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Oxidized albumin in blood reflects the severity of multiple vascular complications in diabetes mellitus

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ABSTRACT

Albumin has an oxidized form, known as non-mercaptalbumin (HNA), which reflects systemic oxidative stress. The association between serum HNA levels and diabetic complications are yet to be reported. In this cross-sectional study, we investigated 164 diabetic subjects to assess the correlation between HNA% (the proportion in the total albumin) and various clinical parameters. HNA% was significantly associated with the severity of multiple complications including neuropathy (23.3 ± 4.1% vs 26.2 ± 5.1%) and nephropathy (24.1 ± 3.9%, 24.6 ± 4.2%, 28.5 ± 6.1%, 31.3 ± 5.7%, 37.8 ± 2.9%, stage 1/2/3/4/5, respectively). These findings highlight the universal importance of oxidative stress, indicating HNA% potential as a versatile marker of the severity of diabetic complications.

Energy production in living organisms depends on oxidative phosphorylation in mitochondria, where oxygen acts as an electron acceptor. In the course of metabolism, some oxygen molecules become reduced to superoxide or reactive oxygen species (ROS), which can potentially damage DNA. Superoxide dismutase (SOD) eliminates ROS to protect mammalian macromolecules from oxidative damage. An imbalance in this process causes oxidative stress, leading to functional disorders in tissues and organs. Oxidative stress has been shown to play a key role in the pathogenesis of vascular complications of diabetes [1]. Chronic hyperglycemia enhances oxidative stress due to increased ROS generation [2] and impaired antioxidant defense [3]. Previous hyperglycemia has a long-standing impact on the subsequent development of diabetic complications (known as the “legacy effect”). Epigenetic changes have been implicated in this effect [4] and these changes may, theoretically, be mediated by ROS [1]. Various biomarkers of oxidative stress, such as 8-hydroxydeoxyguanosine (8-OHdG) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo) levels in urine and thiobarbituric acid reactive substance (TBARS) levels, catalase activity, and SOD activity in plasma, have been used in clinical trials. However, these biomarkers are rarely applied in clinical practice [5,6].

Albumin is the most abundant protein in the plasma. Several reports have shown that Cys34-cysteinylated albumin (human non-mercaptalbumin: HNA) may regulate redox status in humans [7–9]. Although some studies have reported a correlation between HNA levels and disease severity [10–13], there are no reports assessing their association with specific diseases, such as diabetes mellitus. Until recently, the accurate measurement of HNA levels in blood was technically difficult because of the complex and time-consuming procedures involved. Instability of the oxidation status of the specimen is the major challenge when investigating the relationship between HNA and diabetes complications. HNA gradually increases over time after blood sampling. The recent

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development of a novel high-performance liquid chromatography (HPLC) method by our group [14] has enabled the rapid and accurate measurement of the degree of oxidized albumin (HNA%). Using this method, HNA% was stable at room temperature for 25 h. Because of stability and simple procedure, large numbers of samples can be analyzed in a clinical setting, with greater ease and precision compared to conventional procedures for general oxidative stress markers.

1. Methods

1.1. Study subjects

In this single-center cross-sectional study, the subjects were inpatients diagnosed with diabetes mellitus, who were admitted to the Department of Diabetes and Metabolic Diseases at The University of Tokyo Hospital between July 17th, 2016 and March 31st, 2017. Patients were excluded if they were pregnant, were lactating, had acute organ failure (e.g., pneumonia, acute myocardial infarction, acute cerebral infarction, diabetic ketoacidosis, and hyperosmolar hyperglycemic state), or had congenital cognitive disorders. This study was performed according to the principles outlined in the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the University of Tokyo (No. 11171) and informed consent was obtained from all patients. For those patients who scored less than 22 out of 30 on the Mini Mental State Examination (MMSE), consent to participate was obtained from their legal representatives. This analysis was part of an exploratory study that aimed to examine factors related to cognitive function in patients with diabetes mellitus (Study S).

We investigated the correlation between HNA% and the following parameters: sex, age, disease duration, body mass index (BMI), smoking, alcohol consumption, family history of diabetes mellitus, coronary artery disease, stroke, neuropathy, retinopathy, nephropathy, systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure, diabetic treatment, use of antihypertensive drugs, use of lipid-lowering drugs, HbA1c, glycoalbumin (GA), GA/HbA1c ratio, homeostasis model assessment-insulin resistance (HOMA-IR), fasting C-peptide immunoreactivity (CPR), 2-h-after-meal CPR, C-peptide Index (CPI), fasting plasma glucose (FPG), serum albumin (Alb), uric acid (UA), triglycerides (TG), calculated LDL cholesterol (c-LDL), blood urea nitrogen (BUN), creatinine (Cre), estimated glomerular filtration rate (eGFR), and Mini Mental State Examination (MMSE) score. Neuropathy was diagnosed when the patient has at least one of the following findings: (1) coefficient of variation of R-R intervals (CVR-R) under 2%, (2) reduction in Achilles tendon reflex, (3) decreased lower limb vibration sensing, (4) and the presence of obvious sensory impairment. Retinopathy was diagnosed and classified into normal (-), simple diabetic retinopathy (SDR), pre-proliferative diabetic retinopathy (PPDR), or proliferative diabetic retinopathy (PDR), according to the Davis classification [15]. Nephropathy stage was determined by urinary albumin excretion and eGFR, according to the Classification of Diabetic Nephropathy 2014 proposed by the Joint Committee on Diabetic Nephropathy in Japan [16]. Patient background information was collected on the day of hospitalization and fasting blood samples were collected soon after obtaining informed consent.

