General Cardiology

Oxidative Stress is Associated with Urinary Albumin Excretion in Taiwanese Women

Hsin-Hua Chou,[1] Lung-An Hsu,[2] Ching-Hsiu Peng,[3] Semon Wu,[4] Ming-Sheng Teng,[5] Pi-Yueh Chang[6] and Yu-Lin Ko[1,7]

Background: Both albuminuria and oxidative stress predict future cardiovascular disease and atherosclerosis. Mounting evidence indicates that oxidative stress plays an important role in the development of albuminuria in diabetes and hypertensive patients. We aimed to test oxidative stress as a potential early risk factor of microalbuminuria by examining the statistical associations between albuminuria and oxidative stress in a Taiwanese population.

Methods: A sample population of 469 Taiwanese subjects (241 men and 228 women) was enrolled. Urinary albumin concentrations were presented as urinary albumin-to-creatinine ratio (ACR). Oxidative stress was estimated by measuring urinary 8-hydroxydeoxyguanosine (8-OHdG) using enzyme-linked immunosorbent assay.

Results: During univariate analysis stratified by gender, urinary ACR was significantly positively correlated with blood pressure and homeostasis model assessment of insulin resistance (HOMA-IR) in men and correlated with blood pressure, total cholesterol levels, triglyceride levels, LDL-cholesterol level, body mass index and urinary 8-OHdG in women. In the multivariate analysis adjusted for age and smoking, only HOMA-IR ($p = 0.017$) was an important influential factor of urinary ACR in men. On the contrary, urinary 8-OHdG ($p = 0.010$) was positively associated with urinary ACR in women.

Conclusion: Oxidative stress may serve as a risk factor for the presence of albuminuria in Taiwanese women.

Key Words: Albuminuria • Oxidative stress • 8-hydroxydeoxyguanosine

INTRODUCTION

It has become apparent that microalbuminuria is associated with a greater risk for future cardiovascular disease[1,2] and mortality.[3,4] Microalbuminuria has been identified as a marker of incipient nephropathy in patients with type 1 and type 2 diabetes mellitus.[5,6] Furthermore, albuminuria at a level far below the current definition of microalbuminuria has also been identified as a risk factor of cardiovascular disease and mortality,[7,8] indicating that there is a prognostic value of urinary albumin excretion at a level far below the current definition of microalbuminuria. To support this idea, it has been shown that urinary albumin excretion rate is associated with systemic dysfunction of vascular endothelium, which may contribute to overall cardiovascular disease.[9,10] Therefore, efforts have been focused on identifying risk factors for developing albuminuria. This may allow for the early recognition of people who are likely to develop albuminuria and the subsequent risks of cardiovascular events, thus prevention and treatment may be initiated earlier.

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[1]Division of Cardiology, Department of Internal Medicine, Buddhist Tzu Chi General Hospital, Taipei Branch; [2]The First Cardiovascular Division, Department of Internal Medicine, Chang Gung Memorial Hospital, Chang Gung University College of Medicine; [3]Division of Nephrology, Department of Internal Medicine, Buddhist Tzu Chi General Hospital, Taipei Branch; [4]Department of Life Science, Chinese Culture University; [5]Department of Medical Research, Buddhist Tzu Chi General Hospital, Taipei Branch; [6]Department of Pathology, Chang Gung Memorial Hospital, Taipei; [7]Tzu Chi University College of Medicine, Hualien, Taiwan.

Address correspondence and reprint requests to: Dr. Yu-Lin Ko, Division of Cardiology, Department of Internal Medicine, Buddhist Tzu Chi General Hospital, Taipei Branch, No. 289, Jianguo Road, Xindian City, Taipei 231, Taiwan. Tel: 886-2-6628-9779 ext. 5709; Fax: 886-2-6628-9009; E-mail: yulinkotw@yahoo.com.tw

Oxidative stress, defined as a situation in which an increased level of reactive oxygen species generation overwhelms the antioxidative defense capacity, can cause endothelial dysfunction[11,12] and is involved in the pathogenesis of atherosclerosis.[12,13] Cumulative evidence supports that oxidative stress plays an important role in the pathogenesis of diabetic nephropathy.[14,15] Furthermore, several investigations demonstrate that reduced antioxidant concentrations or increased oxidative stress are associated with the development of albuminuria in diabetic patients.[16,17] In patients with hypertension, increased oxidative stress seems to be a determinant of urine albumin excretion independent of blood pressure levels.[18] Therefore, it is of interest to test the idea that oxidative stress serves as a risk factor of albuminuria in nondiabetic and normotensive subjects by examining the association between oxidative stress and the levels of urine albumin excretion.

