



Oxidatively generated modifications to nucleic acids in vivo: Measurement in urine and plasma

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ABSTRACT

Background: The oxidized guanine nucleosides, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo), derived from DNA and RNA, respectively, were used to investigate the importance of oxidative stress to nucleic acids in vivo. High urinary excretion of 8-oxodG is associated with cancer development, whereas high urinary excretion of 8-oxoGuo is associated with mortality in type 2 diabetes. Like creatinine, these small water-soluble molecules are not reabsorbed in the kidney. Therefore, 8-oxo nucleoside/creatinine reciprocal concentration ratios are identical in plasma and urine. The total amount of 8-oxo guanine nucleosides excreted by the kidneys is the product of plasma concentration and glomerular filtration rate.

Methods: With relevant equations and an estimated glomerular filtration rate, the 24-h urinary excretion of 8-oxodG and 8-oxoGuo was calculated in 2679 subjects with type 2 diabetes, displaying good correlation with the measured urinary 8-oxo nucleoside/creatinine ratio: DNA oxidation $r = 0.86$ and RNA oxidation $r = 0.84$ ($p < 0.05$ for both).

Results: Survival analyses based on the quartiles of the 8-oxodG/creatinine ratio and the quartiles of calculated 24-h urinary excretion rate of the 2679 subjects gave similar hazard ratio estimates for death due to all causes. This finding was similar for the 8-oxoGuo hazard ratio estimates.

Conclusions: This study shows that oxidatively generated modifications to DNA and RNA in vivo can be measured using 1) a spot urine sample, normalized to urinary creatinine, 2) 24-h urine, or 3) a single plasma sample based on concentrations of 8-oxo nucleoside and creatinine and glomerular filtration rate.

1. Introduction

Since 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) formation in DNA was reported by Kasai and Floyd [1,2], 8-oxodG has been investigated as the prototype of DNA oxidation. As a measure of oxidative stress to nucleic acids, 8-oxodG is premutagenic and has been linked to aging and several diseases. A similar guanine lesion, 8-oxo-7,8-dihydroguanosine (8-oxoGuo), has also been found in RNA. To add, a high rate of excretion 8-oxoGuo and also the oxidized nucleobase 8-oxoGua (8-oxo-7,8-dihydroguanine) via urine is a risk factor for cancer development [3,4], whereas 8-oxoGuo does not appear to be a risk factor for cancer [5]. A high rate of urinary 8-oxoGuo excretion is predictive of

death by all causes and cardiovascular causes in patients with type 2 diabetes [6]; this is, however, not found for the high excretion rate of 8-oxodG [6–8]. The differential implication of 8-oxoGuo compared with 8-oxodG appears to be more general as it extends to obesity [9], hemochromatosis [10], and twin-estimated genetic background [11].

A specific and sensitive methodology to measure urinary 8-oxo guanine species given present day analytical demands is available; however, the conceptual framework is unclear, and concentrations in urine or plasma are sometimes inaccurately considered as a measure of “oxidative stress to nucleic acids”, without much consideration given to the physiological context.

The basic concept is that urinary content of 8-oxodG (or 8-oxoGuo)

Abbreviations: 8-oxo-7,8-dihydro-2'-deoxyguanosine, 8-oxodG; 8-oxo-7,8-dihydroguanosine, 8-oxoGuo; glomerular filtration rate, GFR; enzyme-linked immunosorbent assay, ELISA; creatinine, crea

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collected for 24-h will represent all guanines oxidized in DNA/RNA in the body in a similar period. For example, 8-oxodG correlates with the rate of DNA oxidation plus oxidation in the nucleotide pool (i.e., intracellular oxidation in the entire organism) [12,13]. It is calculated by multiplying the urinary concentration of 8-oxoGuo with the volume of the collected urine. Especially in epidemiological research, collecting urine for 24-h is not feasible, and urinary concentration will vary with urine production and water intake. To minimize bias from varying urine volumes for 24-h, a spot urine sample can be analyzed for 8-oxo guanine nucleoside concentration and creatinine. The ratio of 8-oxodG/creatinine concentrations correlates well with a 24-h urine sampling estimate [14,15]. The physiological reasoning for creatinine normalization is the variations in fluid intake and that creatinine excretion is assumed to be constant without much diurnal variation. However, differences in muscle mass [16], dietary pattern [17], kidney function [18], and acute exercise [19] may affect the urinary excretion of creatinine. An alternative correction, although less used, is by the specific gravity of the urine sample. Thus, epidemiological studies should adjust for relevant factors and intervention studies that include one or several of the mentioned interventions should not use creatinine to adjust hydration state.

