

## Oxidative DNA Damage *In Vivo*: Relationship to Age, Plasma Antioxidants, Drug Metabolism, Glutathione-S-transferase Activity and Urinary Creatinine Excretion

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Oxidative DNA modification has been implicated in development of certain cancers and 8-oxodG, the most abundant and mutagenic DNA modification, has for some time been considered a biomarker of this activity. Urinary excretion of 8-oxodG over 24 h has been used to estimate the rate of damage to DNA, and animal studies have supported this rationale. Reported determinants include tobacco smoking, heavy exercise, environmental pollution and individual oxygen consumption.

Samples from three published studies were used to determine the association of urinary 8-oxodG excretion with age, plasma antioxidants, the glutathione-S-transferase phenotype and the activity of the xenobiotic metabolising enzyme CYP1A2. In the age range 35–65 years, age was not related to urinary 8-oxodG excretion, and there were no relations to either the glutathione-S-transferase phenotype or to the plasma antioxidants: vitamin C, alpha-tocopherol, beta-carotene, lycopene or coenzyme Q10. The activity of CYP1A2 showed a significant correlation in two of the three studies, as well as a significant correlation

of 0.26 ( $p < 0.05$ ) in the pooled data set. Regression analysis of CYP1A2 activity on 8-oxodG indicated that 33% increase in CYP1A2 activity would correspond to a doubling of 8-oxodG excretion. This finding needs to be confirmed in independent experiments.

Spot morning urine samples can under certain circumstances be used to estimate 8-oxodG excretion rate provided that creatinine excretion is unchanged (in paired experiments) or comparable (in un-paired experiments), as evaluated from the correlation between 8-oxodG excretion in 24 h urine samples and in morning spot urine samples corrected for creatinine excretion ( $r = 0.50$ ,  $p < 0.05$ ).

We conclude that 8-oxodG excretion is determined by factors like oxygen consumption and CYP1A2 activity rather than by factors like plasma antioxidant concentrations.

**Keywords:** DNA damage, oxidative, 8-oxodG, ageing, antioxidants, vitamin C, beta-carotene, vitamin E, CYP1A2

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*Abbreviations:* 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine; CYP1A2, cytochrome P450 1A2

## INTRODUCTION

Oxidative modification of DNA was first reported about 15 years ago.<sup>[1]</sup> The discovery has led to an intense research effort to test the hypothesis that oxidative DNA modification is linked to the development of human disease and cancer in particular. Still, no direct evidence is available, but a plethora of indirect evidence has been established. This evidence indicates that the most abundant oxidative DNA modification, 8-hydroxy-2'-deoxyguanosine (8-oxodG), can induce GC → TA transversion and that mammalian cells have specific DNA repair mechanisms that recognise and repair 8-oxodG very efficiently (for review see<sup>[2,3]</sup>).

Oxidative DNA modification can result from the endogenous production of free oxygen radicals or a variety of other factors (see<sup>[4]</sup> and references therein). We have reported that gender and tobacco smoking,<sup>[5]</sup> individual oxygen expenditure,<sup>[6]</sup> exercise<sup>[7]</sup> and recently air pollution<sup>[8]</sup> are important determinants for oxidative DNA damage in terms of 8-oxodG excretion. Other determining factors have been reviewed.<sup>[8,9]</sup> Considering the consistent epidemiological findings that a diet rich in antioxidants is associated with low cancer rates<sup>[10]</sup> together with the importance of oxygen radicals for oxidative DNA modification, it seems reasonable to hypothesise that antioxidants can reduce the cancer risk by reducing the oxidative modification of DNA. However, controlled trials with antioxidant intervention have failed to show such effects for beta-carotene, vitamin A and vitamin E.<sup>[11-13]</sup> In accordance with these findings we have not found any effect of beta-carotene, alpha-tocopherol, or vitamin C on the excretion of the oxidised nucleotide 8-oxodG.<sup>[14,15]</sup> In a vitamin deficient population, however, a combination of antioxidants and vitamins decreased the cancer incidence,<sup>[16]</sup> and we have found that a diet rich

