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How stable is oxidative stress level? An observational study of intra- and inter-individual variability in urinary oxidative stress biomarkers of DNA, proteins, and lipids in healthy individuals

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ABSTRACT

Oxidative stress in humans is affected by the health and nutritional status as well as exposure to external environmental factors. To evaluate the effects of external factors, an assessment of baseline levels as well as diurnal variations in oxidative stress status of healthy individuals is needed. In this study, we examined intra- and inter-individual variability of oxidative stress biomarkers (OSBs) of lipids (malondialdehyde [MDA] and four F_2-isoprostane isomers, namely, 8-isoprostaglandin$F_{2\alpha}$ [8-$PGF_{2\alpha}$], 11β-prostaglandin$F_{2\alpha}$ [11-$PGF_{2\alpha}$], 15(*R*)-prostaglandin$F_{2\alpha}$ [15-$PGF_{2\alpha}$], and 8-iso,15(*R*)-prostaglandin$F_{2\alpha}$ [8,15-$PGF_{2\alpha}$]); proteins (*o,o′*-dityrosine [diY]); and DNA (8-hydroxy-2′-deoxyguanosine [8-OHdG]) in urine from healthy individuals. The significance of creatinine correction, which is typically used to account for urinary dilution, on OSB concentrations was evaluated. Analysis of 515 urine samples, collected longitudinally from 19 healthy individuals daily for over a month, showed inter-individual coefficient of variation (CV) in concentrations from 112% for MDA to 272% for 15-$PGF_{2\alpha}$. Intra-individual CV in concentrations ranged from 29% for 8-OHdG to 149% for 15-$PGF_{2\alpha}$. MDA was the most abundant OSB found in urine. The intra- and inter-individual variability in F_2-isoprostane concentrations were higher than the values calculated for diY, 8-OHdG, and MDA. All seven OSB concentrations were significantly correlated with each other and with creatinine. Creatinine normalization of OSB concentrations improved predictability in OSB concentrations over time. Our results suggest that 8-OHdG, showing the highest ICC (0.96), yielded more reproducible measurements with a low CV, and is the most suitable biomarker of OSB in spot urine samples. The measured concentrations and diurnal variability in urinary OSB levels in healthy individuals reported in this study are useful as a benchmark for future toxicological and epidemiological studies.

1. Introduction

Oxidative stress has been an important health marker studied widely in epidemiological and toxicological studies. The extent of oxidative stress in humans is assessed mainly by a quantitative measurement of biomarkers in biospecimens, which can be compared against a baseline value. Oxidative stress biomarkers (OSBs) are produced when excess radical oxygen species (ROS) oxidizes biomolecules as DNA, lipids, and proteins. For OSBs to be useful in the assessment of health status of an individual, they must meet certain requirements: They should be specific products of ROS oxidation, stable and detectable in biological fluids of healthy individuals, and not significantly affected by changes in diet. Moreover, the biomarker concentration should be stable over time, with low within-individual variability (Il'yasova et al., 2012). Although the measurement of OSBs in public health surveillance programs is gaining importance, information regarding reference values and diurnal variability in OSB levels in healthy individuals are scarce. Such information is crucial if we are to establish benchmark values, for comparison among populations, as well as for the interpretation of OSB data in population-based epidemiological studies.

Excess ROS in an organism oxidizes biomolecules, such as lipids, yielding malondialdehyde (MDA) and F_2-isoprostanes; the oxidation of DNA results in 8-hydroxy-2′-deoxyguanosine (8-OHdG), and *o,o′*-dityrosine (diY) is a product of protein oxidation. The products of oxidation by ROS are excreted in measurable concentrations in urine, which can be determined as biomarkers of oxidative stress (Dalle-

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Donne et al., 2006; Il'yasova et al., 2010, 2012; Kadiiska et al., 2005; Lee et al., 2017; Orhan, 2007; Van't Erve et al., 2017; Wang et al., 2018). Although blood and saliva have been used in the analysis of OSBs, urine is a preferred matrix due to the non-invasiveness of sampling and those OSBs excrete primarily in urine. A few studies have examined variability in OSB concentrations in blood (Browne et al., 2008; Goldfarb et al., 2014; Kato et al., 2006) and saliva (Alajbeg et al., 2017; Lettrichova et al., 2016). However, variability in urinary concentrations of OSBs is less well known, especially in healthy individuals.

OSBs have been measured in studies that describe biological mechanisms of cancer, respiratory and cardiovascular diseases, and diabetes, to explain the role of oxidative stress in disease etiology (Ho et al., 2013; Lee et al., 2017; Van't Erve et al., 2017). Moreover, external environmental factors, such as exposure to environmental chemicals (phthalates, bisphenols, polycyclic aromatic hydrocarbons, and triclosan) (Asimakopoulos et al., 2016; Iyer et al., 2018; Kataria et al., 2017; Lu et al., 2016), exercise (Orhan et al., 2004) and tobacco smoke (Lowe et al., 2013), have been associated with urinary OSBs.

