Free Radical Research, February 2009; 43(2): 183–192

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Plasma 3-nitrotyrosine, urinary 8-isoprostane and 8-OHdG among healthy Japanese people

NORIKO SAKANO[1], NORIKO TAKAHASHI[1], DA-HONG WANG[1], RANI SAURIASARI[1], KEI TAKEMOTO[1], SAKIKO KANBARA[2], YOSHIE SATO[3], TOMOKO TAKIGAWA[1], JIRO TAKAKI[1], & KEIKI OGINO[1]

[1]*Department of Public Health, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan,* [2]*University of KinDAI Himeji, School of Nursing, Japan, and* [3]*Okayama University Graduate School of Health Sciences, Okayama, Japan*

(Received 19 October 2008)

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Abstract

To examine the relation between lifestyle factors and oxidative stress biomarkers, this study analysed plasma 3-nitrotyrosine (3-NT), urinary 8-isoprostane and 8-hydroxy-2′- deoxyguanosine (8-OHdG) of 323 healthy Japanese without any disease. Plasma 3-NT was significantly increased by excessive exercise ($p = 0.010$), but it was not significantly different in terms of sex, age (<40, $\geqq 40$), BMI (<18.5, 18.5–24.9, $\geqq 25.0$), smoking (non-smokers, smokers) and alcohol drinking per week (non-drinkers, <10 units, $\geqq 10$ units). Urinary 8-isoprostane was significantly associated with alcohol drinking ($p < 0.01$) and sex ($p < 0.01$), although it had no significant relevance to age and exercise. Moreover, urinary 8-OHdG was positively associated with age ($p < 0.05$) and negatively associated with BMI ($p < 0.05$) and fasting insulin ($p < 0.001$). However, it was not related with sex, smoking, alcohol drinking and exercise. In conclusion, the present results suggest that 3-NT, 8-isoprostane and 8-OHdG seem to be useful biomarkers for early prediction of lifestyle-related disease risk at the population level.

Keywords: *3-Nitrotyrosine, 8-isoprostane, 8-OHdG, biomarkers, lipid peroxidation, reactive oxygen species*

Introduction

As a result of oxygen metabolic processes, cells continuously produce free radicals and reactive oxygen species (ROS) such as superoxide anion (O_2^-) and hydroxyl radicals ($OH^\cdot$) [1]. These free radicals are generally neutralized by the antioxidant defense system comprising enzymes including catalase, superoxide dismutase, glutathione peroxidase and low-molecular-weight antioxidants such as β-carotene and tocopherol [1]. Oxidative stress is defined as a situation in which an increased level of ROS generation overwhelms the antioxidative defense capacity, resulting in oxidative damage to lipids, DNA and proteins [1]. Oxidative stress is suspected to contribute to the initiation and progression of many diseases and even to the normal ageing process. Since ROS themselves are very reactive and have an extremely short half-life, direct determination of them in tissue or body fluids is generally impracticable [1], the measurement of biomarkers of oxidatively modified cellular constituents in biological samples as 'intermediate endpoints or early-outcome predictors' of disease development provides a promising strategy in the public health field [2].

3-Nitrotyrosine (3-NT) has been known as a biomarker of nitrosative stress such as peroxynitrite ($ONOO^-$) [3], generated by the reaction of nitric oxide (NO) and O_2^-. NO is produced by three NO-synthase (NOS) isoforms. O_2^- can be generated by several mechanisms, such as via NAD(P)H

Correspondence: Keiki Ogino, MD, PhD, 2-5-1, Shikata-cho, Okayama 700-8558, Japan. Tel: +81-86-235-7184. Fax: +81-86-226-0715. Email: kogino@md.okayama-u.ac.jp

ISSN 1071-5762 print/ISSN 1029-2470 online © 2009 Informa UK Ltd.
DOI: 10.1080/10715760802663124

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oxidase, xanthine oxidase (XO) and release from mitochondria [4]. 3-NT was generated by a peroxidase-dependent system of neutrophils and eosinophils [5].

F_2-isoprostanes, a group of bioactive prostaglandin F_2-like compounds generated by oxidatively catalysed reaction of arachidonic acid, are considered as the reliable marker of lipid peroxidation *in vivo* [6]. The 8-isoprostane (8-iso-prostaglandin $F_{2\alpha}$; the major F_2-isoprostane), the well known compound belonging to the F_2-isoprostane class, is usually quantified in urine instead of plasma for practical use because of the short half-life of plasma F_2-isoprostane [6]. Elevated levels of plasma and/or urinary 8-isoprostane have been reported in several diseases such as diabetes [7,8], alcohol liver disease [9] and cardiovascular disease [10].

