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Longitudinal Study of Urinary 8-Hydroxy-2′-Deoxyguanosine Excretion in Healthy Adults

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Numerous studies have investigated the urinary excretion of 8-hydroxy-2′-deoxyguanosine (8-OHdG) as a biomarker for the assessment of oxidative DNA damage in humans. In this study, we performed six consecutive series of measurement of urinary levels of 8-OHdG in 68 healthy probands, in order to provide information on the intra- and inter-individual variability of 8-OHdG and to estimate the influence of smoking, age, sex, body weight and body mass index (BMI) on the excretion of 8-OHdG. The intra-individual coefficient of variation (CV) of urinary 8-OHdG/24 h ranged from 0.18 to 1.06 (mean CV = 0.48). Women excreted significantly lower amounts of 8-OHdG/24 h than men, but the difference lost its significance when the body weight or urinary creatinine were used as covariates. By multiple linear regression analysis significant correlations between the mean individual levels of 8-OHdG/24 h excretion and urinary creatinine ($r_P = 0.61$), and cotinine ($r_P = 0.27$) have been observed, whereas no statistically significant effect of age, body weight and BMI was found. The 8-OHdG/creatinine ratio was found to be significantly increased in 23 smokers (1.95 ± 0.40 μmol/mol) opposed to 45 non-smoking probands (1.62 ± 0.50 μmol/mol), which is in good agreement with previously published data. No effect of passive smoking on the excretion of 8-OHdG was found. From our data we conclude that the intra-individual variability of urinary 8-OHdG excretion has been underestimated so far, indicating that values of 8-OHdG measured by single spot monitoring are not representative for individual base levels.

Keywords: 8-hydroxy-2′-deoxyguanosine, oxidative DNA damage, urine

INTRODUCTION

There is good evidence that oxidative DNA damage permanently occurs in living cells.[1] Several oxidative DNA modifications have been shown to be pre-mutagenic,[2–5] and may contribute to the development of cancer.[6] Since 8-hydroxy-2′-deoxyguanosine (8-OHdG) is one of the predominant forms of oxidative DNA damage with miscoding potential,[3] it is of practical interest to determine its baseline steady-state level

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in vivo. Gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography with electrochemical detection (HPLC-EC) have been widely used to measure endogenous levels of 8-OHdG (or the base 8-oxo-guanine), with controversial results over a range of two orders of magnitude.[7] This makes it difficult to establish reference values for 8-OHdG as an indicator of oxidative stress and disease predisposition.

Various HPLC based methods for the determination of 8-OHdG[8–12] and 8-oxoguanine[13,14] in urine as putative repair products of oxidative DNA damage have been described. These methods implicate extensive sample clean-up procedures in order to eliminate analytical interference from the complex urine matrix. Despite the analytical difficulties associated with the clean-up of urinary 8-OHdG, this approach offers some advantage as compared to the measurement of oxidative damage in DNA. First, these assays are non-invasive. Second, the problem of artificial formation of 8-OHdG during the sample preparation or the derivatization reaction is absent. Third, 8-OHdG in urine is stable for long time.[15] Fourth, the urinary excretion of 8-OHdG is expected to reflect the "whole body" oxidative DNA damage[16] or its repair from all cells in the organism.

Most studies using urinary 8-OHdG for the assessment of oxidative stress in humans considered tobacco smoking, environmental pollution and plasma antioxidants to influence the rate of oxidative damage.[17] Besides, however, there is evidence for a high biological variability of urinary 8-OHdG, which can be a limiting factor for the assessment of oxidative stress. The aim of the present study was to determine base levels of urinary 8-OHdG in healthy humans, in order to provide data of intra- and inter-individual fluctuations of 8-OHdG, and to identify confounding factors of 8-OHdG excretion. For this purpose we performed a longitudinal study including six consecutive series of measurement of urinary 8-OHdG/24 h in each of 68 subjects.

