

Urinary angiotensinogen is a marker for tubular injuries in the patients with type 2 diabetes

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Abstract

Purpose: Urinary angiotensinogen has been reported as a marker for the activation of intrarenal renin-angiotensin system (RAS) in various kidney diseases. To investigate the importance of urinary angiotensinogen in diabetic nephropathy, we compared the urinary levels of angiotensinogen, albumin and α 1-microglobulin.

Patients and methods: Japanese patients with type 2 diabetes at various stages of nephropathy (n=85) were enrolled and we measured albumin creatinine ratio (ACR) and urinary excretion of angiotensinogen and α 1-microglobulin. We also compared the clinical data of the patients treated with or without angiotensin II receptor blockers (ARB) or angiotensin converting enzyme inhibitors (ACEI) (RAS inhibitors (+), n=51; RAS inhibitors (-), n=34).

Results: Urinary angiotensinogen levels positively correlated with ACR ($r=0.367$, $p=3.48 \times 10^{-4}$) and urinary α 1-microglobulin ($r=0.734$, $p=1.32 \times 10^{-15}$), while they negatively correlated with estimated glomerular filtration ratio (eGFR) ($r=-0.350$, $p=1.02 \times 10^{-5}$) and high density lipoprotein cholesterol (HDL-C) ($r=-0.216$, $p=0.049$). Multiple regression analysis was carried out to predict urinary angiotensinogen levels by employing eGFR, ACR and urinary α 1-microglobulin as independent variables and only urinary α 1-microglobulin entered the regression equation at a significant level. Although ACR was higher in RAS inhibitors (+) group, urinary α 1-microglobulin and angiotensinogen did not show significant increase in RAS inhibitors (+) group.

Conclusion: Urinary angiotensinogen well-correlated with urinary α 1-microglobulin and reflected the tubular injuries which may be associated with the intrarenal RAS activation in the patients with type 2 diabetes.

Key words: diabetic nephropathy, urinary biomarkers, renin-angiotensin system (RAS), angiotensinogen, α 1-microglobulin, albumin

Introduction

The role of local renin-angiotensin system (RAS) in kidney tissues in the pathophysiology of diabetic nephropathy has been emphasized¹. The hyporeninemic hypoaldosteronism observed along with the progression of diabetic nephropathy and the efficacy of RAS inhibitors reducing the albuminuria in such patients also supported the notion that intrarenal RAS plays an important role in the progression of diabetic nephropathy. Sawaguchi et al reported that the baseline urinary excretion of angiotensinogen (10-150 $\mu\text{g/gCr}$) positively correlated with albumin creatinine ratio ($r=0.77$, $p<0.001$) and urinary β 2-microglobulin ($r=0.72$, $p<0.001$) in type 2 diabetes patients with normo- and microalbuminuria. Furthermore, urinary angiotensinogen significantly correlated with annual decline of estimated glomerular filtration rate (eGFR) ($r=-0.51$, $p<0.001$) and higher level of urinary angiotensinogen is a risk for the progression of diabetic nephropathy². Most of the clinical studies measuring urinary angiotensinogen and albumin levels demonstrated the strong correlation between two proteins with comparable molecular weight (67 and 65 kDa). Thus, it is still unknown whether urinary angiotensinogen is a marker of impairment of glomerular permeability like ACR, a marker of tubular interstitial injuries or a biomarker for the activation of intrarenal RAS in diabetic nephropathy. In current study, we measured ACR, urinary α 1-microglobulin and urinary angiotensinogen levels in the patients with various stages of diabetic nephropathy and investigated the clinical significance of urinary angiotensinogen in diabetic nephropathy.