8-Hydroxy-2'-deoxyguanosine (8-OHdG), a product of oxidatively modified DNA base guanine, is the most representative product that may reflect oxidative damage induced by ROS to DNA [1,11]. Urinary 8-OHdG was reported in good association with diabetes mellitus [12], chronic renal failure [13], cancer [14] and some occupational settings [15–17].

These oxidative stress biomarkers were presumed to change in the 'pre-clinical stages of disease' among healthy people because of the influence of unhealthy behaviour related to the lifestyles, such as smoking and alcohol drinking. However, few studies engaged in assessment of these oxidative stress biomarkers for a population who have no disease [18]. Moreover, no data are available regarding the levels of 3-NT by different lifestyle habits in people who are presumably healthy at the time of health check-up.

The present study aimed to examine whether there is any difference in the levels of biomarkers of oxidative stress (3-NT, 8-isoprostane, 8-OHdG) among healthy Japanese people who have different lifestyle habits, in order to find the usefulness of these biomarkers as early predictors of disease risk at population level.

Materials and methods

Subjects

Data were obtained from a worksite lifestyle intervention study in a Japanese city office in which 847 individuals (aged 18–67 years) participated. For the purpose of this study, we excluded subjects who have any history of cancer, stroke, diabetes or ischemic heart disease and who take any kind of medicines or supplements such as vitamin C. Furthermore, the selection criteria was fasting plasma glucose level < 110 mg/dl, triglycerides (TG) < 150 mg/dl, high density lipoprotein cholesterol (HDL-c) $\geqq 40$ mg/dl, systolic blood pressure (systolic BP) < 130 mmHg or diastolic blood pressure (diastolic BP) < 85 mmHg. A total of 323 volunteers aged 20–65 years were selected. All subjects were instructed to fast overnight and not consume any beverage and food except plain water before the measurement. The ethics committee of Okayama University approved the study and all subjects gave informed consent.

Health assessment

The health assessment period was from September–December 2007. Blood samples were taken from the subjects after overnight fasting for at least 10 h. Serum and plasma were preserved at 4°C for the measurement of total cholesterol (TC), HDL-c, TG, low density lipoprotein-cholesterol (LDL-c), uric acid (UA), plasma glucose, insulin, haemoglobin A_{1c} (HbA_1c), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (γ-GT), high-sensitivity C-reactive protein (CRP) and at -80°C for the measurement of 3-NT.

Blood pressure (BP) of each subject was measured by a medical doctor in a sitting position after resting for at least 10 min. Their body composition was evaluated by using the following respective parameters such as body weight, body mass index (BMI) and waist circumference. BMI was calculated by weight/[height]^2 (kg/[m]^2) and BMI over 25 was diagnosed as obesity according to the criteria for Japanese [19]. Their waist circumferences were measured at the umbilical level according to the recommendation of Metabolic Syndrome Diagnostic Criteria in Japan [20]. Information on lifestyles including cigarette smoking, alcohol consumption, exercise and dietary habit was obtained by self-reported questionnaires. The amount of alcohol consumption was defined as one unit was considered to be equivalent to 9–12 g of ethanol [21]. The alcohol intake habit was converted into the number of units per week and 10 units of alcohol consumption per week represented moderate drinking. Information on dietary habit was obtained by a food frequency questionnaire (FFQg) [22].

Analysis of oxidative stress biomarkers

Plasma protein-bound 3-NT, as a marker of nitrosative stress, was determined by a commercial enzyme-linked immunosorbent assay (ELISA) kit (Hycult Biotechnology b.v., Uden, The Netherlands) based on sandwich method. Detection limit was 0.2 nM in 10-times dilution of samples. The intra-assay and inter-assay coefficients of variation (CV) were 4.9% and 8.9%, respectively. Urinary 8-isoprostane and 8-OHdG were determined in spot urine samples stored at -80°C before analysis, because Helmersson and Basu [23] reported that urinary F_2-isoprostanes isomers levels in spot urines showed no significant variation from levels measured in 24-h urine samples in the same healthy individuals by

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radioimmunoassay. Møller and Loft [24] indicated that the correlation coefficient of 8-OHdG measurements by ELISA between spot and 24-h urine sample is 0.87. Urinary 8-isoprostane was analysed by commercially available competitive enzyme immunoassay (EIA) kit (Cayman Chemical Company, Ann Arbor, MI) and the intra-assay and inter-assay CV were 5.4% and 11.0%, respectively. Measurement of 8-OHdG was carried out with an ELISA kit from the Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan [25] and the intra-assay and inter-assay CV were 5.2% and 8.1%, respectively. Values for 8-isoprostane and 8-OHdG were normalized by per milligram of creatinine (Cre) measured in urine (Creatinine test kit, R&D Systems, Minneapolis, MN).