Initially, 8-oxoGua as well as several oxidized bases were not considered as potential biomarkers since their urinary levels were supposed to be diet dependent until this was ruled out a few years later [20]. High 8-oxoGua (8-oxo-7,8-dihydroguanine, the nucleobase) and 8-oxodG (the nucleoside) excretion has been demonstrated as a risk factor for the development of lung cancer and as predictors of survival in colon cancer patients [21,22], 8-oxodG has been demonstrated as a risk factor for development of breast cancer [3], and high 8-oxoGuo excretion has been demonstrated as a risk factor for death and death of cardiovascular disease in patients with type 2 diabetes [6]. Such finding also demonstrates compartmental oxidation of these two molecules, presumably related to the difference in their intracellular location [23].

Plasma or serum concentrations of 8-oxodG have been reported; however, the reported range is rather extensive [24,25]. In contrast, plasma or serum concentrations of 8-oxoGuo have not been reported. Plasma and serum concentrations of 8-oxodG (or 8-oxoGuo) may be identical; however, this needs to be verified. Similar to the concentration of circulating creatinine, theoretically, the concentration of 8-oxodG (or 8-oxoGuo) in plasma or serum is mainly dependent on renal glomerular filtration rate (GFR). The plasma level reflects the balance between formation and elimination and is therefore difficult to interpret as a marker of nucleic acid oxidation. Instead, it reflects kidney function like plasma creatinine.

In this paper, we consider the physiological basis for the use of urine or plasma measurements to estimate meaningful (i.e., interpretable), quantitative measures of oxidatively generated modifications to nucleic acids, that will be suitable for use in clinical practice or large-scale epidemiological research.

2. Material and methods

The Vejle Diabetes Biobank Cohort [26] includes 3320 patients with type 2 diabetes, recruited from 2007 to 2010. In the present investigation, data on 2679 subjects with complete urine 8-oxodG, 8-oxoGuo and creatinine concentrations, as well as plasma creatinine concentrations were available. Age, sex, height, and weight were recorded for all subjects at study entry.

The study was conducted in accordance with the Declaration of Helsinki, and informed consent was obtained from each patient. Study approval for sub-studies on nucleic acid oxidation was granted on April 3, 2013 by the local ethics committee of the Region of Southern Denmark (S-20080097, amendment protocol 37831) and reported to the Danish Data Protection Agency. The Vejle Diabetes Biobank Cohort database contains clinical biochemistry data (i.e., plasma creatinine), analyzed at the Department of Clinical Biochemistry, Vejle Hospital, by

ISO accredited methods by The Danish Accreditation System (DANAK). The data was transferred to Statistics Denmark, and all analyses were performed on servers housed by Statistics Denmark with an encrypted personal identifier and linked with register data (e.g., the National Death Register). Data can be analyzed on an individual level at the secure servers, but only aggregated data on groups can be exported from the servers, to secure individual data protection. By Danish law, studies on such register data on secure servers in Statistics Denmark are characterized as register research, and do not require further ethical or Data Protection Agency permissions or approvals. The urinary 8-oxodG and 8-oxoGuo concentrations were analyzed by ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) methodology as previously reported [6,27,28]. Vital status was assessed using the National Death Registry on December 31, 2016.

2.1. Physiologically-based calculations

The oxidized nucleosides, 8-oxodG and 8-oxoGuo, are chemically similar to creatinine (crea) that is routinely used in the clinic as a measure of kidney function. They are small water-soluble molecules that are excreted by glomerular filtration in the kidneys and are assumed to not be reabsorbed. This means that the ratio of the concentrations of these substances in urine (U) and plasma (P) are identical:

$$P(8\text{-oxodG})/U(8\text{-oxodG}) = P(\text{crea})/U(\text{crea}) \text{ or } P(8\text{-oxodG})/P(\text{crea}) = U(8\text{-oxodG})/U(\text{crea}) \quad (1)$$

