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Temporal variability in urinary pesticide concentrations in repeated-spot and first-morning-void samples and its association with oxidative stress in healthy individuals

Adela Jing Li[a], Maria-Pilar Martinez-Moral[a], Kurunthachalam Kannan[a,b,*]

[a] Wadsworth Center, New York State Department of Health, Department of Environmental Health Sciences, School of Public Health, State University of New York at Albany, Empire State Plaza, P.O. Box 509, Albany, NY 12201-0509, United States
[b] Biochemistry Department, Faculty of Science and Experimental Biochemistry Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia

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ABSTRACT

Exposure of humans to pesticides is widespread. Measurement of urinary levels of pesticides and their metabolites is often used in the assessment of body burdens and exposure doses to these chemicals. An understanding of temporal variability in urinary levels of pesticides within individuals is critical for accurate exposure assessment. We examined within- and between-individual variability in concentrations of nine organophosphate and pyrethroid insecticides as well as two phenoxy herbicides in urine collected consecutively for up to 44 days from 19 individuals. Seven oxidative stress biomarkers also were measured in urine samples to elucidate their relationship with pesticide exposure. Intraclass correlation coefficients (ICCs) were calculated to assess reproducibility in urinary pesticide concentrations from repeated measures. Sensitivity and specificity analyses were performed to evaluate the suitability of spot urine to characterize average exposures. Data analysis was further limited to seven pesticides and their metabolites, which had a detection frequency of > 60%. Poor reproducibility was found for the seven pesticides and their metabolites in both spot (ICCs ≤0.24) and first-morning-void (FMV) samples (ICCs < 0.38) collected during the 44-day study period. Use of single-spot or FMV sample to classify high (top 33%) concentrations showed high specificities (0.73–0.85) but low sensitivities (0.45–0.70). The minimum number of samples (*k*) required per individual to estimate participant-specific mean value for pesticides (within 20% of the "true" values) were 28–140 and 18–119 for spot and FMV samples, respectively. Repeated longitudinal measurements of these pesticides and their metabolites in urine showed considerable within-individual variability in both spot and FMV samples. Urinary concentrations of seven pesticides and their metabolites were significantly correlated with oxidative damage to lipids, proteins, and DNA.

1. Introduction

Pesticides play a crucial role in increasing crop yield and eradicating vectors of human/livestock diseases. In 2012, the global estimated production of pesticides was 5.8 billion lbs. of active ingredients (U.S. Environmental Protection Agency, 2017). The general population is exposed to pesticides through the ingestion of food and water (Lu et al., 2006; McKelvey et al., 2013; Oulhote and Bouchard, 2013). Pesticides have been measured in human urine (Calafat et al., 2017; Garí et al., 2018; Li and Kannan, 2018; Viel et al., 2015; Wang et al., 2016b). Among several classes of pesticides, exposure to organophosphorus (OP) and pyrethroid (PYR) insecticides and phenoxy acid (PA) herbicides, previously referred to as "Universal Pesticides" in the National Health and Nutrition Examination Survey (NHANES) of the Centers for Disease Control and Prevention (CDC), has received considerable attention. Exposure to these pesticides has been associated with impaired reproduction, metabolic disorders, neurobehavioral disorders, macular degeneration, and asthma (Coker et al., 2018; González-Alzaga et al., 2014; Hoppin et al., 2017; Montgomery et al., 2017; Saillenfait et al., 2015).

The half-life of most OP, PYR, and PA pesticides and their metabolites *in vivo* is on the order of a few hours to a few days (World Health Organization, 1986). For example, the serum half-life of malathion was reported to be 3 h (Lyon et al., 1987). Most OP, PYR, and PA pesticides are metabolized by cytochrome P450 enzymes and eliminated in urine as glucuronidated and/or sulfated conjugates. Spot or first-morning-void (FMV) urine specimens have been used in the biomonitoring of exposure to pesticides in the general populations (Calafat et al., 2017;

* Corresponding author at: Wadsworth Center, Empire State Plaza, P.O. Box 509, Albany, NY 12201-0509, United States.
E-mail address: Kurunthachalam.kannan@health.ny.gov (K. Kannan).

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Haines et al., 2017; Li and Kannan, 2018; McKelvey et al., 2013; Naeher et al., 2010). The reliability of single-spot or FMV urine to represent a pesticide exposure profile in individuals over time is questionable due to the episodic nature of exposure and toxicokinetics (i.e., short half-lives of 4–72 h in human bodies). Whereas creatinine correction is often used to offset urine dilution and has been suggested to improve the predictability of exposure to chemicals over time (Martinez-Moral and Kannan, 2019; Wielgomas, 2013; Zhu and Kannan, 2019), creatinine excretion rate is variable and dependent on factors such as age, gender, and physical activity (Wang et al., 2016d; Wang et al., 2019). These issues (i.e., temporal variability in exposure and creatinine correction) can result in exposure misclassification in epidemiological studies (Nassan et al., 2019; Wang et al., 2016d). Studies that report intra-individual variability in pesticide exposures have been focused on specific populations, such as children and pregnant women (Table S1). Two studies reported intra-individual variability in 3-phenoxybenzoic acid (3-PBA) levels in Polish and U.S. populations, but the conclusions were somewhat divergent (Morgan et al., 2016; Wielgomas, 2013). Studies that determine inter- and intra-individual variability in urinary pesticide levels in the general population are needed to evaluate predictability/reliability of a single-spot or FMV urine sample to represent exposure in individuals/populations over time.

