Original Article

Association of urinary 8-hydroxydeoxyguanosine level with arteriosclerosis-related factors in healthy, urban residents aged ≥50 years in Japan

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Previous studies have suggested a relationship between the urinary 8-hydroxydeoxyguanosine level and hypertension, but an association between the urinary 8-hydroxydeoxyguanosine level and systolic blood pressure has not been reported. The aim of this cross-sectional study was to examine the association between the 8-hydroxydeoxyguanosine level, a marker of DNA oxidative damage, and arteriosclerosis-related factors in healthy, urban residents aged ≥50 years who participated in annual health promotion activities in Mitaka City, Tokyo from 2008 to 2018. Arteriosclerosis-related factors were used as independent variables and the urinary 8-hydroxydeoxyguanosine creatinine concentration-corrected level (urinary 8-hydroxydeoxyguanosine/creatinine) as the dependent variable in multiple logistic regression. Two hundred and forty-eight participants were divided into two groups using a cutoff point of 6.2/6.3 ng/mg creatinine, which corresponds to the urinary 8-hydroxydeoxyguanosine/creatinine levels in approximately 80% of the participants. A high urinary 8-hydroxydeoxyguanosine/creatinine level was significantly associated with a body mass index ≥25, obesity, and systolic blood pressure ≥140. Our findings suggest that in healthy individuals aged ≥50 years, arteriosclerosis-related factors such as inappropriate weight management and poor systolic blood pressure control may be associated with the urinary 8-hydroxydeoxyguanosine level.

***Key Words*: urinary 8-hydroxydeoxyguanosine (8-OHdG), arteriosclerosis, healthy adults aged ≥50 years, body mass index, systolic blood pressure**

In recent years, the association between oxidative stress under conditions such as accumulation of reactive oxygen species (ROS) and the pathogenesis of cancer and cardiovascular disease has been increasingly recognized. ROS, produced excessively due to decreased enzyme activity and antioxidant levels, can damage cells and tissues.[1] Macrophages in atherosclerotic lesions have been reported as a source of ROS.[2,3] An indicator of oxidative stress is 8-hydroxydeoxyguanosine (8-OHdG), which is produced during oxidative DNA damage.[4] Thus, it is a marker of oxidative DNA damage. 8-OHdG is a hydroxylated structure at the C-8 position of deoxyguanosine. It is produced when deoxyguanosine, a DNA base, is damaged by the action of ROS. 8-OHdG is excreted in urine during DNA repair.[5] Unlike other biomarkers, the urinary 8-OHdG level remains relatively stable throughout the day.[6] 8-OHdG has been associated with the development of cancer,[7,8] cardiovascular disease,[9,10] hypertension,[11–14] and diabetes.[14,15]

Heart and cerebrovascular diseases have been the second and third leading causes of death in Japan since 1997. Since 2011, cerebrovascular disease has been the fourth leading cause of death. Among heart and cerebrovascular diseases, ischemic heart disease and cerebral infarction are related to arteriosclerosis with metabolic syndrome in the older population. Since April 2008, Japan has implemented nationwide lifestyle health check-ups and health guidance programs, emphasizing the prevention of arteriosclerotic diseases that cause metabolic syndrome. Risk factors for arteriosclerosis, which is a pathological condition of arteriosclerotic diseases, include age, sex, smoking and drinking habits, physical activity, sleep duration, visceral fat obesity, body mass index (BMI), hypertension, diabetes, high-density lipoprotein (HDL) hypercholesterolemia, and hypertriglyceridemia. Urinary 8-OHdG, as a biomarker of oxidative DNA damage, has been associated with these risk factors.[12,14,16–22] Factors that have been suggested to be associated with the urinary 8-OHdG level include age,[17] sex,[14,16,17] smoking habit,[12,14,16,18,19] drinking habit,[20] BMI,[16,18,19,21] physical activity,[18,22] and sleep duration.[6] Men,[14,16] older women,[17] smokers,[12,14,18,19] and individuals with shorter sleep duration[6] tend to have higher urinary 8-OHdG levels. Furthermore, among non-smokers, a previous study has suggested that passive smoking increases urinary 8-OHdG concentrations.[23]