8-hydroxydeoxyguanosine (8-OHdG), an oxidized nucleoside of DNA, is the most frequently detected DNA lesion. Upon DNA repair, 8-OHdG is excreted in the urine.[19] Accumulating data suggest that the urinary 8-OHdG can serve as a sensitive biomarker of oxidative DNA damage.[20] The present study aimed to examine whether urinary albumin excretion is associated with oxidative stress, as estimated by measuring the urinary 8-OHdG, in Taiwanese subjects. Since the levels of oxidative stress are significantly different between females and males in Chinese population,[21] we also aimed to investigate this association stratified by gender.

MATERIALS AND METHODS

Study population

After informed consent was obtained, the study subjects were recruited consecutively during routine health examinations. Only subjects without a known history of major systemic disease or cardiovascular disease were enrolled. Clinical history, including hypertension, diabetes, habitual smoking and drug therapy, were recorded for all participants. Exclusion criteria included age under 18 years old, pregnancy, cancer, and a history of myocardial infarction, stroke, or transient ischemic attack. Furthermore, subjects with diabetes mellitus (defined as blood sugar levels before a meal of ≥ 7.0 mmol/L or the regular use of medications for diabetes mellitus), hypertension (defined as a systolic BP ≥ 140 mm Hg and/or a diastolic BP ≥ 90 mm Hg or regular antihypertensive medication use) and macroalbuminuria (defined as a urinary albumin-creatinine ratio (ACR) more than 300 mg/g) were also excluded. Since statins were known to have an antioxidative effect, subjects who were under lipid-lowering drug treatment were also excluded from this study. There were 469 subjects (241 men with a mean age of 43.3 ± 9.1 years; 228 women with a mean age of 45.3 ± 9.1 years) that were enrolled in this analysis. Current smoker was defined if the subject smoked at least 1 cigarette per day at the time of survey. All participants reported their ethnicity as Han-Chinese origin. The Ethics Committee of the Chang-Gung Memorial Hospital approved the study.

Laboratory measurements

A total of 15 mL of venous blood was collected in the morning after an overnight (8-12 h) fast. All measurements were performed in a central laboratory. Glucose was enzymatically determined by using the hexokinase method. Total cholesterol and triglyceride concentrations were measured by automatic enzymatic colorimetry. High-density lipoprotein (HDL) cholesterol (HDL-C) levels were measured enzymatically after phosphotungsten/magnesium precipitation. Low-density lipoprotein (LDL) cholesterol (LDL-C) levels were calculated from the Friedewald formula, except for patients with triglyceride levels > 400 mg/dL. In these patients serum LDL-C levels were detected with commercial reagents by standard protocol. Serum insulin levels were measured by immunoradiometric assay (Bio-source, Nivelles, Belgium). The intra-assay and inter-assay coefficients of variation were 5.3% and 9.5% respectively. Insulin resistance was estimated by the homeostasis model assessment of insulin resistance (HOMA-IR) index using the following formula: fasting serum insulin (μU/mL) × fasting plasma glucose (mmol/L)/22.5. Highly sensitive C-reactive protein (CRP) was measured using high-sensitivity enzyme-linked immunosorbent assay (ELISA) developed in-house and performed in sandwich format.[22] The inter-assay and intra-assay coefficients of variation were < 5%.

Measurement of urinary ACR

On the same day when blood sample was collected,

a spot urine sample was gathered as the first morning voiding. Urinary albumin concentrations were measured using an in-house ELISA method.[23] The assay sensitivity was 1.6 μg/mL. Within-day and between-day coefficients of variation were 6.6% and 7.6%, respectively. Urine creatinine concentrations were determined with a Hitachi-7600 analyzer (Hitachi Inc., Tokyo, Japan). Microalbuminuria was defined as urinary ACR 30-300 mg/g.

