Nonylphenol exposure is associated with oxidative and nitrative stress in pregnant women

Pei-Wei Wang¹*, Mei-Lien Chen²*, Li-Wei Huang³, Winnie Yang⁴, Kuen-Yuh Wu⁵ & Yu-Fang Huang²,⁶

¹Department of Pediatrics, Taipei City Hospital, Heping Fuyou Branch, Taipei, Taiwan, ²Institute of Environmental and Occupational Health Sciences, National Yang Ming University, Taipei, Taiwan, ³Department of Obstetrics and Gynecology, Taipei City Hospital, Heping Fuyou Branch, Taipei, Taiwan, ⁴Division of Pediatrics, Taipei City Hospital, Yangming Branch, Taipei, Taiwan, ⁵Institute of Occupational Medicine and Industrial Hygiene, College of Public Health, National Taiwan University, Taipei, Taiwan, and ⁶Department of Education and Research, Taipei City Hospital, Taipei, Taiwan

ABSTRACT
Animal studies have shown that exposure to nonylphenol (NP) increases oxidative/nitrative stress, but whether it does so in humans is unknown. This study examines prenatal exposure to NP and its effects on oxidatively/nitratively damaged DNA, lipid peroxidation, and the activities of antioxidants. A total of 146 urine and blood specimens were collected during gestational weeks 27–38 and hospital admission for delivery, respectively. Urinary NP was analyzed by high-performance liquid chromatography (HPLC). Urinary biomarkers of oxidatively/nitratively damaged DNA and lipid peroxidation, including 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), 8-nitroguanine (8-NO2Gua), 8-iso-prostaglandin F 2α (8-isoPF2α) and 4-hydroxy-2-nonenal-mercapturic acid (HNE-MA), were simultaneously analyzed using isotope-dilution liquid-chromatography/electron spray ionization tandem mass spectrometry. The activities of maternal plasma superoxide dismutase and glutathione peroxidase were analyzed by enzyme-linked immunosorbent assay. Urinary NP level was significantly associated with 8-oxodG and 8-NO2Gua levels in late pregnancy, suggesting that NP may enhance oxidatively and nitratively damaged DNA. The adjusted odds ratios for high 8-oxodG level exhibited a significantly dose–response relationship with NP levels, stratified into four quartiles. 8-oxodG appears to be a more sensitive and effective biomarker of NP exposure than 8-NO2Gua. These relationships suggest NP may play a role in the pregnancy complications.

Introduction
Nonylphenol (NP), which is a degradation product of nonylphenol polyethoxylates (NPEOs), is an intermediate chemical widely used for the production of surfactants, detergents, emulsifiers, pesticides, lubricants and oil additives that are used in daily life [1]. Biomonitoring studies revealed significant levels of NP in urine, plasma, placenta and breast milk of pregnant women and newborns in northern Taiwan [2–4]. Based on Müller’s pharmacokinetic study, the half-life of NP in blood is 2–3 h, and the bioactivity of NP is 20% after it is ingested orally or administered intravenously [5]. Despite the rapid metabolism of NP, it can be detected in mother–fetus dyads samples, suggesting that pregnant women and fetuses are repetitively and persistently exposed to NP. Since NP has a similar structure to that of estrogen, it bonds to estrogen receptors in human estrogen-sensitive MCF7 breast tumor cells and exhibits weak estrogen-like properties [6]. Xeno-estrogen exposure may have adverse consequences, especially for pregnant women, who are susceptible and vulnerable, and for their fetuses. NP exhibits reproductive toxicity, in addition to developmental, immune, thyroid and nervous systems effects in the offspring [7]. Our previous studies demonstrated that prenatal NP exposure is associated with small for gestational age (SGA), decreased body length at birth and low neonatal birth weight [3,8]. NP has been shown to induce oxidative/nitrative stress in vitro and in vivo [9–11] and to induce apoptosis and insulin signaling in the liver of rats [12,13]. However, whether NP is related to oxidative and nitrative stress in humans, and especially pregnant women, is unclear.

