

RESEARCH ARTICLE

Short-term biological variation and Reference change values of urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine and 8-oxo-7,8-dihydroguanosine

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Abstract

Aim: The DNA and RNA oxidative damage products urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-odGsn) and 8-oxo-7,8-dihydroguanosine (8-oGsn) have potential use in clinical practice. However, biological variation (BV) and reference change values (RCVs) have not been established. The aim of this study was to establish the short-term between-subject $BV(CV_G)$, within-subject $BV(CV_I)$, and RCVs for urinary 8-odGsn and 8-oGsn.

Methods: First-morning midstream urine specimens were collected from 20 apparently healthy subjects (ten males and ten females) on five consecutive days. 8-odGsn and 8-oGsn were measured using LC-MS/MS, while urine creatinine (U-Cr) was also measured to correct their results. A two-level nested ANOVA was used to estimate the CV_I and CV_G .

Results: The values of CV_G for 8-odGsn, 8-odGsn/U-Cr, 8-oGsn, and 8-oGsn/U-Cr were 31.2%, 39.6%, 35.3%, and 28.8%, respectively, while CV_I for them were 40.5%, 9.0%, 33.5%, and 12.1%, respectively. The RCVs for 8-odGsn, 8-odGsn/U-Cr, 8-oGsn, and 8-oGsn/U-Cr were 112.5%, 26.7%, 93.7%, and 36.5%, respectively.

Conclusion: BV and RCVs were firstly established for 8-oxo-dGsn and 8-oGsn, and can be used in clinical practice.

KEYWORDS

8-oxo-7,8-dihydro-2'-deoxyguanosine, 8-oxo-7,8-dihydroguanosine, biological variation, oxidative damage biomarkers, reference change values

1 | INTRODUCTION

Before a biomarker can be used in clinical practice, two important factors are needed to determine if the change in the value of the biomarker is physiologically significant: biological variation (BV)

and the reference change value (RCV).¹⁻³ In the past few years, a lot of work has been done on establishing BV for biomarkers used in clinical practice,⁴⁻⁷ and European Society of Clinical Chemistry and Laboratory Medicine has formulated the standard for evaluating BV data.^{5,8}

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BV, including between-subject BV (CV_G) and within-subject BV (CV_I), has many uses in laboratory medicine, including analytical quality specifications for imprecision, bias, and total allowable error.^{3,9} A common use of CV_I is to calculate the RCV, which can provide an objective tool for assessment of the significant difference in serial results from an individual.² Though the use of conventional population-based reference intervals (RIs) has many advantages in the interpretation of numerical results in diagnosis, case-finding, and screening, it has limited utility in evaluating the results of an individual since many individuals may have results that are either highly unusual or indicate significant changes for them, but still lie within conventional RIs.^{2,9} The RCV has the advantage of determining whether a change in a biomarker in consecutive samples from an individual represents a physiological change, while index of individuality (II), which can be used to evaluate the suitability of population-based RIs,¹⁰ and II can be used to assess the utility of conventional reference values based on population or RCV. Additionally, objective performance limits are fundamental requirements for the evaluation and effective control of laboratory systems;¹¹ hence, it is important to set APS for a biomarker before it can be used in the clinical laboratory. Using BV data to estimate APS is one of the most commonly used methods to set APS in clinical practice; to ensure test results are of the high quality required for patient care, accrediting organizations require laboratories to establish analytical performance goals (APS).¹¹

However, to our knowledge, there has been no report on the BV or RCV of urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-odGsn) and 8-oxo-7,8-dihydroguanosine (8-oGsn) until now; neither have APS including analytical imprecision and bias been set. 8-odGsn and 8-oGsn are the oxidative products of DNA and RNA, and they have been reported to be linked with various diseases such as diabetes mellitus,¹²⁻¹⁴ Alzheimer's disease,¹⁵ kidney disease,^{16,17} and liver injury.¹⁸ Furthermore, 8-odGsn and 8-oGsn have shown age-dependent accumulation and therefore have been considered novel biomarkers of biological aging in humans.^{19,20} To promote the clinical use of 8-odGsn and 8-oGsn, it is necessary to establish BV and RCV for them; the aim of this study was to derive CV_G , CV_I , and the RCV, as well as setting APS, for urinary 8-odGsn and 8-oGsn, to ensure its future use in clinical practice.