1.2. Measurement of HNA%

HNA% measurements were performed according to a previously described procedure [14]. Briefly, after sample collection, tubes were kept at -80 °C until assayed. After defrosting, oxidative albumin was measured using a basic HPLC system (LabSolutions System; Shimadzu Co. Ltd, Kyoto, Japan) with an anion-exchange column (50*7.6 mm I.D.) containing a polyvinyl alcohol cross-linked gel (9 μm in diameter) reacted with diethyl amine. The HPLC conditions were as follows. Eluent A consisted of a solution of 25 mM phosphoric acid buffer, containing 60 mM sulfuric acid, sodium salt (pH 6.0) and eluent B was a 250 mM high-concentration magnesium chloride solution. The flow rate was 1 mL/min after equilibrating the column for 4.5 min with eluent A. The linear gradient time from eluent A (100%) to eluent B (100%) was programmed at 7.5 min. The total measurement time was 12 min per sample, the sample volume was 3 μL, and the temperature was set at 40 °C. The excitation and emission wavelengths were 280 nm and 340 nm, respectively. In addition, we added citrate buffer (pH.6.0) to the blood collection tubes in advance to avoid proceeding oxidization after blood collection. After blood collection, the amount of citrate buffer was adjusted to a final concentration of 70 mM [17].

1.3. Statistical analysis

Continuous variables are expressed as the mean ± standard deviation (SD). Categorical and ordinal variables are expressed as percentages. For bivariate analyses, we used a simple linear regression analysis, Fisher's exact test or analysis of variance (ANOVA), depending on the scale. Continuous variables were analyzed by paired Student's t-test or Mann-Whitney test. Significant predictors in the univariate analysis were then included in a forward, stepwise multiple logistic regression model to identify the most important risk factors for increased HNA%. Before performing multivariate linear correlation analysis, a stepwise forward selection method was performed, where "In" was defined as a p-value < 0.2 and "Out" was defined as a p-value < 0.1.

To evaluate the impact of HNA% on diabetic complications, we performed bivariate analysis to identify the potential risk factors contributing to diabetic complications. We then eliminated the risk factors that showed multiple collinearity before performing the multivariate linear correlation analysis. For simplicity, we set up dichotomous categorical variables describing each of the following factors and complications: smoking status (current or former versus never), retinopathy (PPDR or PDR versus normal or SDR) and nephropathy (stage 3–5 versus stage 1–2). For nephropathy, we excluded the factors which directly defined renal function due to its redundancy.

A two-sided p-value of less than 0.05 was considered statistically significant. All analyses were performed using JMP Pro 13 software for Windows (SAS Institute Inc, Cary, NC, USA).

2. Results

A total of 235 patients was recruited, of whom 35 did not give consent to participate in this study. Of the remaining 200 participants, 12 were excluded (1 due to a neurological disorder, 1 due to aphasia, 2 due to MMSE refusal, 1 due to impaired fasting glucose/impaired glucose tolerance, 4 due to depression, and 3 due to failure to contact their legal representatives). The remaining 188 patients were eligible for Study S. Blood specimens were not available from 24 patients for HNA% analysis, leaving a total of 164 patients finally enrolled in the study (Study A, Supplemental Fig. S1).163 patients were Japanese and 1 patient was a Chinese. Nine (5.5%) patients were diagnosed with type 1 diabetes and 153 (93.3%) with type 2 diabetes. One (0.6%) patient was suspected to have maturity-onset diabetes of the young (MODY) and one (0.6%) was diagnosed with pancreatic diabetes. One hundred and twenty-three patients scored 28–30 on the MMSE, 40 scored 24–27, and 1 scored below 23. Because of the limited sample size, patients with MMSE scores ≤27 were defined as having mild cognitive

impairment (MCI).

Baseline characteristics of the patients are shown in Table 1. Age was 63.5 ± 13.1 years, whereas the duration of diabetes was 13.4 ± 10.7 years. HbA1c levels were generally high (9.03 ± 1.76 %) since the subjects were all inpatients. Due to the relatively long disease duration, complications were frequently observed; 67.7% of the patients had neuropathy and 21.3% had history of coronary artery disease. HNA% was 25.3 ± 5.0 %.

By bivariate analysis, HNA% was positively correlated with age, disease duration, coronary artery disease, progression of neuropathy, PPDR and PDR, nephropathy, use of GLP-1RA or insulin, use of insulin, use of antihypertensive drugs, use of lipid-lowering drugs, pulse pressure, GA/HbA1c ratio, fasting CPR, CPI, BUN, and Cre, while it was negatively correlated with DBP, Alb, and eGFR (Table 2). Of note, HNA% increased with greater severity of diabetic complications (Fig. 1). The average HNA% was 26.2% and it was approximately 3% higher in the neuropathy (+) group than in the neuropathy (-) group. As for retinopathy, HNA% was not significantly different between the normal and SDR groups; however, HNA% was significantly higher in the PPDR plus PDR groups than normal and SDR groups. The average HNA% of PPDR plus PDR groups was 28.5%, which was 3.8% higher than that of the normal and SDR groups. In patients with nephropathy, HNA% increased gradually according to nephropathy progression, averaging 24.1% at stage 1, 24.6% at stage 2, 28.5% at stage 3, 31.3% at stage 4, and 37.7% at stage 5. HNA% was significantly higher in patients with coronary artery disease by 2.3% than those who did not have coronary artery disease. HNA% was significantly associated with the progression of microvascular complications and cardiovascular disease. Using most anti-hypertensive drug classes (Ca blocker, ARB, β-blocker, diuretic) except ACEI indicated significant associations with elevated HNA%. About lipid-lowering drugs, most classes showed no significant differences in HNA% except for ezetimibe; ezetimibe users exhibited higher HNA% than non-users ($P = 0.0391$).