Materials and Methods

Patients

We recruited Japanese patients with type 2 diabetes ($n=85$, 62.9 ± 11.3 years) into this study at Okayama University Hospital. The current study is sub-study of the report and

shared the recruited patients³. They were treated with oral hypoglycemic agents (n=48) and insulin treatment (n=49). The patients with hypertension (n=59) were treated with angiotensin II receptor blockers (ARB; n=49), angiotensin converting enzyme inhibitors (ACEI; n=13), calcium channel blockers (n=34) and diuretics (n=11). We compared the clinical data of the patients treated with ARB or ACEI (RAS inhibitors (+); n=51) without ARB and ACEI (RAS inhibitors (-); n=34). Dyslipidemia was noted in 53 patients and most of them were treated with statins (n=45). The patients with eGFR < 15 ml/min/1.73 m² or under dialysis were excluded from the current study. All recruited patients with type 2 diabetes agreed to measure urinary levels of α 1-microglobulin and angiotensinogen. The study was conducted in accordance with the ethical principle of the Declaration of Helsinki and approved by ethical committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences. We obtained written informed consent from each patient.

Blood sampling and assays

We measured overnight fasting serum levels of total cholesterol and low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides (Type Wako Triglyceride·H, Wako Chemical, Osaka, Japan), uric acid, creatinine (Cr), and urea nitrogen (UN). HbA1c levels were also measured. Urinary albumin was measured in random spot urine samples by standard immuno-nephelometric assay. The urinary albumin-creatinine ratio (ACR) was calculated. Estimated glomerular filtration rate (eGFR) was calculated by equation; $eGFR \text{ (ml/min/1.73 m}^2\text{)} = 194 \times Cr^{-1.094} \times age^{-0.287}$ in male and $eGFR \text{ (ml/min/1.73 m}^2\text{)} = 194 \times Cr^{-1.094} \times age^{-0.287} \times 0.739$ in female. Urinary excretions of α 1-microglobulin and angiotensinogen were measured with ELISA kits; LZ Test Eiken α 1-M (Eiken Chemical Co., Tokyo, Japan) and Human Total Angiotensinogen Assay Kit (Immuno-Biological Laboratories Co., Ltd., Fujioka, Gunma, Japan).

Statistical analysis

All data are expressed as mean \pm standard deviation (SD) values. Spearman correlation coefficients were used to evaluate whether urinary levels of angiotensinogen correlated with various parameters. To determine the variables independently associated with urinary levels of angiotensinogen in the patients with type 2 diabetes, multiple regression analysis was performed by including estimated glomerular filtration rate (eGFR), ACR and urinary α 1-microglobulin / creatinine ratio as independent variables. Urinary levels of α 1-microglobulin, angiotensinogen and various clinical parameters in RAS inhibitors (-) and RAS inhibitors (+) groups were compared by Mann-Whitney U. *P* values less than 0.05 were considered statistically significant. Statistical analysis was performed with IBM SPSS Statistics Base and IBM SPSS Regression (IBM, Armonk, NY).

Results

Urinary angiotensinogen well-correlated with urinary α 1-microglobulin

The type 2 diabetes patients (n=85) with various albuminuria stages, *i.e.* normo- (n=36), micro- (n=25) and macroalbuminuric (n=24) stages, were recruited into the study and they were classified into RAS inhibitors (-) and RAS inhibitors (+) groups (**Table 1**). At three albuminuria stages, the serum concentrations of total protein (70.4 \pm 4.3, 70.7 \pm 4.8, 66.1 \pm 6.5 g/L; *p*=0.003), albumin (42.9 \pm 2.5, 41.2 \pm 3.2, 35.7 \pm 7.0 g/L; *p*=1.80 \times 10⁻¹⁶), creatinine (66.4 \pm 13.3, 78.3 \pm 26.3, 144.2 \pm 70.3 μ mol/L; *p*=4.86 \times 10⁻¹⁰), urea nitrogen (5.5 \pm 1.5, 7.1 \pm 2.7, 10.0 \pm 3.8 μ mol/L; *p*=5.92 \times 10⁻⁸), uric acid (305.8 \pm 61.5, 352.8 \pm 96.2, 396.2 \pm 68.0 μ mol/L; *p*=9.68 \times 10⁻⁵), HDL-C (1.49 \pm 0.41, 1.35 \pm 0.31, 1.23 \pm 0.39 mmol/L; *p*=0.031), and eGFR (74.5 \pm 16.3, 67.9 \pm 19.2, 42.4 \pm 19.0 mL/min; *p*=6.66 \times 10⁻⁹) significantly changed demonstrated by Kruskal-Wallis test. Urinary proteins also