Oxidative/nitrative stress is caused by the increased production of reactive oxygen and nitrogen species (ROS/RNS) and/or decreased efficiency of antioxidant defense
mechanisms, which involve enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) [14–16]. Oxidative stress in pregnant women has been associated with pregnancy complications and adverse outcomes, such as preeclampsia, birth weight reduction and SGA [17–19]. ROS/RNS reacts with DNA or lipids in the body and can cause oxidatively/nitratively damaged DNA and lipid peroxidation [20]. The frequently measured biomarkers of oxidatively/nitratively damaged DNA are 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), which is formed in the reaction between ROS (e.g., hydroxyl radical) and 2′-deoxyguanosine in DNA, followed by nudix hydrolases, nucleotide excision repair and nucleotide incision repair [21] and 8-nitroguanine (8-NO2Gua) [22], 8-Iso-prostaglandin F2α (8-isoPF2α) and 4-hydroxy-2-nonenal-mercuric acid (HNE-MA) are biomarkers of lipid peroxidation that are derived from the oxidative breakdown of polyunsaturated fatty acids [23,24].

Despite the availability of data on NP exposure with oxidative stress or and the activities of antioxidants in cell and animal studies, to the best of our knowledge, evidence on the simultaneous oxidative/nitrative stress, lipid peroxidation and antioxidant activity of enzymes that are attributable to prenatal exposure to NP is limited. Thus, the hypothesis in this study is that NP may affect oxidative/nitrative stress, lipid peroxidation and the activities of antioxidants. Therefore, NP, 8-oxodG, 8-NO2Gua, 8-isoPF2α, HNE-MA in urine as well as SOD and GPx in blood specimens of pregnant women were analyzed.

Materials and methods

Study population and data collection

A cohort of pregnant women was studied at an obstetrics clinic in northern Taiwan. This study was approved by the Ethics Committee of Taipei City Hospital (TCH), Taipei. Pregnant women from the TCH were recruited during gestational weeks 27–38 (average 28 week). The recruitment period was from May to December 2014. People with occupational exposure to NP were excluded because of NP level was extremely high in textile workers [25]. After receiving informed consent, each woman completed a structured questionnaire to collect data on sociodemographic characteristics (age, weight, height and education level), lifestyle (smoking status, consumption of alcohol and coffee, exercise habit, frequency of use of detergent and plastic products, consumption of health foods and supplements such as vitamins C and E), work history and medical conditions. Lifestyle in the use of detergent and plastic products and work history in occupational NP exposure (e.g. textile and housekeeping workers) were considered to evaluate NP exposure. In total, 150 pregnant women were invited to participate in this study; 146 women agreed to participate and were followed until delivery. Maternal urine and venous blood were collected during clinic visit at gestational weeks 27–38 and in hospital admission for delivery, respectively. Although the duration of labor is associated with oxidative stress, urine sample were collected before delivery (2–13 week prior to labor). Thus, the duration of labor is unrelated with oxidative stress and cannot affect oxidative stress. All samples were immediately chilled and transported to the laboratory. Plasma was fractioned by centrifugation at 3000 rpm for 15 min and all samples were stored at −80°C until further analysis in the laboratory.

Urinary NP analysis

The urine samples were analyzed according to a method in previous studies [2,25]. Briefly, the pH of each 10 mL sample was adjusted to 5.5 using 1 M acetic acid (Merck, Darmstadt, Germany). Next, 1 mL of 1 M ammonium acetate solution (Merck, Darmstadt, Germany) and 125 μL β-glucuronidase (Sigma-Aldrich, St. Louis, MO) were added. The mixture was incubated at 37°C for 15 h in a shaker bath, and acidified to a pH of 3 using 1.0 M hydrochloric acid (Merck, Darmstadt, Germany). Following enzymatic deconjugation, samples were cleaned up using 3 mL Varian PH solid-phase extraction (SPE) cartridges. Into the SPE, cartridges were inserted 2 cm of silanized glass wool and the cartridges were then preconditioned with 20 mL methanol followed by 3 mL pure water that had been acidified with HCl. After the samples were applied to the cartridges, each cartridge was washed with 5 mL of pure water. Finally, analytes were eluted with 3 mL methanol using HPLC coupled with fluorescence detection (Hitachi, Tokyo). The reverse-phase column was a Luna C18-A (150 × 4.6 mm i.d.) with a particle size of 5 μm (Phenomenex, Torrance, CA). The isocratic mobile phase was an acetonitrile/water (75:25, v/v) mixture with a flow rate of 1.0 mL/min. The fluorescence detector was operated with an excitation wavelength of 275 nm and an emission wavelength of 300 nm. The samples were injected in quantities of 20 μL. To extend the lifespan of the HPLC column, all samples were filtered through a 5-μm PTFE membrane filter (Titin, Pullman, WA).

