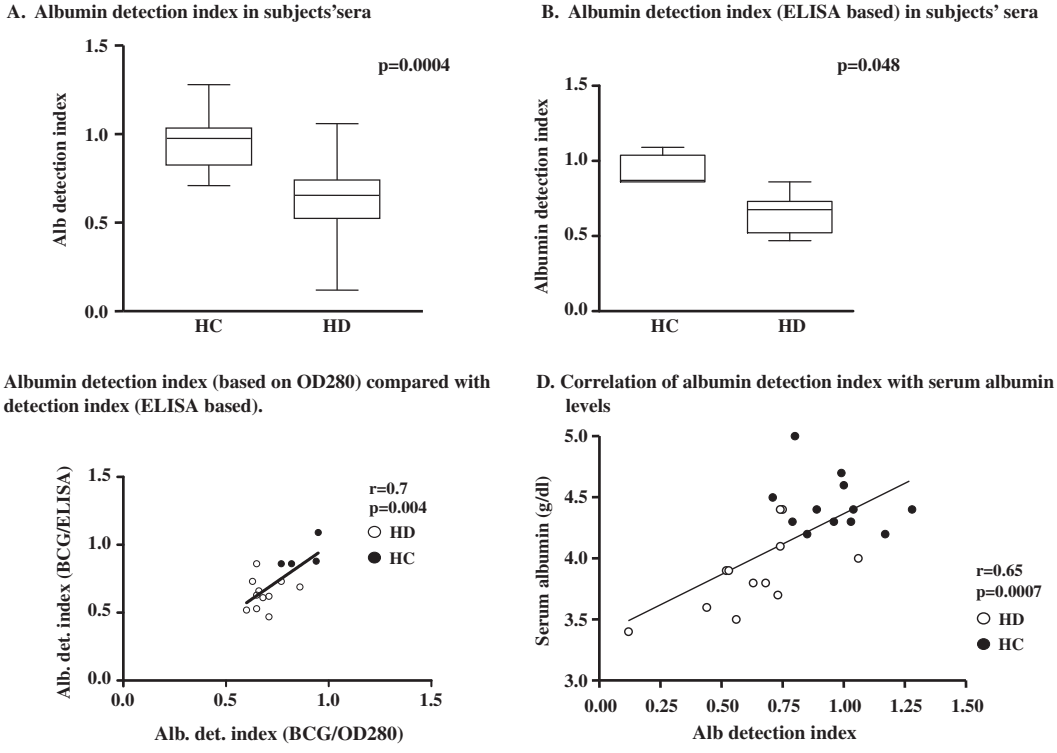

Fig. 1. Albumin-detection index in subjects' sera. Sera samples from patients and controls were fractionated using gel-filtration chromatography. Albumin-containing fractions were identified, and the ratio between HSA levels determined by BCG and by OD_{280} was calculated, giving the albumin-detection index (A). In part of the samples ($n = 15$, 11 patients, 4 controls) the detection index was also calculated using total albumin concentration that was determined by ELISA (B). The indices were compared (C). (D) The albumin-detection index (BCG/OD_{280}) was correlated with serum albumin levels (measured by BCG) of control subjects (●) and patients (○).