Very few earlier studies have measured a wide range of OSBs in a single specimen. We selected seven OSBs, namely, diY, MDA, 8-OHdG, and four F_2-isoprostane isomers, including 8-isoprostaglandin$F_{2\alpha}$ (8-$PGF_{2\alpha}$), 11β-prostaglandin$F_{2\alpha}$ (11-$PGF_{2\alpha}$), 15(R)-prostaglandin$F_{2\alpha}$ (15-$PGF_{2\alpha}$), and 8-iso,15(R)-prostaglandin$F_{2\alpha}$ (8,15-$PGF_{2\alpha}$), which are specific products of oxidation of proteins, DNA, and lipids for analysis in urine. Simultaneous determination of seven OSBs in urine samples, to establish an oxidative stress biomarker profile in healthy individuals, has not been accomplished prior to this study. A couple of studies have examined intra- and inter-individual variability in total urinary F_2-isoprostanes (Wu et al., 2010) and 8-OHdG (Miwa et al., 2004) but not MDA, diY, or individual F_2-isoprostane isomers. Typically, most environmental exposure studies that associate oxidative stress to health outcomes measured a single OSB.

We recently developed a method for simultaneous determination of seven major OSBs of lipids, proteins, and DNA in urine (Martinez and Kannan, 2018). Application of such robust methods enable comprehensive understanding of oxidative stress status of DNA, protein, and lipids and in the selection of most appropriate biomarkers of oxidative stress.

The data presented in this study will contribute to the knowledge of baseline levels of OSBs in healthy individuals, both as volumetric (ng ml^{-1} urine) and creatinine-corrected (μmol mol^{-1} creatinine) concentrations. The suitability of creatinine correction to report OSB levels in urine was explored. In addition, diurnal variability in OSB levels in urine samples collected daily for over a month was examined. Demographic and lifestyle factors, such as age, gender, diet, alcohol, or medications, can affect the levels of OSBs. The main objective of this study was to determine intra- and inter-individual variability in OSB concentrations in healthy individuals, while engaged in normal daily activities, by determining the levels of seven urinary OSBs of lipids, proteins, and DNA.

2. Materials and methods

2.1. Materials

o,o′-Dityrosine (diY) (> 99%) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). 8-hydroxy-2′-deoxyguanosine (8-OHdG) (≥98%), malondialdehyde tetrabutyl-ammonium salt, acetic acid, *n*-hexane, LC-MS grade methanol and water, acetonitrile, ethyl acetate, and synthetic urine were purchased from Sigma Aldrich (St. Louis, MO, USA). 8-isoprostaglandin $F_{2\alpha}$ (8-$PGF_{2\alpha}$) (> 99%), 15(R)-prostaglandin $F_{2\alpha}$ (15-$PGF_{2\alpha}$) (> 98%), 8-iso-15(R)-prostaglandin $F_{2\alpha}$ (8,15-$PGF_{2\alpha}$) (> 98%), 11β-prostaglandin $F_{2\alpha}$ (11-$PGF_{2\alpha}$) (> 98%), and the internal standards (IS) for F_2-isoprostanes, D_4-8-iso-prostaglandin $F_{2\alpha}$ (D_4-8-$PGF_{2\alpha}$) (> 99%), were purchased from Cayman Chemicals (Ann Arbor, MI, USA). 2,4-1,1,3,3-,tetraethoxypropane-1,3-d_2 (> 98%) (IS for MDA) was purchased from C/D/N isotopes (Point-Clair, Quebec, Canada). $^{13}C_{12}$-*o,o′*-dityrosine (IS for diY) and $^{15}N_5$-8-OHdG (IS for 8-OHdG) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Derivatization reagent 2,4-dinitrophenylhydrazine (DNPH) was purchased from Spectrum (New Brunswick, NJ, USA).

Table 1
Demographic data of participants who provided urine samples for this study.

Parameter (range)		N	%
Gender	Male	11	58
	Female	8	42
Ethnicity	Asian	13	68
	White	6	32
BMI* (19.0–36.3)	< 25	16	84
	25–30	1	5
	> 30	2	11
Age (11–56 years)	< 20	2	11
	20–30	5	26
	31–40	6	32
	> 40	6	32

*BMI: Body Mass Index.

2.2. Urine samples

Urine samples were collected from 19 healthy (non-smoker) volunteers (11 male, 8 female) who resided in the Albany area of New York State, USA, during the period February–March 2018. Volunteers were asked to continue their normal daily activities and provide early-morning-void urine samples daily for over a month. Participants' information, such as gender, age, height, weight, and ethnicity, were collected at the beginning of the study. Body mass index (BMI) was calculated, using the online tool from the U.S. National Institutes of Health (NIH). Demographic data for the 19 volunteers are shown in Table 1. Institutional Review Board approvals were obtained for the analysis of OSBs in urine.

Urine was collected in 50-ml polypropylene tubes, frozen after collection and stored at −20 °C at the earliest possible time (within 2 h of collection at the maximum). Over a month, a total of 515 urine samples were collected from 19 participants (daily samples were not available on a few occasions from certain individuals due to travel and other logistical constraints).

2.3. Analysis of OSBs and creatinine in urine

Oxidative stress biomarkers were analyzed in urine by following the previously reported method in our laboratory (Martinez and Kannan, 2018), with minor modifications. Briefly, an internal standard mixture (10 ng/IS) and DNPH reagent were added to a 500-μl urine aliquot. After DNPH derivatization of MDA, samples were extracted by solid phase extraction (SPE) and analyzed by high-performance liquid chromatography (HPLC; Agilent 1260) coupled to tandem mass spectrometry (MS/MS; ABSCIEX 4500).

Concentrations of creatinine were determined by the dilution of urine with Milli-Q water, internal standard addition (D_3-creatinine at 750 ng ml^{-1}) and analysis by HPLC-MS/MS. Urine extraction and instrumental parameters used in the determination of OSBs and creatinine are described in detail in the supplementary materials (Table S1).