The GFR in the kidneys multiplied by plasma concentration of 8-oxodG estimates 8-oxodG excretion by the kidneys into urine; defined units are mL/min for GFR and the preferred unit is nmol/L for plasma and urine concentrations of the oxidized guanine nucleoside. The 24-h urinary excretion, U(oxodG24), is with appropriate units given by

$$U(\text{oxodG24}) = \text{GFR} * P(8\text{-oxodG}) \quad (2)$$

By combining equations (1) and (2), the 24-h urinary excretion of an oxidized guanine nucleoside can be calculated as

$$U(\text{oxodG24}) = \text{GFR} * U(8\text{-oxodG}) * P(\text{crea}) / U(\text{crea}) \quad (3)$$

GFR can be directly estimated by insulin clearance, or alternatively, by Cr-EDTA clearance [29]. However, both are rather cumbersome techniques and are not suited for epidemiological research. In the clinical setting, GFR is routinely estimated from plasma creatinine, sex, and age, thereby yielding an estimated GFR (eGFR) [30] with units mL/min per 1.73 m² from which, a person's GFR can be calculated if race, height, and weight are known. In the present study, we calculated the 24-h GFR, UoxodG24, UoxoGuo24, the U(8-oxodG)/U(crea) ratio, and the U(8-oxoGuo)/U(crea) ratio for all individuals, with appropriate correction factors to provide correct identical units.

2.2. Statistical analyses

All data manipulations and statistical analyses were performed with the statistical software, R version 3.5.0 [31]. Correlations were estimated as Pearson's product-moment correlation and linear regression was performed by the lm function. All-cause mortality (package surv and prodlim) was analyzed by the Kaplan-Meier method and Cox regression analysis was used to analyze the association between UoxodG24, the U(8-oxodG)/U(crea) ratio, and death of all causes. The model was adjusted for age and sex. The level of significance was set at 5%. Calculations for 8-oxoGuo were similarly performed. A separate Cox regression analysis of GFR quartiles and mortality was performed with sex and age as covariates.

Table 1

Baseline values: mean (SD) for the quartiles of 24-h calculated urinary excretion of 8-oxodG (upper part), and quartiles of spot urine 8-oxoGuo/crea (lower part), in 2679 patients with type 2 diabetes. Values are expressed as mean (SD) or N(%).

		24-h Quartile 1 (n = 670)	24-h Quartile 2 (n = 670)	24-h Quartile 3 (n = 669)	24-h Quartile 4 (n = 670)	Total (n = 2679)	p-value
8-oxodG	nmol/mmol creatinine	2.1 (0.5)	2.6 (0.5)	3.0 (0.6)	4.1 (1.7)	3.0 (1.2)	< 1e-04
24-h 8-oxodG	nmol/24-h	19.2 (2.9)	25.6 (1.5)	31.8 (2.1)	46.6 (16.6)	30.8 (13.3)	< 1e-04
Age	years	63.9 (8.7)	63.4 (8.5)	63.5 (8.5)	62.1 (8.8)	63.2 (8.7)	0.0018
Urinary creatinine	mmol/L	8.3 (4.4)	8.5 (4.3)	8.6 (4.4)	8.2 (4.3)	8.4 (4.3)	0.30
P_CREA	micromol/L	90.1 (58.2)	82.2 (22.2)	82.1 (22.2)	82.3 (27.0)	84.2 (35.9)	< 1e-04
eGFR	mL/min/1.73m ²	74.8 (21.1)	80.0 (17.0)	82.2 (16.6)	84.6 (17.3)	80.4 (18.4)	< 1e-04
BMI	kg/m ²	24.3 (4.2)	25.2 (4.2)	26.7 (4.5)	28.6 (5.5)	26.2 (4.9)	< 1e-04
Smoking (missing = 6)	Never (n,%)	242 (36.1)	247 (36.9)	212 (31.7)	200 (29.9)	901 (33.6)	
	no smoke 6 month	294 (43.9)	272 (40.6)	317 (47.4)	334 (49.9)	1217 (45.4)	
	daily smoker	117 (17.5)	124 (18.5)	110 (16.4)	104 (15.5)	455 (17.0)	
	occasional smoker	17 (2.5)	23 (3.4)	30 (4.5)	30 (4.5)	100 (3.7)	
Hba1c (missing = 10)	%	7.0 (1.0)	7.0 (1.0)	7.0 (1.0)	7.1 (1.2)	7.0 (1.1)	0.034

