

## ORIGINAL ARTICLE

## Diurnal variation of urinary markers of nucleic acid oxidation

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## Abstract

**Aims.** Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo) are biomarkers of oxidative stress with clinical potential in a variety of diseases. As part of their clinical validation, this study aimed to investigate whether the urinary excretion of 8-oxodG and 8-oxoGuo undergoes diurnal variation and to evaluate the validity of 6-hour sampling as well as creatinine corrected spot urine sampling. **Methods.** A total of 23 healthy study subjects collecting their 24-h urine in four fractions covering 6 hours each. Urinary 8-oxodG and 8-oxoGuo levels were quantified using a modified version of UPLC-MS/MS. **Results.** No significant difference in excretion levels between the 12-h diurnal and 12-h nocturnal state or between the four 6-h periods during the day was found for either biomarker. A strong linear relationship between the excretion levels in each of the 6-h periods and the 24-h excretion level was shown for both biomarkers. Creatinine correction of the 6-h levels reduced the biological variation of the excretion levels and weakened the linear relationship with the uncorrected 24-h excretion level for both biomarkers. The correlations were strengthened when the 24-h excretion level was expressed per kg body weight. **Conclusion.** The results showed that 8-oxodG and 8-oxoGuo did not undergo diurnal variation in the study population overall and hence that the time of sampling is not crucial. Furthermore, 6-h sampling can be used as a substitute for 24-h sampling, and creatinine corrected sampling may be rational due to the reduction in biological variation of the biomarkers and the reasonable correlation with body weight-adjusted 24-h levels.

**Key Words:** 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine, 8-oxoGuo, 8-oxo-7,8-dihydroguanosine, diurnal variation

## Introduction

8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo) are guanine nucleoside species that are produced upon oxidation of guanine nucleotides in the DNA and RNA, respectively. They are excreted unchanged into the urine from where they can be non-invasively measured as biomarkers of oxidative stress. Oxidative stress is known to be implicated in a variety of diseases in which the compounds 8-oxodG and 8-oxoGuo thus may be of use as biomarkers. In particular, the compounds hold promise as biomarkers for prediction and prognosis in diabetes mellitus [1,2]. As an important part of their clinical validation as biomarkers, it is of interest to assess the biological

and methodological variability of urinary 8-oxodG and 8-oxoGuo excretion. In particular, it is important to assess the diurnal variation of 8-oxodG and 8-oxoGuo excretion. As a part of assessing the methodological variability, it is of interest to evaluate the validity of sampling for less than 24 hours as well as performing creatinine corrected spot urine sampling. While 24-h urine sampling remains the gold standard, creatinine corrected spot urine sampling is the most frequently used method for analysis of urinary compounds in epidemiological research. Establishment of the diurnal variation of the biomarkers and the validity of different sampling designs are crucial in order to establish the proper sampling conditions for analysis of urinary 8-oxodG and 8-oxoGuo.

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The aim of this study was: (i) To investigate whether the urinary excretion of 8-oxodG and/or 8-oxoGuo undergoes diurnal variation using fractional 24-h urine sampling, and (ii) to evaluate the validity of 6-h sampling as well as creatinine corrected spot urine sampling in a group of healthy human volunteers.

## Material and methods

The study was designed as a cross-sectional study. The study subjects were followed for 24 hours where they collected their 24-h urine in four fractions covering 6 h each (T1 = 0–6 h diurnal period [06:00–12:00 h], T2 = 6–12 h diurnal period [12:00–18:00 h], T3 = 12–18 h nocturnal period [18:00–00:00 h], T4 = 18–24 h nocturnal period [00:00–06:00 h]).

Both male and female study subjects were recruited for the study. Study subjects had to be adult (18–70 years of age), healthy volunteers who did not use any medicine. They had to be able to read and write and fully understand the aim and scope of the study. Study subjects were excluded in presence of acute or chronic physiological or mental disease (not including presence of risk factors without recognized disease if well controlled, e.g. well controlled hypertension or well controlled hypercholesterolemia), if they had a regular use of medicine (not including dietary supplements, herbal medicine, contraceptives, and medical treatment for risk factors such as hypertension or hypercholesterolemia) and if they were pregnant (women). Study subjects with a history of cancer who had been recurrence-free for less than five years, were excluded.

