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Urinary excretion of 8-hydroxy-2′-deoxyguanosine measured by high-performance liquid chromatography with electrochemical detection

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Abstract

There is good evidence that oxidative DNA damage permanently occurs in living cells. The oxidative DNA damage product 8-hydroxy-2′-deoxyguanosine (8-OHdG) is one of the predominant forms of radical-induced lesions to DNA, and has therefore been widely used as a biomarker for oxidative stress, either in cellular DNA or as DNA repair product in urine. In this paper we describe the use of a high-performance liquid chromatographic procedure with electrochemical detection for the measurement of urinary 8-OHdG. Our study has addressed the questions (i) of baseline urinary levels of 8-OHdG in spot urine and 24-h urine, (ii) of inter- and intra-individual variation of this biomarker, and (iii) of confounding factors for the excretion of 8-OHdG. No significant difference between the mean group levels of 8-OHdG/creatinine in spot urine (2.03 ± 1.21 μmol/mol, $n=148$) and in 24-h urine (1.86 ± 1.09 μmol/mol, $n=67$) was observed. However, when only 24-h urine was used for analysis, 8-OHdG was found to be statistically significantly higher in smokers. By multiple linear regression analysis, urinary creatinine was identified as the only predictor of 8-OHdG/24 h ($r_p=0.33$, $P=0.007$). High intra-individual coefficients of variation of 8-OHdG/24 h were observed in two healthy subjects over a period of 10 consecutive days (37 and 57%, respectively), indicating that the intra-individual fluctuation of urinary 8-OHdG has so far been underestimated. Therefore, we suggest that single values of 8-OHdG should be considered with caution, in particular in small study groups and when spot urine is used.

Keywords: 8-Hydroxy-2′-deoxyguanosine

1. Introduction

Reactive oxygen species (ROS) are generated permanently in living cells, and their ability to modify cellular structures and functions has led to the implication of their contribution to a number of diseases including cancer [1,2]. DNA is an important target for ROS, resulting in a variety of DNA damage patterns [3]. Although more than 20 different oxidative modifications of DNA bases have been identified, most attention has focused on 8-hydroxyguanine or its deoxynucleoside derivative 8-hydroxydeoxyguanosine (8-OHdG) [4]. This is related (i) to the significant role of 8-OHdG in the induction of mutations [5], (ii) to its quantitative abundance, and (iii) to the availability of sensitive methods for its determination in different biologic matrices.

Four repair systems are involved in protection

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against persist 8-OHdG in DNA (reviewed in Ref. [6]). These are base excision repair, nucleotide excision repair (NER), mismatch repair and the sanitization of the nucleotide pool. Glycosilase activity provides the main mechanism of removal of oxidised guanine from DNA, resulting in urinary 8-oxoguanine, whilst the action of a specific endonuclease and NER may, in addition to the nucleotide pool, contribute to the amount of the oxidised nucleoside 8-OHdG in urine. On this basis, the excretion of 8-OHdG into urine is considered to reflect ''whole body'' oxidative damage [7], and numerous methods for the determination of urinary 8-OHdG [8–14] have been described. Most of these methods are based on high-performance liquid chromatography with electrochemical detection (HPLC–EC), requiring extensive sample clean-up procedures in order to eliminate analytical interference by the complex urine matrix. Despite the analytical difficulties associated with the clean-up of urine, the determination of urinary 8-OHdG offers some advantage as compared to the measurement of oxidative damage in DNA: (i) these assays are non-invasive; (ii) there is no artefactual formation of 8-OHdG during the sample preparation or the derivatization reaction; and (iii) 8-OHdG is not degraded in the circulation or through incubation in urine [15] and is stable for a long time [16].

Although there is a broad range of workplace exposures that might contribute to oxidative DNA damage, urinary 8-OHdG has so far been rarely used in occupational health studies. Experimental protocols vary dramatically among the studies on 8-OHdG, and there is still a need for identification and characterisation of limiting factors for the use of 8-OHdG as a marker of oxidative stress, especially with regard to its biological variability. The aim of the present study was to determine levels of 8-OHdG in spot urine and in 24-h urine of healthy subjects, to provide data of inter- and intraindividual variation, and to determine confounding factors of urinary 8-OHdG.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical reagent grade quality. Water, acetonitrile and methanol of chromatographic grade were obtained from Merck (Darmstadt, Germany). Bond Elut LCR solid-phase extraction columns (C_{18}/OH, 500 mg, 2.8 ml) were obtained from Varian (Harbor City, CA, USA), and 8-OHdG was from Wako (Neuss, Germany).