To identify the significant factors contributing to HNA%, we performed multiple regression analysis. For stepwise analysis, applied variables were the followings; gender, age, disease duration, BMI, smoking, amount of alcohol consumed, diabetes family history, coronary artery disease, stroke, neuropathy, retinopathy, nephropathy, diabetic treatment, using depressor drugs, lipid-lowering drugs, SBP, DBP, pulse pressure, HbA1c, GA, GA/HbA1c ratio, FPG, HOMA-IR, 2-h-after-meal CPR, TG, and eGFR. Among the variables tested, BMI, GA/HbA1c ratio, and eGFR remained significantly independent, with the following formula: HNA % = 24.019 + 0.204 × BMI +1.442 × GA/HbA1c - 0.117 × eGFR. Fig. 2 indicates the relationship between predicted values based on the formula and measured values. The coefficient of determination (R^2) was 0.44.

The bivariate analysis showed neuropathy was significantly correlated with age, disease duration, current smoker (versus never or former), diabetes treatment oral or GLP-1 or insulin (versus no drug), GLP-1 or insulin (versus no drug or oral), insulin (versus no drug or oral or GLP-1), antihypertensive drug use, lipid-lowering drug use, SBP, pulse pressure, c-LDL, BUN, Cre, eGFR<60 mL/min/1.73 m^2 and HNA%. Likewise, retinopathy was significantly correlated with disease duration, diabetes treatment GLP-1 or insulin (versus no drug or oral), insulin (versus no drug or oral or GLP-1), antihypertensive drug use, SBP, pulse pressure, GA/HbA1c ratio,

Table 1
Baseline characteristics of patients.

Characteristic	Value	Laboratory data	Value
Male	106 (64.6)	Systolic blood pressure (mmHg)	122 ± 16
Age	63.5 ± 13.1	Diastolic blood pressure (mmHg)	66 ± 11
Disease duration (years)	13.4 ± 10.7	Pulse pressure (mmHg)	56 ± 15
BMI (kg/m^2)	27.1 ± 5.6	HbA1c (%) (mmol/mol)	9.03 ± 1.76 (75 ± 4.3)
Smoking (never/former/current)	70/68/26	GA (%)	23.6 ± 6.9[a]
Alcohol consumption (g)	6.04 ± 14.2	GA/HbA1c ratio	2.61 ± 0.56[a]
Diabetes mellitus family history	101 (61.6)	FPG (mg/dL)	152 ± 48
Coronary artery disease	35 (21.3)	HOMA-IR	3.23 ± 2.57[a]
Stroke	17 (10.4)	CPR: fasting (ng/mL)	1.58 ± 1.08
Neuropathy	111 (67.7)	CPR: 2 h after meal (ng/mL)	3.69 ± 2.17
Retinopathy (-/SDR/PPDR or PDR)	95/14/35	CPI	1.11 ± 0.80
Nephropathy (1/2/3/4/5)[b]	89/44/21/8/2	Alb (mg/dL)	4.08 ± 0.37
Diabetes treatment (no drug/oral/GLP-1/insulin)	19/77/8/60	UA (mg/dL)	5.33 ± 1.40
Antihypertensive drug use	96 (58.5)	TG (mg/dL)	171 ± 170
Calcium blocker	65 (39.9)	c-LDL (mg/dL)	101 ± 33
ACE inhibitor	13 (7.9)	BUN (mg/dL)	17.4 ± 7.94
ARB	64 (39.0)	Cre (mg/dL)	0.98 ± 0.84
β-blocker	22 (13.4)	eGFR (mL/min/1.73 m^2)	69.2 ± 26.0[a]
Diuretic	22 (13.4)	MMSE	28.4 ± 1.54
Others	4 (2.4)	HNA%	25.3 ± 5.0
Lipid-lowering drug use	97 (59.1)		
Strong statin	71 (43.3)		
Standard statin	10 (6.1)		
Fibrate	9 (5.5)		
EPA/DHA	11 (6.7)		
Ezetimibe	10 (6.1)		
Others	4 (2.4)		

Values are expressed as n (%) or mean ± SD; n = 164.
BMI: body mass index, SDR: simple diabetic retinopathy, PDR: proliferative diabetic retinopathy, PPDR: pre-proliferative diabetic retinopathy, ACE: Angiotensin converting enzyme, ARB: Angiotensin II receptor blocker, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, GA: glycoalbumin, FPG: fasting plasma glucose, HOMA-IR: homeostasis model assessment-insulin resistance, CPR: C-peptide immunoreactivity, CPI: C-peptide Index, Alb: serum albumin, UA: uric acid, TG: triglyceride, c-LDL: calculated LDL cholesterol, BUN: blood urea nitrogen, Cre: creatinine, eGFR: estimated glomerular filtration rate, MMSE: Mini Mental State Examination, HNA%: percentage of human non-mercaptalbumin.
[a] Data are missing for some patients. GA (n = 162), GA/HbA1c ratio (n = 162), HOMA-IR (n = 105), eGFR (n = 163).
[b] Nephropathy stage was determined according to the Classification of Diabetic Nephropathy 2014 proposed by the Joint Committee on Diabetic Nephropathy in Japan [16]. Stage 1: microalbuminuria <30 mg/g Cre and eGFR ≥30 mL/min/1.73 m^2, stage 2: microalbuminuria 30–299 mg/g Cre and eGFR ≥30 mL/min/1.73 m^2, stage 3: macroalbuminuria ≥300 mg/g Cre or continuous proteinuria ≥0.5 g/g Cre and eGFR ≥30 mL/min/1.73 m^2, stage 4: eGFR <30 mL/min/1.73 m^2, and stage 5: requiring dialysis.