All procedures regarding study subjects were in accordance with the guidelines set out by the Declaration of Helsinki and approved by the Local Ethics Committee.

The diurnal variation of 8-oxodG and 8-oxoGuo was assessed by (1) analyzing the difference in biomarker excretion levels between pooled 12-h urine samples, representing the diurnal and nocturnal state, respectively, and (2) analyzing the difference in biomarker excretion levels between the four 6-h urine fractions.

As an evaluation of the validity of 6-h sampling, the relationship between biomarker excretion levels in individual 6-h fractions and the total 24-h urine was assessed. Similarly, as an evaluation of the validity of creatinine corrected spot urine sampling, the relationship between biomarker excretion levels in individual creatinine corrected 6-h fractions and the total 24-h urine was assessed.

Four plastic containers for urine collection were handed out and study subjects were carefully instructed how to appropriately collect their urine over a period of 24 h in four fractions.

Study subjects were instructed to follow their usual daily routine during the 24-h collection period without any change in their fluid intake or their normal diurnal activity. Study subjects were instructed to empty their bladder before beginning the collection and before switching containers. In order to ensure compliance, study subjects were asked to agree to receive text messages throughout the 24-h collection period that reminded them to begin the collection, empty their bladder, and to change containers. Acceptable compliance was defined as the collection of a 24-h urine sample set in which all fractions were at least 75% complete.

### Urine samples

Upon retrieval, the volumes of the filled urine containers were recorded and 1 mL from each fraction was transferred to an Eppendorf tube. The fractions were then pooled to cover 12-h (T1 + T2 and T3 + T4) and 24-h (T1 + T2 + T3 + T4) periods, respectively. The urine samples were immediately frozen at  $-20^{\circ}\text{C}$  to ensure stability. The samples from each of the original and pooled fractions were used for analysis of 8-oxodG and 8-oxoGuo as well as creatinine.

### Biomarker quantification

All laboratory analyses were performed at the Laboratory of Clinical Pharmacology. Samples were analyzed after last-person-last-visit. The urinary content of the oxidized nucleosides 8-oxodG and 8-oxoGuo was quantified using a modified version of an ultra performance liquid chromatography and tandem mass spectrometry (UPLC-MS/MS) assay, described in detail elsewhere [3]. Briefly, the frozen urine samples were thawed, mixed and heated to  $37^{\circ}\text{C}$  for 5 min to re-dissolve possible precipitate and centrifuged at 10,000 *g* for 5 min. All further sample preparation was performed using a Biomek 3000 robot (Beckman Coulter, CA, USA). 110  $\mu\text{L}$  of each urine sample or calibration standard were mixed with 90  $\mu\text{L}$  100 mM lithium acetate buffer and 90  $\mu\text{L}$  of 50 nM internal standard. The chromatographic separation was performed on an Acquity I-class UPLC system (Waters Corp., Milford, USA) using an Acquity UPLC BEH Shield RP18 column (1.7  $\mu\text{m}$ ,  $2.1 \times 100$  mm; Waters Corp.) with a column temperature of  $4^{\circ}\text{C}$ . The mass spectrometry detection was performed on a Xevo-TSQ triple quadrupole mass spectrometer (Waters Corp., Milford, USA), using electrospray ionization in the positive mode for 8-oxodG and negative ionization mode for 8-oxoGuo. Calibration standards ranged from 1–60 nM. As internal standards, stable isotope-labeled 8-oxodG and 8-oxoGuo, [ $^{15}\text{N}_5$ ]8-oxodG and [ $^{15}\text{N}_5$ ]8-oxoGuo, were used. To confirm the presence of the analyte and the absence of false contributions from co-elution