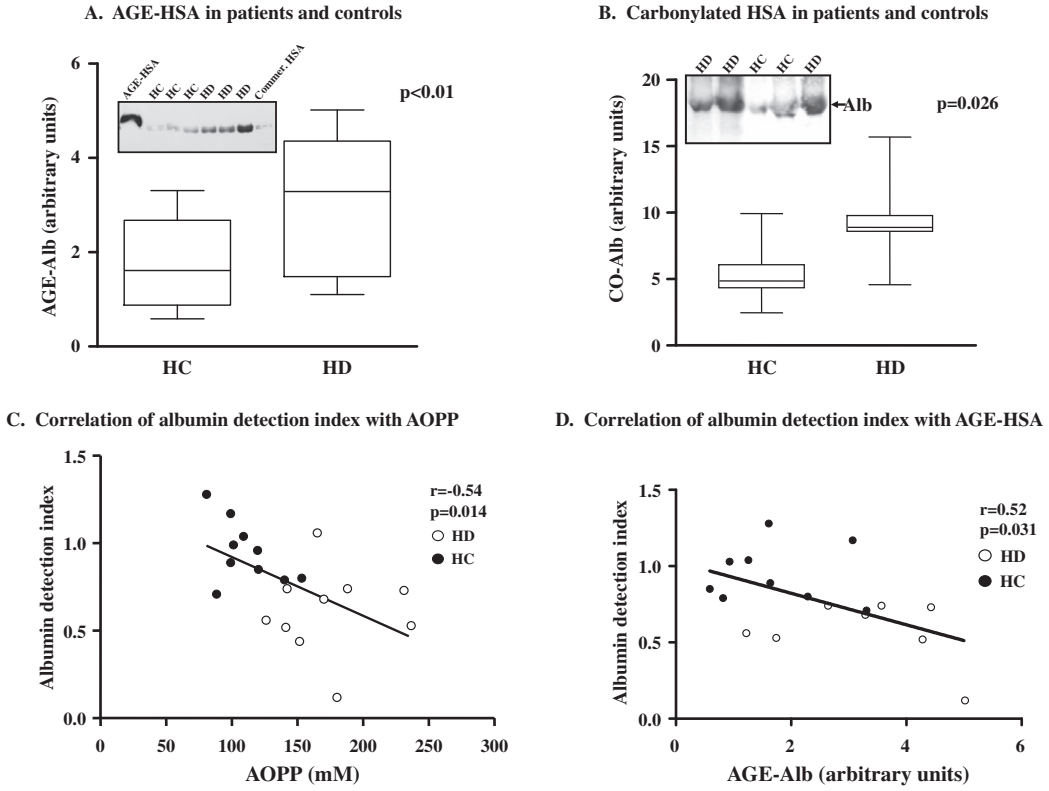

Fig. 2. Determination of oxidized albumin in serum. (A) The levels of glycoxydized-albumin were followed in the gel-filtration chromatography fractions (from 9 HC and 9 HD subjects) using Western analysis with antibodies against advanced glycoxidation end products (anti-AGE). Equal albumin amounts (based on calculated OD_{280} of albumin, described in "Materials and Methods") were loaded. Samples from three representative patients (HD) and three healthy control subjects (HC) are shown. Commercial human serum albumin that was glycoxydized *in vitro* (AGE-HSA) and untreated commercial human serum albumin (Commer. HSA), served as positive and negative controls, respectively. The Western analysis signals of control subjects (●) and HD patients (○) were quantified by densitometry, normalized according to silver-stained protein signals and compared. (B) The levels of carbonylated-albumin (from 8 HC and 7 HD subjects) were followed in gel-filtration chromatography fractions, quantified and compared as for AGE-HSA. The albumin-detection index (determined in fractionated sera by BCG/OD_{280}) was correlated with serum levels of AOPP (C) and with the AGE-HSA (D).

(Fig. 2D). Albumin levels in serum (BCG-measured) also correlated negatively with AOPP levels ($r = -0.52$, $p = 0.0195$), altogether suggesting that the albumin-detection efficiency and the albumin levels in serum may be influenced by oxidative stress.

Interestingly, an increase in the molecular mass of AGE-HSA positive control was observed (Fig. 2A). This may have resulted from the massive addition of sugar moieties (AGEs) to the protein during its preparation *in vitro*.

None of the investigated modifications were detected on the non-oxidized commercial HSA (negative control, Fig. 2A), and immunodetection with anti-human albumin antibodies did not indicate any degree of fragmentation (data not shown), suggesting that the commercial albumin was free of carbonyl and AGE modifications.

3.3. Detection of in vitro oxidized albumin

The *in vivo* results showing decreased HSA detection in HD patients, and its correlation to oxidative stress markers, stimulated us to support our hypothesis with *in vitro* experiments. After oxidation of commercial HSA by HOCl (mainly formation of AOPP-HSA), Fe3+/ascorbate (formation of CO-HSA) and glycoxidation (AGE-HSA), HSA concentrations were determined by the BCG assay and OD_{280}. The OD_{280} measurements of albumin samples were virtually unaffected by oxidation (Fig. 3A) with no significant differences in their absorption spectra (Fig. 3B), while the BCG-based measurements were clearly flawed by Fe3+/ascorbate oxidation and glycoxidation (Fig. 3A). The calculated albumin-detection index (Fig. 3C) showed significant decreases of 50.3%, 24.3% and 7.7% for glycoxidation, Fe3+/ascorbate oxidation and HOCl-mediated oxidation, respectively. In control samples (non-oxidized HSA), the mean albumin-detection index was 1.03 ± 0.09, as predicted.

3.4. Effects of glycoxidation on albumin structure and function

Albumin's function as a supporter of oncotic pressure was investigated in the *in vitro* oxidation system that showed the most remarkable effects, i.e., glycoxidation. Glycoxidation decreased the oncotic pressure by 30.0% (Fig. 4). In parallel, SDS–PAGE and silver-staining showed that glycoxidation caused some degradation of AGE-HSA, evident by a "smeared" appearance and a fainter signal, and an increase in its molecular mass, compared to control samples (Fig. 4).