2 | MATERIALS AND METHODS

2.1 | Subjects and samples

The subjects were 20 apparently healthy volunteers, 10 men, aged 23-66 years, and 10 women, aged 27-64 years. All subjects were told to maintain their normal lifestyles, which did not include severe exercise, nor did any have a terrible cold throughout the study. All subjects were without systemic diseases, such as acute or chronic infections, digestive diseases, kidney disease, metabolic and nutritional disease, rheumatic diseases, endocrine disease, or circulatory system diseases by performing an enrollment questionnaire.

Once a day for five consecutive days, the first midstream specimen of urine in the morning between 5:30 AM and 7:30 AM was collected in a clean-catch white cap tube. The urine was then separated and stored frozen at -70°C until analysis in less than ten days. After all the samples were collected, assays were performed as one batch to avoid inter-batch variation.

The study was reviewed and approved by the Ethics Committee of Peking Union Medical College Hospital (S-T483). All studied individuals were informed in writing of the intended use of their samples and each provided written consent.

2.2 | Analytical methods

8-odGsn and 8-oGsn were analyzed using LC-MS/MS using a Waters Xevo TQ-XS mass spectrometer (Milford, MA, USA) by modifying the method of Gan et al.¹⁹ Generally, after the urine samples were thawed, mixed, and equilibrated to room temperature ($24-26^{\circ}\text{C}$) for 2 hours, 50 μL of urine sample, quality control solution, or calibrating solution was added into a well of a 96-well plate, and then 20 μL of combined isotope-labeled internal standards (8-odGsn- d_3 and 8-oGsn- d_3) and 430 μL ultra-pure water were added to each sample, resulting in a ten-fold dilution. Ten microliters of the diluted samples were transferred into the LC-MS/MS platform. The chromatographic separation was performed using a Waters ACQUITY UPLC BEH C18 column (2.1×50 mm, $1.7 \mu\text{m}$). The mobile phase A was comprised of water with 0.1% formic acid, while mobile phase B was methanol. Using a flow rate of 0.4 mL/min, the gradient was as follows: 0-1 minutes: 1% B; 1-2.5 minutes: 1-3% B; 2.5-3.5 minutes: 3-10% B; 3.5-4.5 minutes: 50% B, 4.5-5 minutes: 50-1% B. The MS/MS detection was operated in the positive electrospray ionization mode, and the multiple reaction monitoring mode used for each analyte was m/z 284.1-168 (8-odGsn), 287.1-171 (isotope-labeled internal standard for 8-odGsn), 300-168 (8-oGsn), and 303-171 (isotope-labeled internal standard for 8-oGsn). Other MS parameters included a cone voltage of 40 V, capillary voltage of 1 kV, desolvation temperature of 600°C , flow of 1100 L/h, nitrogen desolvation gas, and argon collision gas. A representative chromatogram of the LC-MS/MS is shown as Figure S1.

As urinary 8-odGsn and 8-oGsn levels are often affected by drinking water, the values can be corrected by urine creatinine (U-Cr). Therefore, when calculating the values of BV, their ratios with U-Cr were also calculated. U-Cr was measured using an automated chemistry analyzer (Beckman Coulter AU2700, Beckman Coulter; Brea, CA, USA). All samples were measured twice, and the duplicate results were used to calculate CV_I and CV_G .