in Brussels sprouts reduces the excretion of 8-oxodG.<sup>[17]</sup> Furthermore, in a smoking cessation study we found that an increase in vitamin C after smoking cessation.<sup>[18]</sup> was mirrored by a decrease in urinary 8-oxodG excretion.<sup>[19]</sup> Another study with multiple antioxidant intervention showed improvement in oxidative DNA damage in lymphocytes measured by the Comet assay.<sup>[20]</sup>

In this paper we report on the relationship between plasma antioxidant concentrations and the urinary 8-oxodG excretion.

## MATERIAL AND METHODS

The data presented here originate from a population based sample of 40–64 year old population of Copenhagen and suburbs ( $n=83$ ),<sup>[5]</sup> from a randomised smoking cessation study on 182 men and women aged 35–65 years and smoking more than 15 cigarettes per day<sup>[19]</sup> and from a controlled intervention study for 8 weeks with three antioxidants on 142 subjects aged 35–65 years and smoking more than 10 cigarettes per day.<sup>[21]</sup> Experimental details are described in these publications.

Plasma antioxidant concentrations and coenzyme Q10 at baseline of the three studies were determined by HPLC methods.<sup>[22-25]</sup> Determination of urinary caffeine metabolites by HPLC was used for calculation of cytochrome P4501A2 activity, N-acetyl transferase activity and xanthine oxidase activity,<sup>[26]</sup> cytochrome P4503A activity was determined from the urinary 6beta-hydroxycortisol/cortisol ratio.<sup>[27]</sup> Glutathione-S-transferase phenotype was determined by a commercial available kit, Mukit<sup>®</sup>. Urinary excretion of 8-oxodG was quantified by a three-dimensional HPLC method.<sup>[5]</sup> In 1997 we re-analysed the samples stored from 1991 at  $-20^{\circ}\text{C}$  by the same HPLC method, although now calibrated by a commercially available 8-oxodG standard.<sup>[28,29]</sup>

Statistical analysis, correlation and regression analysis, were performed by Statistica<sup>®</sup> for

Windows version 5.1 F, StatSoft, Inc. 1997, Tulsa, Oklahoma, USA. *P*-values less than 0.05 are considered statistically significant.

## RESULTS

Regression analysis of urinary 8-oxodG excretion on age for each of the three data set S from the three studies<sup>[5,14,19]</sup> did not reveal significant slopes ( $-0.074$ ,  $+0.36$ ,  $-0.23$  nmol/24 h year; *p*-values 0.56, 0.14 and 0.051, respectively). Analysis of the pooled data set gave a non-significant slope of  $-0.10$  nmol/24 h year (sd = 0.065, *p* = 0.12).

The analysis of relationship between the most common antioxidants, vitamin C, beta-carotene, coenzyme Q10 and lycopene, to urinary excretion of 8-oxodG did not reveal significant relationships evaluated from the regression slopes. A statistically marginal significant slope of  $-0.0012$  (sd = 0.00068, *p* = 0.04) was found for the regression of beta-carotene concentration ( $\mu\text{mol/L}$ ) on urinary excretion of 8-oxodG (nmol/24 h), however if corrected for performing several tests on the same data set by the method of Bonferoni, the statistical significance disappears (Figures 1 and 2).