of similar compounds in the urine samples, two specific fragments of each analyte were included in the analysis. Retention time for 8-oxoGuo was 11.4 min and mass transitions 298/208 (20 V), 298/165 (24 V) and 303/213 (20 V) for quantifier, qualifier and internal standard (dwell time 400 ms). Values for 8-oxodG were retention time 12.7 min; mass transition 284/168 (14 V), 284/140 (32 V), 289/173 (14 V) (dwell time 400 ms). Values for 8-oxodG were retention time 12.7 min; transition (positive ionization) transition times 284/168 (14 V), 284/140 (32 V), 289/173 (14 V) (dwell time 400 ms). All samples were analyzed in a single run with simultaneous measurement of 8-oxodG and 8-oxoGuo. The average within-day variation (RSD, %) estimated from the method validation was 2.3% for 8-oxoGuo, and 3.8% for 8-oxodG. The average recovery was 103.7% and 104.8%, respectively. The urinary creatinine concentration was quantified by Jaffe's reaction.

### Statistical analysis

The urinary excretion level of each biomarker was expressed as nmol excreted per time period in hours. The excretion rates for each time period were then calculated by dividing the excretion levels with the time period in hours. Furthermore, creatinine corrected biomarker excretion levels for the 6-h fractions were calculated based on the biomarker and creatinine concentrations, giving a unit of nmol/mmol creatinine.

The difference in urinary excretion levels of 8-oxodG and 8-oxoGuo between the two pooled 12-h periods from each study subject was analyzed using Student's *t*-test for paired samples. The difference in urinary excretion levels of 8-oxodG and 8-oxoGuo between the four 6-h periods was analyzed by way of repeated measures analysis of variance (rANOVA).

The correlation between biomarker excretion levels in each of the 6-h fractions and the pooled 24-h urine for both biomarkers was assessed by Pearson's correlations coefficient.

The between-individual coefficient of variation (CV) in urinary excretion of the biomarkers was calculated from the standard deviation (SD) and mean of individual averages [4]. The within-individual CV in urinary excretion levels of the biomarkers within one day was calculated as the square root of the mean of squared individual CVs, the root mean square approach. The assumption of normal distribution was validated graphically by normal quantile-quantile (QQ) plotting and formally by the Shapiro-Wilk test. For the rANOVA, the assumption of sphericity was tested formally by Mauchly's Test of Sphericity.

Statistical significance was in all analyses defined as  $p < 0.05$  and all statistical tests were two-tailed. The statistical tests were carried out with the R statistical package, version 2.13.1 and with Statistica® version 7.0.

## Results

### Study population

A total of 27 potential study subjects were recruited during the four weeks set aside for recruitment. Of the 27 subjects, 26 (96%) met the eligibility criteria. The reason for non-eligibility was newly discovered disease. Of the 26 enrolled participants, 23 (88%) completed the study satisfactorily. Data from these 23 subjects was used for the data analysis. The subjects who did not complete the study satisfactorily were all withdrawn due to non-compliance. The clinical and demographic characteristics of the study subjects who successfully completed the study can be seen in Table I. The subjects were non-smoking, 25–67 years of age, and 11 out of 23 were men. All study subjects were Caucasian.

### Diurnal variation of 8-oxodG and 8-oxoGuo

The urinary excretion levels of 8-oxodG and 8-oxoGuo in the respective 6-, 12-, and 24-h periods are given in Table II. Also the mean creatinine corrected excretion levels in the four 6-h periods are given.

No statistically significant difference in urinary excretion levels of 8-oxodG between the two 12-h periods was found (mean difference 0.5 nmol/12 h, 95% CI:  $-0.6$ – $1.7$ ,  $p = 0.32$ ). Similarly for 8-oxoGuo, the difference in urinary excretion levels between the two 12-h periods was found not to be statistically significant (mean difference 0.6 nmol/12 h, 95% CI:  $-1.1$ – $2.2$ ,  $p = 0.48$ ).

Table I. Clinical and demographic characteristics of the study population.