The method limits of detection (LODs) were 0.03 ng ml^{-1} for diY and 8-OHdG, 0.024 ng ml^{-1} for MDA, 0.010 ng ml^{-1} for 8-$PGF_{2\alpha}$, 0.013 ng ml^{-1} for 8,15-$PGF_{2\alpha}$, 0.016 ng ml^{-1} for 11-$PGF_{2\alpha}$, and 0.012 ng ml^{-1} for 15-$PGF_{2\alpha}$. The method limits of quantification (LOQs) were 0.10 ng ml^{-1} for diY and 8-OHdG, 0.08 ng ml^{-1} for MDA, 0.03 ng ml^{-1} for 8-$PGF_{2\alpha}$, 0.05 ng ml^{-1} for 11-$PGF_{2\alpha}$, and 0.04 ng ml^{-1} for 15-$PGF_{2\alpha}$ and 8,15-$PGF_{2\alpha}$. Target compounds were not found in

procedural blanks, except for MDA, which was found at 0.42 ng ml^{-1}. The concentrations of MDA reported in samples were subtracted from blank values. Instrumental calibration curves were prepared by fortifying synthetic urine with target chemicals and ISs at concentrations that ranged from LOQ (0.03–0.1 ng ml^{-1}) to 100 ng ml^{-1} and processed along with the samples. Quality assurance/quality control (QA/QC) samples, including procedural blanks and analysis of spiked synthetic and pooled urine samples (spiked at 1.0 ng ml^{-1}) were included in the analysis. A duplicate sample was analyzed after every 25 samples as a check for method repeatability. Detailed results of QA/QC are shown in Table S2 in the supplementary materials.

2.4. Statistical analysis

Statistical analyses were performed using SPSS statistics v. 24. Microsoft Excel Version 2010 was used to draw plots. Values below the LOQ were considered as estimated values and the concentrations below the LOD were substituted with the LOD value divided by the square root of 2. The urinary OSB concentrations followed a log-normal distribution, and therefore, statistical analyses were performed on logarithm-transformed concentrations. Intra- and inter-individual variance was studied by one-way ANOVA, and the coefficients of variation (CV) were calculated from the standard deviation and mean values. Intraclass correlation coefficients (ICCs) were calculated to evaluate inter-individual variability in OSB concentrations by using the one-way model: ICC = $\sigma_B^2 / (\sigma_B^2 + \sigma_W^2)$ (σ_B^2: between groups variance; σ_W^2: within group variance) (Wu et al., 2010), with the interpretation of ICC values > 0.75 for excellent reproducibility, 0.75–0.40 for moderate reproducibility, and ICC < 0.40 for poor reproducibility (Alajbeg et al., 2017; Morgan et al., 2018). Spearman's correlation analysis was performed to elucidate relationships among seven urinary OSBs and between OSBs and creatinine.

3. Results

3.1. OSB concentrations in urine

Urinary concentrations of diY, 8-OHdG, MDA, and four F_2-isoprostane isomers measured in 515 samples (maximum, median, minimum, quartiles, and variance) are shown in Table 2. All OSBs were found in urine, at varying detection frequencies (DF); 8-OHdG and MDA were detected in all urine samples (100% DF); diY was detected in 99.8% of the samples, and 8-PGF$_{2\alpha}$, 11-PGF$_{2\alpha}$, 15-PGF$_{2\alpha}$, and 8,15-PGF$_{2\alpha}$ were detected in 78, 74, 93, and 97%, of the samples, respectively. DiY concentrations varied from below LOQ to 19.8 ng ml^{-1} in urine. The creatinine-corrected values for diY in urine were up to 2.43 μmol mol^{-1} (creatinine). 8-OHdG concentrations in urine ranged from 0.05 to 38.6 ng ml^{-1} with creatinine-corrected values in the range of 0.03 to 4.78 μmol mol^{-1}. Urinary MDA concentrations ranged from 0.95 to 198 ng ml^{-1} urine (0.79 to 126 μmol mol^{-1} creatinine), which was, therefore, the most abundant OSB found at a wider range of concentrations in healthy individuals. F_2-isoprostane was not detected at the lowest level in any of the samples. The highest urinary concentrations of 8-PGF$_{2\alpha}$, 11-PGF$_{2\alpha}$, 15-PGF$_{2\alpha}$, and 8,15-PGF$_{2\alpha}$ were 1.72, 7.66, 22.5, and 7.43 ng ml^{-1} (creatinine-corrected concentrations 0.61, 1.21, 3.53, and 0.72 μmol mol^{-1} creatinine), respectively. The sum concentrations of F_2-isoprostane isomers ranged from 0.14 to 23.9 ng ml^{-1} (0.06 to 4.38 μmol mol^{-1} creatinine) (Table 2). The overall CVs of volumetric (in ng ml^{-1}) and creatinine-adjusted concentrations (in μmol mol^{-1}) of OSBs in 515 urine samples are presented in Fig. 1. For volumetric concentrations, the CV ranged from 89% (diY and 8-OHdG) to 122% (MDA), whereas that for creatinine-corrected concentrations ranged from 40% (8-OHdG) to 86% (F_2-isoprostanes).

3.2. OSB levels in relation to gender, age, ethnicity, and BMI

The variability in OSB concentrations was examined between gender, age, ethnicity, and BMI groups. The concentrations and CV in OSB concentrations between gender, age, and ethnic groups were not significantly different. However, volumetric concentration and CV in OSB concentrations were higher in individuals with a high BMI (Fig. S1 and Table S3). This trend was not observed for creatinine-adjusted values.

