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*Article*

# Association between Albumin Alterations and Renal Function in Patients with Type 2 Diabetes Mellitus

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**Abstract:** Diabetic kidney disease (DKD) is a major cause of morbidity and mortality of type 2 diabetes mellitus (T2DM). Aim of this study was to investigate whether albumin structural alterations correlate with DKD severity and evaluate whether native and reduced albumin concentrations could complement the diagnosis of DKD. To this aim, one hundred and seventeen T2DM patients without (n=42) and with (n=75) DKD (DKD I-III upon KDIGO classification) were evaluated; total albumin concentration (tHA) was quantified by bromocresol green assay, while structural alterations were profiled assessed by liquid chromatography-high resolution mass spectrometry (LC-HRMS). The concentrations of albumin in native (eHA, effective albumin) and reduced (rHA) forms were then assessed. HRMS analyses showed a reduced relative amount of native albumin in DKD patients along with an increased abundance of altered forms, especially those bearing oxidative modifications. Accordingly, both eHA and rHA values varied along the stages of progressive renal failure, and alterations dose-dependently correlated with renal dysfunction. ROC curves analysis showed a significantly higher sensitivity and specificity of eHA and rHA with respect to tHA for the diagnosis of DKD. Importantly, at multivariable logistic regression analysis, eHA was identified as independent predictor of DKD.

**Keywords:** diabetic kidney disease; effective albumin; reduced albumin; structural alterations; oxidative damages; high resolution mass spectrometry

## 1. Introduction

Diabetic kidney disease (DKD) is one of the most common chronic complications and a major cause of morbidity and mortality of diabetes mellitus (both type 1, T1DM, and type 2, T2DM)[1–3]. Among 400 million T2DM patients worldwide, 50% show evidence of chronic kidney disease (CKD) mainly related to DKD. In regional studies, the prevalence of DKD in the diabetic population ranges from 30% to 80% and more[4]. Moreover, DKD is considered the leading cause of end-stage kidney disease (ESKD) with DKD patients accounting for 25-45% of all patients enrolled in ESKD programs[2]. This is particularly worrying considering that the prevalence of diabetes mellitus (especially T2DM) has dramatically risen worldwide; in 2021, 11% of the global population had diabetes and this prevalence is expected to reach 12% by 2045[4]. Early identification of DKD is a primary unmet clinical need, not only to predict and prevent disease progression, but also to improve patients' survival and reduce associated morbidities. In clinical practice, the diagnosis of kidney

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damage in diabetic patients is primarily based on the observation of persistent (3-6 months) and elevated urinary protein excretion (proteinuria) (> 150 mg/24 h measured with a 24 h hour urine protein test, or > 30 mg/g measured with urine albumin-to-creatinine ratio [UACR]) and impaired renal function, expressed by a reduced estimated glomerular filtration rate (eGFR < 60 mL/min/1.73 $m^2$). These parameters are frequently associated with elevated blood pressure and cardiovascular complications and are a major cause of morbidity and mortality. Diagnostic renal biopsy is only required when non-diabetic renal disease is suspected[2]. In 2012, a cross-sectional study carried out on a cohort of 15,773 T2DM patients suggested that patients with significant albuminuria predominantly experience microvascular complications – the kidney being the main target of microvascular damage in diabetes –, while cardiovascular complications were principally associated with reduced eGFR alone[5].

The prototype of DKD is characterized by an early stage of glomerular hyperfiltration, followed subsequently by the onset of albuminuria and later by the progressive decline of the eGFR. However, additional phenotypes have now been identified, some of which are characterized by the presence of only microalbuminuria or the absence of urinary protein excretion. These phenotypes are often marked by a rapid decline in renal function[6]. In both cases, it is clear that albuminuria per se is not sensitive enough as biomarker in the early phase of DKD, whereas eGFR was proven to risk stratify DKD patients only when < 60 mL/min/1.73 $m^2$, i.e., when almost half of kidney function was lost[7],[8].

In this scenario, more sensitive biomarkers able to diagnose DKD and prompt appropriate therapeutic intervention are urgently needed, and their identification has been object of intense investigations. In the past decades, several biomarkers, mainly proteins, have been proposed[7,9–11]; most of them lack rigorous external validation in adequately powered studies with renal endpoints[9].

A recent study[12] in patients with liver cirrhosis showed that the decrease in total serum albumin concentration (tHA), a common feature of the disease, is accompanied by significant structural alterations, mainly oxidation at the only free cysteine residue (Cys34), and truncations. Due to the high plasma concentration of albumin, the reduce form of Cys34 represents the main plasma reservoir of free thiol groups which are endowed with scavenging capacity[13]. As a result of the structural alterations, the native form of the protein (nHA) is decreased to a greater extent than tHA. From this observation, the concept of effective albumin concentration (eHA), namely the concentration of albumin in its native form, was introduced[14],[12]. In the context of decompensated cirrhosis, eHA has been shown to be endowed with greater diagnostic and prognostic power than tHA. As in the case of liver cirrhosis, hypoalbuminemia is considered a reliable clinical indicator of DKD and is associated with impaired renal function and poor prognosis in T2DM patients with renal damage[15–18]. Previous studies have also shown that albumin structure is partially altered in T2DM patients with renal impairment and proposed the oxidized form of the protein as a marker for disease progression[19,20].