Conversely, moderate drinking[20] and moderate exercise[18,22] have been associated with lower urinary 8-OHdG levels. The relationship between BMI and the urinary 8-OHdG level appears to be complex, with some studies suggesting a decrease in the 8-OHdG level with increasing BMI,[18,19] whereas others proposed a U-shaped relationship.[21] Recent studies on this aspect include longitudinal studies[17] and multivariate analyses.[6,13–15] However, only few studies have included healthy older people aged ≥75 years. In 2018, individuals aged >65 years accounted for 28% of the population in Japan, and the urinary 8-OHdG level might be an important marker for the early detection of arteriosclerotic diseases such as cerebral infarction and ischemic heart disease, which are common causes of death in Japanese. Therefore, the aim of this study was to investigate the relationship between the urinary 8-OHdG level and arteriosclerosis-related factors in healthy, middle-aged and older individuals residing in urban areas of Japan.

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Patients and Methods

This was a cross-sectional study. Individuals were recruited from participants of an exercise consultation for older residents, a health promotion activity that was held annually in Mitaka City, Tokyo, from 2008 to 2018.

This health promotion program aims to maintain and improve the health of adults aged ≥50 years living in Mitaka City. From 1993 to 2019, under this program, physical fitness measurements and medical checks were conducted in approximately 40 applicants each year, and urinary 8-OHdG measurement was started in 2008. The individuals who participated after reading the annual event guide underwent medical interviews (pertaining to medical history, lifestyle habits, and medication status), medical examinations, body measurements, blood pressure measurements, blood tests, urine tests (including 8-OHdG measurement), and exercise load tests using a bicycle ergometer and other test equipment. All participants underwent an exercise stress test using a bicycle ergometer (Health-guard Active 10 II; Takei Scientific Instruments Co., Ltd., Kamo, Japan), which indicated that they maintained sufficient cardiac function to perform their daily activities.

Between 2008 and 2018, 259 residents (78 men and 181 women) participated in the project. Of these, 11 residents were excluded because of a lack of data or consent. A total of 248 adult residents (75 men and 173 women) were included in the analysis. Of them, 65 residents participated more than once (2–9 out of 11 times) in the health-promotion program from 2008 to 2018. Data used for the analysis were from the first year for each participant.

Measurement of urinary 8-OHdG level. Urinary 8-OHdG has been reported to be involved in the development of various diseases; thus, it is expected to be useful for detecting pre-disease conditions before the onset of symptoms. The urinary 8-OHdG level may be affected by exercise;[18,22] therefore, we collected 1.0 ml of partial urine sample in a dedicated urine-collection tube immediately after the arrival of the project participants at the venue or before the exercise load. The collected urine specimens were submitted to SRL Co., Ltd., which was commissioned to measure the urinary 8-OHdG level.

SRL Co., Ltd. Measured the urinary 8-OHdG level using high-performance liquid chromatography (HPLC).[24,25] HPLC separates and detects components in a liquid using the difference in the interaction between the stationary and mobile phases. Using HPLC, measurement of the urinary 8-OHdG level was achieved via a two-stage separation method (Kasai method). In the first step, 8-OHdG was extracted using 8-OHG as a marker; only trace amounts of 8-OHdG were measured using HPLC in the second step.[25] The measured urinary 8-OHdG level was used for analysis after conversion to a urinary 8-OHdG-creatinine concentration-corrected value (urinary 8-OHdG/creatinine level). The urinary creatinine concentration was corrected because the urinary 8-OHdG level measured in partial urine specimens can be influenced by variations in urinary creatinine concentrations. However, it is known that the 24-h excretion of urinary 8-OHdG can be estimated by adjusting for urinary creatinine concentration. This correction accounts for differences in urine dilution and provides a more accurate assessment of the urinary 8-OHdG level.