3.3. Intra- and inter-individual variability in OSB concentrations and creatinine correction

Intra- and inter-individual variabilities in OSB concentrations were examined on volumetric and creatinine-adjusted concentrations (Table 3). The inter-individual CVs decreased following creatinine correction of OSB concentrations. The CV of diY concentrations in urine was 285% on a volumetric basis, and this value decreased to 115% after creatinine adjustment. Similarly, the CV of urinary concentrations of 8-OHdG was 399% on a volumetric basis, which was reduced to 152% on a creatinine basis; the CV of MDA concentrations was reduced from 311% to 112%; 8-PGF$_{2\alpha}$, from 184% to 179%; 11-PGF$_{2\alpha}$, from 398% to 163%; 15-PGF$_{2\alpha}$, from 432% to 272%; and 8,15-PGF$_{2\alpha}$, from 226 to 184%, following creatinine normalization. These results suggest that creatinine normalization decreased intra- and inter-individual variabilities in urinary OSB concentrations. Creatinine concentrations were

Table 2
Concentrations of seven oxidative stress biomarkers in 515 urine samples from 19 healthy individuals collected over a month.

Detection frequency	N	diY	8-OHdG	MDA	8-PGF$_{2\alpha}$	11-PGF$_{2\alpha}$	15-PGF$_{2\alpha}$	8,15-PGF$_{2\alpha}$	ΣPGF$_{2\alpha}$	Creatinine, μg ml^{-1}
	515	99.8	100	100	78.1	73.8	93.4	97.1		100
Concentration in urine, ng ml^{-1}										
Minimum	515	< LOD	0.05	0.95	< LOD	< LOD	< LOD	< LOD	0.14	85
1st quartile		1.16	2.45	5.91	0.04	< LOD	0.24	0.19	0.85	831
Median		2.13	3.65	11.5	0.16	0.17	0.55	0.38	1.56	1239
3rd quartile		3.73	5.64	20.2	0.29	0.34	1.29	0.82	2.73	2128
Maximum		19.8	38.6	198	1.72	7.66	22.5	7.43	23.9	8291
Variance		6.5	19.0	437	0.05	0.40	3.04	0.49	5.16	$1.86 \cdot 10^6$
LOD		0.03	0.03	0.02	0.01	0.02	0.01	0.01		0.1
Creatinine corrected concentration, μmol mol^{-1}										
Minimum	515	< LOD	0.03	0.79	< LOD	< LOD	< LOD	< LOD	0.06	
1st quartile		0.35	0.90	9.15	0.02	< LOD	0.07	0.06	0.25	
Median		0.51	1.16	13.4	0.04	0.05	0.14	0.09	0.37	
3rd quartile		0.73	1.47	20.3	0.07	0.08	0.26	0.16	0.55	
Maximum		2.43	4.78	126	0.61	1.21	3.53	0.72	4.38	
Variance		0.13	0.25	126	0.003	0.008	0.12	0.012	0.16	

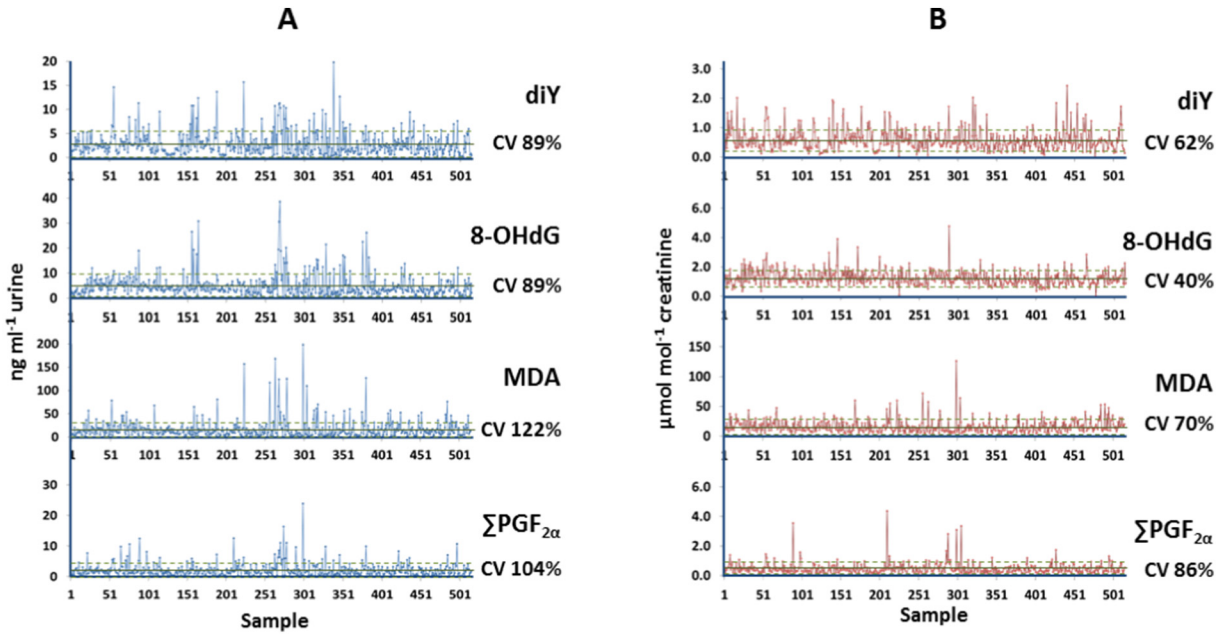

Fig. 1. Concentrations of oxidative stress biomarkers (OSBs) in 515 urine samples from healthy individuals (x-axis is sample number from 1 to 515). Red line indicates mean value and spotted lines are at ± SD (CV indicated). A. Volumetric concentrations, B. Creatinine corrected concentrations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly positively correlated ($\alpha = 0.01$) with all OSBs analyzed in this study, with a Spearman's correlation coefficient of 0.689 for diY, 0.816 for 8-OHdG, 0.734 for MDA, and 0.708 for the sum of F_2-isoprostanes (Fig. S2). Based on these results, only creatinine-corrected values were considered for further discussion.