The present study aims to evaluate eHA and rHA (namely, the concentration of all albumin forms with Cys34 bearing a free thiol group) in a cohort of T2DM patients with and without DKD in order to assess whether these parameters can complement the (early) diagnosis of the renal impairment.

## 2. Results

### *2.1. Subject population*

One hundred and seventeen patients were enrolled between 2018 and 2021. Patients were classified as control or DKD group according to their albuminuria and eGFR values following the current Clinical Practice Guideline for Diabetes Management in Chronic Kidney Disease (KDIGO)[21]. Patients with eGFR greater than 60 mL/min/1.73 $m^2$ were tested as controls, while patients with eGFR < 60 mL/min/1.73 $m^2$ and UACR ≥ 30 mg/g were considered as having DKD. Anthropometric and clinical characteristics of enrolled patients with or without DKD are reported in

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Table 1. Briefly, patients with DKD were older and more likely to be on blood pressure lowering and diuretic therapies. In addition, DKD patients were characterized by lower cholesterol, LDL and HDL, and higher triglycerides levels. As expected, DKD patients were also characterized by a more impaired renal function, as indicated by higher creatinine with lower eGFR value and higher UACR (Table 1).

**Table 1.** Anthropometric and clinical data of T2DM patients with (DKD) and without (No DKD) renal impairment.

| | **No DKD** n = 42 | **DKD** n = 75 | **p value** |
|---|---|---|---|
| **Anthropometric data** | | | |
| Age (years) | 63 (56-67) | 70 (64-74) | <0.001 |
| Male sex (n, %) | 29 (69) | 52 (69) | 1.000 |
| BMI | 28.7 (25.8-34.4) | 32.3 (27.4-36.5) | 0.114 |
| **Drug therapy** | | | |
| Anti-hypertensives (n, %) | 31 (74) | 62 (83) | 0.340 |
| *ACE inhibitors (n, %)* | *15 (48)* | *29 (47)* | *0.883* |
| *Angiotensin receptor blockers (n, %)* | *13 (42)* | *27 (44)* | *0.882* |
| *Diuretics* (n, %) | *6 (14)* | *32 (43)* | *0.002* |
| Metformin (n, %) | 29 (70) | 42 (56) | 0.175 |
| Insulin (n, %) | 15 (36) | 40 (53) | 0.083 |
| Other glucose-lowering therapies (n, %) | 22 (52) | 43 (57) | 0.699 |
| *Sulfonylureas (n, %)* | *4 (18)* | *10 (23)* | *0.222* |
| *DPP-4 inhibitors (n, %)* | *7 (32)* | *13 (30)* | *0.896* |
| *GLP-1 Receptor agonists (n, %)* | *8 (36)* | *21 (49)* | *0.298* |
| *SGLT-2 Inhibitors (n, %)* | *5 (12)* | *3 (4)* | *0.067* |
| Statin/fibrates (n, %) | 26 (62) | 50 (67) | 0.687 |
| **Biochemical parameters** | | | |
| HbA1c (%) | 7.0 (6.4-7.6) | 7.1 (6.2-7.6) | 0.952 |
| Glucose (mg/dL) | 133 (110-155) | 123 (110-151) | 0.496 |
| Total cholesterol (mg/dL) | 172 ± 35 | 149 ± 31 | 0.001 |
| HDL (mg/dL) | 47 ± 9 | 41 ± 9 | 0.005 |
| LDL (mg/dL) | 101 ± 31 | 79 ± 27 | 0.002 |
| Triglycerides (mg/dL) | 122 (87-177) | 166 (116-204) | 0.032 |
| Creatinine (mg/dL) | 0.8 (0.8-0.9) | 1.4 (1.2-1.7) | <0.001 |
| UACR (mg/g) | 7 (5-10) | 66 (17-229) | <0.001 |
| eGFR (mL/min/1.73m$^2$) | 89 (77-97) | 48 (34-56) | <0.001 |

BMI: body mass index; ACE: angiotensin converting enzyme; DPP-4: Dipeptidyl Peptidase IV; GLP-1: Glucagon-like peptide-1; SGLT-2: Sodium-glucose Cotransporter-2; HDL: high density lipoprotein; LDL: low density lipoprotein. UACR: urinary albumin to creatinine ration. Data are reported as mean and standard deviation, median and interquartile range or absolute number and frequencies.