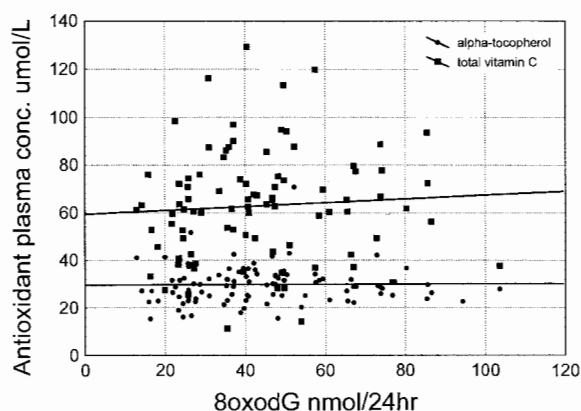


FIGURE 1 Relationship between plasma antioxidant concentrations, alpha-tocopherol and total vitamin C, in  $\mu\text{mol/L}$ , versus urine excretion of 8-oxodG nmol/24 h. The slopes of the regression lines are not different from null, *p* > 0.05. Data are baseline values from a randomised placebo controlled antioxidant intervention study.<sup>[14]</sup>

The urinary excretion of 8-oxodG did not differ between the two glutathione-S-transferase phenotypes, mean values 44.9 nmol/24 h (sd = 20.0) and 39.3 (sd = 18.2), *p* = 0.09 by Students *t*-test for unpaired data.

The activity of the cytochrome P4501A2 isozyme (CYP1A2), responsible for metabolism of a number of xenobiotics, correlated significantly with 24 h urinary 8-oxodG excretion in two of the three studies, correlation coefficients (*r*) being 0.33, 0.21 and 0.09 with *p*-values of < 0.05, < 0.05 and > 0.05, respectively. The correlation coefficient of the pooled data set also gave a significant correlation, *r* = 0.26 (*p* < 0.05). Regression analysis gave a slope of 0.0039 (*p* < 0.05) (Figure 3). The drug metabolising enzyme activity of N-acetyl transferase showed no correlation to the urinary excretion of 8-oxodG; i.e. in all cases the slopes of the regression of the activity for the enzyme and 8-oxodG were not significant different from null, *p* > 0.23. The same was the case for the slope of the regression of activity of xanthine oxidase on 8-oxodG, *p* = 0.29.

Re-analysis of the urine sample concentration of 8-oxodG in samples stored since 1991 at  $-20^{\circ}\text{C}$  revealed a slight but significant difference, slope

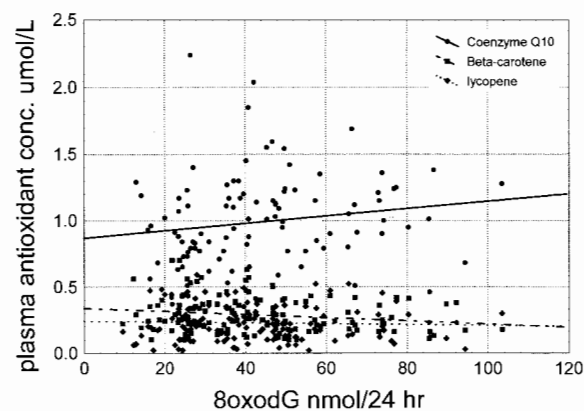


FIGURE 2 Relationship between plasma antioxidant concentrations, coenzyme Q10, beta-carotene and lycopene ( $\mu\text{mol/L}$ ) and urinary excretion of 8-oxodG (nmol/24 h). Data are baseline values from a randomised placebo controlled antioxidant intervention study.<sup>[14]</sup> The slope of the beta-carotene regression line is marginally significant, *p* = 0.04, while the two other slopes are not significant (*p* > 0.10).

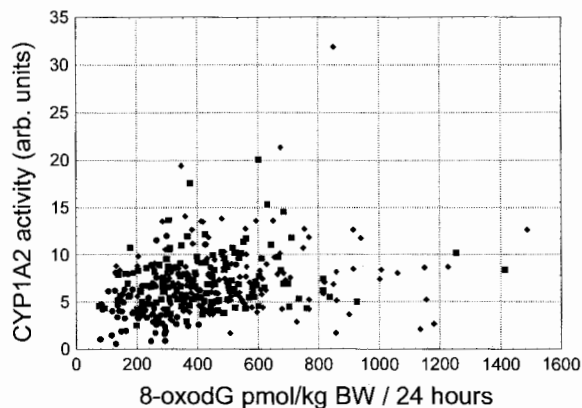


FIGURE 3 Relationship between urinary excretion of 8-oxodG, nmol/24 h and CYP1A2 activity. Data are pooled baseline data from 3 studies.<sup>[5,14,19]</sup> The correlation coefficient ( $r$ ) is 0.26 ( $p < 0.05$ ), the slope of the regression line is 0.0039.