2.3 | Statistical analysis

Linear regression analysis was performed to identify whether the concentrations of the analytes for each individual were in a steady state.²¹ 95% CI of the slope that did not include the zero was

considered as drift exists. Individuals with a consistent increasing or decreasing trend were excluded. A double-sided Grubbs test was performed to detect and eliminate outlier values among all results, as well as for replicates and subjects. A Shapiro-Wilk test was used to verify the normal distribution of the total samples, while Levene's test for homogeneity of variance was performed to evaluate the homogeneity of variance for each subject. The data that did not have a normal distribution were log-transformed before analysis. The differences between 8-odGsn and 8-oGsn and their ratio with U-Cr in the male and female subgroups were examined using the Student t test. A two-level nested ANOVA was used to estimate the CV_I and CV_G ,³ while confidence intervals (CIs) of BVs were calculated using the method of Burdick et al.²²

The index of individuality (II), which was used to evaluate the suitability of population-based RIs, was calculated as follows: $II = CV_I/CV_G$.¹⁰

RCVs were calculated using the formula $RCV = 2^{0.5} \times Z \times (CV_A^2 + CV_I^2)^{0.5}$, where Z is the number of standard deviations appropriate to the desired probability (here we used Z-scores of 1.96 for $P < .05$, ie, probabilities at 95%), and analytical CV (CV_A) was calculated from the duplicate measurements for the samples.

APS for analytical imprecision (CVaps) and analytical bias (Bias) were estimated using the following formulas²³: $CVaps = CV_I/2$; $Bias = 0.25(CV_I^2 + CV_G^2)^{0.5}$.

3 | RESULTS

3.1 | Subject demographics

Table 1 summarizes the demographics, mean levels of 8-odGsn and 8-oGsn, and the U-Cr corrected value at the initial visit. Linear regression analysis for the ratios of 8-odGsn/U-Cr and 8-oGsn/U-Cr showed that all individuals were in a relatively steady-state situation (line charts with markers are presented in Figure S2); thus, no individuals were excluded because of a consistent increasing or decreasing trend. The double-sided Grubbs test for 8-odGsn/U-Cr and 8-oGsn/U-Cr showed no outlier values among the results (Figure 1).

3.2 | Short-term biological variation

CV_G for 8-odGsn, 8-odGsn/U-Cr, 8-oGsn, and 8-oGsn/U-Cr were 31.2% (20.1%-49.5%), 39.6% (30.0%-58.0%), 35.3% (24.9%-54.0%), and 28.8% (21.6%-42.5%), respectively, while the corresponding

CV_I values were 40.50% (35.1%-47.9%), 9.0% (7.8%-10.7%), 33.5% (29.0%-39.6%), and 12.1% (10.4%-14.3%), respectively (Table 2). Females had higher mean CV_I values than males for all monitored analytes, but the difference was not significant considering their confidence interval(CI) overlaps. Generally, the mean CV_I values were much smaller for 8-odGsn/U-Cr and 8-oGsn/U-Cr than for results that were not corrected by U-Cr, however, with overlapped CI; besides, mean CV_G values corrected by U-Cr were not always smaller than those results corrected by U-Cr. The mean CV_G for 8-odGsn/U-Cr was a little higher than that of 8-odGsn, possibly because the mean CV_G for U-Cr in females was very high.

3.3 | Application of biological variation

3.3.1 | II and RCV

The II values for 8-odGsn/U-Cr and 8-oGsn/U-Cr were <0.6 (Table 2) among all subjects, in both males and females. In contrast, for 8-odGsn and 8-oGsn, the II values were all higher than 0.6 but lower than 1.4; hence, the RCVs were suitable for evaluating whether the changes in 8-odGsn/U-Cr and 8-oGsn/U-Cr for an individual were abnormal.

Using duplicate measurements from the 20 individuals, the CV_A values were calculated for 8-odGsn, 8-odGsn/U-Cr, 8-oGsn, and 8-oGsn/U-Cr were 2.5%, 3.0%, 4.8%, and 5.3%, respectively, and the RCVs were calculated from the CV_A results (Table 2).