DKD patients were further classified according to the degree of renal damage following the CGA scheme proposed by KDIGO, where CGA stands for Cause, GFR category (G1–G5), and Albuminuria category (A1–A3), into moderately increased risk (DKD-I), high risk (DKD-II) and very high risk (DKD-III) of poor prognosis (see details in Figure 1).

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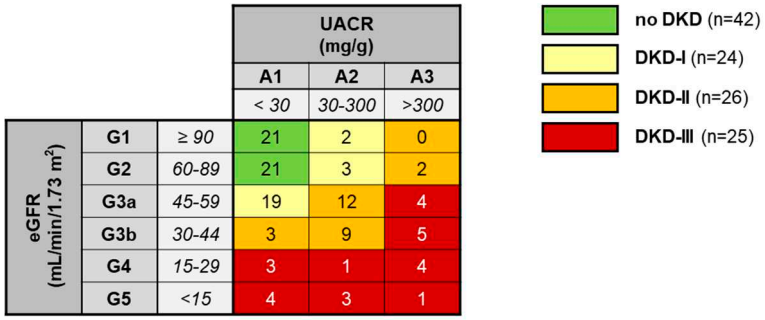


**Figure 1.** Classification of patients adapted from CGA scheme by KDIGO[21]. Green boxes: control, low risk (no CKD); yellow boxes: DKD-I group, moderately increased risk; orange: DKD-II group, high risk; red boxes: DKD-III group, very high risk. The number of enrolled patients for each eGFR category (G1–G5), and Albuminuria category (A1–A3) is reported.

Anthropometric and clinical characteristics of DKD patients grouped according to the risk category are listed in Table S1. The groups DKD I-III of subjects were similar with respect to age, sex, BMI, and most of pharmacologic treatments, except for glucose-lowering drugs. The three groups also had a significantly different lipid profile (Supplementary Table 1). Within the DKD-III group, 8 patients (25%) were under dialysis therapy at the time of the enrollment (patients with eGFR < 15 included in G5, Table 1).

*2.2. Evaluation of albumin structure*

The MS-analysis highlighted a significant higher number of structural alterations in circulating HA from DKD patients and showed that alterations primarily involve the redox state of Cys34 (Figure 2). Specifically, in patients with kidney damage the percentage of HNA1 increased by about 9% (from 16.4 (13.7 - 19) % to 25.7 (19.8 - 30.9) %; $p<0.0001$). Consistently, a significant reduction of the native form, i.e. nHA, (from 59.0 (57.1 - 60.6) % to 52.5 (48.3 - 56.7) %; $p<0.0001$) and HMA (form 75.7 (73.2 - 78.4) % to 67.1 (62.5 - 72.7) %; $p<0.0001$) was observed. A slight but significant decrease of HNA2 was also encountered (Table 2). Apart from the redox state of Cys34, no significant difference in the abundance of truncated forms was observed. Conversely, a slight increase (from 10.8 (9.2 - 12.2) % to 12.5 (10.5 - 14.7) %, $P = 0.0028$) in the relative abundance of glycated albumin was detected (Table 2). To note, carbamylated HA was previously annotated as altered form of HA in DKD patients[22] however, in our samples, no significant amount of carbamylated HA was detected. Considering that both HA concentration and structural integrity were altered in the presence of renal damage and that most alterations involved the redox state of Cys34 we focused our attention on the serum concentration of HMA, i.e., the concentration of all HA forms carrying a reduced Cys34. It is worth noting that the observed decrease of HMA (-9%) in DKD patients paralleled the increase of HNA1 (+9%) (Table 2) as these two forms are both related to the redox state of Cys34. Therefore, further investigations were conducted considering only rHA, being aware that similar results of the opposite sign could be obtained with the concentration of HNA1 forms.

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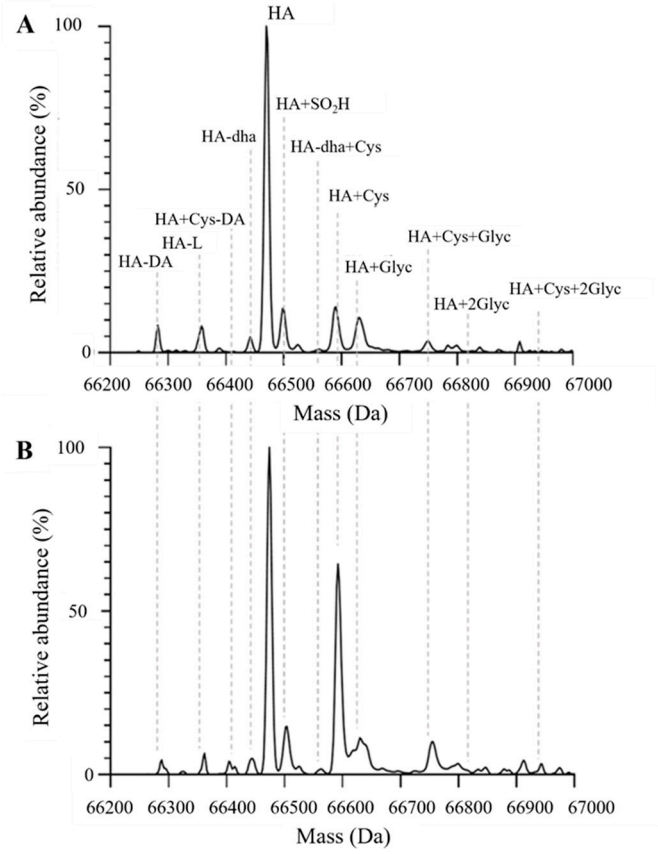