2.2. Urine samples

Spot urine samples were collected from 148 healthy subjects, and 24-h urine samples from 67 healthy participants, within a period of 8 weeks. The characteristics of the study groups are shown in Table 1. In addition, two probands (male, 35 and 31 years old) collected their 24-h urine on 10 consecutive days. The diet was not controlled in the study subjects. Urine samples were adjusted to pH 4–5 by the addition of 2 *M* HCl and 5-ml aliquots were kept frozen until clean-up procedure at −20°C. To show the reproducibility of the method and the stability of urinary 8-OHdG, nine samples were measured on 2 consecutive days and another nine samples within a time span of 6 months.

Table 1
Descriptive data of the study groups

	Spot urine group		24-h Urine group	
	Female (n=76)	Male (n=72)	Female (n=27)	Male (n=40)
Age (years)	35.3±9.6	38.3±11.9	35.3±10.6	35.1±10.7
Body weight (kg)	61.9±9.3	81.1±9.7	66.0±8.8	81.7±10.3
Body mass index (kg/m^2)	22.5±3.3	25.3±2.8	23.6±2.5	25.5±2.8
Non-smokers/smokers	35/41	35/37	20/7	24/16

2.3. Measurement of urinary 8-OHdG

The clean-up of urine samples included a two-step solid-phase extraction (SPE). The analysis of 8-OHdG was performed by HPLC–EC as described previously [11]. Briefly, urine fractions were thawed in a 45°C water bath. After centrifugation, 2 ml urine was spiked with 8-OHdG (Wako, Neuss, Germany), adsorbed twice on preconditioned Bond Elut C_{18}/OH cartridges (Varian, Harbor City, CA, USA), and eluted with 50 m*M* KH_2PO_4 (pH 7.5) containing 15 and 20% methanol, respectively. Subsequently, the sample was evaporated to remove methanol and brought to a final volume of 1.5 ml with HPLC solvent. For analysis a Hewlett-Packard (Waldbronn, Germany) Series 1050 pump system equipped with pulse damper and autosampler was used, connected to a Hewlett-Packard 1049A amperometric detector. The electrochemical cell was equipped with a glassy carbon working electrode, operated at +0.6 V versus a Ag/AgCl reference electrode. The system was operated at 50-nA full range deflection. Data acquisition was performed by Hewlett-Packard HPLC 2D ChemStation software. The separation of 8-OHdG was carried out on two EcoCart (125×3 mm I.D.) analytical columns attached in series, packed with 4-μm Supersphere 100 RP-18 (endcapped) (Merck, Darmstadt, Germany). A LiChroCART (4×4 mm I.D.) packed with 5-μm LiChrospher 100 RP-18 (endcapped) (Merck) was used as guard column. The mobile phase used for isocratic elution of 8-OHdG was composed of 50 m*M* KH_2PO_4 (pH 3.5), 2.5% acetonitrile (gradient grade, Merck) and 1% methanol (gradient grade, Merck). Each injection was done after a defined pretreatment procedure for the analytical column and the EC-electrode. The retention time of 8-OHdG was 14.0 min. The recovery was determined by the use of a spiked urine pool (pooled urine fractions from ten individuals). All results were corrected for the recovery rate. Standards were used after every fifth sample to monitor the accuracy.

2.4. Urinary creatinine measurement

Urinary creatinine was determined using a kit from Merck (Darmstadt, Germany) according to Jaffe's picric acid method [17].

2.5. Determination of urinary cotinine

Urinary cotinine was analysed by gas chromatography connected to a nitrogen specific detector (GC–NPD) as described by Feyrabend and Russel [18]. The limit of detection was 1 ng/ml.

2.6. Statistical analysis

Normality of the distributions was tested by Kolmogoroff-Smirnoff test and equality of variances was tested by Bartlett test. Data were analysed by ANOVA and two-sided Student's *t*-test. Significance was set at $P<0.05$. Bivariate correlation between variables and forward stepwise multiple regression analysis were examined. Analyses were conducted using STATISTICA 5.0 software package.