MATERIALS AND METHODS

Subjects

Sixty-eight subjects (28 female and 40 male), aged 18–71 years, were selected randomly under study applicants to obtain equally sized groups for non-smokers, passive smokers and smokers. Ex-smokers who had quit smoking for more than one year were classified as non-smokers. Only healthy subjects without medical drug use were included. All probands gave their informed consent for participating in the study. A questionnaire was used for data collection, to obtain information on age, sex, body weight, height and smoking habits. Twenty two probands reported environmental tobacco smoke at workplace or at home, and were therefore classified as passive-smokers. The probands were examined within six consecutive series of measurement at intervals of 4–8 weeks from March to October 1996. Table I shows the descriptive data of the study subjects.

Sample Collection

Twenty-four hour urine was collected after voiding the first morning urine from the day before measurement until the next morning. The sample was stored in a cooling box until it was returned to the laboratory. In the laboratory, pH and volume were determined within 2 h, before the sample was divided into several fractions, and frozen at −20 °C.

TABLE I Descriptive data of the study subjects

	All	Women	Men
No. of subjects	68	28	40
Age (yr)	35 ± 11	36 ± 11	35 ± 11
Weight (kg)	75 ± 13	66 ± 9	82 ± 10
BMI (kg/m^2)	24.7 ± 2.8	23.6 ± 2.5	25.5 ± 2.8
No. of non-smokers	23	11	12
No. of passive smokers	22	10	12
No. of smokers	23	7	16
Smokers: cigarettes/day	18 ± 10	20 ± 12	18 ± 9
Vol. of 24 h urine (ml)	1736 ± 566	1751 ± 638	1726 ± 518

Measurement of 8-OHdG in Urine by HPLC-EC

The samples were analysed batch-wise for each series of determination. The clean-up of urine samples included a two-step solid phase extraction. The analysis of 8-OHdG was performed by high-performance liquid chromatography with electrochemical detection (HPLC-EC) as described previously.[11] Briefly, acidified urine fractions stored at −20 °C 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 mM 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 puls 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 vs 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 mm × 3 mm I.D.) analytical column attached in series, packed with 4 µm Supersphere 100 RP-18 [endcapped] (Merck, Darmstadt, Germany). A LiChroCART (4 mm × 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 mM 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 expression of 8-OHdG relative to creatinine was based on 24 h urine collection.

Urinary Creatinine Measurement

Urinary creatinine was determined using a kit from Merck (Darmstadt, Germany) according to Jaffe's picric acid method.[18]

Determination of Urinary Cotinine

Urinary cotinine was analysed by gas chromatography connected to a nitrogen specific detector (GC/NPD) as described by Feyerabend and Russell.[19] The limit of detection was 1 ng/ml.

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 $p < .05$. Bivariate correlation between variables and forward stepwise multiple regression analysis were examined. Analyses were conducted using STATISTICA 5.0 software package.

Figure 1 describes basic statistical parameters resulting from the series of measurement. The inter-individual coefficient of variation (CV) of urinary 8-OHdG was calculated within each series as the standard deviation (SD) devided by the mean level of the study group. The inter-series CV resulted from the differences between the six mean group levels obtained from each series. The intra-subject CV was derived from the individual values of 8-OHdG excretion measured in each subject in the six series.

RESULTS

The group levels of urinary 8-OHdG/24 h in 68 probands within six consecutive series of measurement over a period of eight months are summarized in Figure 2. One female non-smoker dropped out of the study at series 4. The mean group level of urinary 8-OHdG/24 h was found