A growing number of studies indicate that exposure to OP, PYR, and PA pesticides provokes oxidative stress (Guyton et al., 2015; Wang et al., 2016c). In occupationally exposed populations, significant positive correlations were found between pesticide exposure and urinary oxidative stress biomarkers (OSBs), such as 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 8-isoprostaglandin $F_{2\alpha}$ (8-$PGF_{2\alpha}$) (Lerro et al., 2017; Wang et al., 2016a). Biomarkers of oxidative damage to lipids, proteins and DNA have been linked to adverse health outcomes (Li et al., 2019; Wang et al., 2019). Studies that relate pesticide exposure with oxidative damage in general populations, however, are limited.

To address the knowledge gaps described above, we conducted a longitudinal study of pesticide exposure in 19 healthy individuals with the following objectives: (1) to assess inter- and intra-individual variability in urinary concentrations of 11 pesticides and their metabolites in spot and FMV urine samples collected consecutively for up to 44 days; (2) to evaluate sensitivity and specificity of urinary measurements of pesticides in individuals for exposure classification; and (3) to examine the relationships between urinary concentrations of 11 pesticides and their metabolites and biomarkers of oxidative damage.

2. Materials and methods

2.1. Study population and sample collection

From February to April 2018, 19 volunteers were recruited to participate in a study designed to examine the variability in urinary levels of OSBs (Martinez-Moral and Kannan, 2019). The volunteers were healthy, nonsmoking men (57.9%) and women (42.1%) who lived in the Albany area of New York State, USA (Table 1). The participants were 11 to 56 years of age (mean ± standard deviation, 33.8 ± 12.4 years), with a body weight of 67.6 ± 17.4 kg and a height of 168 ± 9.64 cm. The average body mass index (BMI) was 23.6 ± 4.44 kg/m^2, with 16% of the individuals having a BMI of ≥25. Of the participants, 68% were Asian, and 32% were Caucasian. None of the participants was a habitual alcohol consumer, and none had occupational exposure to pesticides. The urine samples archived from a previous study on OSBs (Martinez-Moral and Kannan, 2019) were used for pesticide analysis in this study. The Institutional Review Board of the New York State Department of Health approved this study.

The volunteers provided FMV and spot urine samples in 50-mL polypropylene (PP) tubes daily for up to 44 days. The samples were devoid of personal identifiers and were frozen and stored at −20 °C until further analysis. A total of 515 urine samples from 19 participants were available for analysis (average number of samples per person: 27): 4 of 19 (21%) participants provided 13–19 urine samples, 7 of 19 (37%) provided 21–30 samples, and 8 of 19 (42%) provided 31–40 samples (Table S1). Among the total 515 urine samples, there were 243 FMVs. FMV refers to those samples of urine that were collected immediately after wake up in the morning. For the purpose of data analysis, all 515 samples were included in the category of spot urine, whereas 243 samples that were exclusively identified as FMVs were grouped in that category.

Table 1
Demographic characteristics of 19 participants who provided repeated urine samples consecutively for up to 44 days.

Characteristic	Statistics
Gender	
Male (*n*/%)	11 (57.9%)
Female (*n*/%)	8 (42.1%)
Age (mean ± SD; year)	33.8 ± 12.4
< 30 (*n*/%)	7 (36.8%)
30–40 (*n*/%)	6 (31.6%)
> 40 (*n*/%)	6 (31.6%)
Body weight (mean ± SD; kg)	67.6 ± 17.4
Height (mean ± SD; cm)	168 ± 9.64
BMI (mean ± SD; kg/m^2)	23.6 ± 4.44
BMI < 25 (*n*/%)	16 (84.2%)
BMI ≥25 (*n*/%)	3 (15.8%)
Ethnicity	
Asian (*n*/%)	13 (68.4%)
Caucasian (*n*/%)	6 (31.6%)
Dietary supplements	
No (*n*/%)	10 (52.6%)
Yes (*n*/%)	9 (47.4%)
How many times of exercise per week	
0 (*n*/%)	5 (26.3%)
1–2 (*n*/%)	6 (31.6%)
≥3 (*n*/%)	8 (42.1%)
How often in alcohol consumption	
Never (*n*/%)	4 (21.1%)
Seldom (*n*/%)	10 (52.6%)
Occasionally (*n*/%)	5 (26.3%)
Smoking status	
No (*n*/%)	19 (100%)
Yes (*n*/%)	0 (0%)

SD, standard deviation; BMI, body mass index.