**Figure 2.** Representative MS spectra from a T2DM patient without (panel A) and from a T2DM patient with (panel B) renal impairment. *Abbreviations:* ***HA-DA****: truncation at the N-terminal portion;* ***HA-L****: truncation at the C-terminal portion;* ***HA+Cys-DA****: N-terminal truncated form cysteinylated at the Cys34;* ***HA****: native albumin;* ***HA-SO2H****: albumin sulfonylated at the Cys34;* ***HA+Cys****: cysteinylation at the level of the Cys34;* ***HA+Glyc****: mono-glycation;* ***HA+Cys+Glyc****: cysteinylated form carrying one glycation;* ***HA+2Glyc****: di-glycation;* ***HA+Cys+2Glyc****: cysteinylated form carrying two glycations.*

**Table 2.** Relative abundances (%) of native and altered forms of human albumin (HA) in diabetes patients without renal damage (no DKD n=42) and with renal damage (DKD, n=75) determined by LC-ESI-MS analysis. Data are statistically expressed as median and interquartile range.

| HA forms | Relative Abundance (%) | | p value* |
|---|---|---|---|
| | no DKD n = 42 | DKD n = 75 | |
| nHA | 59.0 (57.1 - 60.6) | 52.5 (48.3 - 56.7) | < 0.0001 |
| HMA | 75.7 (73.2 - 78.4) | 67.1 (62.5 - 72.7) | < 0.0001 |
| HNA1 | 16.4 (13.7 - 19) | 25.7 (19.8 - 30.9) | < 0.0001 |
| HNA2 | 8.9 (8.1 - 9.6) | 7.9 (7 - 8.6) | < 0.0001 |
| Truncated | 6.5 (5.1 - 7.9) | 5.2 (4.4 - 7) | 0.0242 |
| Glycated | 10.8 (9.2 - 12.2) | 12.5 (10.5 - 14.7) | 0.0028 |

*Mann-Whitney U test. Abbreviations: HMA: mercaptoalbumin; HNA1: non-mercaptoalbumin type 1; HNA2: non-mercaptoalbumin type 2.

*2.3. Total, effective, and reduced albumin concentration*

The levels of total albumin concentration (tHA) in DKD patients [4.1 (3.9 - 4.4)] g/dL were slightly lower than those in control patients [4.3 (4.1 - 4.5)] g/dL (P=0.001) (Table 3). In addition, the levels of both eHA, representing the absolute concentration of nHA, and rHA, representing the

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absolute concentration of all albumin forms with reduced Cys34, were significantly lower in T2DM patients with kidney damage and were able to discriminate those patients from control group better than tHA (p<0.001, Table 3).

ROC curves were plotted to assess the sensitivity and specificity of tHA, eHA and rHA for the diagnosis of renal impairment. The AUC (area under the curve) of both eHA and rHA had a higher diagnostic power for renal impairment (AUC-eHA: 0.831, 95% CI, 0.760-0.903; AUC-rHA: 0.823, 95% CI, 0.749-0.898) than tHA (AUC-tHA: 0.676, 95% CI, 0.579-0.772; p<0.001) (Figure 3). At multivariable logistic regression analysis only eHA remained as independent predictor of renal impairment (OR 0.58; 95% CI, 0.46-0.73).

**Table 3.** nHA, tHA and eHA values for patients with or without Diabetic Kidney Disease (DKD). Data were reported as median and interquartile range. The p value as determined by is reported.

| HA forms | Relative Abundance (%) | | *p* value* |
|---|---|---|---|
| | No DKD n = 42 | DKD (I-III) n = 75 | |
| tHA (g/dL) | 4.3 (4.1 - 4.5) | 4.1 (3.9 - 4.4) | 0.001 |
| rHA (g/dL) | 3.3 (3.2 - 3.4) | 2.8 (2.5 - 3.1) | < 0.001 |
| eHA (g/dL) | 2.5 (2.4 - 2.7) | 2.2 (1.9 - 2.4) | < 0.001 |

* Mann-Whitney U test. Abbreviations: tHA, total albumin; rHA: reduced albumin; eHA: native albumin.