The analytical method that was employed in this study was both accurate and precise. No plastic product was used in the pretreatment of the samples. The recovery rate was 77–105% for NP levels of 6–235 ng/mL. The limit of detection (LOD) of NP in urine was 0.20 ng/mL. Urinary NP level was adjusted for creatinine and expressed as μg/g creatinine.
Simultaneous analysis of multibiomarkers for oxidative/nitrative stress and lipid peroxidation

Urinary 8-oxodG, 8-NO2Gua, 8-isoPF2, and HNE-MA were simultaneously analyzed using our newly established isotope-dilution liquid-chromatography–electron spray ionization tandem mass spectrometry (LC-ESI-MS/MS) method [26]. Briefly, 0.5 mL of each urine sample was thawed at room temperature, vortexed until it was thoroughly mixed and then centrifugated at 10,000 rpm/min for 10 min. Next, 100 μL of urine supernatant was diluted with 190 μL deionized water that contained 1 mM of ammonium acetate and was then spiked with 10 μL of 100 ng/mL 15N5-8-oxodG, 8-NO2Gua-4, 8-13C2-7-15N (Santa Cruz, CA) 8-isoPF2-d4 (Cayman, Ann Arbor, MI) and HNE-MA-d4 (Cayman, Ann Arbor, MI) as internal standards. 15N5-8-oxodG was available from Dr. Wu’s laboratory [27]. The mixture was vortexed and passed through a polyvinyl difluoride syringe filter for analysis using LC-MS/MS, which was operated in negative ion and multiple reaction-monitoring mode. The ion pairs monitored for quantification were m/z 194.9—178.1 for 8-NO2Gua, m/z 197.9—181.1 for 8-NO2Gua-4, 8-13C2-7-15N, m/z 318.2—171.1 for HNE-MA, m/z 321.5—174.1 for HNE-MA-d4, m/z 353.4—193.2 for 8-isoPF2, and m/z 357.0—197.2 for 8-isoPF2-d4. The positive ion mode was operated to quantify 8-oxodG by monitoring the ion pairs m/z 284.0—168.1 for 8-oxodG and m/z 289.1—173.2 for 15N5-8-oxodG. The LODs for 8-oxodG, 8-NO2Gua, 8-isoPF2, and HNE-MA in urine were 0.02 ng/mL, 0.03 ng/mL, 0.008 ng/mL and 0.01 ng/mL, respectively. Excellent linearity over the concentration range of 0.1–50 ng/mL was observed with regression coefficients >0.9982. To evaluate the performance of the method, four mixtures of standards that were spiked in urine at three levels (0.5, 5 and 25 ng/mL) were analyzed. The mean accuracy, defined as the percentage ratio of the calculated level of the four standards to the expected spiked concentrations, ranged from 97.8 to 102.2%, and the intraday and interday variations, expressed as relative standard deviation, were in the range of 3.0–8.1% and 3.1–9.3%, respectively. Urinary 8-oxodG, 8-NO2Gua, 8-isoPF2, and HNE-MA levels were adjusted for creatinine and expressed as μg/g creatinine.

Determination of concentration of creatinine

The measurement of the concentration of urinary creatinine was based on the Hinegard and Tiderstrom modification of the Jaffe reaction [28]. Briefly, 0.1 mL of a urine sample was added to 3 mL 3.3 mM picric acid that was mixed with 0.17 M sodium hydroxide and 26 mM sodium tetraborate. This mixture was then incubated at 37°C for 15 min in a shaker bath. The creatinine level was measured using a spectrophotometer at a wavelength of 510 nm. Fourteen urine samples with a creatinine level of less than 0.3 g/L or greater than 3.0 g/L were excluded from the data analysis.