Categorization of participants. The participants were categorized based on various arteriosclerosis-related factors. The candidate independent variables included age; sex; smoking habit; drinking habit; sleep duration; intensity of daily activities; BMI; systolic blood pressure (SBP); diastolic blood pressure (DBP); hemoglobin A1c (HbA1c), triglyceride (TG), HDL cholesterol, and N-terminal pro-brain natriuretic peptide (NT-proBNP) levels; and electrocardiograph (ECG). The blood sugar level was excluded as it is influenced by meal and beverage consumption and owing to its lack of reliability. The dependent variable was the urinary 8-OHdG/creatinine level.

The participant characteristics were as follows:
Age: categorized into three groups—50–64 years old (middle-aged group), 65–74 years old (early older group), and ≥75 years old (late older group).
Sex: divided into male and female individuals.
BMI: divided into three categories—<18.5 kg/m^2 (skinny group), 18.5–25.0 kg/m^2 (normal body type group), and ≥25.0 kg/m^2 (obese group).
Lifestyle factors were classified as follows:
Smoking habit: divided into non-smokers (non-smoking group) and current smokers (smoking group).
Drinking habit: divided into non-drinkers (non-drinking group) and current drinkers (drinking group).
Sleep duration: divided into ≥7 h (normal sleep time group) and <7 h (short sleep time group).
Daily activity intensity: divided into walking fast for an average of over 30 min per day (strength group) and walking fast for an average of under 30 min per day (non-strength group).

The standard values for each indicator related to arteriosclerosis were based on the following guidelines: Guidelines for the Management of Hypertension 2019 (The Japanese Society of Hypertension) for blood pressure, Guidelines for the Management of Diabetes 2019 (The Japan Diabetes Society) for HbA1c, and Guidelines for the Prevention of Atherosclerotic Cardiovascular Diseases 2017 (The Japan Atherosclerosis Society) for lipids.

Each factor related to arteriosclerosis was divided into two or three categories—SBP: <140 mmHg (normal group) and ≥140 mmHg (high group); DBP: <90 mmHg (normal group) and ≥90 mmHg (high group); HbA1c: <6.2% (normal group) and ≥6.2% (high group); TG: <150 mg/dl (normal group) and ≥150 mg/dl (high group); HDL cholesterol: ≥40 mg/dl (normal group) and <40 mg/dl (low group); NT-proBNP: <125 pg/ml[26] (normal group) and ≥125 pg/ml[26] (high group); ECG: not particular (normal group); right bundle branch block, first-degree atrioventricular block, sinus bradycardia, premature ventricular contractions, non-specific ST-T change (observations group); and atrial fibrillation, multiple premature ventricular contractions, left bundle branch block, left ventricular hypertrophy, old myocardial infarction (heart disease group).

The reference value for the urinary 8OHdG/creatinine level was <12.0 ng/mg creatinine equivalent value (ng/mg creatinine), but only four participants had values higher than this reference value in the present data. Therefore, 6.2/6.3 ng/mg creatinine, which corresponds to the creatinine level of approximately 80% in the participants, was set as the cutoff point, and the participants were divided into the following two categories: high and low (Fig. 1).

Analysis method. A chi-squared test was performed because all analyzed data were categorical data. Multiple logistic regression analysis, with forced entry method, was performed by keeping the urinary 8OHdG/creatinine level as a dependent variable. A total of 13 factors (age; sex; smoking habit; drinking habit; sleep duration; daily activity intensity; BMI; SBP; DBP; HbA1c, TG, HDL cholesterol, and NT-proBNP levels; and ECG) were used as candidate independent variables. Factors with a p value of 0.80 or higher in the chi-squared test were excluded from independent variables in the logistic regression analysis. The IBM SPSS Statistics software for Windows, ver. 26.0 (IBM Corp, Armonk, NY) was used for these analyses. Furthermore, results with $p<0.05$ were considered statistically significant.