Measurement of urinary 8-OHdG

Urinary 8-OHdG was measured using a competitive ELISA described elsewhere[19] and was presented as urinary 8-OHdG-to-urinary creatinine ratio. Briefly, bovine serum albumin-conjugated 8-hydroxyguanosine (8-OHG) was coated on the microplate in this assay. Urinary 8-OHdG and monoclonal anti-8-OHdG antibody were then incubated together in the microwell. During incubation, urinary 8-OHdG and the coated bovine serum albumin-conjugated 8-OHG compete for the monoclonal antibody. Final quantification of bound 8-OHdG antibody was estimated by the addition of horseradish peroxidase-conjugated sheep-anti-mouse antibody. The sensitivity of the assay was 0.5 ng/mL. The within-day precision and day-to-day precision were 5.9% and 8.0%, respectively. This ELISA kit compared well with the commercial kit from Japan Institute for the Control of Aging with a correlation coefficient of 0.9.[19]

Anthropometric measurements

Anthropometrics were obtained with the participants in light clothing, no footwear and after 12 h of fasting. Body weight was measured to the nearest kilogram using a digital scale, and height was measured to the nearest centimeter in the standing position using a wall standiometer. Body mass index (BMI) was computed as the ratio of weight to the square of height (kg/m^2). Obesity was defined as a BMI of 25 kg/m^2 or more according to the Asian criteria.[24]

Statistical analysis

The clinical characteristics of the participants were expressed as means ± SD and percentages. Data with skewed distribution, such as urinary ACR, 8-OHdG, HOMA-IR, CRP, triglycerides and HDL-C, were presented as median value (interquartile ranges) and log-transformed in all analysis. The chi-square test or chi-square test for trend was used to determine the significance of differences between genders in the distribution of categorical data. The clinical characteristics that were continuous variables were compared between genders by independent *t* tests. Pearson's correlation analysis was performed to examine the relationships between urinary ACR, 8-OHdG and factors of interest. Multiple linear regression analysis with the enter method was used to determine the relationships between urinary ACR, 8-OHdG and the variables that showed association with urinary ACR in the Pearson's correlation analysis, adjusted for age and smoking. A p value < 0.05 using a two-sided test was considered statistically significant. All statistical analyses were conducted with the SPSS statistical package for Windows version 12.0 (SPSS, Chicago, IL, USA).

RESULTS

Clinical and biochemical characteristics of subjects stratified by sex

A summary of demographic features, clinical and lipid profiles, anthropometric measurements, urinary 8-OHdG and urinary ACR stratified by gender is shown in Table 1. The analysis showed that many variables were statistically significantly different between men and women. The male subjects were younger than female ($p = 0.019$). There was a higher percentage of current smokers ($p < 0.001$) and obesity ($p < 0.001$) among men than women. Systolic blood pressure ($p = 0.011$), diastolic blood pressure ($p < 0.001$), triglyceride levels ($p < 0.001$), LDL-C levels ($p = 0.008$), fasting glucose ($p < 0.001$), CRP levels ($p = 0.02$), BMI ($p < 0.001$) and HOMA-IR index ($p < 0.001$) were also significantly higher in men than in women. In contrast, HDL-C levels ($p < 0.001$), urinary 8-OHdG ($p = 0.002$) and urinary ACR ($p = 0.001$) were significantly lower in men than in women.

Relationship between urinary ACR and variables of interest

The results of Pearson's correlation analysis between urinary ACR, oxidative stress and factors of interest are presented in Table 2. During univariate analysis,

Table 1. Baseline characteristics of the study subjects according to gender

	All subjects (n = 469)	Men (n = 241)	Women (n = 228)	p value
Age (years)	44.3 ± 9.1	43.3 ± 9.1	45.3 ± 9.1	0.019
Smoking (%)	118 (25.2%)	105 (43.6%)	13 (5.7%)	< 0.001
Systolic BP (mmHg)	109.6 ± 13.0	111.1 ± 11.7	108.1 ± 14.1	0.011
Diastolic BP (mmHg)	73.3 ± 8.6	75.2±8.3	71.3 ± 8.6	< 0.001
Total cholesterol (mg/dL)	198.1 ± 35.1	200.4 ± 34.0	195.0 ± 35.9	0.094
Triglycerides (mg/dL)*	107.1 (72.6-156.6)	130.1 (91.2-196.5)	86.7 (63.7-126.5)	< 0.001
HDL cholesterol (mg/dL)*	54.1 (45.2-66.4)	47.9 (42.9-55.6)	62.2 (52.1-71.0)	< 0.001
LDL cholesterol (mg/dL)	115.4 ± 31.7	119.3 ± 31.7	111.6 ± 31.3	0.008
Fasting glucose (mg/dL)	92.3 ± 8.1	94.4 ± 8.3	90.5 ± 7.6	< 0.001
CRP (mg/L)*	0.54 (0.25-1.12)	0.64 (0.29-1.12)	0.46 (0.21-1.07)	0.02
Body mass index (kg/m^2)	23.9 ± 3.3	24.6 ± 3.0	23.1 ± 3.4	< 0.001
HOMA-IR*	1.76 (1.36-2.41)	1.91 (1.49-2.51)	1.68 (1.26-2.33)	< 0.001
Obesity (%)	164 (35.0%)	104 (43.1)	60 (26.3)	< 0.001
Urinary 8-OHdG (ng/mg Cr)*	32.32 (24.24-44.29)	30.7 (22.68-42.15)	34.59 (26.47-46.38)	0.001
Urinary ACR (mg/g)*	4.37 (2.85-7.02)	3.74 (2.48-6.29)	4.93 (3.52-8.10)	0.001