<i>Demographic characteristics</i>	
Age (years)	27 [26–31]
Male sex	11/23 (47.8%)
<i>Clinical characteristics</i>	
Body weight (kg)	65 [59.7–85.5]
BMI (kg/m <sup>2</sup> )	22.6 [21.0–24.9]
Smoking:	
Current	0/23 (0%)
Former	7/23 (30.4%)
Never	16/23 (69.6%)
Physical activity level:	
5 + times/week	3/23 (13.0%)
3–4 times/week	9/23 (39.1%)
1–2 times/week	5/23 (21.7%)
< 1 time/week	6/23 (26.1%)
Diuresis (L/24 h)	2.2 [1.5–2.8]
Urine density (g/cm <sup>3</sup> )	1.013 [1.006–1.02]
<i>Medical history</i>	
Previous diagnosis of cancer	1/23 (4.3%)
Hypertension	2/23 (8.7%)
Hypercholesterolemia	1/23 (4.3%)

Clinical and demographic characteristics of the study population ( $n = 23$ ). Only data from subjects that were not withdrawn during the study was used ( $n = 23$ ). Values are presented as the median [interquartile range] or in absolute numbers (percent of total).

Table II. Urinary excretion levels of 8-oxodG and 8-oxoGuo in the different time periods of the day.

	T1	T2	T3	T4	T1 + T2	T3 + T4	24-h urine
8-oxodG	7.0 (3.0)	7.2 (3.6)	7.4 (3.4)	6.4 (3.0)	14.3 (6.7)	13.7 (6.1)	27.7 (12.3)
8-oxodG/creatinine	1.8 (0.6)	1.8 (0.6)	1.8 (0.6)	1.7 (0.6)	–	–	–
8-oxoGuo	8.4 (2.9)	8.9 (3.6)	9.0 (3.0)	7.8 (2.4)	17.2 (5.9)	16.6 (5.1)	33.1 (10.0)
8-oxoGuo/creatinine	2.2 (0.6)	2.3 (0.6)	2.1 (0.6)	2.1 (0.6)	–	–	–

Mean urinary excretion levels (standard deviation) of 8-oxodG and 8-oxoGuo, respectively, in the different time periods of the day as measured for 23 healthy humans. Values are in nmol per time period in hours for the uncorrected levels and in nmol/mmol creatinine for the creatinine-corrected levels. T1 = 0–6 hour diurnal period, T2 = 6–12 hour diurnal period, T3 = 12–18 hour nocturnal period, T4 = 18–24 hour nocturnal period, T1 + T2 = diurnal 12-hour period, T3 + T4 = nocturnal 12-hour period.

There was no statistically significant difference in urinary excretion levels of 8-oxodG (rANOVA,  $F(3, 66) = 2.434$ ,  $p = 0.07$ ) and 8-oxoGuo (rANOVA,  $F(3, 66) = 2.398$ ,  $p = 0.08$ ) between the four 6-h periods. Mean plots of the diurnal variation in the two markers are shown in Figure 1.

The between-subject and within-subject CV was calculated on the basis of the urinary excretion levels of 8-oxodG and 8-oxoGuo in the four 6-h periods for each individual. The between-subject CV was estimated to 43.3% for 8-oxodG and 31.0% for 8-oxoGuo. The within-subject CV was estimated to 19.6% for 8-oxodG and 19.1% for 8-oxoGuo.

#### Diurnal variation of creatinine

rANOVA did not reveal any significant difference in creatinine excretion levels between the four 6-h periods;  $F(3, 66) = 1.884$ ,  $p = 0.14$  (Figure 1). The between-individual CV and within-individual CV for creatinine excretion levels were calculated on the basis of the creatinine excretion levels in the four 6-h periods for each individual. The between-individual CV was estimated to 32.8% and the within-individual CV within a day was estimated to 21.4%.

#### Validity of sampling design

There was a strong linear relationship between the urinary excretion levels of 8-oxodG in each of the 6-h periods and the 24-h urinary excretion level of 8-oxodG (Table III). The relationships were in all cases statistically significant. Furthermore, the correlation coefficients were not significantly different from each other (data not shown).

For 8-oxoGuo, the linear association between the 24-h excretion level and the urinary excretion levels in each of the four 6-h periods was equally strong and in all cases statistically significant (Table III). The correlation coefficients were not significantly different from each other (data not shown).

No significant differences in excretion rates between each of the 6-h fractions and the total

24-h urine were found for either biomarker (Table IV).