Ethics approval. This study was approved by the Ethics Committee of Faculty of Health Sciences in Kyorin University

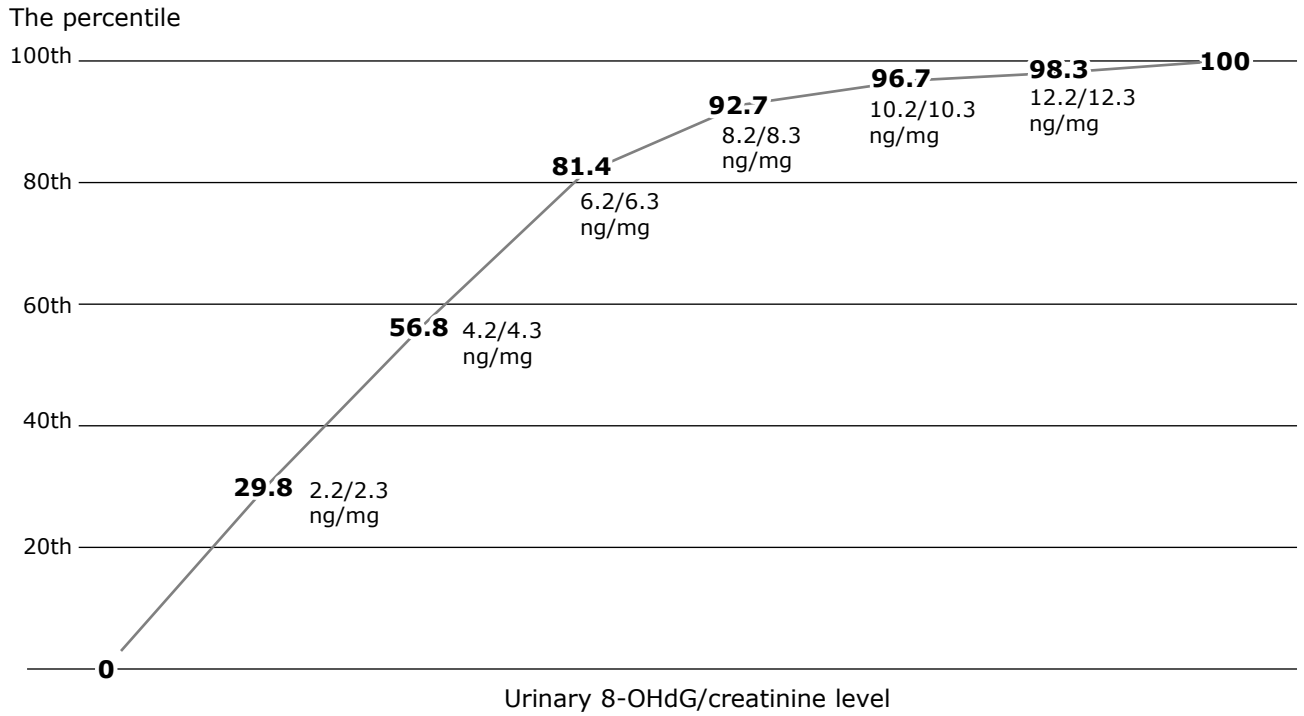

Fig. 1. Cumulative percentage value and urinary 8-OHdG creatinine equivalent among the participants.

(Approval No. 30-2). Written informed consent was obtained from all study participants. Furthermore, this study complied with the provisions of the 2008 Declaration of Helsinki.[27]

Results

Two hundred and forty-eight participants were included in the study: 202 participants in the low urinary 8-OHdG/creatinine level group and 46 in the high group. No association was observed between the three age groups and the two groups of urinary 8-OHdG/creatinine levels (Table 1). Among arteriosclerosis-related factors, smoking habit; drinking habit; sleep duration; daily activity intensity; HbA1c, TG, HDL cholesterol, and NT-proBNP levels; and ECG showed no significant association with the urinary 8-OHdG/creatinine level (Table 1). However, the BMI, SBP, and DBP were significantly associated with the urinary 8-OHdG/creatinine level (Table 1).

Among the candidate independent variables, sex, smoking habit, TG level, and ECG were excluded from the analysis as they had *p* values greater than 0.80 based on the chi-squared test. The variance inflation factor for each of the ten remaining independent variables was less than 5.0, indicating the absence of multicollinearity. The Hosmer–Lemeshow's test result showed a *p* value of >0.05, indicating a good fit for the multiple logistic regression analysis (Table 2). After adjusting for other factors, the results revealed that an increase in the urinary 8-OHdG/creatinine level was significantly associated with a BMI of 25.0 or higher [odds ratio (OR) = 2.75, 95% confidence interval (CI): 1.17–6.46, $p = 0.02$] and an SBP of ≥140 mmHg (OR = 2.24, 95% CI: 1.04–4.84, $p = 0.04$; Table 2). There was a tendency of the BMI <18.5 (OR = 2.57, $p = 0.09$) and late-elderly group (OR = 2.14, $p = 0.13$) to be associated with an increase in the urinary 8-OHdG/creatinine level. The other variables did not show a significant association with an increase in the urinary 8-OHdG/creatinine level.