significantly increased with the progression of albuminuria stages; ACR (12.7 ± 6.0 , 114.3 ± 72.6 , 1424 ± 996 mg/gCr; $p=1.81 \times 10^{-16}$), urinary α 1-microglobulin (4.24 ± 4.03 , 6.30 ± 5.12 , 17.83 ± 18.08 μ g/gCr; $p=8.84 \times 10^{-9}$), and urinary angiotensinogen (6.8 ± 11.6 , 8.5 ± 9.9 , 73.3 ± 95.2 μ g/gCr; $p=2.88 \times 10^{-4}$).

By simple regression analyses of these parameters, urinary angiotensinogen levels significantly and positively correlated with ACR ($r=0.376$, $p=3.84 \times 10^{-4}$) and urinary α 1-microglobulin ($r=0.734$, $p=1.32 \times 10^{-15}$), while they significantly and negatively correlated with eGFR ($r=-0.350$, $p=3.84 \times 10^{-4}$) and HDL-C ($r=-0.216$, $p=0.049$) (**Table 2, Figure 1**). We next investigated the correlations of urinary angiotensinogen, ACR and urinary α 1-microglobulin in various albuminuria stages. At all three albuminuria stages, urinary angiotensinogen levels did not correlate with ACR; however urinary angiotensinogen levels significantly and highly correlate with α 1-microglobulin at normoalbuminuria stage ($r=0.840$, $p=1.40 \times 10^{-10}$) (**Figure 2**). By multiple regression analysis by including all parameters, eGFR, ACR and urinary α 1-microglobulin, as independent variables, only urinary α 1-microglobulin was a significant independent variable (**Table 3, Model 1**). Furthermore, in stepwise multiple regression analysis, only urinary α 1-microglobulin was employed as independent variable (**Table 3, Model 2**).

Urinary angiotensinogen and α 1-microglobulin levels were not altered in RAS inhibitors (+) and (-) groups

We next compared various clinical parameters in RAS inhibitors (+) and (-) groups. In RAS inhibitors (+), ACR was significantly higher compared with RAS inhibitors (-) group (368.2 ± 863.8 v.s. 489.8 ± 779.8 mg/gCr, $p=0.006$). In contrast, urinary α 1-microglobulin and angiotensinogen did not show significant differences between two groups (**Figure 3**). In clinical practice, the type 2 diabetes patients with higher ACR seem to be

preferentially treated with ARB or ACEI. Since urinary α 1-microglobulin and angiotensinogen levels were not significantly higher in RAS inhibitors (+) group, tubular damage and subsequent intrarenal RAS activation may be ameliorated by the administration of ARB or ACEI in RAS inhibitors (+) group.

Discussion

Proximal tubular angiotensinogen, collecting duct renin, and tubular angiotensin II type 1 (AT1) receptors are positively augmented by intrarenal angiotensin II (Ang II)⁴. The infusion of ¹²⁵I-Ang II into pigs demonstrated that steady-state concentrations of ¹²⁵I-Ang II in cortical and medullary tissue were 4- and 2-fold higher than arterial plasma and the tissue concentrations of endogenous Ang II were 100- and 60- times higher than arterial plasma⁵. Thus, it suggested that most renal AT1 receptors are exposed to locally generated Ang II rather than Ang II from circulation. In rodent models, the urinary excretion of angiotensinogen in streptozotocin-induced diabetic mice at 3 days after the induction of diabetes was significantly higher ($349.6 \pm 89.1 \mu\text{g/day}$) than control mice ($15.9 \pm 2.2 \mu\text{g/day}$) and the authors demonstrated the up-regulation of angiotensinogen mRNA and protein expression in the mouse kidneys⁶. The data suggested that urinary excretion of angiotensinogen is a biomarker for the activation of RAS in the kidney under diabetic states. In human, plasma angiotensinogen reaches urine via glomerular filtration like albumin and normal urinary angiotensinogen levels in human are $\sim 0.2 \text{ pmol/mL}$ versus $\sim 1200 \text{ pmol/mL}$ in plasma. Thus, the urinary angiotensinogen levels range from 0.01 to 0.1% of the plasma levels in human. In contrast, urinary angiotensinogen levels in rodents are much higher and range from 0.1 to 400 pmol/mL, implying that the urinary angiotensinogen levels in rodents sometimes higher than their plasma levels¹. <100 -fold higher urinary angiotensinogen levels in rodents suggested the concept, in which urinary angiotensinogen is exclusively plasma-derived in humans,