1.10 ( $p < 0.05$  for slope different from 1), intercept 2.31 ( $p < 0.05$  for intercept different from 0) and a correlation ( $r$ ) of 0.87 ( $p < 0.05$ ). In 1991 we found that the correlation coefficient between 24 h urine 8-oxodG excretion and a creatinine corrected 8-oxodG excretion in an independent morning spot urine sample was not significant, Figure 4(a),  $p > 0.05$ . However as can be seen in Figure 4 panels there is a correlation of 0.50 ( $p < 0.05$ ) for comparing a 24 h urine sample with creatinine corrected 8-oxodG in an independent morning urine sample after reanalysis of the samples in 1997 Figure 4(b), and similar high correlations for comparison of 24 h urine in 1991 versus 1997 (Figure 4(c),  $r = 0.05$ ,  $p < 0.05$ ) and comparing spot samples analysed in 1991 versus 1997 (Figure 4(d),  $r = 0.64$ ,  $p < 0.05$ ).

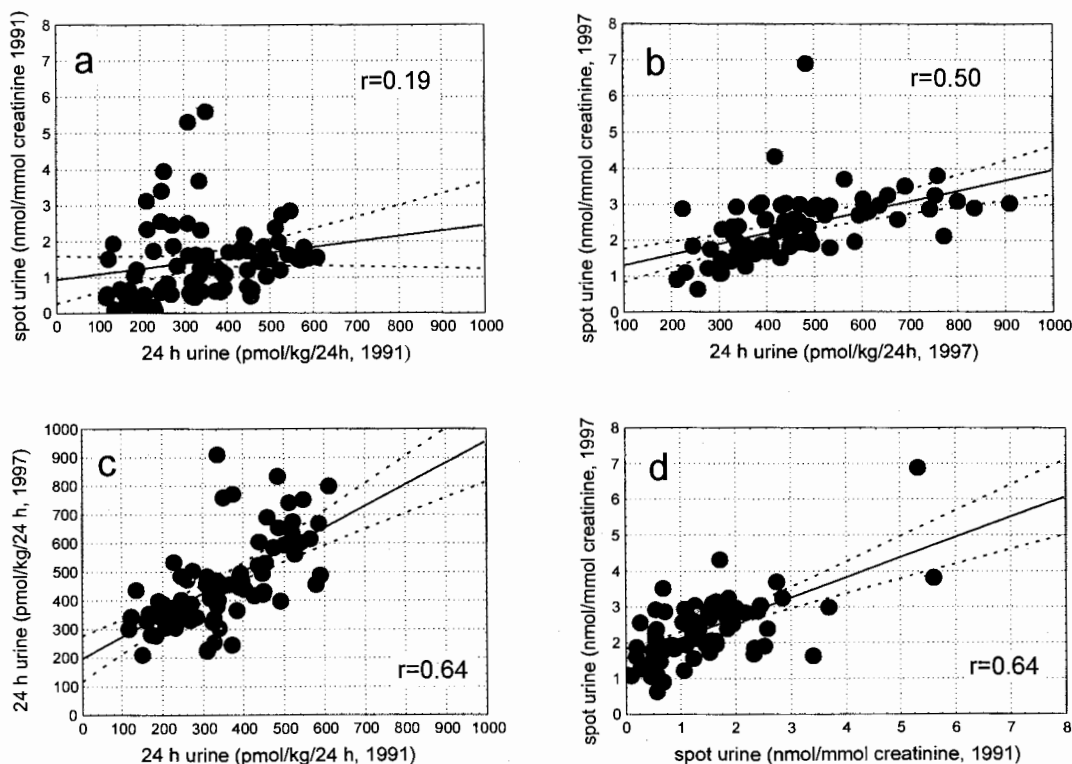


FIGURE 4 Relationship between morning spot urine samples and 24 h urine samples for analysis of spot urine creatinine corrected 8-oxodG versus 24 h excreted 8-oxodG. Samples originate from our previous study<sup>[5]</sup> analysed in 1991 and re-analysed in 1997. The correlation coefficient for the relationship is given in each of the four panels (a, b, c and d). The lines indicate the regression line (solid line) and the 95% confidence lines for the regression. The sample and the year of analysis is given by the text on the graph axes.

## DISCUSSION

Measurement of DNA oxidation products is a promising tool for use in molecular epidemiology for investigating ageing, cancer development and cancer prevention, and presumably also some other degenerative diseases.<sup>[31]</sup> Presently, the value of biomarkers as measurements of DNA oxidation is unknown, and a variety of basic information still needs to be acquired. From a theoretical point of view, measurement of DNA oxidation is quite simple and adheres to the simple model depicted in Figure 5. The body pool of oxidised nucleobases will arise from a balance that results from formation and elimination. Presently, it has been clearly established that formation of oxidised nucleobases occurs and that there are specific enzymes repairing the oxidative DNA damage. It is presently not clear to what degree the nucleotide pool is oxidised, and quantitative aspects of the contribution from