Table 2
Bivariate analysis of candidate parameters and HNA%.

Variable	β coefficient	95% CI	Standardized regression coefficient	*p*-value
Male	0.014	−1.597−1.626	0.001	0.9862
Age	0.102	0.045−0.159	0.268	0.0005 *
Disease duration	0.158	0.090−0.226	0.339	<0.0001 *
BMI	0.069	−0.068−0.206	0.078	0.3217
Smoking				
Former or current smoker (versus never)	0.644	−0.910−2.199	0.064	0.4144
Current (versus never or former)	−1.364	−3.463−0.7352	−0.100	0.2013
Alcohol consumption	0.027	−0.027−0.081	0.077	0.3283
Diabetes mellitus family history	1.322	−0.249−2.892	0.129	0.0985
Coronary artery disease	2.377	0.533−4.221	0.196	0.0119 *
Stroke	−0.458	−2.984−2.069	−0.0281	0.721
Neuropathy	2.907	1.322−4.491	0.274	0.0004 *
Retinopathy				
PPDR or PDR (versus - or SDR)	3.739	1.857−5.621	0.313	0.0001 *
Nephropathy				
Stage 3 or 4 or5 (versus stage 1 or 2)	5.568	3.800−7.337	0.439	<0.0001 *
Diabetes treatment				
Oral or GLP-1 or insulin (versus no drug)	1.161	−1.240−3.561	0.075	0.3411
GLP-1 or insulin (versus no drug or oral)	2.301	0.778−3.823	0.228	0.0033 *
Insulin (versus no drug or oral or GLP-1)	2.256	0.696−3.817	0.219	0.0049 *
Antihypertensive drug use	3.701	2.253−5.161	0.368	<0.0001 *
Lipid-lowering drug use	1.68	0.134−3.225	0.166	0.0334 *
Blood pressure				
Systolic	0.043	−0.0045−0.091	0.138	0.078
Diastolic	−0.094	−0.164~-0.0234	−0.202	0.0093 *
Pulse pressure	0.103	0.052−0.154	0.301	<0.0001 *
HbA1c	−0.248	−0.686−0.191	−0.087	0.266
GA	0.047	−0.066−0.160	0.065	0.413
GA/HbA1c ratio	1.547	0.181−2.913	0.174	0.027 *
FPG	−0.009	−0.025−0.0070	−0.087	0.269
HOMA-IR	−0.112	−0.417−0.193	−0.072	0.467
CPR: fasting	0.888	0.185−1.591	0.192	0.0136 *
CPR: 2 h after meal	0.058	−0.300−0.414	0.025	0.719
CPI	1.076	0.120−2.031	0.172	0.0277 *
Alb	−3.102	−5.096~-1.109	−0.235	0.0025 *
UA	1.15	0.626−1.673	0.323	<0.0001 *
TG	−0.00095	−0.0055−0.00359	−0.032	0.6803
c-LDL	−0.0219	−0.0459−0.00215	−0.145	0.074
BUN	0.3418	0.260−0.423	0.545	<0.0001 *
Cre	2.859	2.050−3.668	0.481	<0.0001 *
eGFR	−0.117	−0.140~-0.0930	−0.608	<0.0001 *
MMSE	−0.191	−0.691−0.310	−0.059	0.4532

CI: confidence interval, *$p < 0.05$.

BUN, Cre, eGFR<60 mL/min/1.73 m^2 and HNA%. Nephropathy was significantly correlated with disease duration, diabetes treatment GLP-1 or insulin (versus no drug or oral), insulin (versus no drug or oral or GLP-1), antihypertensive drug, lipid-lowering drug, SBP, pulse pressure, Alb, UA, BUN, Cre, eGFR<60 mL/min/1.73 m^2 and HNA%. Coronary artery disease was significantly correlated with age, disease duration, diabetes treatment insulin (versus no drug or oral or GLP-1), antihypertensive drug use, lipid-lowering drug use, SBP, pulse pressure, c-LDL, BUN, Cre, eGFR<60 mL/min/1.73 m^2 and HNA% (Table 3). Multivariate linear correlation analysis showed that HNA%, age, diabetes treatment and lipid-lowering drug use were significantly associated with neuropathy. Likewise, HNA%, antihypertensive drug use and SBP were associated with nephropathy. As for retinopathy and coronary artery disease, HNA% showed relatively weaker association than the other factors (Table 4).

3. Discussion

In this study, we showed that HNA% was significantly associated with the presence and/or the severity of multiple diabetic complications, including neuropathy, retinopathy, nephropathy, and coronary artery disease. In a multivariate analysis, eGFR had a strong negative correlation with HNA%, whereas BMI and the surrogate index of glycemic variability, GA/HbA1c ratio, were independently, significantly and positively correlated with HNA%. HNA% had a stronger correlation with neuropathy and nephropathy than other factors.

The pathogenesis of diabetes and its complications are influenced by oxidative stress [1]; however, there is little clinical data supporting this. This study showed that HNA% had a strong negative correlation with eGFR and a strong positive correlation with diabetic complications. Previous reports have shown that HNA% increases in hepatic and/or renal failure [10−13], suggesting that oxidized albumin may reflect systemic oxidation status. It has also been reported that HNA% may be elevated in patients with cardiovascular disease [12].