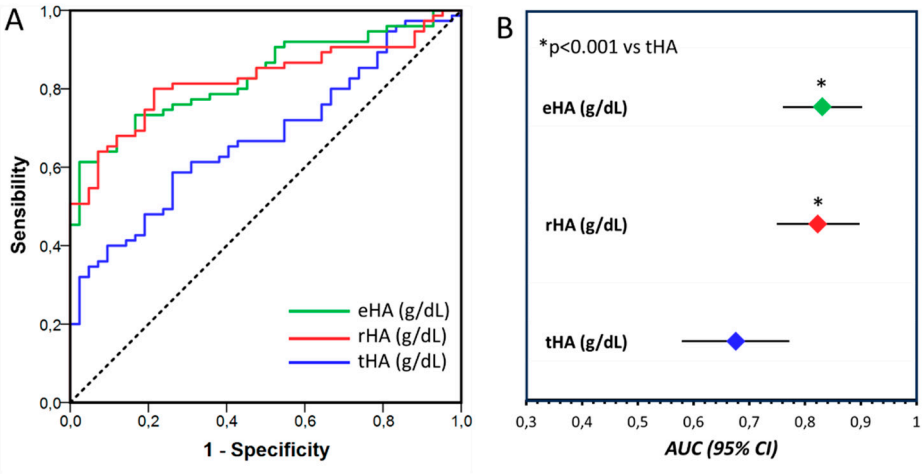


**Figure 3.** Panel A. Receiver operating characteristics curve analysis of total HA concentration (tHA), reduced HA concentration (rHA) and native albumin concentration (nHA) for diagnosis of Diabetic Kidney Disease; Panel B. Area Under the Curve (AUC) and 95% confidence interval. AUC were compared according to the DeLong method.

*2.4. Correlation of tHA, rHA and eHA with biochemical parameters*

Correlations between tHA, rHA and eHA and currently used biochemical parameters of DKD severity, i.e., creatinine, albuminuria and eGFR, were also evaluated. At baseline, all albumin-related parameters, namely tHA, rHA and eHA, were negatively correlated with creatinine and albuminuria levels, and positively with eGFR values (Table 4). After 1 year, significant correlations with biochemical parameters were maintained for rHA, eHA, while tHA was only associated with the amount of albuminuria level (Table 4).

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**Table 4.** Correlation of tHA, rHA and eHA, assessed at the baseline, with creatinine, albuminuria and eGFR levels (n = 117). Analysis was performed with data collected both at the baseline and at one year follow up.

| | | tHA | rHA | eHA |
|---|---|---|---|---|
| | | *Rho (p value)* | *Rho (p value)* | *Rho (p value)* |
| **Baseline** | Creatinine (mg/dL) | -0.260 (0.006) | -0.567 (<0.001) | -0.548 (<0.001) |
| | eGFR | 0.337 (<0.001) | 0.659 (<0.001) | 0.649 (<0.001) |
| | Albuminuria | -0.497 (<0.001) | -0.546 (<0.001) | -0.554 (<0.001) |
| **Follow-up** | Creatinine (mg/dL) | -0.172 (0.077) | -0.508 (<0.001) | -0.509 (<0.001) |
| | eGFR | 0.172 (0.076) | 0.562 (<0.001) | 0.584 (<0.001) |
| | Albuminuria | -0.363 (<0.001) | -0.461 (<0.001) | -0.407 (<0.001) |

Abbreviations: tHA, total albumin; rHA: reduced albumin; eHA: native albumin.

*2.5. Albumin and severity of diabetic kidney disease*

The association of tHA, rHA and eHA with the progression of the DKD was also evaluated. Eight patients under dialysis therapy at enrollment, who were previously included in group III, for the aim of this analysis were considered as a separate group.

As shown in Figure 4, tHA was different between patients with minimally-impaired renal function (DKD-I) and those with DKD-II, DKD-III and dialysis groups ($p<0.05$), but failed to disentangle more severe renal disease stages; rHA was able to distinguish dialysis group from DKD-I and DKD-II ($p<0.05$), as well as DKD-III from DKD-I ($p<0.05$); finally, eHA was able to discriminate DKD-III and dialysis group from DKD-I. Summarizing, rHA and eHA provided additional information to classify patients according to the stages of their renal disease.

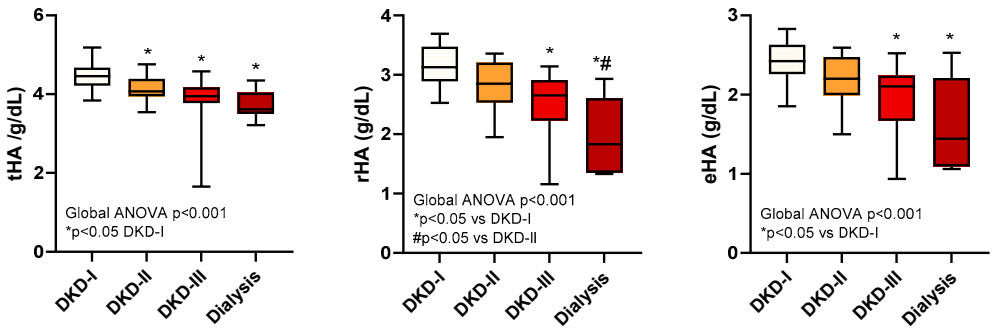


**Figure 4.** tHA, rHA and eHA in subgroups of diabetic patients with renal impairment (DKD-I, DKD-II, DKD-III and dialysis group).