Statistical analysis

Data were presented as mean ± standard error (SE) unless stated otherwise. We used non-parametric analysis because of the skewed distribution of the data. Mann-Whitney U-test and Kruskal-Wallis test were used to examine the differences in levels of oxidative stress biomarkers among the variables such as sex, age, smoking, BMI, alcohol drinking and exercise. Spearman's correlation analysis was performed to examine the relation between oxidative stress biomarkers and the variables. A stepwise multiple regression analysis was performed to test the relationship between oxidative stress biomarkers with the variables that had significant correlation tested by the Spearman's correlation and the variables that presumably confound to oxidative stress biomarkers such as age, sex, BMI, exercise, smoking and alcohol drinking were also included in the analysis models. The difference was considered statistically significant when the p-value was < 0.05. All analyses were performed using Statistical Package of SPSS 12.0 for Windows.

Results

Subject characteristics

The characteristics of the subjects in this study are presented in Table I. Their average age was 38.3 years. Smokers accounted for 30% and the alcohol consumption rate was 63%. The proportion of the subjects who engage in exercise was 43%.

Oxidative stress biomarkers according to the life style

The concentration of oxidative stress biomarkers according to the lifestyles are described in Table II. Concentration of plasma 3-NT in subjects whose 3-NT level was over the limit of detection ($n = 68$) was 93.3 ± 31.2 nM. Regarding BMI, the concentration of 3-NT in obesity was the highest and that in underweight was the lowest, although there was no

Table I. Characteristics of subjects.

Variable		n (%)
Total		323 (100.0)
Sex	Male	103 (31.9)
	Female	220 (68.1)
Age (year)	<40	187 (57.9)
	≥40	136 (24.1)
BMI (kg/m^2)	<18.5	47 (14.6)
	18.5–24.9	252 (78.0)
	≥25.0	24 (7.4)
Smoking	Never	226 (70.0)
	Past smokers	20 (6.2)
	Current smokers	77 (23.8)
Alcohol drinking (units/week)	No	121 (37.5)
	<10	173 (53.6)
	≥10	29 (9.0)
Exercise (times/week)	No	185 (57.3)
	1–5	121 (37.5)
	6 or over	17 (5.3)

significant correlation between plasma 3-NT and BMI (Figure 1). The 3-NT concentration was higher in smokers and drinkers who drank alcohol over 10 units per week. The 3-NT concentration was the lowest in drinkers who drank under 10 units per week, an appropriate amount for health. As for exercise, the concentration of 3-NT in persons who did exercise nearly every day was the highest ($p < 0.05$) (Figure 2).

The mean concentration of urinary 8-isoprostane ($n = 320$) was 0.74 ± 0.03 ng/mg Cre. Three samples under the limit of detection were excluded from the data analysis. The concentration of urinary 8-isoprostane was significantly higher in males as well as in smokers (Figure 3) and it was positively correlated with the value of BMI (Figure 1). Higher concentration of urinary 8-isoprostane was observed among the subjects who consumed a great amount of alcohol (Table II).

The mean level of urinary 8-OHdG was 9.09 ± 0.23 ng/mg Cre ($n = 323$). The concentration of urinary 8-OHdG in persons who were over 40 years old was significantly higher than that in persons who were under 40. There was significantly negative correlation between urinary 8-OHdG and BMI (Figure 1). The concentration of 8-OHdG in smokers and drinkers who drink alcohol over 10 units per week was higher, whereas 8-OHdG was the lowest among the subjects who do exercise less than five times a week.

Relationship between oxidative stress biomarkers and health examination variables

The results of Spearman's correlation analysis between oxidative stress biomarkers and health assessment data are presented in Table III. Urinary 8-isoprostane was significantly positive correlated with

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Table II. Characteristics of subjects by oxidative stress markers.