3.3.2 | Estimation of APS

The desirable APS showed lower values of CVaps and Bias for 8-odGsn/U-Cr and 8-oGsn/U-Cr than for the corresponding values not corrected by U-Cr (Table 2). All of the analytical CV_A values derived from duplicate measurements from the 20 individuals in this study met the desirable goals for CVaps.

4 | DISCUSSION

It has been reported that oxidative damage to DNA occurs earlier than damage to proteins and lipids, and RNA is more vulnerable to oxidative stress than DNA because it is single-stranded and lacks protective histones.^{14,16} Damage to DNA and RNA can cause transversion-type mutations and transcriptional errors that

TABLE 1 Demographics and initial levels of 8-odGsn and 8-oGsn

Individuals	N	Mean age (Range)	8-odGsn ($\mu\text{mol/L}$)	8-odGsn/U-Cr ($\mu\text{mol/mol}$)	8-oGsn ($\mu\text{mol/L}$)	8-oGsn/U-Cr ($\mu\text{mol/mol}$)
All	20	41.3 (23-66)	0.021 \pm 0.012	1.86 \pm 0.81	0.022 \pm 0.010	1.95 \pm 0.62
Male	10	41.0 (23-66)	0.016 \pm 0.006	1.47 \pm 0.36	0.022 \pm 0.009	1.94 \pm 0.62
Female	10	41.5 (25-64)	0.025 \pm 0.014	2.24 \pm 0.96	0.022 \pm 0.011	1.97 \pm 0.65

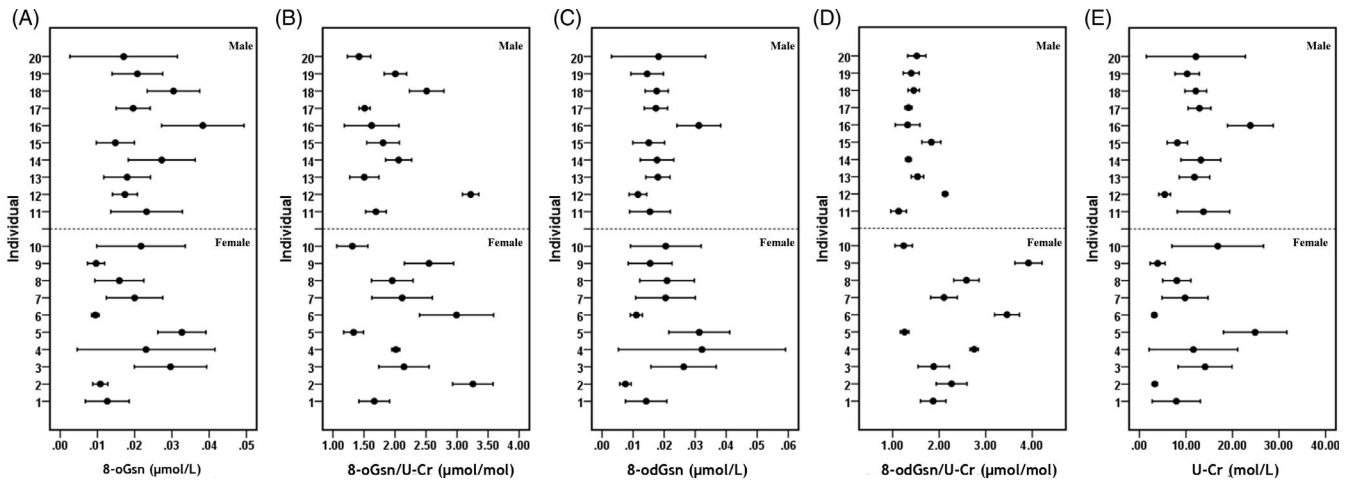


FIGURE 1 Short-term concentrations of biomarkers. A, 8-oGsn, (B) 8-oGsn/U-Cr, (C) 8-odGsn, (D) 8-odGsn/U-Cr, and (E) U-Cr Data are presented as the 95% confidence interval for the mean for each individual. For each panel, individuals below the divider are female, and those above the divider are male