## 3. Discussion

The study shows that HA structure is impaired in DKD patients with a prevalence of oxidative damages progressively increased with the severity of renal damage, likely resulting from the pro-oxidant environment associated with diabetes. This alteration occurs independently of the metabolic control, considering that no significant differences in HbA1c were demonstrated in the present setting, albeit achieved with different pharmacologic treatment, but glycated albumin was moderately increased in DKD.

CKD can itself be considered a chronic inflammatory disease independently of the presence of DM. In fact, a persistent, low-grade inflammation is now widely acknowledged as a pivotal factor in the pathophysiology of renal disease. This inflammatory state assumes a distinctive role, not only contributing to the progression of DKD but also playing a crucial role in the increased risk of

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cardiovascular events and all-cause mortality associated with this condition. Furthermore, this chronic inflammatory milieu is implicated in the genesis of protein-energy wasting, further exacerbating the complexities of DKD management. A multitude of factors contributes to the chronic inflammatory state in DKD. These include an increased production and decreased clearance of pro-inflammatory cytokines, oxidative stress, acidosis, chronic and recurrent infections, altered metabolism of adipose tissue, and intestinal dysbiosis. The level of inflammation is directly correlated with eGFR in CKD and intensifies in dialysis patients[23,24].

HA is an acute-phase reactant which undergoes several structural modifications during its circulatory life[25]. Although these modifications are also encountered in healthy patients, it is known that their extent is significantly larger in patients with chronic diseases characterized by increased proinflammatory and pro-oxidant circulatory microenvironment as is the case of DKD and T2DM-induced KD[26–31]. Indeed, reduction in tHA is considered a threatening parameter for long-term survival in several clinical settings, as well as a strong biomarker of poor outcome in several diseases[32]. Furthermore, HA plasma levels (tHA) have shown a consolidated prognostic power in liver diseases and malabsorption syndromes.

Given the clinical relevance of alterations in both HA plasma levels and structure in acute or chronic pathological conditions[32], we focused our attention on evaluating whether DKD severity in T2DM patients is associated with alterations in HA structure by exploiting a MS-based analytical approach that allowed the fine characterization of HA microheterogeneity (Fig. 2, Table 2). Consistently with previous investigations, the results showed that DKD is accompanied by a higher prevalence of altered forms and that most changes involved the redox state of Cys34[19,33,34] (i.e., HNA1 and HNA2, Table 2). Cys34 is a key residue of HA since it represents the major plasma reservoir of free thiols groups and acts as a scavenger of reactive oxygen species (ROS), thus contributing to a large part of plasma antioxidant capacity[13]. Indeed, the significant increase of the oxidized forms of circulating HA, i.e. HNA1, was paralleled by a significant decrement of nHA and HMA, in DKD patients is in line with an increased oxidative stress[29] and may imply a lower "buffering capacity" towards further ROS-related damages.

Along with the increase of HNA1, a slight but significant decrease of HNA2 was also observed. Similar data were previously reported by Baldassarre et al. who showed that the sulfynylated form of albumin was slightly lower in hospitalized cirrhotic patients compared to liver disease outpatients[12].

HA structure impairment was accompanied by a significant decline of tHA, in agreement with previous evidence showing that, in disease states accompanied by increased inflammatory processes, as it is the case of DKD, albumin levels are decreased as a consequence of reduced hepatic synthesis, increased catabolism and vascular permeability[16].

Due to the key physiological role of reduced HA as antioxidant agent, along with rHA (serum concentration of all HA forms reduced at the level of the Cys34) also eHA, i.e., the serum concentration of native HA, was evaluated. This evaluation is supported by the promising results previously achieved in the field of decompensated cirrhosis[12].

A comparison of tHA, rHA and eHA values showed that both rHA and eHA were significantly decreased in T2DM patients with DKD. More importantly, both rHA and eHA were able to discriminate the stage of renal damage better than tHA. This observation suggests the importance of considering not only the quantity of circulating protein, but also its structural integrity and prompted us to investigate the diagnostic capacity of these parameters.

The promising diagnostic power of rHA and eHA was confirmed by the analysis of the ROC curves; indeed, sensitivity and specificity of eHA and rHA was significantly higher than that of tHA for the diagnosis of renal impairment. Finally, a multivariable logistic regression analysis showed that eHA, but not rHA, was an independent predictor of renal impairment.