whereas it reflects angiotensinogen release from renal tissues, possibly proximal tubules, in rodents. Since albumin is produced in the liver and urinary albumin is entirely derived from plasma, the comparison of urinary albumin and angiotensinogen would demonstrate to what degree of urinary angiotensinogen is plasma-derived in human. In fact, several studies^{2, 7-9} demonstrated tight correlations between urinary albumin and angiotensinogen without exception.

In current investigation, we simultaneously measured ACR, urinary α 1-microglobulin and urinary angiotensinogen levels in the patients with type 2 diabetes and various stages of diabetic nephropathy. Urinary angiotensinogen levels significantly and positively correlated with ACR ($r=0.376$, $p=3.84 \times 10^{-4}$) as previous reports and we also found further tight correlation with urinary α 1-microglobulin ($r=0.734$, $p=1.32 \times 10^{-15}$). Tight correlation of urinary α 1-microglobulin and angiotensinogen levels was also true both in microalbuminuria and macroalbuminuria stages; however, ACR did not show significant correlation with urinary angiotensinogen at normo-, micro- and macroalbuminuria stages. The high correlation between urinary α 1-microglobulin and angiotensinogen levels at normoalbuminuric stage supported the concept that tubular damage promotes the intrarenal RAS activation and production of tubular angiotensinogen, since the normoalbuminuric patients would not be expected to have leakage of plasma angiotensinogen into urinary space.

Urinary α 1-microglobulin is filtered freely through glomerular capillaries and reabsorbed by the proximal tubules¹⁰. Thus, urinary α 1-microglobulin is a marker for proximal tubule dysfunction and the increased levels of urinary α 1-microglobulin has been reported in normoalbuminuric patients with type 1 diabetes^{11, 12} and type 2 diabetes^{10, 13, 14}. The assessment of proximal tubule dysfunction in the course of diabetes by urinary α 1-microglobulin allows the early diagnosis of diabetic nephropathy

in prior to the appearance of microalbuminuria and also predicts the progression of diabetic nephropathy. Plasma angiotensinogen is filtered through glomerular capillaries and urinary angiotensinogen is mainly derived from plasma. Subsequently, urinary angiotensinogen is largely removed via endocytotic uptake in tubules in a megalin-dependent manner. Endocytotic angiotensinogen is subsequently degraded and the contents of angiotensinogen in the proximal convoluted tubules tightly correlated with plasma levels of angiotensinogen¹⁵. Thus, previous studies also support the idea that urinary excretion of angiotensinogen reflects not only abnormalities of glomerular filtration barrier but also proximal tubular functions. Actually it has been reported that urinary angiotensinogen levels increase before glomerular injuries in the patients¹⁶ as well as in rodents⁶.

Yamamoto et al. demonstrated that administration of losartan reduced urinary and plasma angiotensinogen levels in the patients with chronic kidney disease¹⁷. In hypertensive patients with a preserved kidney function, RAS blockers decreased the urinary angiotensinogen and the decrease was comparable to that in urinary albumin⁷. Furthermore, in IgA nephropathy, the treatment with valsartan also reduced urinary angiotensinogen^{18, 19}. As various kidney diseases, RAS inhibitors suppressed urinary angiotensinogen levels with type 2 diabetes²⁰. One can speculate that reduction of urinary angiotensinogen by RAS blockers is due to the amelioration of glomerular hyperfiltration and suppression of enhanced filtration of various proteins including angiotensinogen through glomerular capillaries. Although ACR was higher in RAS inhibitors (+) group, urinary α 1-microglobulin and angiotensinogen did not show significant increase in RAS inhibitors (+) group. Thus, we speculate that RAS inhibitors may ameliorate the tubular injuries and intrarenal RAS activation which was reflected by no significant increase in urinary α 1-microglobulin and angiotensinogen levels.