Intra-individual CVs were 60% for diY, 29% for 8-OHdG, and 67% for MDA. For F_2-isoprostane isomers, intra-individual CVs were 109, 139, 149, and 79% for 8-$PGF_{2\alpha}$, 11-$PGF_{2\alpha}$, 15-$PGF_{2\alpha}$, and 8,15-$PGF_{2\alpha}$, respectively, and 83% for total F_2-isoprostanes. The ICCs yielded excellent reproducibility (ICC > 0.75) for the inter-individual concentrations of diY, 8-OHdG, MDA, 8-$PGF_{2\alpha}$ 15-$PGF_{2\alpha}$, 8,15-$PGF_{2\alpha}$, and total F_2-isoprostanes. The ICC values showed moderate reproducibility for 11-$PGF_{2\alpha}$. The intra- and inter-individual variability in F_2-isoprostanes were higher than the values calculated for diY, 8-OHdG, and MDA. The intra- and inter-individual variations in OSB concentrations in 19 individuals for urine samples collected daily for a month are shown in Fig. 2.

3.4. OSB profiles of healthy individuals

The profiles of seven oxidative stress biomarkers in the urine of healthy individuals are presented in Fig. 3. The distribution profile was similar across 19 individuals, with the predominance of MDA accounting for 80–93% of the total OSB concentrations (µmol mol^{-1} of creatinine). 8-OHdG, a biomarker of DNA oxidation, accounted for 4–12%, followed by dityrosine at 2–6%, of the total OSB concentrations in urine. The sum of four F_2-isoprostanes accounted for 0.7–2% of the total OSB concentrations measured. The concentrations and profiles of OSBs were not significantly affected by gender, age, or BMI (Figs. S3 and S4B).

Table 3
Inter- and intra-individual variance in seven oxidative stress biomarker concentrations in 19 healthy individuals collected over a month. Data are presented on a volumetric basis and creatinine corrected basis.

		OSB concentration, ng ml^{-1}			Creatinine corrected OSB concentration, µmol mol^{-1}		
		Variance	ICC	CV %	Variance	ICC	CV %
diY	Inter-individual	64	0.94	285	0.47	0.80	115
	Intra-individual	4.4		73	0.12		60
8-OHdG	Inter-individual	353	0.98	399	3.48	0.96	152
	Intra-individual	6.9		54	0.13		29
MDA	Inter-individual	2620	0.88	311	331	0.74	112
	Intra-individual	358		110	119		67
8-$PGF_{2\alpha}$	Inter-individual	0.14	0.75	184	0.008	0.76	179
	Intra-individual	0.05		109	0.003		109
11-$PGF_{2\alpha}$	Inter-individual	1.6	0.81	398	0.011	0.58	163
	Intra-individual	0.36		192	0.008		139
15-$PGF_{2\alpha}$	Inter-individual	19	0.89	432	0.35	0.76	272
	Intra-individual	2.4		145	0.11		149
8,15-$PGF_{2\alpha}$	Inter-individual	1.8	0.81	226	0.054	0.84	184
	Intra-individual	0.43		107	0.010		79
Σ$PGF_{2\alpha}$	Inter-individual	43	0.92	308	0.53	0.78	159
	Intra-individual	3.8		89	0.15		83