Moderate linear relationships between creatinine corrected 8-oxodG excretion levels in each of the 6-h periods and the uncorrected 24-h urinary excretion level of 8-oxodG were found (Table V). The correlations were in all cases statistically significant, and they were not significantly different from each other (data not shown).

For 8-oxoGuo, the linear associations between the creatinine corrected urinary excretion levels in each of the 6-h periods and the 24-h urinary excretion level were of weak to moderate strength. The relationship was statistically significant only in the case of the first diurnal 6-h period (T1) ( $p = 0.04$ ). However, the correlation coefficients were not significantly different from each other (data not shown).

When 24-h excretion was given per kg body weight, the analyses revealed a closer association to the creatinine corrected 6-h levels (Table V). Strong linear relationships between creatinine corrected 8-oxodG excretion levels in each of the 6-h periods and the weight-adjusted 24-h urinary excretion level of 8-oxodG were shown. The correlations were in all cases statistically significant, and they were not significantly different from each other (data not shown).

For 8-oxoGuo, the linear associations between the creatinine corrected urinary excretion levels in each of the 6-h periods and the weight-adjusted 24-h urinary excretion level were of moderate strength. The correlations were in all cases statistically significant, and the correlation coefficients were not significantly different from each other (data not shown).

The between-individual CV and within-individual CV for the creatinine corrected urinary excretion levels was calculated on the basis of the creatinine corrected excretion levels in the four 6-h periods for each individual. The between-individual CVs was estimated to 32.3% and 25.2% for 8-oxodG and 8-oxoGuo, respectively, and the within-subject CVs within a day were estimated to 14.8% and 14.7%, respectively.