	3-Nitrotyrosine (nM)			8-Isoprostane (ng/mg Cre)			8-OHdG (ng/mg Cre)		
Variable	*n*	mean ± S.E.		*n*	mean ± S.E.		*n*	mean ± S.E.	
Total	68	93.3 ± 31.2		320	0.74 ± 0.03		323	9.09 ± 0.23	
Sex									
Male	27	106.3 ± 53.4		103	0.94 ± 0.06	**	103	8.60 ± 0.32	
Female	41	84.8 ± 38.4		217	0.65 ± 0.03		220	9.32 ± 0.30	
Age (year)									
<40	48	53.9 ± 24.0		185	0.76 ± 0.04		187	8.46 ± 0.26	**
≥40	20	188.0 ± 87.0		135	0.72 ± 0.04		136	9.96 ± 0.40	
BMI (kg/m^2)									
<18.5	10	61.7 ± 47.9		47	0.68 ± 0.06		47	9.35 ± 0.63	
18.5–24.9	51	91.7 ± 36.0		250	0.75 ± 0.03	**	252	9.21 ± 0.26	*
≥25.0	7	150.2 ± 144.5		23	0.79 ± 0.13		24	7.28 ± 0.69	
Smoking									
Never	50	91.7 ± 36.8		225	0.68 ± 0.03		226	9.04 ± 0.29	
Smokers	18	98.0 ± 60.5		95	0.90 ± 0.04		97	9.21 ± 0.36	
Alcohol drinking (units/week)									
No	26	120.9 ± 59.8		120	0.66 ± 0.05		121	9.21 ± 0.39	
<10	37	55.0 ± 28.2		171	0.74 ± 0.03	**	173	8.94 ± 0.32	
≥10	5	233.5 ± 209.1		29	1.09 ± 0.08		29	9.51 ± 0.58	
Exercise (times/week)									
No	35	18.9 ± 4.5		183	0.71 ± 0.04		185	9.40 ± 0.33	
1–5	27	108.3 ± 54.8	*	120	0.79 ± 0.04		121	8.52 ± 0.31	
6 or over	6	460.1 ± 207.4		17	0.81 ± 0.16		17	9.81 ± 0.92	

*p <0.05, **p <0.01 (Sex, Age, Smoking by Mann Whitney U-test. BMI, Alcohol drinking, Exercise by Kruskal-Wallis test).

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serum AST, ALT, γ-GT, UA, fasting plasma glucose and negatively correlated with the percentage of dietary energy from fat and protein. There was significant negative correlation between urinary 8-OHdG and fasting insulin. Nevertheless, no significant correlation between plasma 3-NT and health assessment variables was observed.

Multiple regression analysis for 3-NT demonstrated that exercise was an important influential factor of plasma 3-NT (Table IV) independent of sex, age, BMI, number of cigarette smoking and alcohol drinking. That is to say, the more they exercised, the higher the concentrations of plasma 3-NT were. This model explained 7.6% of the variation of plasma 3-NT.

In the model for urinary 8-isoprostane, sex and alcohol drinking were important influential factors of urinary 8-isoprostane after adjustment for age, BMI, number of cigarette smoking, frequency of exercise, AST, γ-GT and the percentage of dietary energy from fat. Male participants showed significantly higher concentration of urinary 8-isoprostane than females, which is in agreement with the result by Spearman's correlation (data not shown). This model explained 10.2% of the variation of urinary 8-isoprostane.

In the model for urinary 8-OHdG, fasting insulin, age and BMI were important influential factors of urinary 8-OHdG after adjustment for sex, number of cigarette smoking, alcohol drinking and frequency of exercise, which explained 9.0% of the variation of urinary 8-OHdG. The urinary 8-OHdG was negatively correlated with fasting insulin and BMI and positively correlated with age. There was no association between dietary intake and urinary 8-OHdG.

Discussion

The present study demonstrated the association of oxidative stress biomarkers such as 3-NT, 8-isoprostane and 8-OHdG with lifestyle in healthy Japanese people. 3-NT is a well-known biomarker of protein tyrosine modification by $ONOO^-$ and peroxidase-dependent nitrite oxidation and then 8-isoprostane and 8-OHdG are markers of oxidative damage of DNA and membrane lipid by ROS. There were few reports that evaluated these three oxidative stress-related biomarkers for a healthy population in association with lifestyle and several clinical laboratory examinations.

During exercise, elevated plasma 3-NT was detected and its values were associated with the frequency of exercise. Moreover, multiple regression analysis also showed the relation of 3-NT with exercise. However, exercise was not associated with other oxidative stress biomarkers such as 8-isoprostane and 8-OHdG. Generation of 3-NT is influenced by the generation of $ONOO^-$, which is formed by NO and O_2^- and by peroxidase-dependent oxidation of nitrite (NO_2^-) [3]. During exercise, neuronal NOS (nNOS, NOS1) and endothelial NOS (eNOS, NOS3) in skeletal muscle and NOS3 in vascular endothelial cells contributed in the formation of $ONOO^-$ and upregulation of NO [3,26]. Especially,