		Spot Quartile 1	Spot Quartile 2	Spot Quartile 3	Spot Quartile 4	Total (n = 2679)	p-value
8-oxoGuo	nmol/mmol creatinine	1.9 (0.2)	2.5 (0.1)	3.1 (0.2)	4.0 (1.6)	3.0 (1.2)	< 1e-04
24-h 8-oxoGuo	nmol/24-h	21.3 (4.7)	27.0 (5.4)	31.5 (7.1)	43.4 (18.3)	30.8 (13.3)	< 1e-04
Age	years	60.6 (9.4)	63.1 (5.4)	64.5 (7.8)	64.6 (8.2)	63.2 (8.7)	< 1e-04
Ucrea_all	mmol/L	9.8 (4.7)	8.9 (4.4)	8.0 (4.1)	6.9 (3.7)	8.4 (4.3)	< 1e-04
P_CREA	micromol/L	92.2 (55.3)	84.8 (25.8)	81.3 (23.0)	78.3 (28.1)	84.2 (35.9)	< 1e-04
eGFR	mL/min	79.6 (20.3)	80.0 (17.8)	80.0 (17.1)	81.9 (18.4)	80.4 (18.4)	0.092
BMI	kg/m ²	26.19 (4.5)	26.3 (4.6)	26.1 (5.1)	26.3 (5.4)	26.2 (4.9)	0.78
Smoking (missing = 6)	Never (n,%)	210 (31.3)	213 (31.8)	230 (34.4)	248 (37.0)	901 (33.6)	
	no smoke 6 month	325 (48.5)	301 (44.9)	295 (44.1)	296 (44.2)	1217 (45.4)	
	daily smoker	118 (17.6)	125 (18.7)	117 (17.5)	95 (14.2)	455 (17.0)	
	occasional smoker	17 (2.5)	30 (4.5)	25 (3.7)	28 (4.2)	100 (3.7)	
Hba1c (missing = 10)	%	7.1 (1.0)	7.0 (1.1)	7.0 (1.1)	7.0 (1.2)	7.0 (1.1)	0.088

3. Results

On December 31, 2016, 499 (18.3%) deaths occurred since the study's inception. Median observation time was 9.2 yr (range 14 days–11 yr). Baseline characteristics of the 2679 subjects with type 2 diabetes and their complete data are given in Table 1.

A formal test of the differences between nucleotide levels in the quartile groups based on the spot urine method versus the 24-h calculated values was not performed because of their differences in units. However, nominally, the quartile groups had only minor differences in other variables. The correlation between the calculated 24-h urinary excretion of 8-oxodG and the 8-oxodG/creatinine ratio was 0.86 (95% confidence interval: 0.85–0.87, $p < 0.05$); the corresponding correlation for 8-oxoGuo was 0.84 (95% confidence interval: 0.83–0.85, $p < 0.05$). Fig. 1 shows the individual data and regression line of the spot urine measurements versus the 24-h calculated excretion for 8-oxodG and 8-oxoGuo.

An international inter-laboratory standardization [32] reported values for 24-h urinary excretions of 8-oxodG that agree with the calculated 24-h excretion presented in Table 1.

To determine whether the calculated 24-h urinary values for 8-oxoGuo are consistent with published values, we searched PubMed. As shown in Table 2, average values for 8-oxoGuo excretion align with published values. Besides our lab, only one was found to use chromatographic methodology and report 8-oxoGuo in 24-h urine.

To evaluate the performance or proficiency of the calculated 24-h urinary excretion, we performed survival analyses comparing the quartiles of the 2679 observations of the 24-h calculated data and spot urine data. For the 8-oxodG spot urine data and the calculated 24-h urinary excretion of 8-oxodG, the same tendency was found for the association between high values and high survival (survival plots are not shown). Table 3 contains the results of the Cox regression survival analyses. A similar survival analysis on a sub-dataset (age 60 and