Conclusion

In summary, we demonstrated the significant and positive correlation of urinary angiotensinogen levels with ACR and urinary α 1-microglobulin, and the negative correlation with eGFR. By multiple regression analysis by including all parameters, eGFR, ACR and urinary α 1-microglobulin, as independent variables, only urinary α 1-microglobulin was a significant independent variable. The current cross-sectional clinical study revealed that urinary angiotensinogen is a marker for the tubular injuries of early stage of diabetic nephropathy in the patients with type 2 diabetes. Since the current study was cross-sectional clinical investigation, the limitation was that it is still unknown the significance of urinary angiotensinogen for predicting long-term course of diabetic nephropathy. In the future prospective study, the significance of urinary angiotensinogen in predicting the progression of diabetic nephropathy needs to be demonstrated.

Acknowledgements: This work was supported by JSPS Grant-in-Aid for Scientific Research, Grant numbers (23390241, 25126716) and Health Labour Sciences Research Grant, Japan. K.I., T.T. and J.W. designed and performed most of the experiments. J.E., A.N., S.T., K.M., D.O., T.T., and A.K. recruited the patients. All authors contributed to conception and design, acquisition of data, or analysis and interpretation of data. In addition, all authors drafted the article, revised it critically for important intellectual content and gave final approval of the version to be published.

Disclosures: JW is a consultant for Boehringer Ingelheim, receives speaker honoraria from Novartis. HM is a consultant for Teijin, AbbVie and Astellas, receives speaker honoraria from Astellas, MSD, Takeda, and Tanabe Mitsubishi, and receives grant support from Astellas, Daiichi Sankyo, Dainippon Sumitomo, MSD, Novo Nordisk and Takeda.

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Figure legends

Figure 1 Simple correlation of urinary excretion of angiotensinogen with urinary albumin creatinine ratio (ACR), estimated glomerular filtration ratio (eGFR), urinary α 1-microglobulin and high density lipoprotein cholesterol (HDL-C) in the patients with

diabetic nephropathy (n=85). Spearman correlation coefficients are used.

Figure 2 Simple correlation of urinary excretion of angiotensinogen with urinary albumin creatinine ratio (ACR) and urinary α 1-microglobulin in the type 2 diabetes patients (n=85) with normo- (n=36), micro- (n=25) and macroalbuminuric (n=24) stages. Spearman correlation coefficients are used.

Figure 3 Urinary albumin creatinine ratio (ACR), estimated glomerular filtration ratio (eGFR), urinary excretion of α 1-microglobulin, and urinary excretion of angiotensinogen in the patients with various stages of diabetic nephropathy (n=85). Urinary levels of albumin, α 1-microglobulin, angiotensinogen and eGFR in RAS inhibitors (-) and RAS inhibitors (+) groups are compared by Mann-Whitney U.

Table 1 Comparison of various parameters in the type 2 diabetes patients (n=85) with normo- (n=36), micro- (n=25) and macroalbuminuric (n=24) stages. The patients are classified whether they are treated with or without angiotensin receptor blockers or angiotensin converting enzyme inhibitors.