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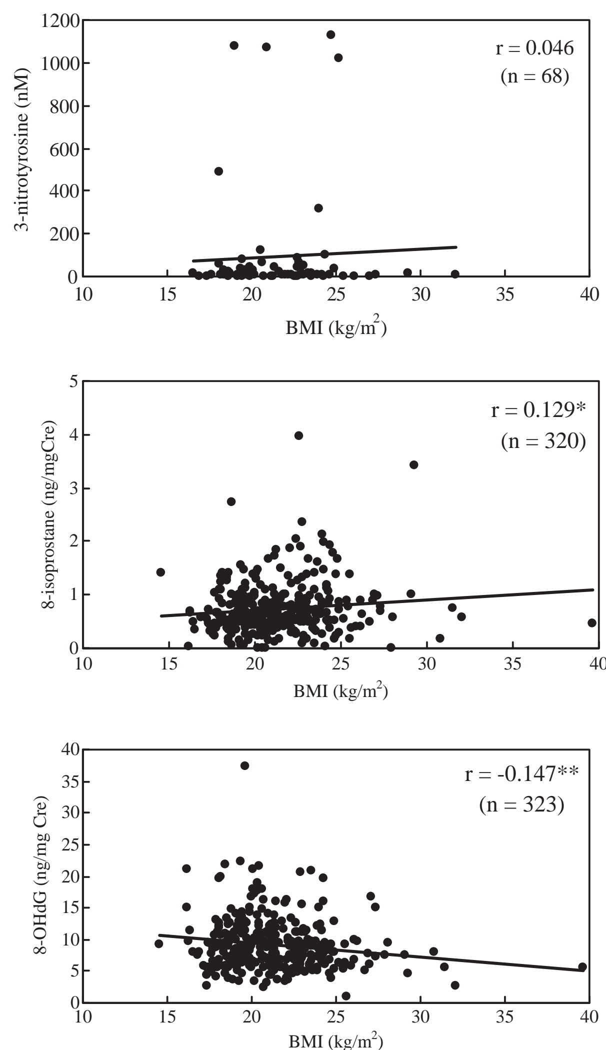

Figure 1. Relationship between oxidative stress biomarkers and BMI. No correlation between the plasma concentration of 3-NT and BMI was found in 68 healthy Japanese people. Urinary 8-isoprostane was significantly positive correlated with BMI in 320 healthy Japanese people. Urinary 8-OHdG was significantly negative correlated with BMI in 323 healthy Japanese people (* $p < 0.05$, ** $p < 0.01$).

exercise activates NOS3 via phosphorylation of serine residues [26]. Moreover, an increase in O_2^- production in exercise is said to contribute to XO, complexes of NAD(P)H oxidase and O_2^- release from mitochondria and O_2^- generation from uncoupled NOS are due to insufficient supply of tetrahydrobiopterin (BH_4) [27]. An alternative generation pathway for 3-NT has been postulated, which is due to NO_2 formed by peroxidase-dependent oxidation of NO_2^- in the presence of hydrogen peroxide (H_2O_2) in myeloperoxidase of neutrophils and eosinophil peroxidase in eosinophils [5]. Therefore, 3-NT by peroxidase-dependent pathway can not be ruled out as a candidate for the origin of elevated 3-NT in plasma proteins during exercise, because activated neutrophils in the blood stream during exercise can produce O_2^- and H_2O_2 [28]. In addition, it is known

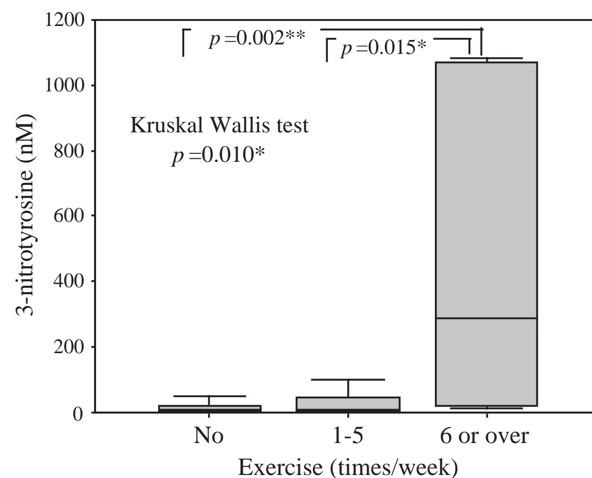

Figure 2. Plasma 3-nitrotyrosine (3-NT) concentrations by exercise frequency. Values are expressed as median (25th percentile, 75th percentile). None indicates group that did not engage in any exercise in the leisure-time per week ($n = 35$); 1–5, doing exercise from 1–5 days per week ($n = 27$); 6 or over, doing exercise for 6 days or over per week ($n = 6$). Difference in 3-NT among three groups was analysed by Kruskal Wallis test, then Mann-Whitney U-test was used for analysis of difference between groups.