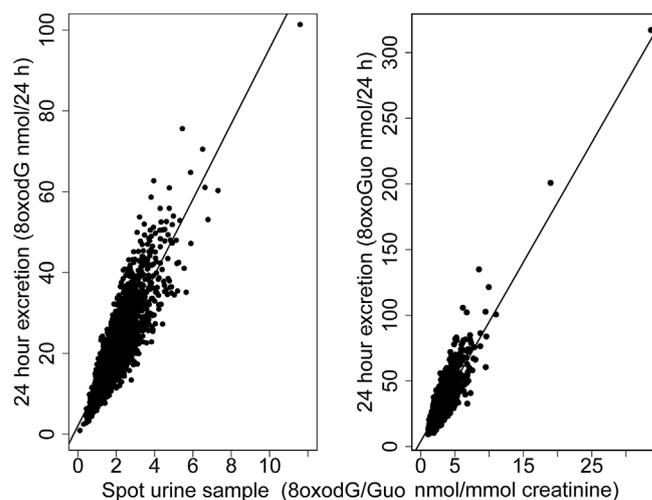


Fig. 1. Left panel contains the plot of the individual data for 2679 patients with type 2 diabetes: the oxidized nucleoside 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) by the spot urine method (nmol 8-oxodG/mmol creatinine) versus the calculated 24-h urinary excretion in nmol. Right panel contains values for the oxidized RNA nucleoside 8-oxo-7,8-dihydroguanosine (8-oxoGuo). The straight line indicates the regression line.

higher) with shorter observation time has been reported previously [6], with a focus on death from cardiovascular disease.

Because of the clear association between mortality and high urinary excretion of 8-oxoGuo in spot urine level corrected by creatinine [6], we performed similar survival analyses with 8-oxoGuo quartiles.

The association between death of all causes and the 24-h calculated data and spot urine for the 8-oxoGuo data is given in Table 3 as hazard ratios (HRs) from Cox regression analysis, with age and sex as

Table 2
Published urinary 24-h values of 8-oxoGuo excretion.

	8-oxoGuo nmol/24-h	N	M/F
Present study	30.8 (sd = 13.2)	2679	1626/1053
Exercise [44] ^a	30.2 (sd = 3.6)	11	11/0
Obesity with/without hypertension [9]	40.6 (sd not given)	20	20/0
Normal males [45]	33.1 (sd = 10.0)	27	11/23

^a Calculated from data in the paper, sd assumed to be 12%.

covariates. The results obtained with the two methods are consistent, cumulative incidence curves in Fig. 2 are consistent, and the interpretation is identical to that of our previous study [6].

To evaluate the association between GFR and mortality, we constructed a sex and age adjusted Cox regression analysis of the quartiles (Q4, Q3, Q2, Q1) of GFR and death of all causes, with Q4 (highest GFR) as reference: HR Q3 0.92 (95% CL: 0.66–1.27, $p = 0.60$); HR Q2 0.91 (95% CL: 0.66–1.27, $p = 0.59$); HR Q1 1.69 (95% CL: 1.59–2.32, $p < 0.001$). The result demonstrates that an association only existed in Q1 GFR quartile (i.e., only severely reduced kidney function is associated with death of all causes, as one would expect).

4. Discussion

Oxidative stress has been advocated as an important mechanism in many diseases and in aging. The basic chemical and biological processes related to oxidative stress have been well characterized; however, translation into clinical use is limited.

In this study, we derived a physiological-based model to calculate the 24-h urinary excretion of 8-oxodG from the urinary concentrations of 8-oxodG and creatinine, plasma concentration of creatinine and the eGFR from sex, age, height, and weight. This was extended to the corresponding RNA lesion in guanine, 8-oxoGuo. Furthermore, we could estimate the expected plasma concentration of the oxidized nucleosides that has not been presently established.

Table 3
Cox regression survival analyses of 8-oxodG and 8-oxoGuo in quartiles.

	Spot urine method			Calculated 24-h excretion		
	HR	CI.95	p-value	HR	CI.95	p-value
Oxidatively generated DNA modification (8-oxodG)						
Quartile 1	Ref			Ref		
Quartile 2	0.78	[0.61; 0.99]	0.039	0.83	[0.65; 1.05]	0.12
Quartile 3	0.62	[0.48; 0.80]	< 0.001	0.58	[0.45; 0.75]	< 0.001
Quartile 4	0.84	[0.66; 1.06]	0.13	0.72	[0.57; 0.93]	0.010
Sex (ref = female)	1.83	[1.50; 2.24]	< 0.001	2.01	[1.64; 2.47]	< 0.001
Age < 49	Ref			Ref		
Age 50-59	2.96	[1.35; 6.51]	0.0069	2.82	[1.28; 6.20]	0.0010
Age 60-69	5.77	[2.72; 12.27]	< 0.001	5.38	[2.53; 11.43]	< 0.001
Age > 70	13.23	[6.23; 28.09]	< 0.001	11.96	[5.62; 25.43]	< 0.001
Oxidatively generated RNA modification (8-oxoGuo)						
Quartile 1	Ref			Ref		
Quartile 2	1.09	[0.83; 1.45]	0.53	0.98	[0.74; 1.28]	0.86
Quartile 3	1.21	[0.92; 1.60]	0.17	1.12	[0.86; 1.47]	0.39
Quartile 4	2.00	[1.54; 2.59]	< 0.001	1.52	[1.17; 1.97]	0.0015
Sex (ref = female)	2.09	[1.70; 2.56]	< 0.001	1.69	[1.38; 2.09]	< 0.001
Age < 49	Ref			Ref		
Age 50-59	2.80	[1.27; 6.16]	0.010	3.00	[1.37; 6.60]	0.0062
Age 60-69	5.35	[2.52; 11.37]	< 0.001	5.97	[2.81; 12.69]	< 0.001
Age > 70	11.50	[5.40; 24.45]	< 0.001	13.78	[6.49; 29.27]	< 0.001