2.2. Determination of pesticides and their metabolites

Analytical standards (2-isopropyl-4-methyl-6-hydroxypyrimidine, IMPY; malathion dicarboxylic acid, MDA; *para*-nitrophenol, PNP; 3,5,6-trichloro-2-pyridinol, TCPY; 3-PBA; 4-fluoro-3-phenoxybenzoic acid, 4F-3PBA; 2,4-dichlorophenoxyacetic acid, 2,4-D; 2,4,5-trichlorophenoxyacetic acid, 2,4,5-T; *trans/cis*-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropane-1-carboxylic acid, *trans/cis*-DCCA and *cis*-3-(2,2-dibromovinyl)-2,2-dimethyl-cyclopropane-1-carboxylic acid, *cis*-DBCA) of 95.5–99.5% purity and corresponding isotopically labeled internal standards ($^{13}C_4$-IMPY, D_6-MDA, D_4-PNP, $^{13}C_3$-TCPY, $^{13}C_6$-3-PBA, $^{13}C_6$-4F-3PBA, $^{13}C_6$-2,4-D, $^{13}C_6$-2,4,5-T, $^{13}C_2$-*trans*-DCCA and $^{13}C_2$-*cis*-DCCA) of 99–99.5% purity were purchased from Dr. Ehrenstorfer (Augsburg, Germany), Cambridge Isotope Laboratories (Andover, MA, USA), and Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, acetone, hexane, acetic acid (ACS reagent grade), and water (HPLC grade) were purchased from J.T. Baker (Center Valley, PA, USA). Methanol (LC/MS grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). β-glucuronidase from *Helix pomatia* (68,800 units/mL β-glucuronidase and 199 units/mL sulfatase) was purchased from MP Biomedicals, LLC (Solon, OH, USA).

Urine samples were extracted by a solid phase extraction (SPE) method, as described earlier, with some modifications (Li and Kannan, 2018). Briefly, urine samples (500 µL) were spiked with stable

isotopically labeled internal standards, vortexed, and subjected to enzymatic deconjugation to liberate glucuronide- and sulfate-bound pesticides or their metabolites. The hydrolysates were extracted by Oasis® HLB (3 mL) SPE cartridges. The eluates (acetone and hexane) were dried under a gentle stream of nitrogen and reconstituted with 250 μL of acetonitrile:water (1:1, *v/v*) for analysis by high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS).

Pesticides and their metabolites were chromatographically separated by a Waters ACQUITY Class I HPLC system (Waters, Milford, MA, USA), connected to a Betasil C18 column (100 × 2.1 mm, 5 μm; Thermo Electron Corp., Waltham, MA, USA), which were then detected by an ABSCIEX 5500 electrospray triple quadrupole mass spectrometer (ESI-MS/MS; Applied Biosystems, Foster City, CA, USA) in either a positive or negative ionization mode.

A total of 25 urine samples were analyzed in duplicate for the evaluation of method precision. The relative standard deviation (RSD) in measured concentrations from duplicate analysis was < 19% for all target analytes. The recoveries of target chemicals through the analytical method were examined by spiking a known amount of target pesticides and their metabolites (1.0, 10, and 100 ng/mL) into a synthetic urine sample. The recoveries of target analytes ranged from 84% to 115%, with a RSD of < 16%. The method limit of detection (LOD) for the 11 target analytes was in the range of 0.025 to 0.05 ng/mL. Trace concentrations (ng/mL) of select target compounds (0.44 of MDA, 0.47 of PNP, 0.03 of 2,4-D, 0.02 of 3-PBA, 0.02 of 4F-3PBA, and 0.01 of *trans*-DCCA) were found in procedural blanks, and these concentrations were subtracted from those measured in samples (Table S2).

2.3. Determination of oxidative stress biomarkers

Seven OSBs, namely o,o′-dityrosine (diY), 8-OHdG, malondialdehyde (MDA), 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-$PGF_{2\alpha}$), were analyzed in urine samples by following the method described in detail elsewhere (Martinez-Moral and Kannan, 2019); the method is summarized in the Supporting Information.

2.4. Determination of urinary creatinine

Urinary creatinine concentrations were determined by ACQUITY HPLC (Waters, Milford, MA, USA), coupled to ACQUITY TQD MS/MS (Waters, Milford, MA, USA) by following the method described earlier (Martinez-Moral and Kannan, 2019).

2.5. Data analysis

Data were analyzed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Concentrations below the LOD were replaced with a value of LOD divided by the square root of 2. Before statistical analyses, data were log-transformed ($\chi + 1$) to normalize their distributions. Mixed regression models were conducted to explore associations between urinary concentrations of pesticides/metabolites and OSBs.

Intraclass correlation coefficients (ICCs, a ratio of between-individual variance to total variance) were calculated as a measure of reproducibility/reliability of measurements made over time within individuals. The ICC values were categorized as excellent (ICC ≥ 0.75), fair to good (0.75 > ICC ≥ 0.40), and poor (ICC < 0.40) for reproducibility (Wang et al., 2016d). Between- and within-individual variances were calculated by a linear mixed effects model, using maximum likelihood estimation, as implemented in SPSS. Akaike Information Criterion (AIC) values were used to assess the fitness of models for urinary concentrations reported as uncorrected values, creatinine-corrected values, and creatinine as a covariate. The lower the AIC value, the better the fitness of the model. We also evaluated the sensitivity (a true positive rate to measure the proportion of actual positives that are correctly identified as such) and specificity (a true negative rate to measure the proportion of actual negatives that are correctly identified as such) of one, two, or three randomly selected spot/FMV urine samples from each participant as predictors of high (top 33%) exposure based on their 44-day average concentrations in comparison with the distribution of "true" and "predicted" levels (Wang et al., 2016d). To examine whether FMV urine samples represent exposure better than do spot urine samples, 243 FMV urine samples were analyzed separately from spot urine samples. For the "true" levels, arithmetic means of log-transformed ($\chi + 1$) concentrations of pesticides and their metabolites from each participant were calculated for samples collected during the 44-day period. The values were categorized into tertiles, and the "true" top 33% exposure levels were identified. For the "predicted" levels, ten data sets, with each containing one randomly selected spot and FMV sample per person, were generated. Within each randomly selected data set, concentrations were identified for the "predicted" top 33% exposure levels. The average sensitivity and specificity observed across the ten separate randomly selected samples were calculated for spot and FMV samples. To examine whether a collection of multiple urine samples would improve the sensitivity, we repeated the analysis, using arithmetic means of two or three samples randomly selected from each participant collected on different days. The minimum number of spot or FMV samples (k) required to estimate the participant-specific mean concentration for pesticides and their metabolites to be within 20% of the "true" values (at a probability of 95%) was calculated after log-transformation ($\chi + 1$) of measured concentrations, using the following equation:

$$k = (1.96 \times CV/20)^2,$$

where CV is the within-individual coefficient of variation (Kim et al., 2014; Wang et al., 2016d).

3. Results and discussion

3.1. Profiles of urinary pesticides and their metabolites

A total of 515 urine samples collected consecutively from 19 participants (13–40 samples per person) during February–April 2018 were analyzed. The available demographic information of the participants is presented in Table S3. The detection frequency, distribution, and volume-based and creatinine-adjusted concentrations of pesticides and their metabolites in spot and FMV urine are presented in Tables S4 and S5. The frequency of detection varied, depending on the pesticides. IMPY, PNP, TCPY, 3-PBA, *trans/cis*-DCCA, and 2,4-D were the most frequently detected pesticides/metabolites (> 89%). Several of the pesticide metabolites, including 4F-3PBA and *cis*-DBCA (metabolites of cyfluthrin, flumethrin, and deltamethrin) and MDA (a specific metabolite of malathion), were detected in 38–65% of the samples analyzed. 2,4,5-T was not detected in any urine sample. Further analyses of the data were restricted to those analytes that had a detection frequency of > 60% in all samples to exclude possible bias arising from substitution of values for samples below the LOD (Li and Kannan, 2018; Li et al., 2019).

Creatinine-corrected concentrations of pesticides and their metabolites were compared among gender, age (< 30, 30–40, and > 40 years), BMI (< 25 and ≥ 25 kg/m^2) and ethnic categories (Asian and Caucasian) (Fig. 1). The overall distribution of pesticide concentrations did not vary significantly among different demographic categories. The sum concentrations of three OPs, IMPY (metabolite of diazinon), PNP (metabolite of parathion and methyl parathion), and TCPY (metabolite of chlorpyrifos and chlorpyrifos-methyl) collectively accounted for 38–64% of the sum of seven pesticide concentrations. Pyrethroid metabolites (i.e., 3-PBA and *trans/cis*-DCCA) accounted for 29–56% of the sum of seven pesticide concentrations. Such a pattern of higher OP concentrations than those of PYRs in urine has been reported previously (Calafat et al., 2017; Garí et al., 2018; Li and Kannan, 2018). In particular, PNP and TCPY were the dominant pesticide metabolites in urine

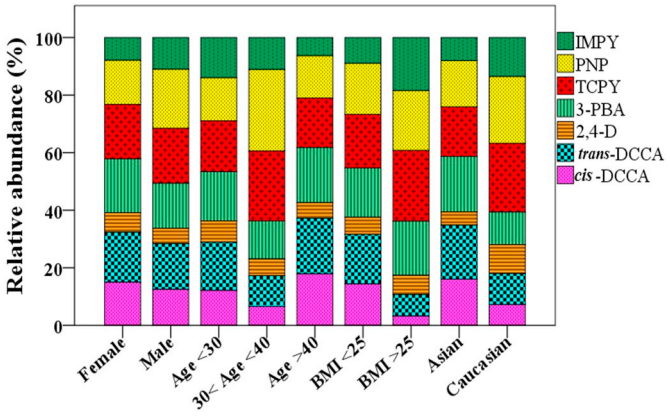

Fig. 1. Composition profile of urinary pesticide concentrations (to the sum of seven concentrations, ng/g creatinine) collected consecutively from 19 participants during a 44-day study period, stratified by gender, age, body mass index (BMI) and ethnicity. IMPY, 2-isopropyl-4-methyl-6-hydroxypyrimidine; PNP, *para*-nitrophenol; TCPY, 3,5,6-trichloro-2-pyridinol; 3-PBA, 3-phenoxybenzoic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; *trans/cis*-DCCA, *trans/cis*-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropane-1-carboxylic acid.

of the 19 participants (accounting for 32–53% of the sum of seven pesticide concentrations), suggesting the prevalence of exposure to parathion, methyl parathion, and chlorpyrifos. Although the use of OPs for indoor and garden pest control was banned in the USA in 2000 (Barr et al., 2010), these pesticides are still commonly used in agriculture, accounting for 33% (20 million lbs.) of all insecticides used in the USA in 2012 (EPA, 2017). It is worth noting that a higher proportion of sum PYR concentrations than that of OP concentrations was observed in female (51% vs. 42%; $p < 0.05$), > 40 years of age (56% vs. 38%; $p = 0.05$), and Asian (54% vs. 41%; $p > 0.05$) donors.