HDL, high-density lipoprotein; LDL, low-density lipoprotein; CRP, C-reactive protein; HOMA-IR, homeostasis model assessment of insulin resistance; 8-OHdG, 8-Hydroxy-2'-deoxyguanosine to urinary creatinine ratio; ACR, albumin-to-creatinine ratio.
*Data with skew distribution were logarithmically transformed before statistical testing to meet the assumption of normal distribution and presented as median (interquartile range).

Table 2. Pearson correlation coefficients of urinary ACR with variables of interest

Variables	All (n = 469)		Men (n = 241)		Women (n = 228)	
	r	p value	*r*	p value	*r*	p value
Age	0.166	< 0.001	0.170	0.008	0.131	0.048
Systolic BP	0.164	< 0.001	0.204	0.001	0.170	0.010
Diastolic BP	0.120	0.009	0.149	0.021	0.176	0.008
Total cholesterol	0.055	0.237	-0.014	0.831	0.157	0.018
Triglycerides	0.051	0.273	0.090	0.166	0.135	0.041
HDL cholesterol	0.042	0.365	0.007	0.912	-0.068	0.306
LDL cholesterol	0.016	0.729	-0.065	0.315	0.152	0.022
CRP	0.039	0.399	0.073	0.260	0.038	0.571
Body mass index	0.087	0.059	0.120	0.062	0.137	0.039
HOMA-IR	0.144	0.002	0.219	0.001	0.125	0.059
Urinary 8-OHdG	0.131	0.005	0.025	0.697	0.204	0.002

Acronyms are as in Table 1.
Urinary ACR, 8-OHdG, HOMA-IR, triglycerides, HDL cholesterol and CRP were logarithmically transformed before statistical testing to meet the assumption of normal distribution.

urinary ACR was significantly positively correlated with age, systolic blood pressure, diastolic blood pressure, HOMA-IR and urinary 8-OHdG. When the analysis was further stratified by gender, urinary ACR was significantly positively correlated with age, systolic blood pressure, diastolic blood pressure and HOMA-IR in men. On the other hand, urinary ACR was significantly positive correlated with age, systolic blood pressure, diastolic blood pressure, total cholesterol levels, triglyceride levels, LDL-C levels, BMI and urinary 8-OHdG in women. However, neither HDL-C nor CRP levels was correlated with urinary ACR in both sexes.

Multiple linear regression analysis for urinary ACR

The results of multiple linear regression analysis for

urinary ACR are shown in Table 3. Urinary 8-OHdG and variables that showed association with urinary ACR in the Pearson's correlation analysis were entered into the multivariate analysis and were adjusted for age and smoking, stratified by gender. The model for men demonstrated that only HOMA-IR (p = 0.017) was an important influential factor of urinary ACR. That is to say, urinary ACR tended to be higher in male subjects who had higher insulin resistance. On the other hand, the model for women revealed that only urinary 8-OHdG (p = 0.010) was an important influential factor of urine ACR. When the minorities of participants with microalbuminuria were excluded from the analysis, the positive association of urinary ACR and urinary 8-OHdG in female subjects was unchanged (p = 0.012, data not shown), but the association of urinary ACR and HOMA-IR in male subjects was attenuated (p = 0.083, data not shown). This result strongly suggests that female subjects who have increased oxidative stress tend to have higher urinary ACR.