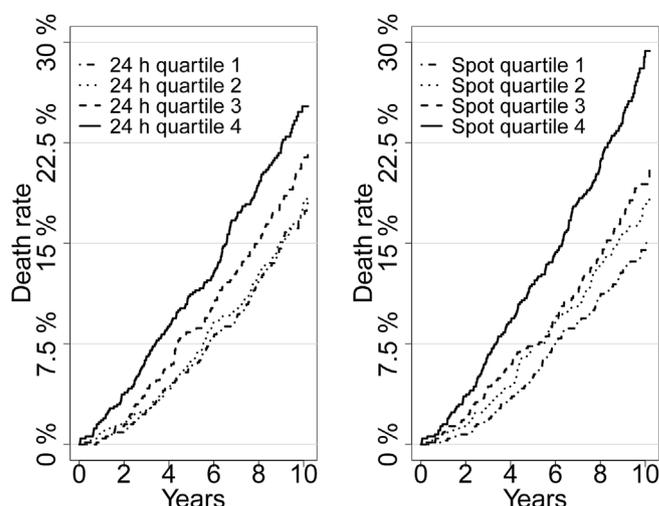


Fig. 2. Cumulative incidence (death of all causes); left hand panel is spot urine data as quartiles of 8-oxoGuo/creatinine, nmol/mmol, while right hand panel is the quartiles of the calculated 24-h urine excretion of 8-oxoGuo, nmol/24-h. Y-axis is the cumulative death rate for death from all cause.

With the 8-oxoGuo 24-h excretion values, we estimated that the model closely aligns with data from ours and other researchers 24-h urine collections, thereby validating our results. A high correlation also existed between the calculated 8-oxodG 24-h excretion and urinary 8-oxodG/creatinine ratio, the latter is the most used marker for the 24-h urinary excretion of 8-oxodG. We also found high consistency between spot urine data and the calculated 24-h excretion of 8-oxoGuo. Previously, we reported a high correlation between the measured 24-h excretion and this ratio for 8-oxodG. Thereafter, by using the data from a large cohort of subjects with type 2 diabetes, we compared survival analyses data using the quartiles of spot urine measurement and quartiles of the calculated 24-h excretion. As a result, consistency was identified with the previously-reported predictive value for death of all causes [6–8]. In addition, the HR estimates were found to be consistent

for both nucleosides. Taken together, we concluded that the physiological model is valid, and the three methods for estimating “oxidative stress” are similar and interchangeable.

Kasai and Floyd [1,2] reported the use of liquid chromatography coupled with electrochemical detection for 8-oxodG analysis in tissue DNA. Our group described the application of 3-dimensional liquid chromatography with electrochemical detection to investigate urinary excretion of 8-oxodG in humans and revealed that tobacco smoke increased the urinary excretion of 8-oxodG [12]. Thereafter, we developed the first validated method based on liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) to measure urinary excretion of 8-oxo guanine species [28]. An enzyme-linked immunosorbent assay (ELISA) methodology is commercially available, but it is not specific to discriminate between the 8-oxidized DNA and RNA guanine nucleotides based on the manufacturer, Cayman Chemicals manual. We do not recommend the use of ELISA as its lack of specificity creates failure to detect the increased urinary excretion of 8-oxodG in a meta-analysis of 1172 smokers versus 2496 non-smokers [33]. Others have reported that ELISA does not allow definitive identification and quantification of 8-oxodG [34]. To advance the translational process, clinically-useful, reliable and reproducible methods must be developed.