Analysis of antioxidant enzymes SOD and GPx

The activities of SOD and GPx in plasma were evaluated by enzyme-linked immunosorbent assay (ELISA) using a superoxide dismutase assay kit and a glutathione peroxidase assay kit (Cayman Chemical), according to the manufacturer’s instructions. The activities of SOD and GPx were given as U/mL and nmol/min/mL. All samples were analyzed in duplicate, and the two measurements were averaged for statistical analysis.

Statistical analysis

Data were analyzed using SPSS 17.0 software. Statistical significance was identified as p < 0.05. Before the statistical analyses were performed, Shapiro–Wilk and Kolmogorov–Smirnov normality tests were conducted on the NP level and biomarkers of oxidative stress. The levels of NP, 8-oxodG, 8-NO2Gua, 8-isoPF2, and HNE-MA levels were skewed. Thus, all measurements were subjected to log transformation prior to the statistical analyses. After fitting of the model to the data using the goodness-of-fit test, one female smoker and two female drinkers were excluded for models observations. The correlations among NP exposure, concentrations of biomarkers of oxidative stress and potential confounders such as age and body mass index (BMI), were determined using Spearman’s correlations test. Multivariate regression analyses were performed to investigate the relationships between NP levels and concentrations of 8-oxodG, 8-NO2Gua, 8-isoPF2, HNE-MA and antioxidants after adjustment for confounding factors. The odds ratios (OR) of urinary 8-oxodG, 8-NO2Gua, 8-isoPF2, HNE-MA and antioxidants levels above the median level were estimated for pregnant women with levels of NP that were below the 25th (reference group), between the 25th and 50th, between the 50th and 75th, and above the 75th percentiles, were obtained using logistic regression models.

Results

Characteristics of the study population

Table I shows the characteristics of the expectant mothers who participated in this investigation. In total, 146 pregnant women were followed until delivery. The pregnant women had a mean age of 33.4 years...
Adverse pregnancy outcomes and Occupation

Regular exercise 27 (18.6)
Nutrient supplementation
Coffee consumption
Alcohol consumption
Smoking status
Parity
Primiparous 79 (54.9)
Multiparous 65 (45.1)
Smoking status
Never-user 144 (99.3)
Frequent users (>1 time/wk) 1 (0.7)
Alcohol consumption
Nonuser 143 (98.6)
Frequent users (>1 time/wk) 2 (1.4)
Nutrient supplementation
Vitamins intake 37 (25.3)
Vitamins C & E intake 26 (17.8)
Regular exercise 27 (18.6)
Occupation
Housewife 47 (32.2)
Working women 99 (67.8)
Adverse pregnancy outcomes and medical diseases
Gestational diabetes 3
Gestational hypertension 3
Breast cancer 1
Thalassemia 1

Table I. General characteristics of pregnant women.

| Variable                        | Mother (n = 146) |
|---------------------------------|-----------------|
| Age (y)                         | 33.4 ± 3.3 (24–41) |
| Maternal BMI at delivery (kg/m²) | 26.1 ± 3.2 (18.6–35.8) |
| Educational level               |                 |
| Senior high school              | 18 (12.3)       |
| College                         | 97 (66.4)       |
| Graduate                        | 31 (21.3)       |
| Parity                          |                 |
| Primiparous                     | 79 (54.9)       |
| Multiparous                     | 65 (45.1)       |
| Smoking status                  |                 |
| Never-user                      | 144 (99.3)      |
| Frequent users (>1 time/wk)     | 1 (0.7)         |
| Alcohol consumption             |                 |
| Nonuser                         | 143 (98.6)      |
| Frequent users (>1 time/wk)     | 2 (1.4)         |
| Nutrient supplementation        |                 |
| Vitamins intake                 | 37 (25.3)       |
| Vitamins C & E intake           | 26 (17.8)       |
| Regular exercise                | 27 (18.6)       |
| Occupation                      |                 |
| Housewife                       | 47 (32.2)       |
| Working women                   | 99 (67.8)       |
| Adverse pregnancy outcomes and medical diseases | |
| Gestational diabetes            | 3               |
| Gestational hypertension        | 3               |
| Breast cancer                   | 1               |
| Thalassemia                     | 1               |