The creatinine-corrected concentrations of pesticides and their metabolites (micrograms per gram creatinine) in spot urine samples collected from the 19 participants over the 44-day period are shown in Fig. S1. The concentrations of TCPY, 3-PBA, and *trans/cis*-DCCA varied by two to three orders of magnitude within individuals during the study period. The urinary concentrations of IMPY, PNP, and 2,4-D varied within an order of magnitude for all participants, with the exception of IMPY in one participant (P16) and PNP in another participant (P10) of the study. It is worth noting that all pesticides/metabolites varied within an order of magnitude for three participants (P1–P3), who were all Asian Indians. Variations in pesticide exposure is related to lifestyle, diet, and activity patterns (Oulhote and Bouchard, 2013). The creatinine-corrected concentrations of the seven OSBs in this population have been published previously (Martinez-Moral and Kannan, 2019).

3.2. Creatinine correction of urinary concentrations

Urinary concentrations are often reported in the literature as volume-based or creatinine-based. We examined between- and within-individual variance in urinary concentrations of pesticides and their metabolites by three different models, namely uncorrected concentrations (i.e., volume-based), creatinine-corrected concentrations, and creatinine as a covariate (Table 2). The lowest AIC values were found for creatinine-corrected concentrations of most analytes (i.e., PNP, TCPY, 3-PBA, 2,4-D, and *trans/cis*-DCCA) and when creatinine was used as a covariate (for IMPY). The apportionments of between-individual and within-individual variances in target chemical concentrations were similar for creatinine-corrected values and when creatinine was used as a covariate. Because creatinine-corrected concentrations of most compounds presented the lowest AIC values, we used creatinine-corrected values in subsequent analyses.

3.3. Intraclass correlation coefficients and reproducibility of concentrations

The within- and between-individual variance in creatinine-corrected concentrations of pesticides and their metabolites in spot and FMV samples from the 19 individuals collected consecutively during the 44-day study period is shown in Table 3. Most of the variance was attributed to within-individual variability in both spot and FMV samples. The low ICC values for both spot and FMVs indicated poor reproducibility of overall exposures from a single measurement. This may be attributed to episodic nature of exposure to pesticides analyzed in this study. These results suggest the need for repeated sampling to accurately characterize an individual's exposure to the target pesticides. The ICC values for the creatinine-corrected concentrations of seven OSBs were reported in our previous study, and that ranged from 0.58 to 0.96 (Martinez-Moral and Kannan, 2019).

Poor reproducibility was found for pesticides and their metabolites with ICCs in the range of 0.05–0.24. The within-individual variance (76–95%) was much higher than that of the between-individual variance (5–24%). This finding implies that measurements of these pesticides/metabolites in a single spot urine sample may not adequately represent exposure over a long period of time. Similar to what was found in our study, an earlier study reported poor reproducibility of TCPY and 3-PBA analyzed in spot urine from pregnant women in the USA ($n = 528$; ICCs: 0–0.06) (Barkoski et al., 2018). Low ICC values were reported for pesticides across different population groups: children, pregnant women, and adults (Attfield et al., 2014; Barkoski et al., 2018; Morgan et al., 2016). A few studies, however, reported fair to excellent reproducibility in pesticide concentrations (ICCs: 0.41–0.85) measured in spot urine (Fortenberry et al., 2014; Morgan et al., 2008; Whyatt et al., 2009; Wielgomas, 2013;). Nevertheless, these earlier studies comprised fewer samples. The divergence in ICC values between studies may be attributable to differences in study design (e.g., sample size, duration of collection), and physiological conditions of participants (e.g., pregnancy status) (Smolders et al., 2014; Wang et al., 2016d).

In comparison to those of spot urine samples, concentrations of PNP (ICC = 0.38) determined in FMVs showed higher reproducibility. In general, reproducibility of PNP and TCPY concentrations in urine was greater than that of other target compounds, which could be related to the frequency of exposures. PNP and TCPY were the dominant OP metabolites found in urine from eight countries (Li and Kannan, 2018). No earlier study has reported the reproducibility of PNP, IMPY, and *cis*-DCCA in urine. The high within-individual variance in FMV urinary pesticide concentrations (62–100%) suggests that FMV does not provide an accurate estimate of exposure over a month. Several studies have examined the suitability of FMV to evaluate individual exposure to pesticides, but the results are somewhat conflicting (Table S1). Whereas poor reproducibility was reported for MDA, TCPY, 3-PBA, and *trans*-DCCA (ICCs: 0.02–0.34) in some studies (Attfield et al., 2014; Barkoski et al., 2018; Morgan et al., 2016), fair to good reproducibility was reported for MDA, TCPY, and 3-PBA (ICCs: 0.40–0.55) in other studies (Adgate et al., 2001; Egeghy et al., 2005; Meeker et al., 2005; Wielgomas, 2013).