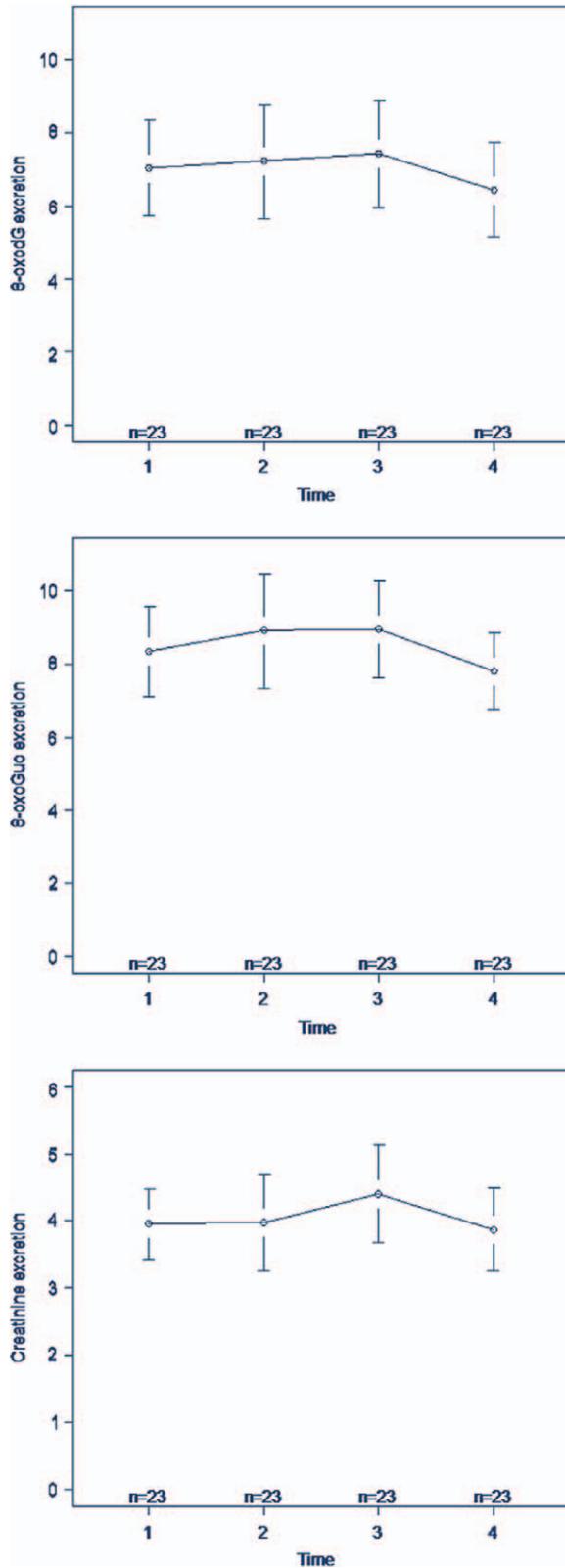


Figure 1. Mean plot of the diurnal variation in (A) 8-oxodG, (B) 8-oxoGuo, and (C) creatinine excretion in four 6-hour periods as assessed in 23 healthy humans. Bars represent 95% confidence intervals and dots represent mean values. 8-oxodG and 8-oxoGuo excretion levels are in mmol/6 hours while creatinine excretion levels are in mmol/6 hours. T1 = diurnal 0–6 hour period, T2 = diurnal 6–12 hour period, T3 = nocturnal 12–18 hour period, T4 = nocturnal 18–24 hour period.

Table III. Correlation between the 24-hour urinary excretion level and the urinary excretion level in each of the 6-hour periods for 8-oxodG and 8-oxoGuo.

	T1	T2	T3	T4
8-oxodG	0.97	0.93	0.93	0.93
	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
8-oxoGuo	0.88	0.89	0.85	0.87
	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$

Pearson's correlation coefficients,  $r$ , for the correlation between the 24-hour urinary excretion level and the urinary excretion level in each of the 6-hour periods for 8-oxodG and 8-oxoGuo, respectively, as assessed in 23 healthy humans. T1 = 0–6 hour diurnal period, T2 = 6–12 hour diurnal period, T3 = 12–18 hour nocturnal period, T4 = 18–24 hour nocturnal period.

## Discussion

Our study showed that urinary excretion of 8-oxodG and 8-oxoGuo did not differ significantly between the diurnal 12-h period and the nocturnal 12-h period, or between four 6-h periods during the day. In addition, we found a strong correlation between 24-h excretion and biomarker excretion in each of the 6-h samples and lack of difference in excretion rates between 6-h samples and 24-h urine.

Conversely, urine levels in creatinine corrected 6-h urine fractions were found to be somewhat poorer representatives of the full 24-h excretion levels, but reasonable representatives when the 24-h excretion levels were given per kg body weight.

### Diurnal variation of 8-oxodG and 8-oxoGuo

Overall, analysis of the biomarker excretion from fractional 24-h urine showed that the urinary excretion of 8-oxodG and 8-oxoGuo do not undergo a statistically significant diurnal variation in the study subjects. This means that on average, the biomarker excretion does not significantly differ during the day. The observations are in accordance with previous

Table IV. Comparison of the urinary excretion rates of 8-oxodG and 8-oxoGuo in each of the 6-hour periods and in the total 24-hour urine.

	T1	T2	T3	T4	24-hour urine
8-oxodG	1.2 (0.5)	1.2 (0.6)	1.2 (0.6)	1.1 (0.5)	1.2 (0.5)
	$p = 0.47$	$p = 0.30$	$p = 0.07$	$p = 0.06$	–
8-oxoGuo	1.4 (0.5)	1.5 (0.6)	1.5 (0.5)	1.3 (0.4)	1.4 (0.4)
	$p = 0.79$	$p = 0.11$	$p = 0.05$	$p = 0.08$	–

Mean urinary excretion rates (standard deviation) of 8-oxodG and 8-oxoGuo, respectively, in the different time periods of the day and the total 24-hour urine, and p-values for the difference in excretion rates between individual 6-hour periods and 24-hour urine as measured for 23 healthy humans. Values are in nmol/hour for the excretion rates. T1 = 0–6 hour diurnal period, T2 = 6–12 hour diurnal period, T3 = 12–18 hour nocturnal period, T4 = 18–24 hour nocturnal period.

Table V. Correlation between the 24-hour urinary excretion level and the creatinine corrected urinary excretion level in each of the 6-hour periods for 8-oxodG and 8-oxoGuo.