CV: Coefficient of variation; ICC: Intraclass correlation coefficient

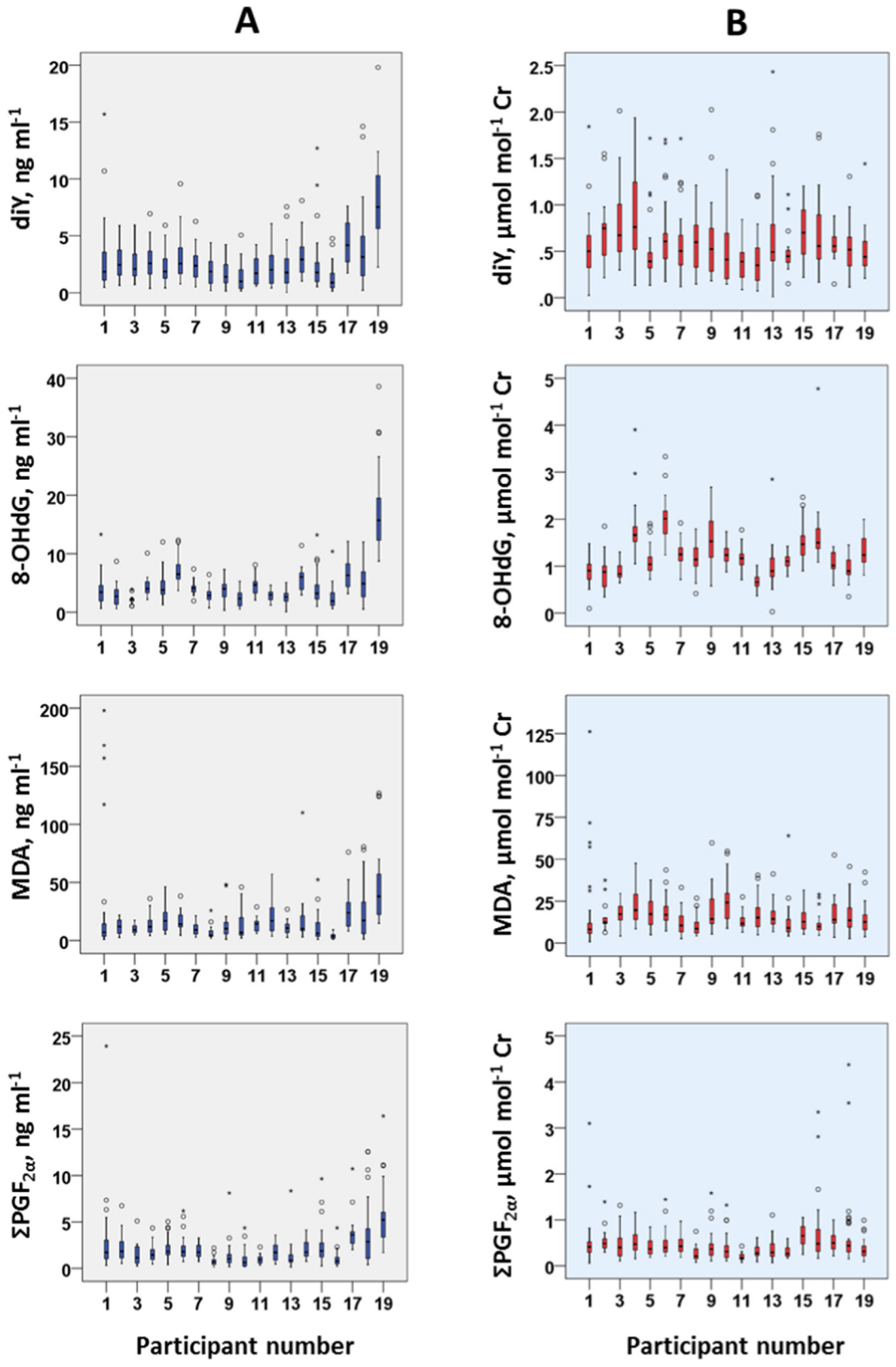

Fig. 2. Box-plots of oxidative stress biomarkers in urine of 19 healthy individuals collected over a month showing intra- and inter-individual variability. A. Volumetric concentrations, B. Creatinine (Cr)corrected OSBs concentrations.

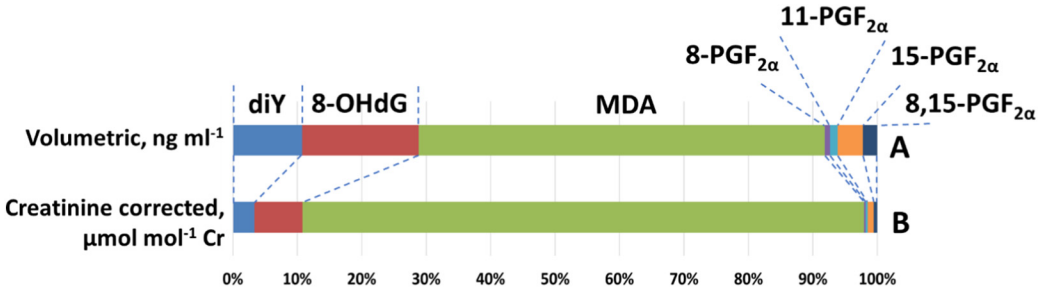

Fig. 3. Profiles of seven oxidative stress biomarkers in urine from healthy individuals collected daily for over a month. A. Volumetric concentration. B. Creatinine (Cr) corrected concentration.

3.5. Correlations among seven OSBs

Spearman's correlations showed significant positive correlations among seven urinary OSB concentrations (Table S4). diY, 8-OHdG, MDA, and total F_2-isoprostanes were significantly positively correlated ($p = 0.01$) with Spearman's correlation coefficients, ranging from 0.131 to 0.474.

4. Discussion

MDA, diY, 8-OHdG, and F_2-isoprostanes have been widely used as biomarkers of oxidative stress, although studies that describe their urinary levels and variability in healthy individuals have rarely been presented. This is the first study to report simultaneous analysis of seven OSBs of lipids, DNA, and proteins in healthy individuals' urine and to examine variability in concentrations over time. Wide ranges of age and BMI were represented between male and female volunteers in this study (Table 1).

A positive correlation between creatinine and OSBs was found in this study. Creatinine-corrected concentrations of OSBs were less variable, and showed a low CV. For example, Participant 19 (a White male in the age group of 31–40 years and BMI > 30) presented the highest total OSB volumetric concentration (Fig. S4A), which was 8-fold higher than that found for Participant 16 (an Asian male in the age group of 20–30 years and BMI < 25), who showed the lowest volumetric concentration of OSBs. The OSB profiles of creatinine-corrected concentrations, however, were similar between the two individuals (Fig. S4B). Similarly, the volumetric concentrations of OSBs for various BMI groups showed a higher median and CV of all biomarkers with an increase in BMI (Fig. S1 and Table S3), whereas creatinine-corrected concentrations showed similar values among all BMI groups. Urinary creatinine levels are associated with several factors, such as muscle mass and kidney disease, and are generally higher in males than in females (Sugita et al., 1992).