Discussion

In the multiple logistic regression analysis, two factors were found to be associated with an increase in the urinary 8-OHdG/creatinine level: obesity with a BMI of 25.0 or higher and hypertension with an SBP of 140 mmHg or higher. The former finding was similar to that of a previous study.[21] Some studies have reported that individuals with a low BMI have higher urinary 8-OHdG levels.[18,19,21] The higher urinary 8-OHdG level in skinny individuals is thought to be due to the higher metabolic rate than that of obese individuals.[18] On the contrary, the increased urinary 8-OHdG level in obese individuals may be attributed to enhanced oxidative DNA damage. However, the underlying mechanism remains unknown. Considering that increased urinary 8-OhdG levels were observed in individuals with chronic obesity, it is important to maintain a normal body weight within the BMI standard value through lifestyle habits.

Previous studies have suggested that sex,[14,16,17] smoking,[12,14,16,18,19] drinking,[20] physical activity,[18,22] and sleep duration[6] could be related to the urinary 8-OHdG level. However, the present study results showed no association between these lifestyle habits and the urinary 8OHdG level. The reason for no association between smoking habit, which is known to be a risk factor for arteriosclerotic diseases such as cerebral infarction and ischemic heart disease, and the urinary 8OHdG level could be the small number of smokers, being only 11. In addition, a previous study suggested a relationship between passive smoking and urinary 8-OHdG concentrations.[23] However, we could not examine the influence of passive smoking in our current study because no smoking data for family members living in the same household were available. Future studies should include data on passive smoking as well as smoking history.

Previous studies have suggested a relationship between hypertension, a risk factor for cardiovascular disease, and the urinary 8-OhdG level.[14,15] However, only few studies have examined the relationship between the 8-OhdG level and blood pressure after dividing it into SBP and DBP.[13] In this study, a possible associa-

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Table 1. Characteristics, lifestyle, and biomarkers related with arteriosclerotic disease in the 248 participants