	T1	T2	T3	T4
8-oxodG	0.68	0.57	0.62	0.57
	$p=0.0004$	$p=0.004$	$p=0.002$	$p=0.004$
8-oxodG/kg	0.84	0.76	0.82	0.78
	$p<0.0001$	$p<0.0001$	$p<0.0001$	$p<0.0001$
8-oxoGuo	0.43	0.25	0.13	0.15
	$p=0.04$	$p=0.24$	$p=0.56$	$p=0.48$
8-oxoGuo/kg	0.62	0.56	0.45	0.45
	$p=0.001$	$p=0.006$	$p=0.03$	$p=0.03$

Pearson's correlation coefficients for the correlation between the 24-hour urinary excretion level and the creatinine corrected urinary excretion level in each of the 6-hour periods for 8-oxodG and 8-oxoGuo, respectively, as assessed in 23 healthy humans. T1 = 0–6 hour diurnal period, T2 = 6–12 hour diurnal period, T3 = 12–18 hour nocturnal period, T4 = 18–24 hour nocturnal period.

studies using spot urine sampling for assessment of the diurnal variation of 8-oxodG and 8-oxoGuo [4,5]. Furthermore, a very recent study employing fractionated 24-h urine sampling similarly proposes that 8-oxodG does not undergo significant diurnal variation [6].

The estimated mean difference in 8-oxodG and 8-oxoGuo excretion between the diurnal and nocturnal state of approximately 4% was not statistically significant and is not considered biologically relevant. The 95% CIs of  $-1.1$ – $-2.2$  and  $-0.6$ – $-1.7$  nmol/12 h for 8-oxodG and 8-oxoGuo, respectively, suggest the probability of a diurnal variation of 12% at the maximum. This means that any true diurnal variation is most likely not biologically relevant. In accordance, the excretion rates for each 6-h period did not differ significantly from the 24-h excretion rate for both 8-oxodG and 8-oxoGuo.

Our study revealed a relatively high and random within-individual variation in biomarker excretion during the day with a CV of around 20% for both 8-oxodG and 8-oxoGuo. Also, the study revealed a relatively high between-individual variation for both 8-oxodG and 8-oxoGuo with a CV of  $>30\%$  for both biomarkers. Any discrepancies in the variation between individuals from other reports may be due to differences in study population and their exposure and/or due to experimental variation.

Although no significant difference was found, a tendency towards a higher-than-average excretion during the first nocturnal 6-h period (T3 = 12–18 hours) and a lower-than-average excretion during the second nocturnal 6-h period (T4 = 18–24 hours) was observed for both 8-oxodG and 8-oxoGuo. The trend in diurnal variation of 8-oxodG paralleled the observations by Kanabrocki et al. who also employed fractional 24-h sampling [7]. In the present study, however, the variation was not statistically significant. This may be due to the difference in study

design, including the difference in analytical method.

#### Diurnal variation of creatinine

Creatinine excretion did not undergo a statistically significant diurnal variation in the study subjects overall ( $p=0.14$ ). As seen in Figure 1, a tendency towards a higher-than-average excretion during the first nocturnal 6-h period (T3 = 12–18 hours) and a lower-than-average excretion in the second nocturnal 6-h period (T4 = 18–24 hours) was observed, yet without reaching statistical significance. This paralleled the trends seen for 8-oxodG and 8-oxoGuo excretion. Similarly, a relatively high within- and between-individual variation was observed in the same magnitude as the variation of 8-oxodG and 8-oxoGuo excretion levels (approximately 20% and 30%, respectively). Diurnal variation in creatinine excretion has previously been reported with a higher excretion in the later afternoon compared to overnight and early morning that in some studies reached statistical significance [8,9]. The difference in statistical significance may be due to difference in study designs, difference in study populations or in power of the study. The within- and between-individual CVs for creatinine excretion found in this study are of similar magnitude as reported elsewhere [8,10]. Similarly, the parallel between the daily profile of creatinine excretion and 8-oxodG and 8-oxoGuo excretion has been reported in previous studies [5,11], and also in the case of other urinary analytes [12].