Factors such as urine collection time, diet, medication, health status, and daily activities, including exercise and other environmental factors, can affect ROS levels in the human body and, therefore, the levels of urinary OSBs. The 19 individuals were followed for a month, and urine samples were collected daily from > 70% of the participants. The intra-individual CV in concentrations was between 29% (for 8-OHdG) and 149% (for 15-$PGF_{2\alpha}$). These values should be taken into consideration when using spot urine samples from an individual for exposure assessment or epidemiologic studies. The inter-individual variability was found between 112% for MDA and 272% for 15-$PGF_{2\alpha}$. Inter-individual variability was 1.2- to 5.2-fold higher than that of intra-individual variability, which can be explained by personal factors, such as lifestyle, health, and dietary preference.

The reported values for urinary OSBs in healthy individuals are shown in Table 4. Urinary concentrations of diY in healthy individuals ranged from 7.4 to 33.5 μmol mol^{-1} of creatinine in a study that linked exercise and oxidative stress (Orhan et al., 2004). The reported diY concentration in a population in Japan was 8.8 ± 0.6 μmol mol^{-1} of creatinine (Kato et al., 2009). A mean diY concentration of 3.9 ± 1.0 nmol mol^{-1} creatinine was reported for a population in the United States (Bhattacharjee et al., 2001). There have been inconsistencies in reports of urinary concentrations of diY of healthy individuals, which could be attributed to analytical variations. Lack of inclusion of internal or native standards in the analysis could explain some of the inconsistencies in previous data. In this study, commercially available standards for diY as well as isotopically labeled diY standards were used. In our study, diY concentrations in 515 samples ranged from below LOQ to 4.2 μmol mol^{-1} creatinine, which is in the same order of magnitude as those reported from an LC-MS method, but higher than the values determined by the GC–MS methods.

8-OHdG has been measured in urine mainly by ELISA or LC-MS methods. In this study, urinary 8-OHdG concentrations ranged from 0.03 to 4.8 μmol mol^{-1} creatinine. Our values are on the same order of magnitude as those previously reported from the Netherlands (from 0.7 to 4.2 μmol mol^{-1} creatinine) (Orhan et al., 2004), Japan (2.0 to 6.0 μmol mol^{-1} creatinine) (Miwa et al., 2004), Spain (from 1.3 to 14.1 μmol mol^{-1} creatinine) (Campos et al., 2011), China (0.65 to 13.8 μmol mol^{-1} creatinine) (Lu et al., 2016), and Saudi Arabia (0.019 to 5.8 μmol mol^{-1} creatinine) (Asimakopoulos et al., 2016). The intra-day variability in 8-OHdG concentrations has been reported to be high in comparison to 24-h pooled urine sample ($n = 5$) (Miwa et al., 2004). No significant differences were found between the mean concentrations of 8-OHdG in spot urine samples and 24 h composite samples, with an inter-individual CV of 58% and intra-individual CV of 37% during a 10-day period that involved healthy participants (Pilger et al., 2002). In our study, the intra-individual CV was 29%, and inter-individual CV was 152%.

MDA was the predominant OSB found in urine, with concentrations that ranged from 0.79 to 126 μmol mol^{-1} of creatinine. These values were on the same order of magnitude as those previously reported from China (1.91 to 229 μmol mol^{-1} of creatinine) (Lu et al., 2016) or the Czech Republic (69 to 119 μmol mol^{-1} of creatinine) (Syslova et al., 2009). Interestingly, the highest and lowest MDA concentrations were found in urine samples from the same participant in this study (Asian male, with BMI < 25 in the age group of 31–40 years), who did not indicate any unusual activity on the day before the urine collection.

F_2-isoprostanes have been widely used as OSBs of various disease conditions. Wu et al. (2010) studied variability in F_2-isoprostane levels for over a year in healthy individuals and reported an ICC of 0.69 for total F_2-isoprostanes, which suggested that a spot urine sample is representative of the F_2-isoprostane levels for a year period. Keaney et al. (2003) analyzed urinary 8-$PGF_{2\alpha}$ and found a wide range of concentrations (from 0.028 to 5.20 μmol mol^{-1} of creatinine). In our study, we found ICCs for inter-individual variability at 0.76, 0.58, 0.76, and 0.84 for 8-$PGF_{2\alpha}$, 11-$PGF_{2\alpha}$, 15-$PGF_{2\alpha}$, and 8,15-$PGF_{2\alpha}$ respectively, which suggested excellent reproducibility of F_2-isoprostane measurements in healthy individuals (except for 11-$PGF_{2\alpha}$). The concentrations of 8-$PGF_{2\alpha}$ in urine have been reported for a pediatric population, establishing a reference value for healthy individuals at 0.16 μmol mol^{-1} of creatinine (Xiao et al., 2016). The urinary concentrations of 8-$PGF_{2\alpha}$

Table 4
Reported concentrations of urinary oxidative stress biomarkers in healthy individuals.