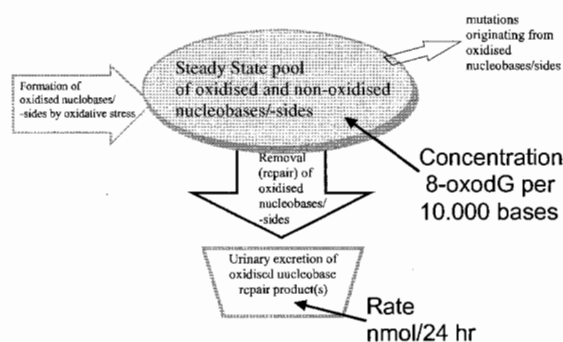


FIGURE 5 Oxidation of nucleobases in DNA can be viewed as an input to the pool of nucleobases of oxidised bases. Repair will eliminate damaged nucleobases from the pool and they can be found in urine. Measurement of oxidised nucleobases in urine will reflect their removal from the organism. Assuming no major age changes (see text) and stable steady state concentrations of oxidised nucleobases in tissues, input (formation of oxidised nucleobases) and output (elimination of oxidised nucleobases) will be equal. Measurement of urine samples will consequently represent a measure of the rate of DNA oxidation whereas a tissue sample will measure the concentration of oxidised nucleobases reflecting the balance between formation and repair. It should be noted that if both formation and repair increase the tissue levels of oxidised nucleobases need not change.

mitochondrial DNA, nuclear DNA and the nucleotide pool remains to be established. Support for a balance between nuclear DNA oxidation products and urinary excretion is indirect. 8-oxodG is eliminated and fully recovered after injection<sup>[30]</sup> and the excess organ nuclear DNA levels of 8-oxodG after inducing oxidative damage with 2-nitropropane correspond to the excess 8-oxodG excretion into urine.<sup>[31]</sup>

The data presented in this paper show no major change in urinary excretion of 8-oxodG with age, but this does not exclude decreased repair and increased levels with age. Recent studies have shown accumulation of mitochondrial 8-oxodG with age.<sup>[32]</sup> If repair is decreased and tissue levels increased, there might be a higher chance for mispairing of 8-oxodG with subsequent mutation as a consequence.