DISCUSSION

In this cross-sectional study, we found a positive association of urinary ACR with urinary 8-OHdG in Taiwanese women, but not in men. Importantly, this association was unchanged when the minorities of participants with microalbuminuria were excluded from the analysis, indicating that urinary ACR was associated with urinary 8-OHdG even in Taiwanese women with so-called normoalbuminuria. On the contrary, HOMA-IR was the only predictor of higher urinary ACR in male subjects. These associations were independent of the traditional cardiovascular risk factors that were reported to be associated with albuminuria, such as obesity,[25,26] hypertension,[26-28] hyperlipidemia,[26,27] insulin resistance,[29] and smoking.[26] Our study provides an attractive explanation for the development of albuminuria in Taiwanese subjects, which has enabled us to initiate possible prevention and treatment strategies earlier, therefore reducing the risk of future cardiovascular disease.

It is well known that oxidative stress plays an important role in the development of diabetic nephropathy. In diabetic subjects, hyperglycemia results in the generation of reactive oxygen species as well as the depletion levels of antioxidants through glycation of the scavenging enzymes.[14] Increased oxidative stress or reduced levels of antioxidants, in turns, activate signal transduction cascades (protein kinase C, mitogen-activated protein kinase, and janus kinase/signal transducers and activators of transcription) and transcription factors (nuclear factor-κB, activated protein-1, and specified protein-1), leading to upregulation of genes and proteins involved in the glomerular mesangial expansion and tubulointerstitial fibrosis.[14,15] Animal studies show that reactive oxygen species can cause torrential proteinuria by inducing a molecular size-selectivity defect of the glomerular capillary wall, without any identifiable structural changes in the glomerular filtration barrier.[30] An

Table 3. Multiple linear regression analysis of the relationship between urinary ACR, 8-OHdG and variables associated with urinary ACR in the Pearson's correlation analysis, according to sex (*)

	All subjects		Men		Women	
	β coefficient	p value	β coefficient	p value	β coefficient	p value
Systolic blood pressure	0.002	0.247	0.004	0.161	0.001	0.691
Diastolic blood pressure	< 0.001	0.273	< 0.001	0.947	0.002	0.651
Total cholesterol	0.002	0.174	< 0.001	0.521	< 0.001	0.924
Triglycerides	-0.039	0.650	0.017	0.890	0.078	0.535
LDL-cholesterol	-0.002	0.172	-0.002	0.356	0.001	0.666
Body mass index	0.002	0.386	0.005	0.560	0.005	0.512
HOMA-IR	0.255	0.009	0.323	0.017	0.101	0.484
8-OHdG	0.190	0.021	0.026	0.827	0.290	0.010

* Adjusted variables: age and smoking.

Acronyms are as in Table 1.

Calculations of urinary ACR, 8-OHdG, HOMA-IR and triglycerides were based on log-transformed value.

increase in oxidative stress is also associated with endothelial dysfunction,[11,12] which has been linked to the development of microalbuminuria.[31] These lines of evidence link oxidative stress with the development of microalbuminuria in diabetic patients. Our study clearly demonstrates a significant association between urinary ACR and urinary 8-OHdG in Taiwanese women but not in men. One possibility of this gender interaction between urinary ACR and oxidative stress is that women have higher oxidative stress and lower incidence of traditional cardiovascular risk factors that are associated with albuminuria such as smoking, obesity, hypertension, insulin resistance and hyperlipidemia. Thus, the influence of oxidative stress on the albuminuria may be more evident and significant in women. On the other hand, within male subjects increased incidence of these risk factors may all overwhelm the oxidative stress "strength of signal", and hence, the oxidative effect on albuminuria may be lower than in the female subjects.

In the present study, levels of urinary 8-OHdG were higher in women than in men. This gender difference of urinary 8-OHdG remained significant after adjustment for age, smoking, blood pressure, BMI and HOMA-IR ($p = 0.020$, data not shown). Our data is compatible with that of some previous reports that showed higher urinary 8-OHdG in the postmenopausal women.[32-34] It has been postulated that, during menopause, a decreased production of estrogen may be involved in the elevation of urinary 8-OHdG.[33,34] Recently, evidence has shown that oxidative DNA damage, as measured by urinary 8-OHdG, is associated with exposure to cooking oil fumes (COFs) in Chinese restaurant workers,[35] and female restaurant workers had a greater oxidative stress response to COFs than male restaurant workers, providing additional evidence of the link between lung cancer in Chinese women and exposure to COFs. Thus, overexposure to COFs in Chinese women may be another plausible mechanism that causes the gender difference of oxidative stress in the present study.