Characteristics, lifestyles, and biomarkers related with arteriosclerotic disease	All subjects $n = 248$		Low group of urinary 8OHdG/creatinine level $n = 202$		High group of urinary 8OHdG/creatinine level $n = 46$		Chi-squared test
Age: 51–86 years old							
51–64 years old (middle-aged group)	74	29.8%	63	85.1%	11	14.9%	
65–74 years old (early elderly group)	113	45.6%	95	84.1%	18	15.9%	$p = 0.09$
75–86 years old (late-elderly group)	61	24.6%	44	72.1%	17	27.9%	
Sex:							
Male group	75	30.2%	62	82.7%	13	17.3%	$p = 0.86$
Female group	173	69.8%	140	80.9%	33	19.1%	
Smoking habit							
Non-smoking group	237	95.6%	193	81.4%	44	18.6%	$p>0.99$[b]
Smoking group	11	4.4%	9	81.8%	2	18.2%	
Drinking habit:							
Non-drinking group	122	49.2%	101	82.8%	21	17.2%	$p = 0.63$
Drinking group	126	50.8%	101	80.2%	25	19.8%	
Sleeping hours: 3–10 h							
7 h or more (normal sleep group)	130	52.4%	104	80.0%	26	20.0%	$p = 0.63$
Less than 7 h (short sleep group)	118	47.6%	98	83.1%	20	16.9%	
Daily activity intensity:							
Walking fast for an average of over 30 min per day (strength group)	125	50.4%	104	83.2%	21	16.8%	$p = 0.52$
Walking fast for an average of under 30 min per day (non-strength group)	123	49.6%	98	79.7%	25	20.3%	
BMI (body type): 14.1–30.5 kg/m^2							
Under 18.5 (skinny group)	24	9.7%	18	75.0%	6	25.0%	
18.5 to 25.0 (normal body type group)	188	75.8%	160	85.1%	28	14.9%	$p = 0.02$
25.0 or higher (obese group)	36	14.5%	24	66.7%	12	33.3%	
SBP[a]: 88–177 mmHg							
Less than 140 mmHg (normal group)	169	68.1%	146	86.4%	23	13.6%	$p<0.01$
140 mmHg or higher (high group)	79	31.9%	56	70.9%	23	29.1%	
DBP[a]: 50–108 mmHg							
Less than 90 mmHg (normal group)	217	87.5%	181	83.4%	36	16.6%	$p<0.05$
90 mmHg or higher (high group)	31	12.5%	21	67.7%	10	32.3%	
HbA1c: 4.9–9.1%							
Less than 6.2% (normal group)	224	90.3%	183	81.7%	41	18.3%	$p = 0.78$[b]
6.2% or higher (high group)	24	9.7%	19	79.2%	5	20.8%	
TG: 38.0–674.0 mg/dl							
Less than 150 mg/dl (normal group)	152	61.3%	123	80.9%	29	19.1%	$p = 0.87$
150 mg/dl or higher (high group)	96	38.7%	79	82.3%	17	17.7%	
HDL cholesterol: 36.0–112.0 mg/dl							
40 mg/dl or higher (normal group)	239	96.4%	195	81.6%	44	18.4%	$p = 0.67$[b]
Less than 40 mg/dl (low group)	9	3.6%	7	77.8%	2	22.2%	
NT-proBNP: 7.0–5,270.0 pg/ml							
Less than 125 pg/ml (normal group)	185	74.6%	153	82.7%	32	17.3%	$p = 0.45$
125 pg/ml or higher (high group)	63	25.4%	49	77.8%	14	22.2%	
ECG:							
Not particular (normal group)	193	77.8%	157	81.3%	36	18.7%	
RBBB, sinus bradycardia, PVC, non-specific ST-T change (observations group)	45	18.1%	36	80.0%	9	20.0%	$p = 0.82$
Af, multiple PVC. LBBB, LVH, OMI (heart disease group)	10	4.0%	9	90.0%	1	10.0%	

[a]Conforms to the definition of office hypertension in the Japanese Society of Hypertension "Hypertensive Treatment Guidelines 2019". [b]Fisher's exact test. Af, atrial fibrillation; BMI, body mass index; DBP, diastolic blood pressure; ECG, electrocardiograph; HbA1c, hemoglobin A1c; HDL, high density lipoprotein; LBBB, left bundle branch block; LVH, left ventricle hypertrophy; NT-proBNP, N-terminal pro-brain natriuretic peptide; OMI, old myocardial infarction; PVC, premature ventricular contraction; RBBB, right bundle branch block; SBP, systolic blood pressure; TG, triglyceride.

tion between SBP and the urinary 8-OhdG level was observed. Both SBP and DBP are known to be influenced by peripheral vascular resistance and the autonomic nervous system. After the age of 50 years, the compliance of large arteries such as the aorta decreases, leading to an increase in SBP and a decrease in DBP.

These findings suggest that oxidative DNA damage may be

Table 2. Results of multiple logistic regression analyses with forced entry method