that 8-isoprostane from free radical catalysed peroxidation of arachidonic acid on plasma membrane phospholipids and 8-OHdG from oxidative DNA damage increase with exercise [29,30]. Our results that did not show the association of 8-isoprostane or 8-OHdG with exercise did not coincide with previous reports. Generally, oxidative stress is associated with acute intense exercise [31]. During long-term moderate exercise, these oxidative stress biomarkers did not increase because of up-regulation of antioxidant enzymes such as superoxide dismutase, catalase and

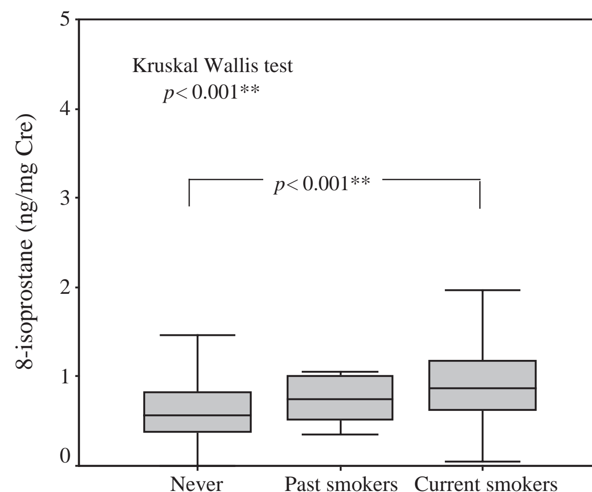

Figure 3. Urinary 8-isoprostane concentrations by smoking. Values are expressed as median (25th percentile, 75th percentile). Never indicates group that never have smoked ($n = 225$); Past smokers, smoked in the past but currently not smoking ($n = 20$); Current smokers, smoking cigarettes currently ($n = 75$). Difference in 8-isoprostane among three groups was analysed by Kruskal Wallis test, then Mann-Whitney U-test was used for analysis of difference between groups.

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Table III. Spearman's correlation of oxidative stress markers with each parameter.

Variable	3-Nitrotyrosine ($n=68$) r	3-Nitrotyrosine ($n=68$) p	8-Isoprostane ($n=320$) r	8-Isoprostane ($n=320$) p	8-OHdG ($n=323$) r	8-OHdG ($n=323$) p
Systolic BP (mmHg)	0.067	0.585	0.067	0.234	−0.048	0.391
Diastolic BP (mmHg)	−0.058	0.640	−0.057	0.311	−0.021	0.709
AST (IU/L)	−0.035	0.775	0.132	0.018*	0.028	0.611
ALT (IU/L)	−0.120	0.328	0.140	0.012*	−0.008	0.882
γ-GT (IU/L)	0.021	0.866	0.290	<0.001**	0.042	0.449
UA (mg/dL)	0.026	0.834	0.164	0.003**	0.025	0.657
TC (mg/dL)	−0.106	0.390	0.068	0.223	0.061	0.277
TG (mg/dL)	−0.133	0.279	0.020	0.722	0.034	0.543
HDL-C (mg/dL)	0.202	0.099	−0.002	0.969	0.063	0.258
LDL-C (mg/dL)	−0.213	0.081	0.057	0.306	0.028	0.621
Fasting insulin (μU/mL)	−0.057	0.643	−0.010	0.856	−0.255	<0.001**
Fasting plasma glucose (mg/dL)	−0.145	0.238	0.118	0.035*	0.011	0.846
HbA1c (%)	−0.180	0.141	−0.088	0.115	−0.064	0.252
High-sensitivity CRP (mg/dL)	−0.146	0.234	−0.013	0.818	0.103	0.064
Dietary intake						
Energy (kcal)	−0.053	0.666	−0.016	0.772	0.003	0.964
Carbohydrate (%)	0.058	0.641	−0.002	0.975	0.009	0.872
Fat (%)	−0.063	0.609	−0.137	0.014*	−0.035	0.533
Protein (%)	0.080	0.516	−0.121	0.031*	−0.060	0.284
Retinol (μg)	0.053	0.669	−0.083	0.140	0.037	0.506
β-Carotene (μg)	0.064	0.605	−0.059	0.294	−0.007	0.894
α-Tocopherol (mg)	−0.001	0.992	−0.020	0.724	−0.051	0.363

*$p<0.05$, **$p<0.01$.

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glutathione peroxidase [31]. Therefore, urinary 8-isoprostane or 8-OHdG were not associated with exercise in this population because exercise in this study may be long-term.