These results are consistent with those reported by Maruyama's group, showing that HNA1, which indirectly describes the antioxidant capacity of albumin, is the parameter that best correlates with the diagnosis of renal damage[35]. Moreover, the fact that eHA, which describes the concentration of native and fully functional albumin, is the only independent predictor of renal

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impairment suggests that other functions than the antioxidant capacity of Cys34 (such as binding and detoxification) may be impaired as the disease progresses.

The ability of HA to snapshot the clinical condition of DKD was further confirmed by the significant correlations between tHA, rHA and eHA and the biochemical parameters commonly used in clinical settings, i.e., creatinine, eGFR and albuminuria. This means that both the structural integrity and the amount of albumin are affected by the severity of kidney damage. Interestingly, a similar association was observed when the same parameters were assessed at one year's follow-up – albeit in a limited number of cases –, suggesting that rHA and eHA may be associated with disease progression.

In terms of clinical impact, a better understanding of the overall status of T2DM patients with different stage of renal damage might also help clinicians in the decision-making process. Hence, the discriminating power of tHA, rHA and eHA was evaluated. This comparison confirmed that both rHA and eHA significantly varied along the stages of progressive renal failure, supporting the idea that the initial stage of the disease is characterized by a decrease in serum albumin concentration, while oxidative damage prevails and impacts the oxidative status of Cys34 as renal damage progresses.

In this study, tHA is the only biomarker that significantly decreases in the early stages of the disease, while rHA and eHA values decrease significantly with the progression of renal damage. Interestingly, only rHA undergoes a further significant reduction in the terminal stage of the disease. Given this perspective, the diagnostic power of tHA in the early stages of CKD and DKD is intriguing, especially when used in conjunction with traditional markers of renal damage such as eGFR and albuminuria. Conversely, rHA and eHA seem to exhibit improved diagnostic efficacy in the intermediate-advanced stages of the diseases, enabling better risk stratification of patients. This may allow the identification of those at a higher risk of disease progression in which a more aggressive pharmacological approach could be beneficial.

Concluding, in this study we demonstrated that rHA and eHA were significantly altered in DKD patients, in a dose-dependent correlation with renal dysfunction, and might be exploited to complement the diagnosis of kidney damage. eHA was identified as independent predictor of renal impairment. The results prompt for further studies more deeply addressing biochemical processes leading to albumin changes and the clinical utility of this parameter for early diagnosis and prognosis of T2DM-related kidney disease.

## 4. Materials and Methods

### *4.1. Patients and study design*

Patients were screened for study enrollment among those attending the outpatient clinic of the Metabolic Diseases & Clinical Dietetics Unit and the Nephrology, Dialysis and Transplantation Unit of the IRCCS Azienda Ospedaliero-Universitaria di Bologna (Italy). The inclusion criteria were age between 18 and 70 years, a diagnosis of T2DM for at least 1 year and renal function at various stages (G1-5, with/without albuminuria), allowing coverage of DKD complications. Subjects were classified into four groups: NO-DKD (non-renal impairment), DKD-I (moderately increased risk), DKD-II (high risk), and DKD-III (very high risk) on the basis of the severity of renal dysfunction, using a combination of albuminuria levels, measured as urine albumin-creatinine ratio (UACR), and eGFR category, as recommended by KDIGO guideline [21]. Demographic, anthropometric and blood pressure parameters were assessed, and a medical history was collected to survey drug therapy. Blood samples (plasma and serum) were collected on fasting and were centrifuged at 3,000 g for 10 minutes; serum was aliquoted into cryotubes (Corning Inc., Corning BV, Amsterdam, The Netherlands) and stored at –80°C until analysis. Renal function parameters were also recorded after 1-year from study inclusion in all subjects. The study protocol was approved by the local ethical committee and written informed consent was obtained from all patients before enrolment, according to the 1975 Declaration of Helsinki.

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*4.2. Bromo cresol green (BCG) colorimetric assay*

Total albumin serum concentration (tHA) was determined by BCG colorimetric assay, adapting on a smaller scale the well-established method currently used in clinic[36]. BCG reagent contains 0.2 mM BCG, 0.1 mM succinate buffer pH 4.2 and 0.8% v/v Tween®20. Serum samples were diluted 5 folds in ultrapure water. A 5 µL aliquot of diluted serum samples was added to 200 µL of BCG reagent and taped lightly to mix. Samples were incubated for 5 minutes at room temperature. Blank solutions were prepared in parallel and contained all components except plasma sample. 200 µL of each sample and blank were transferred to a well of a clear 96-well flat-bottom microplate and absorbance in the range 570-670 nm (peak absorbance at 620 nm) was measured using a Spark® multimode microplate reader (Tecan, Austria). HA quantitation was performed by interpolating absorbance value at OD620nm in a calibration curve, built using HA standard solutions at increasing concentrations (5, 7,5, 10, 15 e 20 mg/mL). A standard curve was run with each set of assays. All assays were performed in triplicate.