The antioxidant mechanisms in the body include the essential dietary antioxidant vitamins. The quantitative role they play in the antioxidative defence network is unknown. As pointed out in the introduction, trials with antioxidant supplementation in well nourished groups have failed. In the present study we measured the major antioxidant vitamins in plasma for correlation with the oxidative DNA damage and found no correlations of significance. The range of plasma concentrations for e.g. alpha-tocopherol and vitamin C varied between 20 and 130  $\mu\text{M}$ , which should be a sufficient span representing close to deficiency and saturation (Figure 1). On the other hand, depletion of several antioxidants may be necessary before an effect on 8-oxodG formation can be expected. Similarly, there were no correlations between 8-oxodG urinary excretion and the plasma antioxidants that occur in much lesser concentrations, coenzyme Q10, beta-carotene and lycopene (Figure 2).

The cytochrome P450 system metabolises a variety of xenobiotics and during that process can produce hydrogen peroxide in varying amounts. In this context it is quite interesting that we found a positive correlation between the

activity of CYP1A2 and the urinary 8-oxodG excretion. However, the biological importance is difficult to evaluate, but it is intriguing that an increase in CYP1A2 activity from 7.5 to 10.0 can be estimated to increase the urinary 8-oxodG from 602 to 1248 nmol/kg BW/24 h (regression equation is  $y = 5.12 + 0.0038x$ ). Other drug metabolising enzymes such as CYP3A did not show this effect, neither does the enzyme xanthine oxidase (data not shown). The observation of a relation between CYP1A2 and oxidative DNA damage needs to be confirmed in independent experiments, preferentially including manipulations with CYP1A2 activity.

Collection of urine for 24 h will provide information of the damage during that period as an average for the total number of cells in the body. Shorter collection periods can be applied, since the elimination of injected 8-oxodG shows a half-life of a few hours.<sup>[30]</sup> Collection of spot urine samples is much more feasible and correction by creatinine excretion to correct for variation in water intake and thereby urine production is a possibility, however our initial analysis of morning spot urine samples and 24 h urine samples for 8-oxodG excretion showed a very poor correlation of 0.19, Figure 4(a). Re-analysis of the samples after 6 years of storage showed a much better correlation coefficient of 0.50, Figure 4(b). The discrepancy between the 1991 and 1997 analysis of spot urine samples is not easily explained. The analysis in 1992 was done over a long period when capacity was available, and the 1997 analysis was done after obtaining many years experience. Taken collectively, we evaluate that the correlation between spot samples and 24 h samples as found in 1997 is correct. Also, correlation between the concentration measured in urine samples with 6 years interval was high, 0.64. The significant intercept of about 200 pmol/kg/24 h can be ascribed to difference in standards. With the present data we feel sure that 8-oxodG is stable for long periods at  $-20^{\circ}\text{C}$ , and that creatinine correction can be a useful tool in special circumstances. Creatinine excre-

tion depends on a variety of factors like weight, muscle mass. Correction with creatinine concentration is particularly valid for repeated measurements on the same individual, but must be considered doubtful for comparison of groups that differ in the factors that determine creatinine excretions, or when creatinine excretion can be thought to change in the same individual. Comparison of a group of patients with a group of healthy subjects by means of e.g. 8-oxodG excretion with creatinine excretion cannot for sure be related to a difference in 8-oxodG.

In conclusion, oxidative damage as measured by urinary excretion of 8-oxodG does not change to any important degree in age from 35 to 65 years. It can also be concluded that urinary excretion of 8-oxodG is not significantly influenced by the plasma concentrations of vitamin C, alpha-tocopherol, beta-carotene, lycopene or coenzyme-Q10. A significant correlation between CYP1A2 activity and urinary excretion of 8-oxodG appears to be an important determinant of oxidative DNA damage, a finding that needs to be confirmed in independent studies. It thus appears that 8-oxodG formation is regulated by processes e.g. oxygen uptake, the activity of CYP1A2, external factors like tobacco smoking and not by plasma concentrations of antioxidants. Under certain circumstances where urinary excretions of creatinine can be assumed comparable, measurement of spot urine 8-oxodG with correction by creatinine concentration can be valid.

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