3. Results

Typical chromatograms of spiked-urine samples and of a standard solution (55 n*M*) are shown in Fig. 1. The SPE did not remove matrix components entirely and the difference in creatinine between the two urine samples (sample A: 0.50 g creatinine/l, sample B: 1.8 g creatinine/l) clearly demonstrates an influence of the matrix concentration on the separation of peaks. Therefore, specimens with concentrations of creatinine above 2.5 g/l were diluted (1+1) before sample clean-up to avoid an overload of the SPE cartridges or insufficient separation. A good reproducibility ($r=0.96$, $y=0.91x+0.53$) of measured 8-OHdG concentrations (2.3–30.9 n*M*) was found when consecutive series of determination within 2 days or 6 months were compared (Fig. 2).

The distribution fittings for 8-OHdG/creatinine in spot urine and 24-h urine are shown in Fig. 3. No significant difference between the mean levels of 8-OHdG/creatinine in the spot urine group (2.03±1.21 μmol/mol, $n=148$) or in the 24-h urine group (1.86±1.09 μmol/mol, $n=67$) was observed. The correction of 8-OHdG for creatinine in 24-h urine did not cause a change in the inter-individual coefficient of variation ($C.V._{crea.}=0.58$, $C.V._{nmol}=0.54$). The intra-individual C.V. of 8-OHdG/24 h in two probands over a period of 10 consecutive days (Fig. 4) was 0.37 and 0.57, respectively.

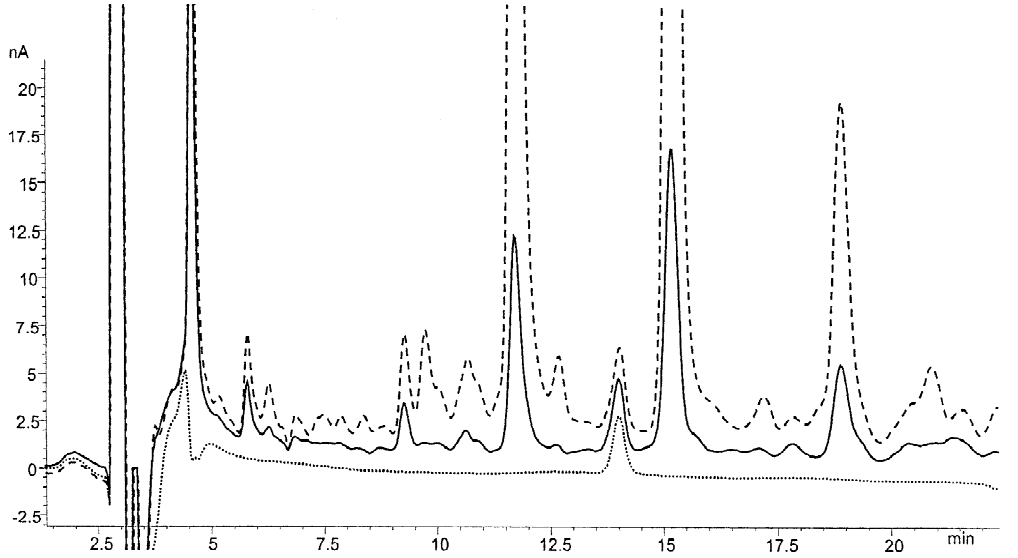

Fig. 1. Chromatograms of a 55 n*M* 8-OHdG standard solution (····), a 55 n*M* spiked urine sample with 0.5 g creatinine/l (—) and a 55 n*M* spiked urine sample with 1.8 g creatinine/l (---).