Most studies of 8-OHdG have simply compared normal subjects with diabetes patients, regardless of the severity or stage of the complications. Suzuki et al. reported that 8-OHdG content in muscle tissue correlates with retinopathy and nephropathy [18]. Nishikawa et al. reported that 8-OHdG levels in urine are associated with HbA1c, microalbuminuria, the occurrence of simple retinopathy, and coronary heart disease risk score [19]. Fukuhara et al. reported HNA% by our method is a strong determinant for activities of daily living disability in elderly patients with diabetes [20]. To our knowledge, our study is the first to report that three major microvascular complications (neuropathy, retinopathy, and

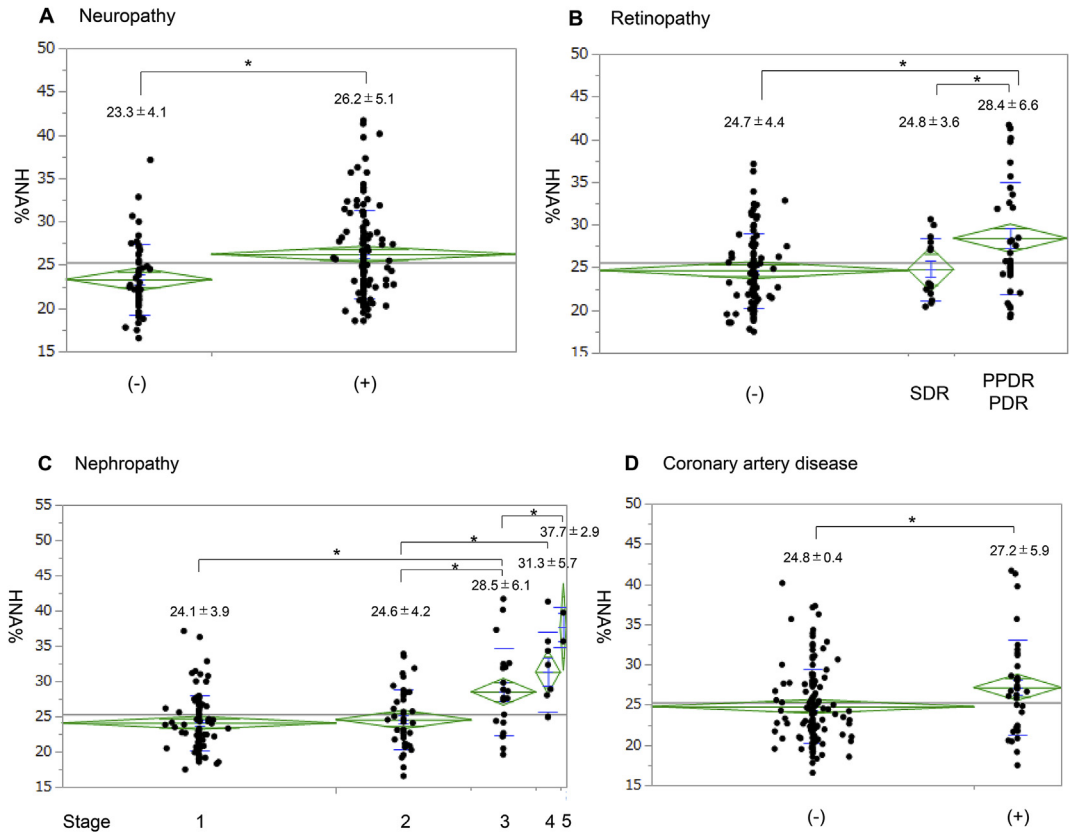
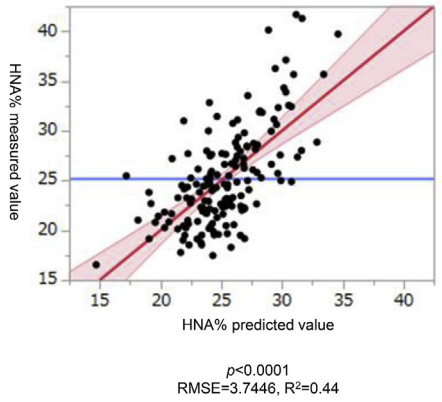


Fig. 1. Distribution of HNA% in the clinical stages of (A) neuropathy, (B) retinopathy, (C) nephropathy, and (D) coronary artery disease in diabetic patients. The diamond shape shows the 95% CI with measured sample size and the shorter lines marking the lower and upper vertex of the diamond indicate the SD. The horizontal diagonal line indicates the average and the two horizontal lines by the average are overlap marks. The numbers over the plots show the average ± SD. The average values of two groups are significantly different if the two overlap marks do not overlap each other. *$p < 0.05$.



	coefficient	SE	p-value
segment	24.019	2.953	<0.0001
BMI	0.204	0.061	0.001
GA/HbA1c ratio	1.442	0.606	0.0185
eGFR	-0.117	0.012	<0.0001

Fig. 2. Clinical parameters affecting HNA% determined by multivariate analysis. Left panel indicates the least-square regression line between the predicted values and the actual measurements of HNA%. Area shaded in red represents the 95% CI confidence interval. The average of the HNA% measurements is displayed as a blue line. The root mean squared error (RMSE) is 3.7446, and the correlation coefficient (R) is 0.663. Right panel shows coefficients and standard errors (SE) of statistically significant parameters. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

nephropathy) and coronary artery disease are all correlated with one oxidative stress biomarker in the blood. Our new method, which enabled the rapid and accurate measurement of HNA in multiple specimens, revealed the relationship between HNA% and diabetic complications.

Using a stepwise method, we narrowed possible predictors of HNA% down to three factors, i.e. BMI, GA/HbA1c ratio, and eGFR. Moderate correlation was observed between the actual values and the predicted values based on the formula.

No correlations were found between HNA% and glycemic markers, such as HbA1c and GA, although some studies have reported positive correlations between HbA1c and oxidative stress markers [19]. However, we found a significant correlation between HNA% and GA/HbA1c ratio. GA is significantly associated with increased ROS production in experimental systems [21]. HbA1c reflects the average plasma glucose level but not the acute elevation

Table 3
Bivariate analysis of risk factors and diabetic complications.