old; their average BMI was 26.1 kg/m² at delivery. Most of the pregnant women (128/146, 88%) had at least a bachelor’s degree and 68% worked during gestation. Fifty-five percent were primiparous. Among the 146 pregnant women, only one smoked cigarettes, two drank alcohol, 16 drank coffee and 27 took regular exercise during gestation. With respect to nutrient supplementation, 25% of women took vitamins and 18% took vitamins C and E. Data from women with pregnancy complications and medical diseases were excluded from the analysis of urinary NP and oxidative/nitrative stress biomarkers: three had gestational diabetes, three had gestational hypertension, one had breast cancer, and one had thalassemia. Accordingly, a total of 138 women had an uncomplicated pregnancy.

Urinary NP and biomarkers of oxidative/nitrative stress in pregnant women

Table II presents the detection rates, geometric mean (GM), geometric standard deviation (GSD), mean ± SD, range and percentiles (25th, 50th, 75th) of maternal NP, biomarkers of oxidative/nitrative stress and antioxidants during the third trimester. Analysis of all samples shows that NP, 8-oxodG, 8-NO₂Gua, 8-isoPF₂, and HNE-MA were detectable in 100% of urine samples. Figure S1 (Supporting Information) shows typical chromatograms of urinary 8-oxodG, 8-NO₂Gua, 8-isoPF₂, and HNE-MA from a pregnant woman. The GM (GSD) levels of urinary NP, 8-oxodG, 8-NO₂Gua, 8-isoPF₂, and HNE-MA were 2.90 (1.50), 3.45 (2.16), 30.06 (2.74), 9.05 (2.30) and 71.26 (3.13) ng/mL; 4.06 (1.77), 5.00 (2.35), 39.74 (2.77), 13.57 (2.04), and 108.57 (3.05) μg/g creatinine, respectively. Also, the SOD and GPx levels in plasma samples were 10.8 ± 4.2 (U/mL) and 289.1 ± 94.6 (nmol/L/min/mL), respectively.

Association among NP exposure, biomarkers of oxidative/nitrative stress, and potential covariates

The correlations among NP exposure, 8-oxodG, 8-NO₂Gua, 8-isoPF₂, HNE-MA, SOD, GPx and potential covariates were analyzed using Spearman correlation coefficients. As shown in Figure 1, significant correlations existed between NP level, stratified into four quartiles (below the 25th, 25th–50th, 50th–75th, above 75th percentiles), and 8-oxodG (r = 0.43, p < 0.0001) and 8-NO₂Gua levels (r = 0.27, p = 0.003). Significant correlations were found between urinary levels of 8-oxodG and 8-NO₂Gua (r = 0.25, p = 0.005) and between 8-isoPF₂ and HNE-MA level (r = 0.20, p = 0.032) (Figure 2). Insignificant correlations existed between urinary NP level and levels of 8-isoPF₂ or HNE-MA and antioxidants activities (Table S1). Significant correlations were found between NP level and BMI at delivery (r = −0.18, p = 0.046), between 8-oxodG level and the taking of vitamin supplements (r = 0.29, p = 0.001), and between 8-NO₂Gua level and regular exercise (r = 0.19, p = 0.037) (Supplementary Table S1).

To study the effects of NP and other predictors that potentially influence urinary levels of 8-oxodG, 8-NO₂Gua, 8-isoPF₂, HNE-MA and the activities of antioxidants, the data were further analyzed using multiple linear regression. Supplementary Table S2 shows that, after controlling for maternal age, BMI at delivery, regular exercise, and the taking of vitamin supplements, an increase in urinary NP in the third trimester significantly increased 8-oxodG level (p < 0.0001) and 8-NO₂Gua level (p = 0.001). However, when the effect of exposure to NP was taken into account, a significant association was found between 8-oxodG level and the taking of vitamin supplements (p = 0.04).