4. Discussion

This study examined the effects of oxidative modifications on albumin measurements in a cohort of patients with established oxidative stress. It demonstrates that the BCG assay, routinely used for serum albumin quantification, under-estimates albumin concentrations when the protein is oxidatively modified. A direct molecular mechanism linking oxidative stress and hypoalbuminemia is provided by this study. This link was only suggested by statistical correlations in previous epidemiological studies [4,11].

The significant negative correlation between the serum albumin levels and AOPP, suggests that protein oxidation may interfere with albumin measurements. Oxidation of albumin *in vivo* by car-

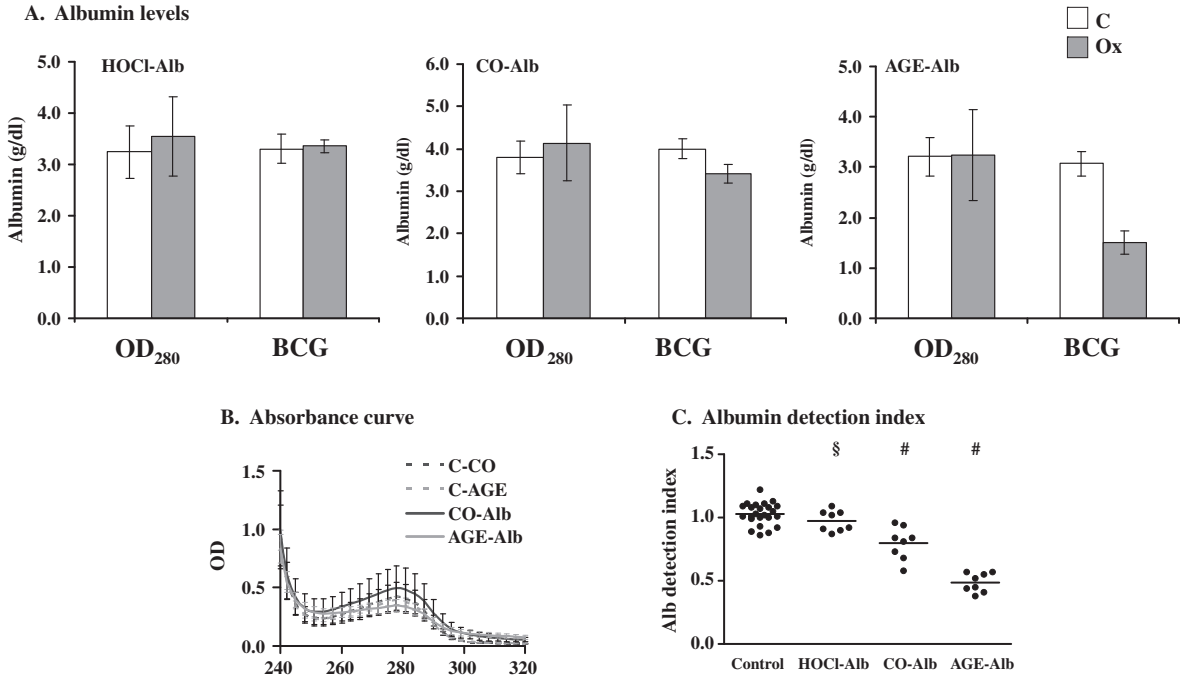

Fig. 3. Detection of *in vitro* oxidized albumin. Commercial HSA was oxidized *in vitro* by: HOCl-mediated oxidation (HOCl-Alb), Fe3+/ascorbate oxidation (carbonylation; CO-Alb) and glycoxidation (AGE-Alb). (A) Albumin concentrations were determined in control (white bars) and oxidized (grey bars) samples by BCG and by OD_{280} (representative results of $n = 3$, $n = 5$ and $n = 7$ for HOCl-med, CO-Alb and AGE-Alb, respectively). (B) The spectral absorbance curve of CO-Alb, AGE-Alb and their corresponding controls (C-CO and C-AGE), at 240–320 nm. Each curve represents the mean of three experiments. (C) Albumin-detection index (determined by BCG/OD_{280}) was calculated. p values (compared to control) are indicated: $^{\#}p = 0.004$, $^{\S}p = 0.026$.