	RAS inhibitors (-)	RAS inhibitors (+)	Total	Mann-Whitney U
Number (male/female)	34 (16 / 18)	51 (33 / 18)	85 (49 / 36)	
Age (years)	63.1±11.7	62.5±11.1	62.9±11.3	0.492
BMI (kg/m ²)	24.5±4.4	25.1±4.7	24.9±4.6	0.618
SBP (mmHg)	122.9±13.4	128.3±19.2	126.2±17.3	0.216
DBP (mmHg)	71.2±10.9	72.8±11.2	72.2±11.1	0.339
HbA1c (%)	7.27±0.68	7.33±0.98	7.31±0.87	0.974
Total protein (g/L)	70.1±5.8	68.7±5.1	69.3±5.4	0.339
Albumin (g/L)	41.4±5.8	39.6±5.0	40.4±5.3	0.032*
Cr (μmol/L)	81.4±53.9	98.8±50.6	91.9±52.3	0.028*
UN (μmol/L)	7.0±3.4	7.4±3.2	7.3±3.3	0.418
Uric acid (μmol/L)	321.3±66.8	360.3±89.8	344.6±83.1	0.034*
T-Cho (mmol/L)	5.32±1.01	4.77±0.88	4.99±0.97	0.011*
TG (mmol/L)	1.76±1.07	1.84±1.37	1.81±1.26	0.904
HDL-C (mmol/L)	1.51±0.45	1.29±0.31	1.38±0.39	0.041*
LDL-C (mmol/L)	3.03±0.84	2.62±0.74	2.79±0.80	0.024
eGFR (mL/min)	68.5±20.7	60.2±23.0	63.5±22.4	0.052
ACR (mg/gCr)	368.2±863.8	489.8±779.8	441.2±812	0.006**
α1-microglobulin (μg/gCr)	7.26±13.2	9.63±10.6	8.68±11.74	0.306
Angiotensinogen (μg/gCr)	13.3±27.5	34.6±71.5	26.1±58.8	0.374

RAS inhibitors (+); The patients treated with angiotensin II receptor blockers or angiotensin converting enzyme inhibitors; BMI, body mass index; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; Cr, serum creatinine; UN, serum urea nitrogen; T-Cho, Total cholesterol; TG, Triglyceride; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; eGFR, estimated glomerular filtration ratio; ACR, albumin / creatinine ratio; *, p < 0.05; **, p < 0.01.

Table 2 Simple correlation of urinary angiotensinogen with various clinical parameters in the patients with type 2 diabetes (n=85)

	Angiotensinogen ($\mu\text{g/gCr}$)
Age (years)	R=-0.040, p=0.713
BMI (kg/m^2)	R=-0.086, p=0.431
SBP (mmHg)	R=-0.033, p=0.764
DBP (mmHg)	R=-0.173, p=0.113
HbA1c (%)	R=-0.039, p=0.726
Total protein (g/L)	R=-0.107, p=0.338
Albumin (g/L)	R=-0.165, p=0.136
Cr ($\mu\text{mol/L}$)	R=0.243, p=0.025*
UN ($\mu\text{mol/L}$)	R=0.362, p=0.001**
Uric acid ($\mu\text{mol/L}$)	R=0.081, p=0.462
T-Cho (mmol/L)	R=-0.109, p=0.325
TG (mmol/L)	R=0.012, p=0.911
HDL-C (mmol/L)	R=-0.216, p=0.049*
LDL-C (mmol/L)	R=-0.051, p=0.646
eGFR (mL/min)	R=-0.350, p=1.02x10 ^{-3**}
ACR (mg/gCr)	R=0.376, p=3.84x10 ^{-4**}
α 1-microglobulin ($\mu\text{g/gCr}$)	R=0.734, p=1.32x10 ^{-15**}

BMI, body mass index; SBP, Systolic Blood Pressure; DPB, Diastolic Blood Pressure; Cr, serum creatinine; UN, serum urea nitrogen; T-Cho, Total cholesterol; TG, Triglyceride; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; eGFR, estimated glomerular filtration ratio; ACR, albumin / creatinine ratio; *, p < 0.05; **, p < 0.01.

Table 3 Multiple linear regression analysis using urinary angiotensinogen as dependent variable in the patients with type 2 diabetes (n=85).