#### Validity of sampling design

Our analyses revealed strong and positive linear relationships between biomarker excretion levels in each of the 6-h samples and total 24-h urine for both 8-oxodG and 8-oxoGuo. This correlated well with the analysis showing that the excretion rates in each of the 6-h periods did not differ significantly from the 24-h excretion rate for both 8-oxodG and 8-oxoGuo. These results imply that 6-h sampling would be a reliable substitute for 24-h sampling.

Concerning the validity of creatinine corrected spot urine sampling, our analyses revealed positive linear relationships between creatinine corrected 6-h excretion and total 24-h excretion of 8-oxodG that were moderate in strength for all four time periods. The strongest association was observed between the biomarker excretion in the first diurnal 6-h period (T1 = 0–6 hours) and the total 24-h excretion ( $r=0.68$ ). However, the difference between the correlation coefficients was not statistically significant. This is in accordance with previous reported correlation coefficient of  $r=0.6$  between first-morning spot urine samples and 24-h excretion of 8-oxodG [6].

For 8-oxoGuo, the linear associations between biomarker excretion in each of the 6-h periods and total 24-h excretion were in all cases positive and weak to moderate in strength. Only the biomarker excretion in the first diurnal 6-h period (T1 = 0–6 hours) showed a statistically significant linear relationship with the total 24-h excretion ( $p = 0.04$ ), however, to a moderate degree ( $r = 0.43$ ), and the difference between the correlation coefficients was not statistically significant.

Interestingly, when the biomarker excretion levels were normalized for creatinine the between-individual CVs decreased from 43.3% to 32.3% and from 31.0% to 25.2% for 8-oxodG and 8-oxoGuo, respectively, while the within-individual CVs decreased from 19.6% to 14.8% and from 19.1% to 14.7%, respectively. The between- and within-individual CVs for creatinine corrected 8-oxodG and 8-oxoGuo excretion are comparable to previous findings [4]. To the best of our knowledge, only the between-individual variation of 8-oxoGuo has been reported previously [11].

Regarding the validity of creatinine corrected spot urine sampling, the correlation between individual 6-h excretion levels and total 24-h excretion was found to be moderate for 8-oxodG and weak for 8-oxoGuo. The reason for the discrepancy between 8-oxodG and 8-oxoGuo results is not known.

The validity of creatinine correction in spot urine sampling was also tested by assessing the diurnal variation of creatinine excretion. Creatinine excretion was found not to undergo significant diurnal variation in the group overall. This suggests that employment of creatinine correction is not affected by the time of day.

It has been suggested that creatinine besides compensating for the differences in diuresis also compensates for between-individual differences in muscle mass and physical workload [5]. This can be explained by the well-studied phenomenon that creatinine, 8-oxodG and 8-oxoGuo excretion are all positively associated with lean body mass [13–15]. In this context, creatinine corrected biomarker levels may be better representatives of the actual body weight-adjusted exposure in biological monitoring and thus of great potential value in environmental health research [12]. This is in accordance with our results showing that when 24-h excretion was expressed per kg body weight the correlation between individual 6-h excretion levels and total 24-h excretion was improved.

### Limitations

As an important note, the creatinine corrected 6-h excretion levels were used in this study as substitutes for true creatinine corrected spot urine levels even though the two are not identical. Spot urine sample values represent only one point in time whereas 6-h sample values represent the average of 6 h. However,

it is generally accepted that the 6-h samples are valid representations of a spot sample taken during that period and hence that assumptions about the validity of creatinine corrected spot urine sampling can be made on this basis.

### Conclusion

In summary, since 8-oxodG and 8-oxoGuo were shown not to undergo diurnal variation in the study population overall, the time of sampling for urinary 8-oxodG and 8-oxoGuo analysis is not crucial. Furthermore, the strong correlation between 24-h excretion and biomarker excretion in each of the 6-h samples combined with the lack of difference in excretion rates between 6-h samples and 24-h urine imply that 6-h sampling can be used as a substitute for 24-h sampling.

Single creatinine corrected spot urine levels were not found to be good representatives of the full 24-h excretion level. However, since creatinine correction reduced the biological variation of 8-oxodG and 8-oxoGuo and biomarker levels in creatinine corrected urine fractions correlated reasonably well with body weight-adjusted 24-h levels, creatinine correction may be rational.

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