Variable	β coefficient	95% CI	Standardized regression coefficient	*p*-value
A. Neuropathy				
Male				0.6661
Age	9.1195	5.0381−13.1998	0.3276	<0.0001 *
Disease duration	4.9794	1.5350−8.4239	0.2189	0.0049 *
BMI	0.4793	−1.3606−2.3193	0.0405	0.6076
Smoking				
Former or current smoker (versus never)				0.5000
Current (versus never or former)				0.0419 *
Alcohol consumption	−3.1466	−7.8111−1.5180	−0.1041	0.1847
Diabetes mellitus family history				1.0000
Stroke				0.5854
Diabetes treatment				
Oral or GLP-1 or insulin (versus no drug)				0.0001 *
GLP-1 or insulin (versus no drug or oral)				0.0188 *
Insulin (versus no drug or oral or GLP-1)				0.0370 *
Antihypertensive drug				0.0271 *
Lipid-lowering drug use				<0.0001 *
Blood pressure				
Systolic	6.4097	1.2057−11.6137	0.1870	0.0161 *
Diastolic	−0.8735	−4.4249−2.6778	−0.0381	0.6278
Pulse pressure	7.2832	2.6001−11.9663	0.2346	0.0025 *
HbA1c	−0.3751	−0.9529−0.2027	−0.1002	0.2017
GA	−0.0584	−2.3464−2.2297	−0.0040	0.9599
GA/HbA1c ratio	0.1058	−0.0805−0.2920	0.0883	0.2637
FPG	5.1795	−10.6675−21.0264	0.0506	0.5196
HOMA-IR	−0.0738	−1.0995−0.9519	−0.0141	0.8868
CPR: fasting	0.0400	−0.3168−0.3967	0.0174	0.8252
CPR: 2 h after meal	−0.3052	−1.0214−0.4109	−0.0660	0.4012
CPI	−0.0444	−0.3078−0.2189	−0.0262	0.7395
Alb	−0.1110	−0.2344−0.0124	−0.1382	0.0776
UA	0.1306	−0.3311−0.5924	0.0439	0.5772
TG	−13.4352	−69.6593−42.7890	−0.0371	0.6377
c-LDL	−11.4970	−22.7638~-0.2301	−0.1624	0.0456 *
BUN	2.9856	0.4018−5.5695	0.1765	0.0238 *
Cre	0.2905	0.0171−0.5638	0.1627	0.0374 *
eGFR	−16.1465	−24.3853~-7.9076	−0.2918	0.0002 *
MMSE	−0.2975	−0.8053−0.2103	−0.0905	0.2491
HNA%	2.9069	1.3224−4.4914	0.2738	0.0004 *
B. Retinopathy				
Male				0.1612
Age	1.9531	−2.7006−6.6068	0.0695	0.4081
Disease duration	8.3780	4.5779−12.1781	0.3435	<0.0001 *
BMI	−0.2052	−2.3405−1.9301	−0.0160	0.8496
Smoking				
Former or current smoker (versus never)				0.4311
Current (versus never or former)				0.0657
Alcohol consumption	−2.4682	−8.2174−3.2811	−0.0710	0.3975
Diabetes mellitus family history				0.1612
Stroke				0.1178
Diabetes treatment				
Oral or GLP-1 or insulin (versus no drug)				0.0392
GLP-1 or insulin (versus no drug or oral)				0.0008 *
Insulin (versus no drug or oral or GLP-1)				0.0029 *
Antihypertensive drug				0.0281 *
Lipid-lowering drug use				0.1680
Blood pressure				
Systolic	9.6860	3.7748−15.5972	0.2623	0.0015 *
Diastolic	−0.3468	−4.4033−3.7097	−0.0142	0.8660
Pulse pressure	10.0328	4.5764−15.4891	0.2918	0.0004 *
HbA1c	−0.2245	−0.8961−0.4471	−0.0554	0.5098
GA	1.8524	−0.7956−4.5003	0.1157	0.1689
GA/HbA1c ratio	0.2620	0.0483−0.4757	0.2000	0.0166 *
FPG	3.3717	−15.0290−21.7724	0.0304	0.7177
HOMA-IR	−0.1977	−1.6355−1.2400	−0.0293	0.7853
CPR: fasting	−0.0302	−0.4588−0.3984	−0.0117	0.8894
CPR: 2 h after meal	−0.7483	−1.5477−0.0511	−0.1535	0.0663
CPI	−0.0326	−0.3443−0.2791	−0.0174	0.8363
Alb	−0.1376	−0.2820−0.0067	−0.1562	0.0615
UA	0.1824	−0.3453−0.7102	0.0572	0.4956
TG	−26.2296	−95.2293−42.7701	−0.0629	0.4536
c-LDL	−3.3586	−15.9849−9.26776	−0.0461	0.5996

Table 3 (*continued*)