3.4. Sensitivity and specificity analysis for predicting urinary concentrations

Table 4 shows the results of sensitivity and specificity analyses to accurately categorize a high-exposure group (top 33% of 44-day average concentrations) based on one, two, or three randomly selected urine samples collected on different days. A trend similar to that observed for ICCs was also found for sensitivity and specificity analysis. The proportion of participants who were correctly classified in the top 33% of average exposure from a single sample was 0.52–0.67 and 0.45–0.70 from spot and FMV samples, respectively. This indicates that data from a single analysis can result in exposure misclassification of pesticides. Analysis of two spot urine samples collected several days

Table 2
Apportionment[a] of variance in log-transformed (χ + 1) pesticide/metabolites concentrations in urine samples collected from 19 participants consecutively for up to 44 days (n = 515).

	IMPY	PNP	TCPY	3-PBA	2,4-D	*trans*-DCCA	*cis*-DCCA
Uncorrected (μg/L)							
AIC[b]	−856	−289	−156	−156	−848	−91	−148
Between-individual σ^2 (%)[c]	0.001 (8)	0.008 (21)	0.011 (23)	0.004 (9)	0.001 (7)	0.004 (7)	0.004 (9)
Within-individual σ^2 (%)[d]	0.010 (92)	0.030 (79)	0.038 (77)	0.040 (91)	0.010 (93)	0.045 (93)	0.040 (91)
Creatinine-corrected (μg/g creatinine)							
AIC	−883	−557	−454	−339	−1064	−265	−311
Between-individual σ^2 (%)	0.001 (5)	0.006 (24)	0.006 (22)	0.003 (10)	0.001 (7)	0.002 (7)	0.003 (9)
Within-individual σ^2 (%)	0.010 (95)	0.018 (76)	0.021 (78)	0.028 (90)	0.007 (93)	0.032 (93)	0.029 (91)
Creatinine as a covariate							
AIC	−916	−400	−250	−188	−910	−114	−156
Between-individual σ^2 (%)	0.001 (13)	0.008 (26)	0.010 (23)	0.004 (11)	0.001 (9)	0.004 (8)	0.004 (10)
Within-individual σ^2 (%)	0.009 (87)	0.024 (74)	0.032 (77)	0.037 (89)	0.009 (91)	0.043 (92)	0.039 (90)

σ^2 = variance.
[a] Age, gender, body mass index, ethnicity, dietary supplement intake, exercise frequency, first morning void and alcohol consumption were included as covariates.
[b] AIC, Akaike Information Criterion values were used to assess the fitness of models.
[c] The proportion of between-individual variance to the total variance.
[d] The proportion of within-individual variance to the total variance.

Table 3
The apportionment[a] of variance in log-transformed (χ + 1) creatinine-corrected concentrations of pesticides and their metabolites in spot and first morning void (FMV) urine samples collected from 19 participants consecutively during a 44-day study period.

	IMPY	PNP	TCPY	3-PBA	2,4-D	*trans*-DCCA	*cis*-DCCA
Spot urine sample (n = 515)							
ICC[b]	0.05	0.24	0.22	0.10	0.07	0.07	0.09
Between-individual σ^2 (%)[c]	0.001 (5)	0.006 (24)	0.006 (22)	0.003 (10)	0.001 (7)	0.002 (7)	0.003 (9)
(95% CI)[d]	(0.000, 0.002)	(0.003, 0.011)	(0.003, 0.012)	(0.001, 0.008)	(0.000, 0.002)	(0.001, 0.007)	(0.001, 0.008)
Within-individual σ^2 (%)[e]	0.010 (95)	0.018 (76)	0.021 (78)	0.028 (90)	0.007 (93)	0.032 (93)	0.029 (91)
(95% CI)	(0.009, 0.011)	(0.015, 0.020)	(0.019, 0.024)	(0.024, 0.031)	(0.006, 0.008)	(0.028, 0.037)	(0.026, 0.033)
First morning urine sample (n = 243)							
ICC	0.01	0.38	0.21	0.06	0.00	0.03	0.05
Between-individual σ^2 (%)	0.000 (1)	0.008 (38)	0.008 (21)	0.003 (6)	0.000 (0)	0.001 (3)	0.002 (5)
(95% CI)	(0.000, 0.006)	(0.003, 0.020)	(0.003, 0.022)	(0.001, 0.010)	–	(0.000, 0.010)	(0.001, 0.011)
Within-individual σ^2 (%)	0.003 (99)	0.013 (62)	0.030 (79)	0.038 (94)	0.004 (100)	0.044 (97)	0.044 (95)
(95% CI)	(0.002, 0.004)	(0.011, 0.016)	(0.025, 0.036)	(0.032, 0.046)	(0.003, 0.005)	(0.037, 0.053)	(0.036, 0.052)

σ^2 = variance. –, not available.
[a] Age, gender, body mass index, ethnicity, dietary supplement intake, exercise frequency and alcohol consumption were included as covariates.
[b] ICC, intraclass correlation coefficient to assess the reproducibility of repeated measurements.
[c] The proportion of between-individual variance to the total variance.
[d] Confidence interval for the variance components.
[e] The proportion of within-individual variance to the total variance.

Table 4
Sensitivity and specificity[a] analysis of urinary concentrations[b] of pesticides and their metabolites for classifying high exposures (top 33%) based on the analysis of one, two and three samples collected over a 44-day period.