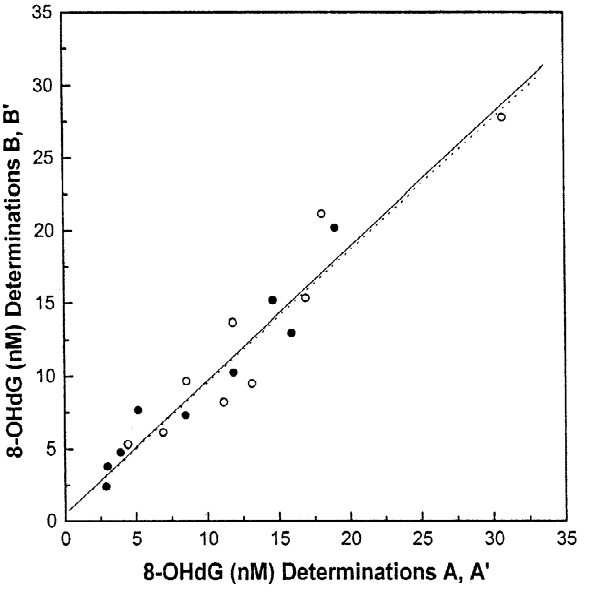

Fig. 2. Repeated measurements of 8-OHdG in 18 samples: ● A, B are two determinations performed on consecutive days (solid line for linear regression curve); ○ A′,B′ are two determinations performed within an interval of 6 months (dotted line for linear regression curve).

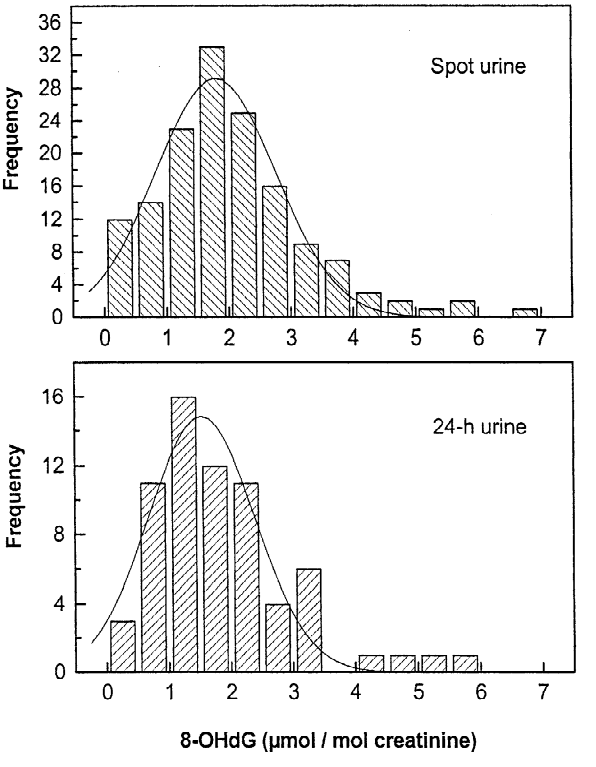

Fig. 3. Histogram of 8-OHdG/creatinine in spot urine and 24-h urine.

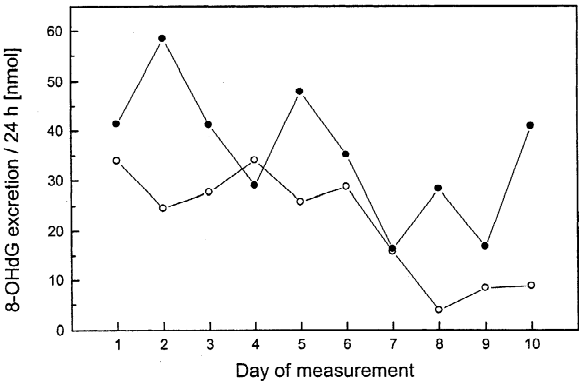

Fig. 4. Intra-individual variation of 8-OHdG/24 h, examined in two subjects over a period of 10 consecutive days.

Data concerning mean levels of 8-OHdG in non-smokers and smokers are shown in Table 2. No significant effect of smoking was evident when spot urine was used for analysis. In contrast, the mean level of excretion of 8-OHdG/24 h was significantly lower in non-smokers as compared to smokers. This difference was even more significant when the amount of 8-OHdG/24 h was related to the excretion of creatinine/24 h. No significant correlation between cotinine and 8-OHdG in smokers or the number of cigarettes per day could be demonstrated. The bivariate analysis showed significant correlations of 8-OHdG/24 h with urinary creatinine ($r=0.38$, $P=0.0017$), body weight ($r=0.32$, $P=0.008$) and body mass index ($r=0.26$, $P=0.030$). No influence of age on 8-OHdG was found. Although not statistically significant, women excreted lower amounts of 8-OHdG/24 h (18.2 ± 11.0 nmol) than men (22.9 ± 11.4 nmol). After stepwise multiple regression analysis the correlations between 8-OHdG and the independent variables body weight or body mass index did not prove to be significant, whereas urinary creatinine remained as the only predictor of 8-OHdG/24 h ($\beta=0.33$, $r_p=0.33$, $P=0.007$).