The risk of increased oxidative and nitrative stress (with the 50th percentile as the cutoff value) among pregnant women was evaluated by stratifying urinary NP level into four quartiles (below the 25th (reference), between the 25th and the 50th, between the 50th and the 75th, and above the 75th percentiles). The adjusted
OR for high 8-oxodG level showed a significant dose-response relationship with NP levels (OR = 1.00, 3.83, 5.93 and 13.30, respectively, all p < 0.05; trend test p = 0.0001) (Table III). The adjusted OR for high 8-NO2Gua were significantly correlated with NP exposure in the second and fourth quartiles compared to the first quartile, after adjustments were made for covariates (OR = 3.67, p = 0.02; OR = 3.41, p = 0.04).

Discussion

Numerous studies have investigated NP levels in urine in the general population and in workers who are occupationally exposed to NP. The GM (range) of NP levels in maternal urine in this study was 2.90 (0.84–7.11) ng/mL or 4.06 (0.75–16.42) mg/g creatinine. This level was consistent with previous studies, which have reported a mean level of 3.74 ng/mL for office workers [25] and 4.10 (0.04–48.45) mg/g creatinine for pregnant women in Taiwan [8], but it exceeds levels of <0.1–1.57 ng/mL obtained for adults in the USA [29,30] and those of <0.3–2.00 ng/mL obtained for adults in Japan [31,32]. However, the NP level in this study was lower than the levels of 23.50 ± 17.34 ng/mL and 42.06 ± 46.63 ng/mL, found in a study of individuals with pre- and postshift occupational NP exposure [25]. Generally, wastewater treatment plants cannot degrade alkylphenols (APs). Chen et al. found high levels of NP in raw water and treated water [33]. The APs levels in Taiwanese rivers and sediments exceeded those in other countries such as Germany and Japan [34]. Inputs of APs into the aquatic environment may account for their presence in aquatic products. Alkylphenols persist in the environment and enter the food chain, eventually accumulating in the lipid-enriched matrix. The high background NP level that arises from the everyday use of NPEOs detergents and contamination of the environment and foods via bioaccumulation may contribute to the high levels of exposure to NP among Taiwanese [33,35]. Some LC-MS/MS methods have been developed for separately quantifying 8-oxodG, 8-NO2Gua, 8-isoPF2a, and HNE-MA [27,36,37]. Those methods have LODs of 0.024 ng/mL for 8-oxodG, 0.001 ng/mL for 8-isoPF2a, 2.5 nM for HNE-MA, and the method herein is comparable, being extremely sensitive to those compounds in all urine samples. To the best of our knowledge, works on the determination of 8-oxodG, 8-NO2Gua, 8-isoPF2a and HNE-MA in human beings are very few, and very limited studies reported the correlation between exposure to NP and the levels of multiple-biomarkers of oxidative and nitrative stress. This study is the first to simultaneously elucidate the associations between exposure to NP and urinary levels of 8-oxodG, 8-NO2Gua, 8-isoPF2a and HNE-MA in human beings are very few, and very limited studies reported the correlation between exposure to NP and urinary levels of 8-oxodG, 8-NO2Gua, 8-isoPF2a and HNE-MA in pregnant women. This study demonstrates that urinary NP level is a significant predictor of 8-oxodG and 8-NO2Gua levels in pregnant women after adjustment for potential covariates. The finding suggests that NP may enhance oxidatively/nitratively damaged DNA. Previous studies have reported that 8-oxodG can cause poor vascularization of the placenta and consequently pre-eclampsia, birth weight reduction and SGA [17–19]. Thus, we suggest NP may play a role in the pregnancy complications. Furthermore, the association between exposure to NP and 8-oxodG level is stronger than that with 8-NO2Gua level, as evidenced by the fact that adjusted OR of high 8-oxodG level showed a significantly dose-response relationship. Thus, 8-oxodG appears to be a more sensitive and effective biomarker of exposure to NP than is 8-NO2Gua.