	IMPY	PNP	TCPY	3-PBA	2,4-D	*trans*-DCCA	*cis*-DCCA
Spot urine sample (n = 515)							
1 sample	0.57 (0.80)	0.60 (0.82)	0.63 (0.83)	0.67 (0.85)	0.63 (0.83)	0.52 (0.78)	0.58 (0.81)
2 samples	0.57 (0.80)	0.73 (0.88)	0.72 (0.87)	0.77 (0.89)	0.70 (0.86)	0.60 (0.82)	0.67 (0.85)
3 samples	0.58 (0.81)	0.80 (0.91)	0.70 (0.86)	0.83 (0.92)	0.67 (0.85)	0.67 (0.85)	0.67 (0.85)
k[c]	31	28	47	58	53	87	140
First morning urine sample (n = 243)							
1 sample	0.48 (0.74)	0.63 (0.81)	0.63 (0.81)	0.70 (0.85)	0.58 (0.79)	0.45 (0.73)	0.50 (0.75)
2 samples	0.45 (0.73)	0.75 (0.88)	0.53 (0.76)	0.78 (0.89)	0.50 (0.75)	0.53 (0.76)	0.70 (0.85)
3 samples	0.65 (0.83)	0.88 (0.94)	0.68 (0.84)	0.83 (0.91)	0.65 (0.83)	0.73 (0.87)	0.65 (0.83)
k	18	22	43	51	46	65	119

[a] Mean sensitivity and specificity were computed based on ten data sets each containing one randomly selected urine sample per participant.
[b] Calculations used creatinine-corrected concentrations (μg/g creatinine) on the log (χ + 1) scale.
[c] The minimum number of urine samples required to estimate participant-specific mean within 20% of the "true" value.

Table 5
Mixed regression models[a] of log-transformed (χ + 1) creatinine-corrected concentrations of urinary pesticide/metabolites and oxidative stress biomarkers.

Type of sample	Mixed regression models β (95% CI[b])						
	IMPY	PNP	TCPY	3-PBA	2,4-D	*trans*-DCCA	*cis*-DCCA
Spot urine sample (n = 515)							
diY	0.09	−0.01	−0.12	−0.03	−0.02	−0.05	−0.01
	(0.03, 0.14)**	(−0.09, 0.07)	(−0.20, −0.03)**	(−0.12, 0.07)	(−0.07, 0.03)	(−0.15, 0.06)	(−0.11, 0.09)
8-OHdG	0.12	0.12	0.07	0.12	0.04	0.15	0.17
	(0.03, 0.21)**	(−0.01, 0.24)	(−0.07, 0.21)	(−0.04, 0.27)	(−0.03, 0.12)	(−0.01, 0.32)	(0.01, 0.33)*
MDA	0.06	0.07	0.01	0.07	−0.04	0.06	0.06
	(0.02, 0.10)**	(0.01, 0.12)*	(−0.05, 0.07)	(0.00, 0.14)*	(−0.07, −0.00)*	(−0.01, 0.13)	(−0.01, 0.13)
8-$PGF_{2\alpha}$	0.23	0.17	0.16	−0.02	0.05	0.00	0.00
	(0.06, 0.39)**	(−0.06, 0.39)	(−0.09, 0.41)	(−0.30, 0.26)	(−0.09, 0.20)	(−0.30, 0.31)	(−0.29, 0.29)
11-$PGF_{2\alpha}$	0.29	0.12	−0.13	−0.03	0.02	−0.07	−0.03
	(0.17, 0.40)**	(−0.04, 0.27)	(−0.31, 0.04)	(−0.23, 0.16)	(−0.08, 0.12)	(−0.28, 0.15)	(−0.23, 0.17)
15-$PGF_{2\alpha}$	0.15	0.02	−0.06	−0.03	−0.01	−0.02	−0.02
	(0.10, 0.21)**	(−0.06, 0.10)	(−0.15, 0.02)	(−0.13, 0.07)	(−0.06, 0.04)	(−0.12, 0.09)	(−0.12, 0.08)
8,15-$PGF_{2\alpha}$	0.04	0.13	0.29	0.11	0.03	0.07	0.06
	(−0.06, 0.15)	(−0.01, 0.27)	(0.13, 0.44)**	(−0.07, 0.28)	(−0.05, 0.12)	(−0.12, 0.26)	(−0.12, 0.23)
First morning urine sample (n = 243)							
diY	0.02	−0.05	−0.28	−0.11	−0.03	−0.14	−0.06
	(−0.02, 0.06)	(−0.15, 0.04)	(−0.42, −0.13)**	(−0.27, 0.06)	(−0.08, 0.02)	(−0.31, 0.03)	(−0.24, 0.11)
8-OHdG	−0.01	0.02	0.01	0.50	0.07	0.39	0.49
	(−0.07, 0.06)	(−0.16, 0.19)	(−0.25, 0.26)	(0.28, 0.72)**	(−0.01, 0.14)	(0.16, 0.63)**	(0.25, 0.73)**
MDA	0.03	0.06	0.03	0.12	−0.02	0.09	0.09
	(0.00, 0.06)*	(−0.01, 0.12)	(−0.07, 0.13)	(0.01, 0.23)*	(−0.05, 0.02)	(−0.03, 0.20)	(−0.03, 0.21)
8-$PGF_{2\alpha}$	0.21	0.03	0.08	0.06	0.04	0.12	0.12
	(0.06, 0.36)**	(−0.31, 0.36)	(−0.41, 0.58)	(−0.49, 0.61)	(−0.14, 0.23)	(−0.47, 0.71)	(−0.47, 0.71)
11-$PGF_{2\alpha}$	−0.02	0.08	−0.29	−0.10	0.07	−0.14	−0.08
	(−0.11, 0.06)	(−0.10, 0.26)	(−0.56, −0.02)*	(−0.41, 0.21)	(−0.04, 0.17)	(−0.47, 0.18)	(−0.41, 0.25)
15-$PGF_{2\alpha}$	0.03	0.04	−0.13	−0.08	−0.00	−0.12	−0.09
	(−0.02, 0.08)	(−0.06, 0.14)	(−0.28, 0.02)	(−0.24, 0.09)	(−0.06, 0.05)	(−0.29, 0.06)	(−0.27, 0.08)
8,15-$PGF_{2\alpha}$	0.06	0.23	0.44	0.08	−0.01	0.01	−0.02
	(−0.01, 0.14)	(0.06, 0.40)**	(0.19, 0.70)**	(−0.21, 0.36)	(−0.10, 0.09)	(−0.30, 0.31)	(−0.33, 0.29)