4. Discussion

The rationale for the measurement of urinary 8-OHdG is based on the assumption that a consequence of repair is the appearance of oxidatively modified nucleosides in urine. From a theoretical point of view, the level of urinary 8-OHdG should reflect the whole body formation of oxidised nucleobases when the capacity of repair is unaffected by functional disturbances. To date, it is not clear to what degree the nucleotide pool contributes to the rate of urinary 8-OHdG, and no significant correlation of urinary 8-OHdG with the levels of 8-OHdG in DNA has been demonstrated. Thus, quantitative aspects of the repair of 8-OHdG from different sources remain to be established, whilst there exists general acceptance of urinary 8-OHdG as an integral marker of oxidative stress. The present study has addressed the question of baseline urinary levels of 8-OHdG in spot urine and in 24-h urine, and of variation of this biomarker.

The data presented in this paper indicate equal levels of group means and ranges of urinary 8-OHdG/creatinine when using spot urine or 24-h urine. This might support the use of spot urine for the comparison of group levels of 8-OHdG, which facilitates the procedure of sample collection. In our study, two different groups of urine donors (spot urine, 24-h urine) were examined. Therefore, we could not test the association between 8-OHdG in spot urine and 8-OHdG/24 h, but a significant correlation of $r=0.5$ ($P<0.05$) for comparing 24-h

Table 2
Excretion of 8-OHdG and cotinine in non-smokers and smokers

	Spot urine		24-h Urine	
	Non-smokers ($n=70$)	Smokers ($n=78$)	Non-smokers ($n=44$)	Smokers ($n=23$)
Cotinine (ng/ml)	23±86	643±519	37±131	1290±663
8-OHdG (μmol/mol crea.)	1.94±1.21	2.11±1.22	1.61±0.95	2.36±1.17**
8-OHdG (nmol/24 h)	–	–	19.1±11.1	24.7±11.1*
8-OHdG (pmol/kg per 24 h)	–	–	257±142	319±140

**$P<0.01$, *$P\leq0.05$ (two-sided t-test); table shows means±SD.

urine samples with creatinine corrected 8-OHdG in morning urine samples has been already demonstrated by Poulsen et al. [16]. However, urinary levels of any oxidative damage product rely on renal function, which raised a controversial discussion about appropriate units of expression of 8-OHdG. Tagesson et al. [19] suggested that urinary creatinine may reflect not only the degree of urine dilution but also other parameters connected to individual characteristics. As a consequence, the adjustment for density was favored by this group. Nevertheless, creatinine is used routinely to correct for variations in urine concentration of various parameters in medicine and our results from multiple regression analysis clearly identify creatinine as a predictor of urinary 8-OHdG.

Although HPLC–EC is most favored for the determination of 8-OHdG, this method is not devoid of analytical difficulties. As indicated in the shown chromatograms, many urine components co-elute with 8-OHdG and may cause interference at high concentrations. This is supported by the data of Bogdanov et al. [13], who recently also reported the presence of co-eluting peaks, identified by multiple channel coulometric electrochemical detection. Despite this issue, in general there is a good inter-laboratory agreement of base levels of urinary 8-OHdG (Table 3) and our data are well in accordance with previously published values.

The inter-individual variation of 8-OHdG was found to be 54–60%. Together with the observed group levels of 8-OHdG, this implies that at least 12 subjects per group would be required to identify a shift of 50% in the mean level of 8-OHdG as statistically significant ($P \leq 0.05$) by the two-sided *t*-test. In addition, our results from monitoring 8-OHdG/24 h over a period of 10 consecutive days indicate that the intra-individual variation of urinary 8-OHdG has been underestimated so far. Thus, single values of 8-OHdG may reflect oxidative DNA base damage at a certain moment, but appear to be not representative for individual long-term excretion rates. This is in good agreement with the finding of high intra-individual fluctuations in subjects of the 24-h urine group who have been examined within six series of monitoring at intervals of 4–8 weeks [20].