The present results showed an elevation of urinary 8-isoprostane in alcohol drinkers, positive Spearman's correlation between urinary 8-isoprostane with serum AST, ALT and γ-GT and the association of alcohol drink with 8-isoprostane by multiple regression analysis. Lee et al. [32] have suggested that serum γ-GT within its normal range is probably an oxidative stress-related enzyme because it has been suggested as a predictor of change in levels of F_2-isoprostane, fibrinogen and CRP in both epidemiological and clinical settings. In human studies, alcohol intake induces increased release of O_2^- and H_2O_2 through induction of the CYP4502E1 isozyme, increased mitochondrial NADH/NAD+ ratios and aldehyde oxidase [33]. In addition to an increase in ROS, ethanol evokes a decrease in glutathione (GSH) by less production of cysteine due to interfering methionine metabolism and direct reaction of acetaldehyde to GSH [33]. It is well known that reduction of GSH by several chemicals can cause lipid peroxidation. Both large doses of ethanol and smaller doses given repeatedly increase lipid peroxidation in plasma and livers of animals and humans, as measured by rises in conjugated dienes, ethane, phosphatidylcholin hydroperoxides, *trans*-4-hydroxy-2-hexenal (HNE) and F_2-isoprostane [34,35]. 8-Isoprostane could have arisen from ROS catalysed products of arachidonic acid [36]. Therefore, 8-isoprostane may be generated from the liver during the metabolism of ethanol and resulting lipid peroxidation by oxidative reaction to arachidonic acid. On the other hand, alcohol intake was not associated with urinary 8-OHdG in our previous study for Japanese healthy people. There are few reports that showed the association between alcohol intake and 8-OHdG, although Yoshida et al. [37] reported that moderate consumption of alcohol caused an increase in UA level and a decrease in 8-OHdG level and they suggested that UA, as an alcohol-induced antioxidant, may suppress DNA damage, resulting in lower 8-OHdG level. In addition, it is not clear why there was no association of 3-NT with alcohol intake in this study and no reports regarding the association of 3-NT with alcohol intake in the literature. Since ethanol can decrease the level of GSH, a specific inhibitor of lipid peroxidation, it seems that the decreased GSH could be blamed for the higher level of 8-isoprostane among alcohol drinkers.

Another noticeable finding of this study was that smokers had a higher level of urinary 8-isoprostane. In particular, male participants smoked a greater number of cigarettes per day and had a longer smoking period than the females. In addition, we also found the excretion of urinary 8-isoprostane among 75 current smokers was positively related with the number of cigarettes smoked per day ($r=0.293$, $p<0.001$) and smoking period ($r=0.289$, $p<0.001$). In long-term smokers, oxidative stress and inflammation are

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Table IV. Multiple regression analysis for oxidative stress markers.

Dependent variable	Explanatory variable	β	p value	Adjusted R^2
3-Nitrotyrosine				0.076
	Exercise	0.300	0.013	
8-Isoprostane				0.102
	Sex	−0.232	<0.001	
	Alcohol drinking	0.165	0.004	
8-OHdG				0.090
	Fasting insulin	−0.167	0.005	
	Age	0.166	0.004	
	BMI	−0.129	0.021	

β indicates standardized partial regression coefficient.
Adjusted variables, for 3-NT: sex, age, BMI, No. of cigarette per day, alcohol drinking; for 8-isoprostane: age, BMI, No. of cigarette per day, exercise, GOT, γ-GT, energy from fat; for 8-OHdG: sex, No. of cigarette per day, alcohol drinking, exercise.

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involved in the development of atherosclerosis and chronic obstructive pulmonary disease (COPD) [33]. ROS production in circulating phagocytes and neutrophils, oxidized and nitrated proteins, lipid peroxidation products such as malonaldehyde and F_2-isoprostane and reduced levels of antioxidants such as vitamin C, β-carotene and glutathione were detected in serum of smokers [38]. Therefore, a significant increase in 8-isoprostane in smokers was a rational result. The relationship between smoking and urinary 8-OHdG is complicated. Several reports showed an association of urinary 8-OHdG with smoking [11,18,39], where urinary 8-OHdG contributed to oxidative damage in lung tissue due to various chemicals in cigarette smoke. However, evidence showed by van Zeeland et al. [40], Yoshida et al. [37] and our previous study [18] did not reveal any distinct correlation between smoking and urinary 8-OHdG. Also, with regard to the correlation between passive smoking and urinary 8-OHdG, negative results have previously been reported [41]. Although the reason for this discrepancy is unknown, the grade of the cigarette filter or differences in the quality of cigarettes among countries may be a factor.