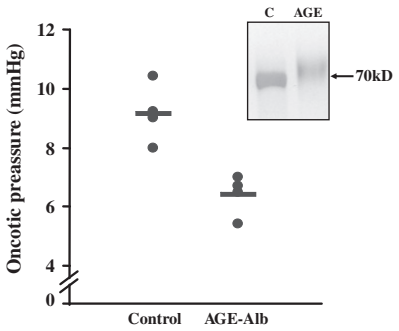

Fig. 4. Effects of glycoxidation on albumin structure and function. Equal amounts of glycoxidized (AGE) and control (C) albumin samples were separated by SDS–PAGE and detected by silver-staining. In part of the *in vitro* glycoxidation experiments ($n = 4$) albumin functions were assessed by measurements of oncotic pressure.

bonyls and AGEs (due to renal failure, dialysis treatments, iron therapy etc.) is supported by previous studies [5–7,11,14,17,18]. Combined with the *in vitro* results, albumin oxidation *in vivo* in the patients is reflected by a deviation of the albumin-detection index from the value of 1.0. Nevertheless, decreased detection of albumin does not rule out other conditions that may affect actual albumin concentrations due to hepatic albumin production, such as acute phase conditions, metabolic acidosis [19] and inflammation [1]. These conditions are associated with high levels of circulating cytokines [20], activating molecular processes that affect both albumin metabolism in the liver and albumin catabolism in the vascular endothelium and other compartments [1,21].

An important aspect is revealed by the significant correlations of the detection index with serum albumin levels, and with oxidative stress markers, concerning the actual levels of functional albumin in serum. One may speculate: should the BCG be replaced by assays measuring total albumin, both functional and oxidized ("low-function") albumin. Conversely, the oxidized ("low-function") albumin probably has an active role in these patients as suggested by the strong association between cardiovascular morbidity, hypoalbuminemia, and oxidative stress [2,11,16,17,22].

There are some weaknesses in the study: (1) the study describes a phenomenon but does not present a practical means to measure total serum albumin levels (oxidized and native) in clinical laboratories; (2) as each albumin molecule may be simultaneously modified *in vivo* by AGEs, carbonyls, AOPPs, oxidized thiols [17,18] and other modifications, each with a different potency to decrease albumin detection, a practical means to predict the decrease in albumin measurements cannot be provided. Hence, quantification of albumin modifications probably cannot assist us in an attempt to calculate total serum albumin (including oxidized and native albumin) indirectly.

Clearly, additional albumin-detection assays, except for those currently used may possibly help in the measurement of absolute albumin concentration in serum. Since such assays, unfortunately, *are not available to date*, the combination of biochemical and molecular techniques, present the strength of this study. The careful study design, the unique method for assessment of the detection index and the selection of straightforward, classical biochemical methods enabled the illumination of a molecular mechanism linking hypoalbuminemia to oxidative stress. Moreover, the focus on the 67 kDa HSA, assuming that, although oxidized, this albumin-fraction maintains its molecular mass mostly unchanged, enabled the investigation of otherwise undetectable molecules. The alternative use of proteomics in this research (and in routine measurements) is impractical, as each serum peptide/protein had to be analyzed to reveal its origin and since MS analysis is not quantitative.

This study implies also to other patient cohorts where hypoalbuminemia and oxidative stress co-exist, as supported by results in the elderly [23,24] and diabetics [25,26]. Although the separate contributions of age and disease to oxidative stress in elderly

populations may be difficult to dissect, serum albumin levels are significantly lower compared to younger subjects [24], pointing out the speculative possibility that albumin modifications occur also in this group. In HD patients, albumin levels do not correlate with patient's age [21], as albumin oxidation processes probably occur also in younger patients on HD therapy. Consequently, age-matching of the patients and control groups is of little importance and should not affect the data obtained.

Our data was supported by studies that showed a significant negative correlation between ischemia modified albumin (IMA), a new oxidative stress marker and serum albumin levels, in pregnancy [27,28]. Hence, we believe that future studies in populations with established oxidative stress may eventually instigate the development of new albumin-determination-assays that are *insensitive* to albumin modifications and will allow accurate albumin measurements.

Another interesting and novel aspect of this study is the decrease in colloidal osmotic pressure in glycoxidized albumin, not studied previously. As the regulation of colloidal osmotic pressure is a major biological function of native albumin [9,10], further studies should elucidate the clinical relevance of this finding.

Since oxidized albumin can trigger endothelial dysfunction [16,29] and promote initiation of atherosclerosis and cardiovascular morbidity, hypoalbuminemia should be interpreted based on the presence or absence of systemic oxidative stress in order to predict long-term clinical outcomes.

5. Conclusion

This study provides a novel, molecular link between oxidative stress and hypoalbuminemia by showing decreased detection of oxidized albumin *in vivo* and *in vitro*. Hence oxidative stress, reflected by hypoalbuminemia, rather than actual albumin levels, may be related to cardiovascular morbidity outcomes in HD patients.

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