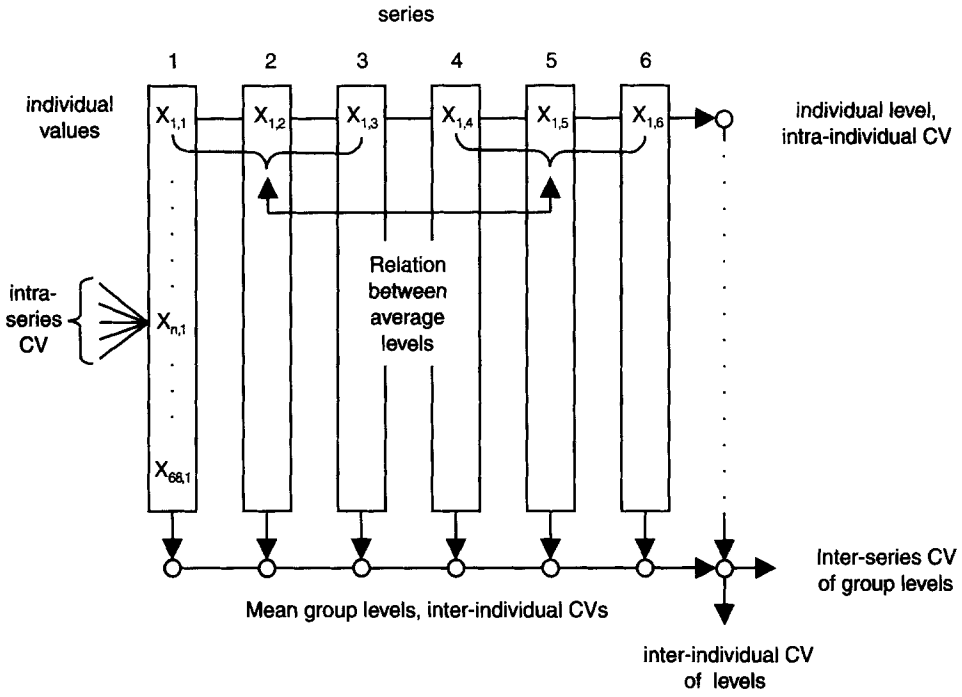

FIGURE 1 Schematic presentation of basic statistical parameters resulting from the series of measurement.

to be significantly decreased in series 3. The inter-individual coefficient of variation (CV) of 8-OHdG/24 h within each series of examination ranged from 0.53 to 0.68 (mean CV = 0.57). The inter-series CV of 8-OHdG/24 h was 0.14. The intra-individual CV (Figure 3) ranged from 0.18 to 1.06 (mean CV = 0.48). The analytical procedure had a CV of 0.14 between series of measurements and a CV of 0.03 within series. From the observed variances of 8-OHdG we calculated that at least three series of monitoring per proband are necessary to identify individuals with a 100% shift of the level of 8-OHdG.

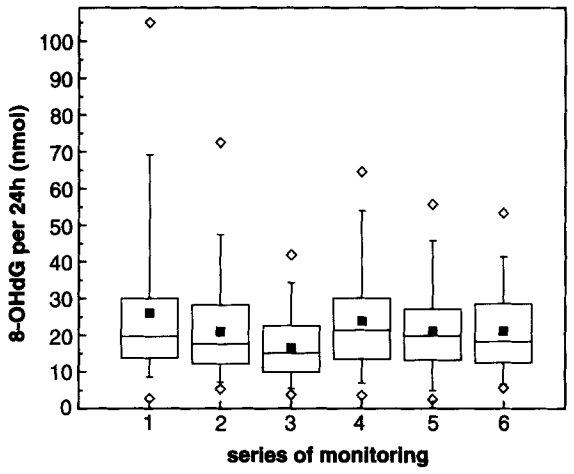

FIGURE 2 Graphical representation of urinary 8-OHdG/24 h (nmol) of 68 healthy probands involved in six consecutive series of measurement (box- and whisker plot). The symbols outside the box mark the 0th, the 1st, the 99th and the 100th percentiles. The bars mark the 5th and 95th percentiles, the edges of the box represent the quartiles, the horizontal line in the box marks the median, and the black square marks the mean.