Finally, we found urinary 8-OHdG had a significantly negative correlation with BMI ($r = -0.147$, $p = 0.008$) and fasting insulin ($r = -0.255$, $p < 0.001$) and positive correlation with age ($r = 0.202$, $p < 0.001$). There was a significant spearman's correlation ($r = 0.187$, $p = 0.001$) between BMI and fasting insulin (data not shown). Inverse association of 8-OHdG with BMI was reported by other authors [42–44]. This study showed lowest BMI (< 18.5) revealed high urinary 8-OHdG. This result was explained that weight loss is related with increased oxidative DNA damage, a state presumably related to an increased risk of cancer [42]. There is no agreement for the association of 8-OHdG with age [18,45,46], although Hofer et al. [44] suggested that a slight non-significant increase in DNA damage for 8-OHdG was noticed using trend-lines, because of the subjects in the narrow age range (19–31 years).

The present study determined plasma 3-NT as 0.28 ng/mg protein (1.24 pmol/mg protein) by ELISA. Frost et al. [47] and Ryberg and Caidahl [48] demonstrated the concentration of 3-NT in plasma protein of healthy subjects was 2.7 ng/mg protein (11.9 pmol/mg protein, $n = 8$) by gas chromatography-mass spectrometry (GC-MS) and 0.60 pmol/mg protein ($n = 12$) by GC-tandem mass spectrometry (GC-MS/MS), respectively. Ryberg and Caidahl [48] have suggested that GC-MS/MS and liquid chromatography (LC)-MS/MS can constantly provide lower basal concentration of 3-NT than the methods lacking selectivity and Frost et al. [47] have proposed that GC-MS is a fully quantitative assay with high specificity and sensitivity. However, time-consuming and high cost may hinder the widespread use of these techniques in population-based studies. The mean level of 3-NT determined in our study was between the above-mentioned values by GC-MS and GC-MS/MS, which did not vastly depart from the values by the recommended methods.

As for the measurement of 8-isoprostane, several researchers pointed out that mass spectrometric methods have high sensitivity and specificity compared with immunoassays [49]. However, at present, no more suitable methods instead of immunoassays are available for the large scaled cross-sectional study. Since many clinical researchers have employed EIA Kit for the measurement of 8-isoprostane [50–52], we used the most popular commercially available competitive EIA kit of Cayman for the detection of urinary 8-isoprostane. The concentrations of urinary 8-isoprostane by mass spectrometric methods in human controls were reported in the range of 0.16–1.88 ng/mg creatinine [53–55]. Therefore, our results, in which the mean urinary 8-isoprostane was 0.58 ng/mg creatinine, did not stray from the values by mass spectrometric methods.

At present, there are two accepted methods for measuring urinary 8-OHdG: the high performance liquid chromatography with electrochemical detection (HPLC-ECD) method [56,57] and ELISA [25,58]. Urinary 8-OHdG data by these two methods showed

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a good correlation ($r = 0.833$; $p < 0.0001$) [59]. For unknown reasons, 10% of the urine samples showed a more than 4-times increase in 8-OHdG value by ELISA in comparison with HPLC-ECD method. There are two commercial kits for quantifying 8-OHdG using a monoclonal antibody N45.1 from the Japan Institute for the Control of Ageing (Fukuroi, Shizuoka, Japan) and another monoclonal antibody (clone 1F7) from Trevigen (Gaithersburg, MD). Chiou et al. [14] found a good correlation between the two kits, with a correlation coefficient of 0.9, although the reason for higher level of 8-OHdG determined by ELISA than by HPLC-ECD was explained by Cooke et al. [11] as that antibody-based urinary 8-OHdG assays possibly also detects oligonucleotides, whereas HPLC-ECD and GC-MS can not detect 8-OHdG derived from DNA repair pathway but only detect monomeric 8-OHdG.

Although the findings are significant, several limitations of the study should be noted. First, the sample size of the study was small. Second, causal relationships could not be determined because this study was a cross-sectional study. Third, some reporting bias may have been introduced because the information on lifestyle habits like smoking, drinking and dietary intakes was obtained via self-reported questionnaires. Fourth, since there is no validated method for measurement of isoprostane at population level, present results should be interpreted with caution. Further studies are needed to examine the analytical methods of 8-isoprostane in comparison with the 'gold standard' mass spectrometric methods and to confirm the urinary level of 8-isoprostane in healthy subjects with increased sample size.

In conclusion, the present results suggest that 3-NT, 8-isoprostane and 8-OHdG seem to be useful biomarkers for early prediction of lifestyle-related disease risk at the population level. However, regarding weight loss, to determine whether urinary 8-OHdG may become an early predictor of cancer risk for an underweight population, further investigations need to be performed to provide supportive evidence.

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Acknowledgements

This work was supported in part by funding from the Junpukai and in part from Health Science Center Foundation. We gratefully acknowledge the technical contribution from Dr Bingling Wang and Ms A. Ohashi.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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