[a] Age, gender, body mass index, ethnicity, dietary supplement intake, exercise frequency and alcohol consumption were included as covariates.
[b] CI, confidence interval.
* Correlation is significant at the 0.05 level.
** Correlation is significant at the 0.01 level.

apart offered a ≤13% increase in sensitivities in comparison to a single sample, whereas the analysis of two FMV urine increased the sensitivity by 8–20% for PNP, 3-PBA, and *trans/cis*-DCCA in comparison to a single sample. When three urine samples collected days apart were analyzed, moderate sensitivities in the ranges of 0.58–0.83 and 0.65–0.88 were obtained for spot and FMV samples, respectively.

The specificity (0.73–0.94) was uniformly higher than the sensitivity (0.45–0.88) for both spot and FMV urine samples. This suggests that ability to predict a false (i.e., specificity) value was higher than that for a true value (i.e., sensitivity). Nevertheless, sensitivity and specificity values may be overestimated due to the contribution of "predicted" values in the calculation (Wang et al., 2016d). The minimum number of urine specimens (k) required from a single person to estimate the participant-specific mean for the seven pesticides and their metabolites within 20% of the "true" values was 28–140 and 18–119 for spot and FMV samples, respectively.

3.5. Association between urinary pesticide/metabolites and oxidative stress biomarkers

We found significant positive correlations between repeated measures of the studied pesticide/metabolites and OSBs, particularly 8-OHdG and MDA′ in spot and FMV samples collected during the 44-day study period (Table 5). The relationships among the seven OSB biomarkers have been described in our previous study (Martinez-Moral and Kannan, 2019). Creatinine-corrected concentrations of all OSBs except for 8,15-$PGF_{2\alpha}$ were significantly correlated with IMPY in spot samples, whereas 8-OHdG concentrations were correlated with those of 3-PBA, and *trans/cis*-DCCA in FMV urine samples. The strength of positive associations (expressed by the β of mixed regression models) at significant levels was 0.03–0.50. Our results showed that exposure to pesticides could induce oxidative damage to lipids, proteins, and DNA in the general population. Significant negative associations in creatinine-corrected concentrations were found between TCPY and diY/11-$PGF_{2\alpha}$, and 2,4-D and MDA. A significant increase in urinary 8-OHdG was reported in Chinese farmers after spraying chlorpyrifos (Wang et al., 2016a). A positive correlation between repeated urinary measures of 2,4-D and 8-OHdG and 8-$PGF_{2\alpha}$ was reported in farmers from Iowa (Lerro et al., 2017). This is the first study to document the relationship between oxidative stress and pesticide concentrations in urine from the general population.

4. Conclusions

Our study demonstrated that the general population is exposed to low levels of OPs, PYRs, and PAs (median concentrations: < 1 ng/mL). Repeated measurements of 11 pesticides and their metabolites in urine showed considerable within-individual variability in both spot and FMV samples. Low ICC values of urinary pesticide concentrations indicated poor reproducibility of overall exposures from a single measurement. To better characterize average exposure over a long period of time, collection and analysis of repeated samples are needed. Positive associations were found between urinary concentrations of pesticides and oxidative stress markers of lipids, proteins, and DNA.

Collection and analysis of a large number of samples consecutively per individual over a 44-day period allowed for a better understanding of temporal variability in urinary measures of pesticide exposure. The strengths of this study include its relatively large sample size collected

over a 44-day period per individual. We also comprehensively and concurrently measured 11 pesticides as well as seven OSBs in urine samples. This study, however, was conducted on a convenience and small sample of participants ($n = 19$). In addition, the participants had a wide range of age (from 11 to 56 years). Other limitations may include lack of actual urine excretion rates, urinary specific gravity concentrations, and potential uncontrolled confounders related to exposure that may induce OSBs. Therefore, the generalizability of pesticide exposures to larger populations warrants caution.

Declaration of Competing Interest

The authors declare no competing financial 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.2019.104904.

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