Dependent variable	Independent variable	Unstandardized		Standardized	t value	P value	Adjusted R ²
		coefficient		coefficient			
		B	Standard Error	Beta			
Model 1 Urinary angiotensinogen ($\mu\text{g/gCr}$)	ACR (mg/gCr)	-0.001	0.007	0.014	0.142	0.888	0.519
	eGFR (mL/min)	0.022	0.246	0.009	0.091	0.928	
	Urinary α 1-microglobulin ($\mu\text{g/gCr}$)	3.647	0.498	0.728	7.324	1.58×10^{-10}	
Model 2 Urinary angiotensinogen ($\mu\text{g/gCr}$)	Urinary α 1-microglobulin ($\mu\text{g/gCr}$)	3.668	0.374	0.732	9.800	1.64×10^{-15}	0.531

Estimated glomerular filtration rate (eGFR) (mL/min), albumin / creatinine ratio (ACR) (mg/gCr) and α 1-microglobulin ($\mu\text{g/gCr}$) are used as independent variables in multiple linear regression analysis. In Model 1, all parameters are included and stepwise multiple linear regression analysis is performed in Model 2. *, $p < 0.05$; **, $p < 0.01$.

Figure 1

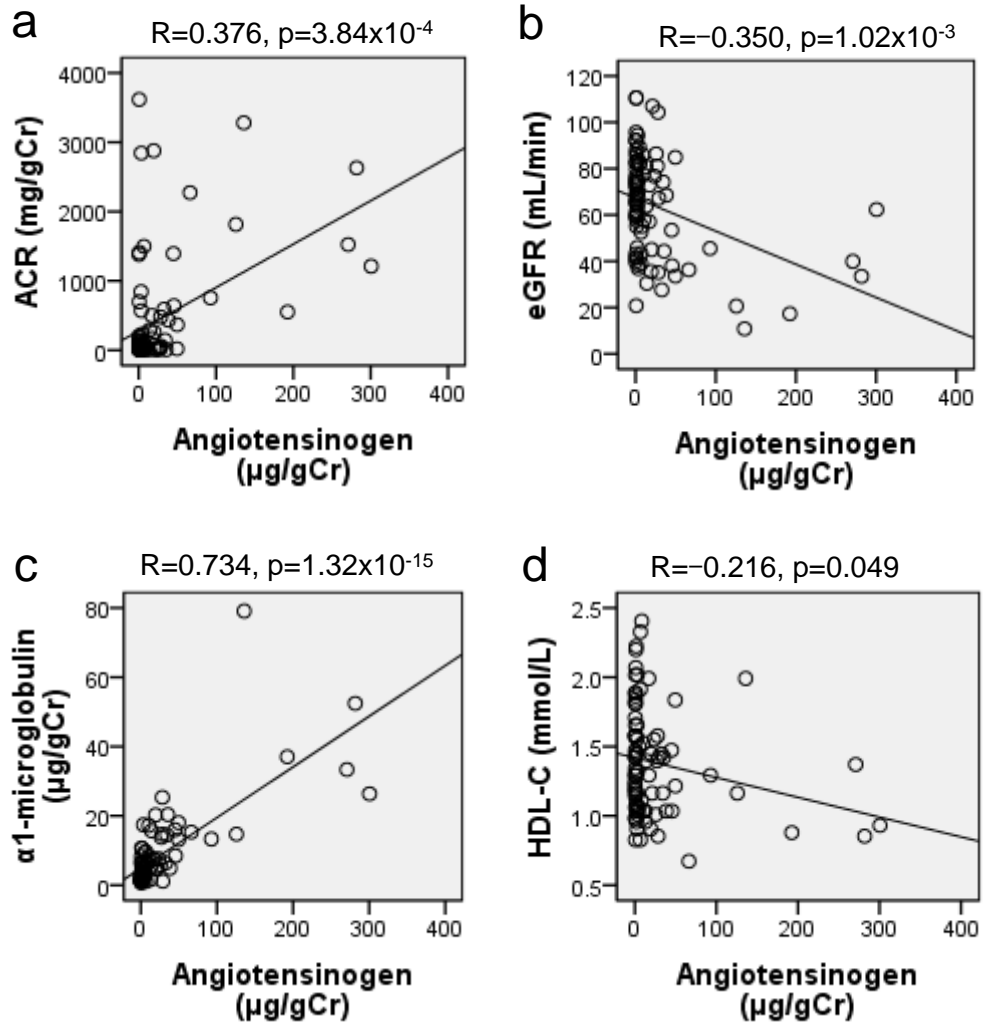


Figure 2

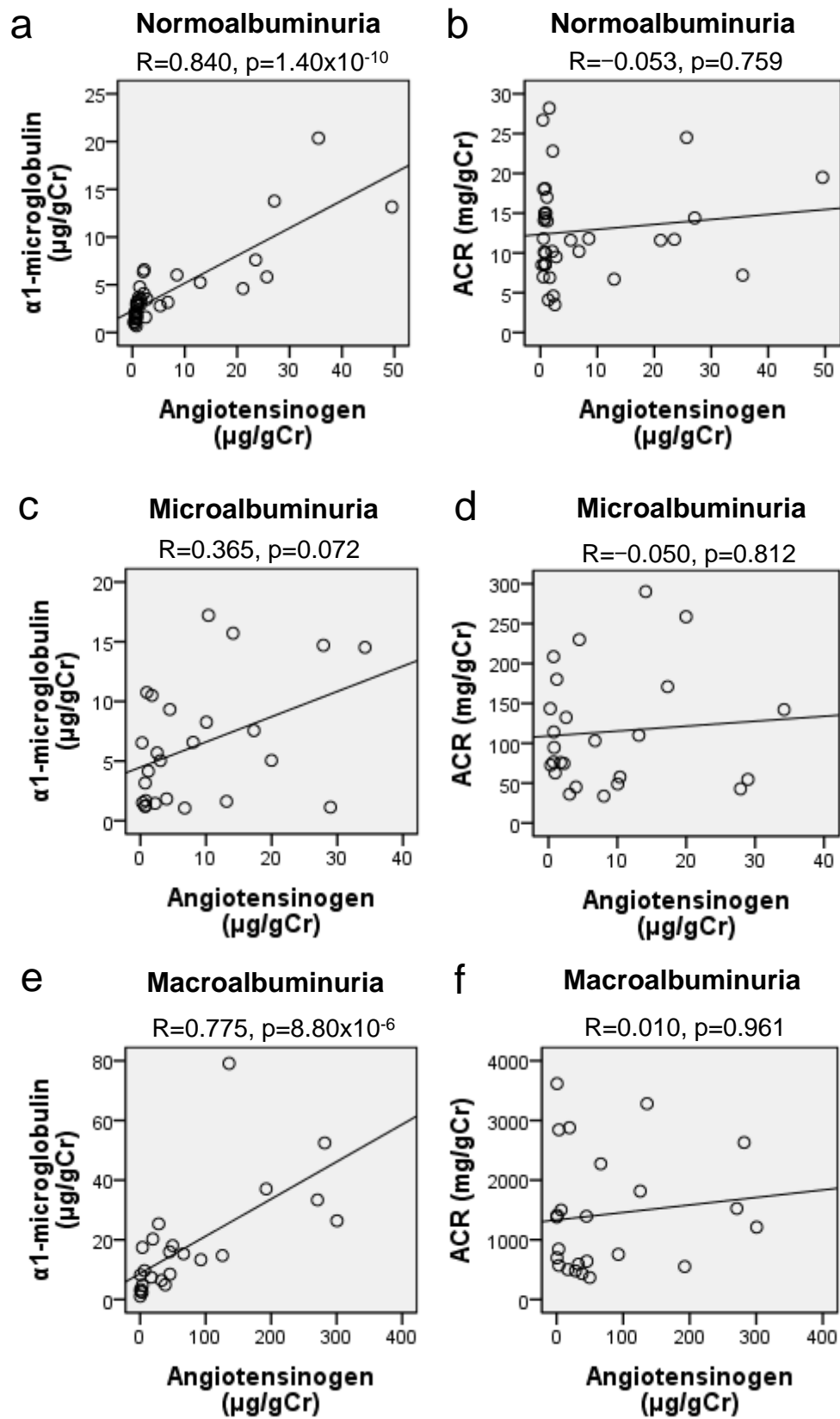


Figure 3