Our physiological model is generally valid and can be used on any substance (e.g., other oxidized nucleic acid breakdown products) that are treated similar in the kidneys. More interestingly, the model provides a means to estimate 24-h excretion from a single plasma concentration of 8-oxoGuo, when GFR can be estimated from age, sex, height, weight, and race and the plasma concentration of creatinine. Presently, eGFR is routinely provided by clinical chemistry laboratories when plasma or serum creatinine concentration is requested. Thus, in the clinical setting, oxidative stress can be estimated from a single blood sample as the product of 8-oxoGuo concentration and GFR, or when age, height, weight, sex, and race are unknown plus the use of eGFR.

The measure of “oxidative stress to DNA and RNA” by urinary excretion of 8-oxodG and 8-oxoGuo has limitations. Instead, it is suited to investigations where one can assume that many cells have increased oxidative stress (e.g., in diabetes [6], severe psychiatric disease [35], haemochromatosis [36], obesity [9], and following drug treatment [37]). For a disease with localized oxidative stress in a small organ (e.g., prostate), it's 8-oxodG and 8-oxoGuo excretion would be minute and undetectable because of the large contribution from the remaining body, even if considerably elevated.

Urinary excretion does not allow for the identification of organ-specific increase in oxidative stress and cannot identify whether oxidation occurs in the nucleotide pool or in the nucleic acid molecule. Therefore, it is mainly a measure of generalized intracellular oxidative stress.

Considering only 8-oxodG or 8-oxoGuo levels in plasma and urine may result in incomplete estimation of oxidatively modified guanine components since 8-oxo-7,8-dihydroguanine (8-oxoGua), the expected released product from DNA through the base excision repair (BER) pathway has been shown to be present in much higher amounts than 8-oxodG in urine [38–41].

There are several reports using a single plasma 8-oxodG value as a marker of “oxidative stress to nucleic acids”. We did not find this approach valid as plasma concentration is defined as the sample of the balance between input and output to plasma. Thus, it is determined mainly by kidney function and similar to creatinine concentration, it is a measure of kidney function.

Our physiologically-based model can be used to estimate the 24-h urinary excretion of 8-oxodG from a blood sample and estimate GFR using current standard methods with clinical variables. The values reported by the ELISA method ranged from 54 pM [42] to 3,382,415 pM [43] (values recalculated to pM) – a range that is greater than 50,000 times, clearly demonstrating the failure of ELISA. With a GFR of 144 L/

24-h (100 ml/min) and a urinary excretion of 8-oxodG of 33.6 nmol/24-h, the expected plasma value of 8-oxodG is 200 pmol/whereas with an 8-oxoGuo 24-h urinary excretion of 33.6 nmol/24-h, its expected plasma value is 233 pmol/L by equation (2). Presently, plasma concentrations of the oxidized nucleosides cannot be measured because a reliable and reproducible method for measuring plasma concentration has yet to be developed.

Besides a validated analytical method and calculation, the assumptions for estimating GFR must be met. In certain groups of patients, this can be challenging as the commonly used creatinine is dependent on muscle mass and thus, in patients with either abnormally high or abnormally low muscle mass or individuals undergoing major physiological changes, such as children, alternative approaches might be necessary. For instance, Cystatin C, which is independent of muscle mass, can be applied to estimate GFR.

In conclusion, we present data validating the potential to equally measure oxidative stress to nucleic acids via three ways:

- 1 A urine spot sample corrected with urinary creatinine
- 2 A 24-h urine collection
- 3 A single plasma sample and an estimated or measured GFR

These equal and alternative methods contribute to the measurement of “oxidative stress to nucleic acids”. To add, they can be readily applied to RNA for large-scale epidemiological studies and in clinical settings, such as for the identification of patients with type 2 diabetes with low risk for complications, intensive treatment, and follow-up, as well as those with a high risk and could be candidates for intensive treatment, follow-up, and participation in clinical trials. In particular, estimation from a single plasma sample represents a novel approach that can easily be applied to large scale epidemiological studies, present plasma biobanks, as well as in clinical settings to identify patients with type 2 diabetes, especially those at high risk.

Contributions

MF devised the physiological model; all authors discussed and planned the study. HEP performed the statistical calculations and drafted the initial manuscript. All authors revised and approved the final manuscript.

Declaration of competing interest

None of the authors have conflicting interest.

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