OSB	N	Location	Urinary levels, μmol mol^{-1} Cr	Method	Reference
diY	4	US	0.0039 ± 0.0010	GC-MS	Bhattacharjee et al., 2001
	23	Japan	8.8 ± 0.6	LC-MS	Kato et al., 2009
	18	Netherlands	7.4–33.5	LC-MS	Orhan et al., 2004
	515	US	< LOD – 2.4	LC-MS	This work
8-OHdG	130	Saudi Arabia	0.019–5.8[b]	LC-MS	Asimakopoulos et al., 2016
	19	Spain	1.3–14.1[b]	ELISA	Campos et al., 2011
	23	Japan	8.8 ± 0.9	ELISA	Kato et al., 2009
	22	China	0.65–13.8[b]	LC-MS	Lu et al., 2016
	5	Japan	2.0–6.0[b]	ELISA	Miwa et al., 2004
	18	Netherlands	0.7–4.2	ELISA	Orhan et al., 2004
	148	Austria	2.03 ± 1.21	LC-EC	Pilger et al., 2002
	515	US	0.03–4.8	LC-MS	This work
MDA	22	China	1.91–229[b]	TBA-LC-Flu	Lu et al., 2016
	10	Czech Republic	69–119[b]	LC-MS	Syslova et al., 2009
	515	US	0.79–126	DNPH-LC-MS	This work
8-$PGF_{2\alpha}$	2828	US	0.028–5.20[b]	ELISA	Keaney et al., 2003
	10	Czech Republic	0.056–0.085[b]	LC-MS	Syslova et al., 2009
	277	US	0.16 ± 0.22[b]	LC-MS	Van't Erve et al., 2017
	123[a]	US	< 0.16	LC-MS	Xiao et al., 2016
	515	US	< LOD – 0.61	LC-MS	This work
11-$PGF_{2\alpha}$	515	US	< LOD – 1.21	LC-MS	This work
15-$PGF_{2\alpha}$	19	Spain	0.11–0.73[b]	ELISA	Campos et al., 2011
	515	US	< LOD – 3.53	LC-MS	This work
8,15-$PGF_{2\alpha}$	515	US	< LOD – 0.72	LC-MS	This work

Flu: Fluorescence; EC: Electrochemical detection.
[a] Pediatric population.
[b] For uniformity, units converted to μmol mol^{-1} of creatinine.

for an adult population in the Czech Republic were up to 0.084 μmol mol^{-1} of creatinine (Syslova et al., 2009). An average concentration of 8-$PGF_{2\alpha}$ in urine from 81 countries was reported to be 0.16 ± 0.22 μmol mol^{-1} of creatinine (Van't Erve et al., 2017). The reported values were comparable to those found in our study (< LOD and up to 0.61 μmol mol^{-1} of creatinine). Limited information is available on the concentrations of other F_2-isoprostane isomers in urine. 15-$PGF_{2\alpha}$ was found at a concentration range of 0.11 to 0.73 μmol mol^{-1} of creatinine in healthy individuals (Campos et al., 2011).

Research that involves simultaneous analysis of more than one OSB has been reported by some authors, showing a positive correlation between the two OSBs. Significant positive correlations ($p < 0.05$) were found between F_2-isoprostanes and other OSBs and inflammation biomarkers, such as 2,3-dinor-5,6-dihydro-15-F_{2t}-isoprostane (F_2-isoprostanes metabolite), prostaglandin E_2 metabolite, and leukotriene E_4 ($r = 0.21$–0.65) (Wu et al., 2010). Significant positive correlations were found between urinary diY and 8-OHdG (Kato et al., 2009), and urinary MDA and 8-OHdG (Orhan et al., 2004). In our study, we found positive correlations between most of the OSBs, including the sum of F_2-isoprostanes ($p = 0.01$) (Table S4). As the ROS in an organism reacts with lipids, DNA, and proteins, all macromolecules appear to be simultaneously oxidized.

Studies have shown that urinary 8-$PGF_{2\alpha}$ levels in women were higher than those in men. Further, OSBs were highly associated with BMI and smoking habits (Keaney et al., 2003). In our study, no significant associations were found between gender, age, or BMI on OSB levels, although this study is limited to 19 individuals (Fig. S3).

Overall, this study evaluates intra- and inter-individual variabilities in oxidative stress among healthy individuals engaged in normal daily activities. It should be noted that factors such as nutrition, medication, exposure to contaminants, second hand smoking and among others that could potentially affect ROS levels were not controlled. Besides urine, other biospecimens have been used in the evaluation of oxidative stress levels. Kato et al. (2006) reported that 8-$PGF_{2\alpha}$ was a reliable OSB in blood samples for up to one year. Goldfarb et al. (2014) reported excellent ICC values (0.785) for protein carbonyls in blood from healthy men. Browne et al. (2008) reported considerable variability in lipid peroxidation products, including MDA, in blood. Regarding salivary markers of OSBs, Lettrichova et al. (2016) reported high intra- and inter-individual variability among 34 healthy volunteers. Alajbeg et al. (2017) found high intra-individual variability in salivary MDA and 8-OHdG of 15 healthy adults. Robust and reliable analytical methods for the analysis of OSBs in blood and saliva are needed. Furthermore, simultaneous analysis of several OSBs in multiple biospecimens (urine, blood, saliva) would shed light on the suitability of these matrices in biomonitoring of health effect biomarkers.

5. Conclusions

Measurements of oxidative stress biomarkers in human specimens provide an excellent way to assess the health status of an individual. The variability associated with daily activities or participant-dependent factors plays an important role in the assessment of OSBs. Creatinine is positively correlated with urinary OSBs, and the creatinine correction of the OSB concentrations reduces both intra- and inter-individual variability. This is the first study to evaluate the diurnal and inter-individual variability in oxidative stress status in healthy individuals and to report the reference values of urinary OSBs of lipids, DNA, and proteins. The ICCs indicate that the seven OSBs analyzed in this study present an excellent inter-individual reproducibility (except for 11-$PGF_{2\alpha}$). 8-OHdG has been detected in 100% of the urine samples, the ICCs are the highest (0.98–0.96), and the CV for intra-individual variability is 29% for creatinine-corrected concentrations and 54% for volumetric concentrations; these values suggest suitability of this biomarker among the seven biomarkers studied here.

Author contributions

This publication was completed with contributions from all authors, who approved the final version of the manuscript.

Notes

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2018.12.009.

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