Cigarette smoke is a well known source of ROS and smoking has consistently been shown to increase urinary 8-OHdG by 16–50% [8,9,21]. In addition, smoking cessation has been found to reduce the excretion rate of 8-OHdG by 21% [22]. Our data support a smoking induced increase in 8-OHdG/creatinine of 47%. However, the effect of smoking was only statistically significant in the group of 24-h urine donors and appears to be of minor importance as compared to the high intra-individual variation of urinary 8-OHdG. In addition, 8-OHdG did not correlate either with urinary cotinine in smokers nor with the number of cigarettes smoked per day.

Our data do not show a change in urinary 8-OHdG with age, but this does not exclude the possibility of a combination of both increased oxidative DNA damage and decreased repair capacity with age. Studies involving different age groups may suggest that the repair of oxidative damage decreases with age [23]. This is in agreement with animal studies [24], which report an age dependent increase of cellular 8-OHdG in rats, that was accompanied by a decrease in urinary 8-OHdG. In addition, mitochondrial 8-OHdG has been shown to accumulate with age [25].

Several occupational exposure studies used urinary 8-OHdG as a biomarker of oxidative DNA damage (reviewed in Ref. [26]). One of the earliest investigations of oxidative stress resulting from workplace exposure was conducted by Tagesson et al. [27], who examined urinary 8-OHdG in workers exposed to asbestos, rubber processing or azo-dyes. In all of these cases urinary 8-OHdG was found to be elevated as compared to unexposed controls. Nilsson et al. [28] showed that although urinary 8-OHdG in benzene exposed workers was not statistically different from controls, 8-OHdG in late-evening urine from exposed workers was significantly increased over pre-shift values. In addition, Lagorio et al. [29] found a significant correlation between urinary 8-OHdG and estimated average yearly benzene exposure in 65 filling station attendants. Industrial art glass work per se did not elevate urinary levels of 8-OHdG, but in female art glass workers the interaction between exposure and smoking was found to contribute significantly to the excretion of 8-OHdG [19]. In urban bus drivers, no significant changes in urinary 8-OHdG between workday and the day off were observed [30]. Urinary 8-OHdG in quartz-exposed workers without silicosis did not differ statistically from silicosis patients, but those silicosis patients with increased 8-OHdG in leukocyte DNA

Table 3
Published levels of urinary 8-OHdG in humans

Subjects	Age	Assay	Mean±SD (*n*)	Ref.
Healthy subjects	32.3±9.4	LC–MS–MS	Smokers: 1.7±0.7 ng/mg crea. (4) Non-smokers: 2.4±0.7 ng/mg crea. (5)	[14]
Healthy subjects	–	HPLC–EC	Males: 4.09±3.16 ng/ml, 3.86±0.48 ng/mg crea. (104), 75.66±2.64 ng/kg per 24 h (36) Females: 3.39±3.43 ng/ml, 3.96±0.37 ng/mg crea. (96), 72.06±4.50 ng/kg per 24 h (35)	[13]
Healthy male subjects	24–55	HPLC–EC	Smokers:1.35±0.50 μmol/mol crea., 20.1±7.3 nmol/24 h, 270±100 pmol/kg per 24 h (30) Non-smokers: 1.16±0.35 μmol/mol crea., 18.4±5.7 nmol/24 h, 250±80 pmol/kg per 24 h (30)	[21]
Healthy smokers after quitting smoking	45±8	HPLC–EC	Baseline: 30.5±13.9 nmol/24 h (58), 4 weeks smoking cessation: 24.1±10.5 nmol/24 h (58)	[22]
Healthy male subjects	22–60	HPLC–EC	Smokers: 7.41±1.47 ng/mg crea. (7) Non-smokers: 3.81±1.93 ng/mg crea. (6)	[34]
Premenopausal women	33±10	HPLC–EC	Smokers: 26.7±11.7 nmol/24 h, 431±168 pmol/kg per 24 h (12) Non-smokers: 18.6±7.8 nmol/24 h, 318±130 pmol/kg per 24 h (21)	[35]
Healthy subjects	51±7	HPLC–EC	Females: 240±106 pmol/kg per 24 h (52) Males: 271±98 pmol/kg per 24 h (31)	[8]
Healthy subjects	–	HPLC	172±79 pmol/kg per 24 h (63)	[40]
Healthy subjects	–	GC–MS	300±100 pmol/kg per 24 h (23)	[41]
Long distance runners	20.1±1.4	HPLC–EC	Control period: 265.7±75.5 pmol/kg per 24 h, 8-day training camp: 335.6±107.4 pmol/kg per 24 h	[38]
Swimmers and runners before and after exercise	20.1±1.2 20.9±2.1	HPLC–EC	Before: 2.27±0.48, after: 2.47±0.44 nmol/mmol crea. (9) Before: 1.42±0.47, after: 1.56±0.46 nmol/mmol crea. (9)	[39]
Quartz-exposed workers vs. silicotics	50±6 62±7	HPLC–EC	2.27±0.91 μmol/mol crea. (63) 2.00±0.92 μmol/mol crea. (42)	[31]
Urban bus drivers	45±8	HPLC–EC	Workday: 172±102 pmol/kg per 24 h (49) Day off: 161±110 pmol/kg per 24 h (51)	[30]
Industrial art glass workers	43.6±2.4	HPLC–EC	Exposed workers: 13.4±6.1 n*M* (181) Unexposed workers: 11.8±5.7 n*M* (49)	[19]
Workers exposed to benzene	20–53	HPLC–EC	Pre-shift: 0.72 nmol/mmol crea. (33) Following evening: 0.99 nmol/mmol crea. (33)	[28]
Filling station attendants	–	HPLC–EC	Smokers: 1.41±0.46 μmol/mol crea. (33) Ex-smokers: 1.29±0.62 μmol/mol crea. (10) Non-smokers: 1.32±0.50 μmol/mol crea. (22)	[29]
Workers exposed to asbestos, rubber, azo-dyes, vs. controls	–	HPLC–EC	1.40±0.56 μmol/mol crea. (30) 1.48±0.57 μmol/mol crea. (28) 1.92±0.85 μmol/mol crea. (30) 1.07±0.41 μmol/mol crea. (41)	[27]