Variable	β coefficient	95% CI	Standardized regression coefficient	*p*-value
BUN	7.0286	4.0748−9.9823	0.3672	<0.0001 *
Cre	0.7683	0.4528−1.0840	0.3743	<0.0001 *
eGFR	−21.6629	−30.4754~-12.8504	−0.3788	<0.0001 *
MMSE	0.0718	−0.5141−0.6577	0.0203	0.8089
HNA%	3.7392	1.8572−5.62114	0.3130	0.0001 *
C. Nephropathy				
Male				0.8353
Age	3.4827	−1.6483−8.6160	0.1047	0.1820
Disease duration	5.9971	1.8844−10.1098	0.2207	0.0045 *
BMI	1.5033	−0.7104−3.7169	0.1051	0.1818
Smoking				
Former or current smoker (versus never)				0.2292
Current (versus never or former)				0.1703
Alcohol consumption	−2.5601	−8.1483−3.0281	−0.0709	0.3670
Diabetes mellitus family history				0.5397
Stroke				1.0000
Diabetes treatment				
Oral or GLP-1 or insulin (versus no drug)				0.1290
GLP-1 or insulin (versus no drug or oral)				0.0004 *
Insulin (versus no drug or oral or GLP-1)				0.0032 *
Antihypertensive drug				<0.0001 *
Lipid-lowering drug use				0.0256 *
Blood pressure				
Systolic	13.3825	7.4040−19.3610	0.3287	<0.0001 *
Diastolic	2.7749	−1.4484−6.9982	0.1014	0.1963
Pulse pressure	10.6076	5.0933−16.1218	0.2860	0.0002 *
HbA1c	0.0867	−0.6069−0.7803	0.0194	0.8054
GA	1.2426	−1.5142−3.9994	0.0702	0.3747
GA/HbA1c ratio	0.0895	−0.1359−0.3149	0.0619	0.4340
FPG	−3.4463	−21.9925−15.9033	−0.0249	0.7514
HOMA-IR	0.3289	−1.1892−1.8469	0.0423	0.6684
CPR: fasting	0.3033	−0.1204−0.7269	0.1104	0.1594
CPR: 2 h after meal	−0.1702	−1.0271−0.6868	−0.0308	0.6954
CPI	0.1714	−0.1421−0.4850	0.0845	0.2820
Alb	−0.2212	−0.3660~-0.0763	−0.2305	0.0030 *
UA	0.6138	0.0699−1.1577	0.1725	0.0272 *
TG	40.5494	−26.3625−107.4612	0.0936	0.2332
c-LDL	0.3487	−13.3428−14.0403	0.0041	0.9599
BUN	9.2095	6.4183−12.0007	0.4557	<0.0001 *
Cre	0.9543	0.6584−1.2502	0.4474	<0.0001 *
eGFR	−29.6886	−38.8731~-20.5040	−0.4494	<0.0001 *
MMSE	−0.3371	−0.9439−0.2697	−0.0859	0.2742
HNA%	5.5685	3.8004−7.3366	0.4390	<0.0001 *
D. Coronary artery disease				
Male				0.4268
Age	5.5732	0.7194−10.4270	0.1754	0.0247 *
Disease duration	9.2283	5.4618−12.9949	0.3553	<0.0001 *
BMI	0.2111	−1.8894−2.3115	0.0156	0.8430
Smoking				
Former or current smoker (versus never)				0.1771
Current (versus never or former)				1.0000
Alcohol consumption	−2.8890	−8.2238−2.4457	−0.0837	0.2865
Diabetes mellitus family history				0.6958
Stroke				0.5310
Diabetes treatment				
Oral or GLP-1 or insulin (versus no drug)				0.0788
GLP-1 or insulin (versus no drug or oral)				0.0017 *
Insulin (versus no drug or oral or GLP-1)				0.0006 *
Antihypertensive drug				<0.0001 *
Lipid-lowering drug use				<0.0001 *
Blood pressure				
Systolic	8.9890	3.1039−14.8735	0.2306	0.0030 *
Diastolic	0.4707	−3.5854−4.5274	0.0180	0.8190
Pulse pressure	8.5181	3.1801−13.8560	0.2403	0.0019 *
HbA1c	−0.1109	−0.7736−0.5518	−0.0260	0.7415
GA	1.3445	−1.3127−4.002	0.0788	0.3192
GA/HbA1c ratio	0.1780	−0.0381−0.3940	0.1276	0.1057
FPG	−0.7710	−18.8829−17.3409	−0.0066	0.9331
HOMA-IR	−0.3655	−1.883−1.1523	−0.0470	0.6340

(*continued on next page*)

Table 3 (*continued*)

Variable	β coefficient	95% CI	Standardized regression coefficient	*p*-value
CPR: fasting	0.1382	−0.2685−0.5449	0.0526	0.5032
CPR: 2 h after meal	−0.5934	−1.4075−0.2207	−0.1124	0.1520
CPI	0.1254	−0.1747−0.4255	0.0647	0.4104
Alb	−0.0551	−0.1971−0.0869	−0.0601	0.4449
UA	−0.1337	−0.6610−0.3935	−0.0393	0.6171
TG	17.5497	−46.6157−81.7151	0.0424	0.5899
c-LDL	−22.6777	−35.1721~-10.1833	−0.2810	0.0005 *
BUN	4.0500	1.1198−6.9794	0.2097	0.0070 *
Cre	0.6195	0.3183−0.9208	0.3040	<0.0001 *
eGFR	−17.0326	−26.4943~-7.5709	−0.2698	0.0005 *
MMSE	−0.3300	−0.9098−0.2498	−0.0880	0.2627
HNA%	2.3766	0.5325−4.2206	0.1961	0.0119 *

*$p < 0.05$.

of plasma glucose levels [22−24], and thus, GA is often used as an alternative measure of acute hyperglycemia [25]. GA has attracted attention recently as a useful marker of postprandial glucose excursion [26,27]. Because acute hyperglycemia or postprandial hyperglycemia may trigger oxidative stress rather than chronic sustained hyperglycemia [28,29], we employed the GA/HbA1c ratio as a surrogate marker of plasma glucose variability. In fact, several recent studies have reported that this ratio is a better marker of glucose excursion than GA or HbA1c alone in continuous glucose monitoring systems [22,27]. The independent association between GA/HbA1c ratio and HNA% identified by a stepwise method suggests that repeated high postprandial glucose levels may affect oxidative stress more than chronic sustained hyperglycemia. This result is consistent with a previous report that HNA% is strongly

Table 4
Logistic regression analysis on diabetic complications.