It has been recognized that insulin resistance is closely associated with microalbuminuria in nondiabetic subjects. The Insulin Resistance Atherosclerosis Study showed that an increasing degree of insulin sensitivity is related to a decreasing prevalence of microalbuminuria. This relationship remained significant after adjustment for age, sex, hypertension, fasting glucose and body mass index, indicating that hypertension, fasting glucose and body mass index didn't fully explain the relationship between microalbuminuria and insulin resistance.[29] It has been proposed that insulin resistance and hyperglycemia may impair endothelial function, resulting in increasing urinary albumin excretion and microalbuminuria.[10] In the present study, we demonstrated a significant positive association between urinary ACR and HOMA-IR in healthy Taiwanese men without macroalbuminuria. However, this association was attenuated if the minorities of participants with microalbuminuria were excluded from the analysis. Further studies are needed to evaluate whether this gender interaction in the relationship between urinary ACR and insulin resistance is consistent among different ethnic groups.

Although obesity, blood pressure, hyperlipidemia and smoking are reported to be associated with albuminuria in some studies,[25-28] the results are not consistent. The association between obesity and development of albuminuria are identified in several studies. However, the results are not concordant. In the WHO MONICA Augsburg survey, the relationship between body mass index and microalbuminuria was only observed in women but not in men.[36] On the other hand, in the Gubbio population study from Italy, positive associations between the body mass index and urinary albumin excretion rate were evident for both males and females in univariate and multivariate analysis.[26] The association between blood pressure and albuminuria could indicate an effect of localized increased pressure in the glomerular vessels, of glomerular dysfunction on blood pressure, or of a third factor favoring increase in blood pressure and albumin excretion. However, a previous epidemiological study[37] showed urinary albumin excretion was not dependent on blood pressure or number of cigarettes smoked, but at blood pressures greater than 140/90 mmHg, urinary albumin excretion rate might become pressure-dependent. The relationship between albuminuria and hyperlipidemia was also not accordant among previous studies. In the Gubbio population study,[26] urinary albumin excretion was significantly associated with plasma total cholesterol and triglyceride levels in simple correlation analysis in both sexes in nondiabetic subjects. During multiple linear regression analysis, however, the association between urinary albumin excretion and triglyceride levels was abolished.

On the contrary, Mykkanen et al.[27] reported that elevated triglyceride levels and decreased HDL-C levels, but not total cholesterol levels, were associated with microalbuminuria in nondiabetic subjects. In the present study, albuminuria was associated with blood pressure, total cholesterol levels, triglyceride levels, LDL-C levels, BMI and urinary 8-OHdG in women during Pearson's correlation analysis. However, most of these associations were abolished during multiple linear regression analysis, especially when 8-OHdG is entered into the model in women.

The strength of this study is that we focused on the participants who did not have diabetes or hypertension, a population in which the association between urinary albumin excretion and oxidative stress has not been fully investigated before. Therefore, our study offers the opportunity to test whether oxidative stress could be a potential early risk factor of developing albuminuria. However, a number of possible limitations of our study merit mention. First, because of the study's cross-sectional nature, our results did not establish causality. A long-term follow-up study of the same population of subjects will illuminate the causal relationship between oxidative stress and urinary ACR. Second, some reporting bias might have been introduced because some information in the medical records such as underlying cardiovascular disease, medication with lipid-lowering agents, and smoking was obtained via self-reported questionnaires. The long-term follow-up study mentioned above will enable us to test this possibility. Third, the subjects we included were all of Han-Chinese origin. Further studies are needed to examine whether the association between urinary albumin excretion and oxidative stress is still significant in different ethnic groups.

CONCLUSION

In conclusion, we have shown that urinary ACR, even when it is within the so-called normal range (i.e. urinary ACR < 30 mg/g), is associated with urinary 8-OHdG in Taiwanese women, but not in men. This association is independent of the traditional cardiovascular risk factors that are reported to be associated with albuminuria, such as obesity, insulin resistance, hypertension, hyperlipidemia and smoking, indicating that oxidative stress plays an important role in the presence of albuminuria in Taiwanese women. Further prospective studies are needed to evaluate whether lowering of the oxidative stress, such as antioxidant treatment, subsequently decreases urinary albumin excretion, and thus reduces the risk of future cardiovascular events, especially in Taiwanese women who have increased urinary albumin excretion.

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