Table 3. Continued

Subjects	Age	Assay	Mean±SD (*n*)	Ref.
Hematological disorder patients vs. healthy controls	28–70 –	ELISA	33.4±41.7 ng/mg crea. (44) 11.9±7.3 ng/mg crea. (10)	[36]
Hemochromatosis patients vs. laboratory staff and medical students	55±13 47±7 28±5	GC–MS	1.39±0.40 μmol/mol crea. (12) 1.33±0.29 μmol/mol crea. (11) 1.58±0.84 μmol/mol crea., 20.6±11.6 nmol/24 h (15)	[32]
Patients with atopic dermatitis vs. healthy individuals	16–36 19–34	ELISA	1.10±0.41 pmol/kg per 24 h (17) 0.46±0.41 pmol/kg per 24 h (17)	[33]
Adriamycin-treated patients	–	HPLC–EC	Before chemotherapy: 3.77±0.58 μmol/mol crea., 34.4±5.09 nmol/24 h (20) After chemotherapy: 3.50±0.52 μmol/mol crea., 35.5±4.59 nmol/24 h (20)	[37]
Cancer patients vs. healthy controls	–	HPLC–EC	18.0±10.9 nmol/24 h, 2.13±1.4 μmol/mol crea. (136) 14.9±7.8 nmol/24 h, 1.11±0.62 μmol/mol crea. (27)	[10]
Patients with malignant and non-malignant disease vs. healthy smokers and healthy non-smokers	43–80 47–82 27–62 22–64	HPLC–EC	3.37±2.84 μmol/mol crea. (10) 1.12±0.45 μmol/mol crea. (7) 1.3±0.38 μmol/mol crea. (10) 1.02±0.35 μmol/mol crea. (24)	[9]

had significantly lower urinary 8-OHdG than the corresponding group of active workers without silicosis [31].

In conclusion, our data indicate that the intra-individual variation of urinary 8-OHdG appears to be largely underestimated so far, and therefore single values of 8-OHdG should be considered with caution, in particular in small study groups and when spot urine is used.

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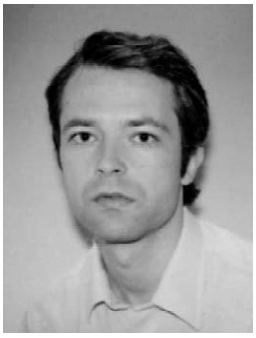

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