	Odds ratio	95% CI	*p*-value
A. Neuropathy			
Age	1.0545	1.0157−1.0948	0.0055
Disease duration	0.9735	0.9270−1.0224	0.2833
Current smoker (versus never or former)	1.2483	0.4033−3.8641	0.7005
Diabetes treatment (oral/GLP-1/Ins use)	0.1373	0.0326−0.5793	0.0069
Antihypertensive drug use	0.9355	0.3770−2.3213	0.8857
Lipid-lowering drug use	0.3205	0.1170−0.8783	0.0269
SBP	1.0166	0.9873−1.0467	0.2708
c-LDL	1.0080	0.9933−1.0228	0.2881
eGFR< 60	1.4002	0.4555−4.3040	0.5569
HNA%	1.1561	1.0467−0.9873	0.0167
B. Retinopathy			
Disease duration	1.0385	0.9918−1.0874	0.1080
Diabetes treatment (GLP-1/Ins use)	0.5007	0.1950−1.2853	0.1504
Antihypertensive drug use	0.9383	0.3051−2.8859	0.9116
SBP	1.0320	1.0018−1.0632	0.0377
GA/HbA1c ratio	1.5656	0.7428−3.2998	0.2387
eGFR< 60	0.3681	0.1279−1.0588	0.0638
HNA%	1.0360	0.9366−1.1461	0.4919
C. Nephropathy			
Disease duration	0.9784	0.9298−1.0295	0.4407
Diabetes treatment (GLP-1/Ins use)	0.3410	0.1111−1.0463	0.0600
Antihypertensive drug use	0.1734	0.0327−0.9187	0.0394
Lipid-lowering drug use	0.9078	0.2653−3.1065	0.8775
SBP	1.0497	1.0154−1.0851	0.0042
Alb	0.5227	2.0144−1.9132	0.3459
UA	1.1461	0.7474−1.7574	0.5318
HNA%	1.1585	1.0270−1.3069	0.0167
D. Coronary artery disease			
Age	0.9882	0.9366−1.0427	0.6644
Disease duration	1.0563	1.0020−1.1136	0.0421
Diabetes treatment (GLP-1/Ins use)	0.3722	0.1294−1.0705	0.0668
Antihypertensive drug use	0.1258	0.0267−0.5925	0.0088
Lipid-lowering drug use	0.5483	0.1433−2.0984	0.3802
SBP	1.0332	1.0008−1.0666	0.0443
c-LDL	0.9805	0.9607−1.0007	0.0588
eGFR< 60	0.9247	0.2793−3.0612	0.8980
HNA%	0.9551	0.8558−1.0659	0.4123

"Retinopathy" was defined as presence of PPDR or PDR. "Nephropathy" was defined as stage 3, 4, or 5.

correlated with daily glucose profile in diabetes patients [30]. As for the diabetic complications, the factors extracted in the bivariate analysis were consistent with those reported in the previous studies (Table 3) [31–34]. As shown in Fig. 1 and Table 3, HNA% was associated with all the diabetic complications; especially neuropathy and nephropathy, suggesting the potentially significant involvement of oxidative stress in the etiology. These findings are consistent with the previous reports indicating that pentosidine (a major advanced glycation end product) levels are relevant to complications in diabetes patients [35,36]. Serum pentosidine levels were increased in diabetic patients with retinopathy and nephropathy [35].

We found that both HNA% and eGFR were useful to predict the status of systemic complications in diabetes. Although they were strongly correlated with each other, HNA% and eGFR represent different statuses. HNA% can be directly altered by redox metabolites, while eGFR is not fundamentally affected by these metabolites. Because of its reversibility, HNA% is potentially a unique biomarker for evaluating the risk of complications in diabetic patients.

There are several limitations in this study. Firstly, there may be a selection bias, as all the subjects were selected from the inpatient pool at a single university hospital, where there tend to be more patients with more complicated medical backgrounds, such as relatively long medical histories. Further multicenter studies are required to enable more general conclusions. Secondly, more information should be collected from not only diabetes patients but also from patients with other diseases to understand the general role of HNA% in predicting oxidative stress in humans. Thirdly, this is a cross-sectional study. We didn't obtain histories about intake of supplements or antioxidative agents. Further investigation is necessary to identify specific agents that influence HNA% and to determine whether decreasing HNA% can be an effective therapeutic strategy to prevent the complications of diabetes.

In conclusion, our newly developed method enabled the rapid and accurate measurement of HNA% in a large number of specimens. HNA% measured with this method reflected the status of multiple diabetic complications. HNA% may have clinical application as a universal marker of the status of systemic complications in patients with diabetes.

CRediT authorship contribution statement

Yuka Kobayashi: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. **Ryo Suzuki:** Conceptualization, Methodology, Formal analysis, Supervision. **Keiko Yasukawa:** Investigation, Resources. **Koji Oba:** Formal analysis. **Toshimasa Yamauchi:** Supervision. **Yutaka Yatomi:** Resources, Supervision. **Takashi Kadowaki:** Conceptualization, Methodology, Supervision.

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Y. K., R. S. and T. K. designed the study. Y. K. performed the experiments. K. Y. measured HNA in blood samples. Y. K., R. S., K. Y., T. Y., Y. Y., and T. K. interpreted the results. Y. K. and R. S wrote the initial manuscript. R.S. and T.K. take responsibility for the content of the paper. K.O. provided advice on statistical analysis. All authors reviewed the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metop.2020.100032.

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