In 8 out of 15 possible cases of inter-series comparison, a significant correlation between individual values of 8-OHdG/24 h was found (Table II), whereas in all cases of comparison of average intra-individual levels of 8-OHdG/24 h obtained from three series significant correlation ($r = 0.33$–0.59) could be demonstrated. Figure 4 shows an example of correlation of average intra-individual levels of 8-OHdG/24 h obtained from three series. Significant correlations between the mean individual levels of 8-OHdG/24 h and

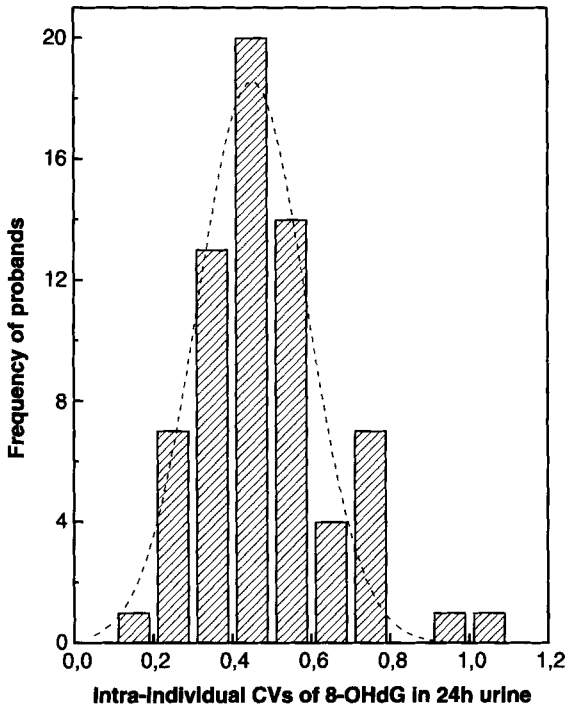

FIGURE 3 Histogram of intra-individual coefficients of variation (CV) of the excretion of 8-OHdG per 24 h in 68 probands.

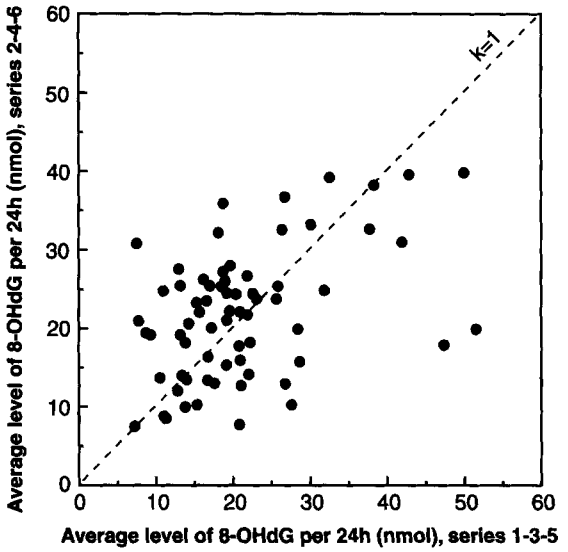

FIGURE 4 Correlation between average intra-individual levels of 8-OHdG/24 h obtained from series 1, 3, 5 and their corresponding levels obtained from series 2, 4, 6 ($r = 0.45$, $p < .0001$).

8-OHdG/kg/24 h ($r = 0.88$, $p < .0001$), and 8-OHdG/kg/24 h vs 8-OHdG/creatinine ($r = 0.71$, $p < .0001$) have been observed. In addition, the bivariate analysis showed significant correlations of the mean individual levels of 8-OHdG/24 h with urinary creatinine ($r = 0.64$, $p < .0001$), height ($r = 0.48$, $p < .0001$), body weight ($r = 0.44$, $p = .0001$) and age ($r = -0.25$, $p = .039$).