TABLE 2 Short-term BV and application of BV

	Subjects/ Samples	CV_A (%) [†]	BV		II	RCV (%)	Desirable specifications of Analytical Performance	
			CV_I (%)	CV_G (%)			CVaps (%)	Bias (%)
Total								
8-oGsn	20/100	4.8	33.5 (29.0-39.6)	35.3 (24.9-54.0)	0.95	93.7	16.7	12.2
8-oGsn/U-Cr	20/100	5.3	12.1 (10.4-14.3)	28.8 (21.6-42.5)	0.42	36.5	6.0	7.8
8-odGsn	20/100	2.5	40.5 (35.1-47.9)	31.2 (20.1-49.5)	1.30	112.5	20.3	12.8
8-odGsn/U-Cr	20/100	3.0	9.0 (7.8-10.7)	39.6 (30.0-58.0)	0.23	26.7	4.5	10.2
U-Cr	20/100	1.6	37.4(32.4-44.2)	48.6 (35.2-73.2)	0.77	103.6	18.7	15.3
Female								
8-oGsn	10/50		38.0 (31.2-48.7)	41.4 (25.2-79.9)	0.92	106.3	19.0	14.1
8-oGsn/U-Cr	10/50		13.6 (11.1-17.4)	29.6 (19.9-54.9)	0.46	40.4	6.8	8.2
8-odGsn	10/50		46.5 (38.2-59.52)	35.4 (17.7-72.1)	1.31	129.1	23.3	14.6
8-odGsn/U-Cr	10/50		8.9 (7.3-11.4)	37.2 (25.4-68.2)	0.24	26.0	4.4	9.6
U-Cr	10/50		44.8 (36.8-57.3)	63.0 (40.5-119.0)	0.71	124.2	22.4	19.3
Male								
8-oGsn	10/50		29.7 (24.4-38.0)	29.5 (17.3-57.5)	1.01	83.4	14.9	10.5
8-oGsn/U-Cr	10/50		9.9 (8.1-12.6)	28.5 (19.3-52.5)	0.35	31.1	4.9	7.5
8-odGsn	10/50		30.9 (25.4-39.5)	25.9 (14.0-51.8)	1.19	85.9	15.5	10.1
8-odGsn/U-Cr	10/50		6.7 (5.5-8.6)	14.7 (9.8-27.1)	0.46	20.3	3.4	4.0
U-Cr	10/50		30.8 (25.3-39.4)	36.1 (22.4-69.2)	0.85	85.5	15.4	11.9

[†]Both male and female used the same analytical coefficient variation (CV_A) that was calculated from the total samples.

lead to diseases. 8-odGsn is by far the most studied DNA oxidative product, while 8-oGsn is the most studied RNA oxidative product. A significant amount of research has shown that 8-odGsn and 8-oGsn are closely linked with various age-associated pathological phenomena; in this small population study, 8-oGsn/Cr was also significantly correlated with age, with a correlation coefficient of 0.543 ($P < .05$), while for 8-odGsn/Cr, the correlation was 0.336

($P = .147$). These metrics have thus shown great potential for use in clinical practice.

Presenting correct BV is not an easy task,²⁴ and here we firstly established the BV data for 8-oGsn and 8-odGsn which may be used in the clinical practice. Determining the BVs of biomarkers is fundamental for setting objective performance limits, which are important for the evaluation and effective control of laboratory systems.¹¹ This

study first established short-term BV and then determined performance limits using the BV data. The 8-odGsn and 8-oGsn values without U-Cr correction are not commonly used because they may be significantly affected by drinking water or by diet. According to our results, females had higher 8-odGsn and 8-oGsn values than males which is consistent with previous report,¹⁹ and females had higher CV_I values than males for all of the monitored analytes, however, with overlapped CI Generally, the CV_I values for 8-oGsn/U-Cr and 8-odGsn/U-Cr were smaller than those results not corrected by U-Cr The 8-odGsn and 8-oGsn values corrected by U-Cr may be more suitable as biomarkers.