Independent variable		*n*	OR	95% CI	*p* value
Age	51–64 years old (middle-aged group)	74	—		
	65–74 years old (early elderly group)	113	1.12	0.44–2.85	0.810
	75–86 years old (late-elderly group)	61	2.14	0.79–5.79	0.130
Drinking habit	Non-drinking group	122	—		
	Drinking group	126	1.29	0.63–2.63	0.480
Sleeping hours	7 h or more (normal sleep group)	130	—		
	Less than 7 h (short sleep group)	118	1.09	0.54–2.19	0.820
Daily activity intensity	Walking fast for an average of over 30 min per day (strength group)	125	—		
	Walking fast for an average of under 30 min per day (non-strength group)	123	1.31	0.65–2.61	0.450
BMI (body type)	18.5–25.0 (normal body type group)	188	—		
	Less than 18.5 (skinny group)	24	2.57	0.87–7.55	0.090
	25.0 or higher (obese group)	36	2.75	1.17–6.46	0.020
SBP	Less than 140 mmHg (normal group)	169	—		
	140 mmHg or higher (high group)	79	2.24	1.04–4.84	0.040
DBP	Less than 90 mmHg (normal group)	217	—		
	90 mmHg or higher (high group)	31	1.71	0.63–4.66	0.300
HbA1c	Less than 6.2% (normal group)	224	—		
	6.2% or higher (high group)	24	0.98	0.32–3.01	0.970
HDL cholesterol	40 mg/dl or higher (normal group)	239	—		
	Less than 40 mg/dl (low group)	9	1.08	0.20–5.93	0.930
NT-proBNP	Less than 125 pg/ml (normal group)	185	—		
	125 pg/ml or higher (high group)	63	1.02	0.45–2.29	0.970

Hosmer and Lemeshow's test: $\chi^2 = 4.91$, df = 8, $p = 0.77$.

associated with pathologies that increase vascular resistance, such as an increase in SBP. Although we adjusted for age as a confounding factor, the findings did not suggest an association between SBP and the urinary 8-OhdG level.

Brain natriuretic peptide (BNP)[28] is a known biomarker for heart failure. In heart failure, the *BNP* gene is expressed in response to myocardial stretching stress. The BNP precursor (proBNP) encoded by the *BNP* gene is cleaved by enzymes such as furin into physiologically inactive NT-proBNP and physiologically active BNP and secreted.[29] NT-proBNP is widely employed in clinical examinations because it is secreted from the myocardium in amounts that are equimolar to BNP, has a longer half-life than BNP,[29] and is physiologically inactive. In failing cardiomyocytes, ROS are produced from mitochondria by neurohumoral factors such as catecholamines and under mechanical stress.[30] However, our results suggested no association with BNP and the urinary 8-OhdG level. In addition, they suggested no association with ECG and the urinary 8-OhdG level.

Obesity and hypertension are known risk factors for arteriosclerosis and have been suggested to be related to the urinary 8-OhdG level.[9,10,18,19,21] Consistent with previous study results, our results suggested an association between obesity and the urinary 8-OhdG level. Additionally, as a new finding, the results suggested an association between SBP and urinary the 8-OhdG level in healthy adults aged ≥50 years. Obesity and elevated SBP in healthy middle-aged and elderly individuals may indicate that arteriosclerosis is associated with the urinary 8-OhdG level.

A major limitation of this study was that the number of individuals was relatively small considering the number of independent variables. Next, the health promotion activity, from which the data for this study were obtained, was not designed to investigate the relationship between the urinary 8-OhdG level and arteriosclerosis. For example, it was not always possible to collect early-morning urine. Furthermore, the existence of a selection bias, such as a type of worker health effect, was also considered. However, our study might be useful for investigating arteriosclerosis-related factors that affect the urinary 8-OhdG level in middle-aged and elderly individuals, including healthy older individuals aged ≥75 years.

Author Contributions

HO: study concept and design; acquisition of data, analysis and interpretation of data; drafting of the manuscript, statistical analysis, obtained funding, administrative, technical, or material support. KT: study concept and design; acquisition of data, analysis and interpretation of data; critical revision of the manuscript for important intellectual content; statistical analysis, study supervision.

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Abbreviations

Af	atrial fibrillation
BMI	body mass index
BNP	brain natriuretic peptide
DBP	diastolic blood pressure
ECG	electrocardiograph
HbA1c	hemoglobin A1c
HDL	high-density lipoprotein
HPLC	high-performance liquid chromatography
LBBB	left bundle branch block
LVH	left ventricle hypertrophy
NT-proBNP	N-terminal pro-brain natriuretic peptide
8-OHdG	urinary 8-hydroxydeoxyguanosine
OMI	old myocardial infarction
OR	odds ratio
PVC	premature ventricular contraction
RBBB	right bundle branch block
ROS	reactive oxygen species
TG	triglyceride

Conflict of Interest

No potential conflicts of interest were disclosed.

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