Using a 2 × 2 between-groups ANOVA design (smoking: 0, 1; gender: female, male), a significant influence of smoking ($F = 5.0$, $p = .028$) and gender ($F = 5.9$, $p = .018$) on the mean individual excretion of 8-OHdG/24 h was found. By contrast, the inclusion of the body weight as a covariate eliminated the effect of gender ($F = 0.2$, $p = .63$) on the excretion of 8-OHdG, indicating a lower body weight in women. A significant increase of 8-OHdG in smokers by 17% ($F = 4.8$, $p = .033$) was found for the adjusted means. A similar increase of 8-OHdG in smokers (18%) was identified after ANOVA with multiple covariates (urinary creatinine, height, body weight, body mass index and age). Table III presents the results of forward multiple stepwise regression for the mean individual levels of 8-OHdG/24 h. The association between the body weight and 8-OHdG/24 h in the bivariate analysis did not prove to be significant in the multiple regression analysis, whereas urinary creatinine and cotinine appeared to be predictors of 8-OHdG excretion.

Data concerning mean levels of urinary 8-OHdG in smokers, passive smokers and non-smokers are shown in Table IV. Passive-smokers showed higher levels of urinary cotinine than

TABLE II Coefficients of correlation obtained from linear regression analysis of individual values of 8-OHdG/24 h from different series

Series	1	2	3	4	5	6
1	1	0.43**	0.23	0.08	0.39**	0.30**
2		1	0.13	−0.06	0.36**	0.29*
3			1	0.00	0.31**	0.22
4				1	0.01	0.32**
5					1	0.41**
6						1

*$p < .05$; **$p < .01$

TABLE III Forward stepwise multiple regression analysis of 8-OHdG in 24 h urine

Mean 8-OHdG (nmol/24 h) dependend variable	r(partial)	β	p
Mean urinary creatinine (g/24 h)	0.61	0.58	< .0001
Mean urinary cotinine (ng/24 h)	0.27	0.21	.029
Age (yr)	−0.13	−0.10	.300
Sex	0.05	0.05	.664
Height (m)	0.03	0.04	.786
Weight (kg)	−0.03	−0.04	.797
BMI (kg/m^2)	−0.07	−0.07	.581

non-smokers, whereas no significant changes in the levels of urinary 8-OHdG have been observed in these individuals as compared to non-smokers. Although urinary cotinine appeared to be a predictor of 8-OHdG/24 h, no significant correlation between 8-OHdG/24 h and cotinine in smokers or the number of cigarettes smoked per day could be demonstrated. Only in determination 2 and 5 a statistically significant increase of 8-OHdG/creatinine in smokers was found. The mean urinary excretion rate of creatinine was found to be higher in men (1.64 ± 0.42 g/day) than in women (1.16 ± 0.23 g/day). No significant difference between the ratios of 8-OHdG/creatinine in women (1.76 ± 0.65 μmol/mol, $n = 21$ non-smoking subjects) and men (1.50 ± 0.41 μmol/mol, $n = 24$ non-smoking subjects) was observed.

DISCUSSION

Urinary 8-OHdG was studied in healthy subjects within six consecutive series of measurement, in order to determine the extent of inter- and intra-individual variability and the influence of various confounding factors. The high intra-individual variation of 8-OHdG in urine ranging from 17% to 106% indicates that endogenous variation of oxidative DNA damage has been underestimated so far, since previous estimations were based on study subjects examined only twice.[20,21] The inter-subject CV within each series was 44%–68%, being in good accordance with previously reported values.[10] The CV of the mean group levels of 8-OHdG was similar to the inter-series CV of the analytical method itself (14%), indicating good reproducibility of group

TABLE IV Urinary base levels of cotinine, creatinine and 8-OHdG in non-smokers, passive-smokers and smokers and (mean ± SD)