It has been reported that when the II is higher than 1.4, it means that the population-based RIs are suitable for determining abnormal values for an individual^{10,23}; when it is lower than 0.6, it means that the population-based RIs have limited capacity to evaluate whether the result for an individual is abnormal.^{10,23} According to our results, the II values for 8-odGsn/U-Cr and 8-oGsn/U-Cr were all <0.6, in both males and females. Hence, population-based RIs may not be suitable for evaluating abnormal values for 8-odGsn/U-Cr and 8-oGsn/U-Cr The RCV has the advantage of determining whether differences between two consecutive detection results of an indicator are significant, rather than being caused by analytical variations and individual biological variations.²³ Hence, the RCV may be more appropriate than population-based RIs for evaluating whether a change in consecutive results of 8-oGsn/U-Cr and 8-odGsn/U-Cr is caused by disease progression or improvement. According to our results, males had a relatively lower RCV than females for 8-odGsn/U-Cr and 8-oGsn/U-Cr If the variation value calculated between two consecutive results from one individual falls in the range of 26.3% (8-odGsn/U-Cr) and 36.5% (8-oGsn/U-Cr), respectively, the variation may be caused by intrinsic analytical variations and individual biological variations rather than pathological changes. After excluding analytical errors and instrument failure, and if the variation values fall outside the RCV for 8-odGsn/U-Cr and 8-oGsn/U-Cr, the change may be caused by disease progression or improvement.

APS include imprecision and bias. These are further divided into desirable, optimal, and minimum.^{1,11} Our methods used in this study met the desirable performance goals, and the established goals can be used in the future to set analytical goals in clinical practice.

A limitation of this study was that the frozen samples were not thawed at 37°C. A previous study²⁵ showed that when using frozen samples thawed at room temperature for 30 minutes followed by centrifugation, levels of 8-odGsn and 8-oGsn in the supernatant may be significantly underestimated; however, if the samples were thawed at room temperature followed by the addition of a fourfold volume of deionized water, the results for 8-odGsn and 8-oGsn were almost the same as those derived from fresh samples, with release efficiencies of 8-oGsn and 8-odGsn of 90%-109% and 87%-101%, respectively. In this study, samples were thawed at room temperature for about 2 hours and then mixed thoroughly. This may also increase the release of the oxidized nucleic acid products from urine precipitates. Another limitation of this study is the limited number

of subjects and the fact that BVs were not evaluated for individuals with various diseases, and though no significant variance was observed for different time point of each single individual, heterogeneity of variances existed for intra-subject; hence, the BV data may not be generalizable to the whole population, and further studies for evaluating the BV data with more samples and different healthy condition are needed.

5 | CONCLUSIONS

Short-term BVs, including CV_A and CV_G, were established for the oxidative damage products of DNA and RNA, 8-odGsn and 8-oGsn, and for their ratio with U-Cr 8-odGsn/U-Cr and 8-oGsn/U-Cr showed smaller BV compared with 8-odGsn and 8-oGsn without correction with U-Cr, and the results validated that 8-odGsn/U-Cr and 8-oGsn/U-Cr were better biomarkers than 8-odGsn and 8-oGsn without U-Cr correction. RCV and APS were established for 8-odGsn and 8-oGsn, as well as for their ratio with U-Cr, and may be used in future clinical practice to set performance goals for analytical instruments in the clinical laboratory.

ACKNOWLEDGMENTS

Thanks to Qianqian Liu and Jing Wen [Waters Technologies (Shanghai) Co., Ltd., Shanghai, China] for providing support in the establishment of the LC-MS/MS method.

CONFLICT OF INTERESTS

The funding organization played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication. The abstract has been presented as a poster in the EuroMedLab 2019 meeting, and the data has been recalculated in this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Zhang R, Yu S, Wang D, et al. Short-term biological variation and Reference change values of urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine and 8-oxo-7,8-dihydroguanosine. *J Clin Lab Anal*. 2020;34:e23522. <https://doi.org/10.1002/jcla.23522>