*4.3. Liquid chromatography-mass spectrometry (LC-MS) analysis*

For the quantitation of albumin structural alterations, the validated high resolution LC-MS method, previously reported by Naldi et al., was employed with minor modifications [37]. The method exploits high-performance liquid chromatography coupled to electrospray ionization/quadrupole time-of-flight mass spectrometry (HPLC-ESI-Q-ToF) and measures the relative amount of i) HA native form (nHA), ii) HA forms with Cys34 in its reduced form (mercaptoalbumin; HMA), iii) HA forms with cysteinylated Cys34 (non-mercaptoalbumin 1; HNA1), iv) HA forms with irreversibly oxidized Cys34 (non-mercaptoalbumin 2; HNA2), v) HA glycated forms, vi) HA forms with truncations at the C- and N-terminals and vii) HA carrying combinations of these alterations (Figure 2, Table 2). Serum samples were diluted 1:100 with ultrapure water and filtered through a 0.22 µm syringe filter (Merck KGaA, Darmstadt, Germany). HPLC analyses were carried out on an Agilent 1200 HPLC System (Walbronn, Germany). The chromatographic separation of HA from other plasma proteins was achieved using a Phenomenex Jupiter C4 column (5 µm, 300 Å, 150 mm × 2.0 mm i.d.). A gradient was developed with mobile phases A [water/acetonitrile/formic acid (99/1/0.1, v/v/v)] and B [acetonitrile/water/formic acid (98/2/0.1, v/v/v)] as follows: 20–70% B, in 5 min; 70% B for 1 min. In between injections, the column was equilibrated for 5 min with starting conditions. The flow rate was set at 0.4 mL/min and the injection volume was 3 µL.

A Quadrupole-Time of Flight hybrid mass analyzer (Q-ToF Micro, Micromass, Manchester, UK) with a Z-spray electrospray ion source (ESI) was employed for the MS analysis. The capillary voltage and cone voltage were set at 3.0 kV and 40 V, respectively. The ESI-Q-ToF source temperature was set to 150°C while the desolvation temperature was set at 300°C. The scan time was set at 2.4 s, while the interscan time was set to 0.1 s. The desolvation gas flow was set at 1,000 L/h, and the cone gas flow was 120 L/h. The total ion current (TIC) chromatograms were acquired in positive polarity in the range of 1,000-1,800 m/z. Using the maximum entropy (MaxEnt1)-based software included with MassLynx software, the HA baseline-subtracted spectrum (m/z 1,084–1,534) was deconvoluted into a genuine mass scale. The output parameters were as follows: mass range 61,500–71,500 Da and resolution 2 Da/channel. The relative abundances of HA forms were estimated from the intensity of each form (obtained from the deconvoluted spectrum) and expressed as percentage of the total intensity of all forms. Microsoft Excel software (Microsoft Corporation, Redmond, WA) was used for data analysis.

*4.4. Assessment of eHA and rHA*

eHA and rHA were calculated using the following formulae: [12]

$$eHA\ \left(\frac{g}{dL}\right) = \frac{tHA\ \left(\frac{g}{dL}\right) \times nHA\ (\%)}{100}$$

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$$rHA\ \left(\frac{g}{dL}\right) = \frac{tHA\ \left(\frac{g}{dL}\right) \times HMA\ (\%)}{100}$$

where nHA is the amount of native albumin, HMA is the amount of albumin forms with reduced Cys34 as assessed by LC-MS analysis, and tHA is total albumin as assessed by BCG assay.

*4.5. Statistical analysis*

Normally distributed data were reported as mean and standard deviation (SD), whereas non normally distributed parameters were summarized by the median and interquartile range. Distribution was preliminarily assessed by the Kolmogorov-Smirnov test. Categorical variables are reported as absolute frequency and percentage. When appropriate, comparisons between groups were tested by the unpaired Student t test or Mann Whitney U test. In the case of comparisons between three or more groups, the Kruskal-Wallis analysis of variance (ANOVA) was performed, followed by a post-hoc analysis in which Bonferroni's correction for multiple comparisons was applied. The association between clinical parameters and nHA, tHA and eHA levels was evaluated by Spearman's correlation analysis, while the sensitivity and specificity of the same parameters in diagnosing kidney damage were determined by the Receiver Operating Characteristics (ROC) curve analysis. The resulting AUC were compared according to the DeLong method. Finally, a multivariable logistic regression analysis with backward selection was performed to compare the predictivity of rHA and tHA against renal damage. All tests were two sided and a p value less than 0.05 was considered as statistically significant. Data were processed using GraphPad Prism 8.4.2 (GraphPad Software, San Diego, CA, USA) and the Statistical Package for Social Sciences (SPSS version 25; IBM Corp., Armonk, NY).

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: Anthropometric and clinical data of patients with DKD I-III.

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**Data Availability Statement:** All data are available from the corresponding author on reasonable request.

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