	Non-smokers (f/m = 11/12)	Passive-smokers (f/m = 10/12)	Smokers (f/m = 7/16)
Cotinine (ng/24 h)	2 ± 2	60 ± 141	2369 ± 1489**
Creatinine (g/24 h)	1.46 ± 0.47	1.38 ± 0.40	1.49 ± 0.42
female	1.13 ± 0.25	1.14 ± 0.23	1.22 ± 0.24
male	1.76 ± 0.42	1.63 ± 0.39	1.60 ± 0.43
8-OHdG (nmol/24 h)	20.5 ± 7.3	19.0 ± 6.8	24.9 ± 8.2**
female	18.3 ± 6.7	16.1 ± 3.2	21.7 ± 4.9
male	22.6 ± 7.5	21.4 ± 8.2	26.3 ± 9.0
8-OHdG (nmol/kg/24 h)	272 ± 79	266 ± 93	321 ± 88*
female	275 ± 78	254 ± 76	328 ± 61
male	270 ± 83	276 ± 107	317 ± 99
8-OHdG/Crea. (μmol/mol)	1.67 ± 0.57	1.57 ± 0.41	1.95 ± 0.40**
female	1.83 ± 0.62	1.68 ± 0.50	2.05 ± 0.33
male	1.51 ± 0.50	1.47 ± 0.32	1.90 ± 0.43**

*$p < .05$; **$p < .01$ (two-sided t-test, [non-smokers + passive smokers] vs [smokers])

levels. We have no ready explanation, however, for the lower 8-OHdG levels observed in the third series. In only 8 out of 15 possible cases of between-series comparison, the correlation of individual values of 8-OHdG/24 h was statistically significant. In contrast, significant correlations between average intra-individual levels of 8-OHdG/24 h obtained from three series were found in any case of comparison. Thus, instantaneous urinary concentrations of 8-OHdG appear to be not representative for individual long term excretion rates and single measurement may not be useful for the assessment of individual base levels of 8-OHdG. The ignorance of the intra-individual variability of urinary 8-OHdG may lead to misinterpretation, especially when (i) the study groups are small; (ii) when small differences between the 8-OHdG levels are observed; and (iii) when the same probands are tested for urinary 8-OHdG before and after exposure to oxidants or supplementation with antioxidants.

In the present study the excretion of creatinine has been identified as a main predictor of urinary 8-OHdG, indicating that 8-OHdG is formed in lean body mass. This is in good agreement with recent data by Pourcelot *et al.*[22] who also reported a dependence of urinary 8-OHdG on creatinine. However, within 76% of the probands we did not find a significant positive correlation between the intra-individual values of urinary creatinine and 8-OHdG. In addition, the correction of 8-OHdG for creatinine did not diminish the high inter- and intra-individual coefficients of variation of 8-OHdG. Thus, creatinine may reflect not only urine concentration but also other factors related to individual characteristics.[23]

Cigarette smoke is a well known source of free radicals[24] and smoking has consistently been shown to elevate the urinary excretion of 8-OHdG by 16–50%.[8,9,22,25] In addition, smoking cessation has been found to reduce the excretion rate of 8-OHdG by 21%.[21] In contrast, conflicting data have been reported on the association of smoking with 8-OHdG formation in DNA of blood leukocytes.[26–28] Our results show an increase in urinary 8-OHdG of smokers by 18% as compared to non-smokers and passive-smokers, which is in good accordance with previously published data.[22] Although smokers showed an overall trend of increase in urinary 8-OHdG, a significant intra-series effect of smoking was observed in only two series. Therefore, the influence of smoking appears to be of minor importance as compared to the high intra-individual variability of urinary 8-OHdG in general. In addition, no correlation between the number of cigarettes smoked per day and the excretion of 8-OHdG has been found.

A relationship between age and oxidative DNA damage in humans is controversial. It has been speculated that the elimination of oxidative DNA damage decreases with age.[17] This is in accordance with animal studies,[29] which report an age-dependent increase of cellular 8-OHdG in rats that was accompanied by a decrease in urinary 8-OHdG. In the present study, we also observed a slightly negative association between age and 8-OHdG excretion, but only when the body weight or urinary creatinine were not taken into account. Similarly, in our study a difference of 8-OHdG levels between women and men lost statistical significance when 8-OHdG was related to body weight or urinary creatinine.

In conclusion, our data indicate that the intra-individual variability of urinary 8-OHdG excretion appears to be largely underestimated as yet, and single spot monitoring of 8-OHdG in urine should be taken with caution, in particular in small study groups.

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