This finding is somewhat in accordance with published studies [9–12,38]. NP has been shown to inhibit the activities of cytochrome P450-1A in rat liver, which leads to increase the levels of ROS (e.g. hydrogen peroxide) in the rats liver [12] and RNS (e.g. nitric oxide) in the mice brain [10] and to induce oxidative damage in the liver, pancreas and kidney of rats [11,12,38]. NP was also shown to increase lipid peroxidation in the...
thiobarbituric acid reactive substance and to depress the activities of SOD and catalase in the liver of rats [12].

The relationship between NP and biomarkers of oxidative and nitrative stress in humans has seldom been investigated. The mean 8-oxodG level in maternal urine in this study was 7.20 μg/g creatinine, which is consistent with those obtained in previous studies of pregnant women, which have reported mean levels of 4.22 μg/g creatinine [18] and 5.30 μg/g creatinine [40] and with a study of maternal exposure to arsenic, which

Figure 1. Correlations between urinary NP level, stratified into quartiles, and (A) 8-oxodG level and (B) 8-NO2 Gua level. The upper and lower bars represent 95% confidence interval.
found a mean 8-oxodG level of 3.48 μg/g creatinine [41]. Few studies have studied 8-NO$_2$Gua levels in pregnant women. Huang et al. reported upon inducible nitric oxide synthase (iNOS) expression and the formation of 8-NO$_2$Gua in the cancer tissues of nasopharyngeal carcinoma patients [42]. Nitroguanosine and nitrite, biomarkers of nitratively damaged DNA, have been found in the placenta and blood of pregnant women [19,43]. With respect to biomarkers of lipid peroxidation, the mean 8-isoPF$_{2\alpha}$ level in maternal urine in this study was 17.20 μg/g creatinine, which exceeds corresponding values obtained that have been elsewhere for pregnant women, including mean levels of 1.31 μg/g creatinine [44] and 3.09 μg/g creatinine [40]. Variations among the groups of subjects in the various studies, in terms of race, age and life style, for example, or/and the assays

Figure 2. Correlations between (A) urinary 8-oxodG level and 8-NO$_2$Gua level and (B) HNE-MA level and 8-isoPF$_{2\alpha}$ level. Dotted lines represent 95% confidence intervals.
Oxidative/nitrative stress can be caused by an endogenous process during normal cell metabolism, as well as by exogenous factors, such as aging, BMI, cigarette smoking, alcohol consumption, dietary intake (including the intake of vegetables and fruits) and supplementation of vitamins C and E [16,24,46]. This study identifies factors other than exposure to NP that influence 8-oxodG, 8-NO₂Gua, 8-isoPF₂a, and HNE-MA levels, by examining the associations between demographic and lifestyle data of pregnant women and urinary biomarkers of oxidative and nitrative stress. Herein, significant correlations were found between 8-oxodG level and the taking of vitamin supplements and between 8-NO₂Gua level and regular exercise. Some studies have shown that oxidative stress increases during pregnancy [44]. Hung et al. indicated that labor is associated with increased placental oxidative stress and influences maternal oxidative stress [47]. However, neither gestational age nor mode of delivery was significantly associated with urinary biomarkers of oxidative or nitrative stress in this study, perhaps because of the relatively small sample size.

There are certain potential limitations to this study. First, the sample size is relatively small, resulting from the challenge of establishing a cohort of pregnant women. Second, paraoxonase genetic polymorphism used for detection, may be responsible for this discrepancy. Very few studies have investigated urinary HNE-MA levels in pregnant women. One study reported the accumulation of HNE in preeclamptic placenta [43]. Another report showed that the mean level of urinary HNE-MA was 3000 μg/g creatinine in a general population of smoking and nonsmoking men and women; this level is higher than that obtained herein [24]. The difference may arise from differences between the subjects of the studies in terms of race, gender and smoking status, for example. The median levels of SOD and GPx of pregnant women in the third trimester herein exceed those in the second trimester, found in a study of Mistry et al. (2015), which were 0.5 U/mL and 3000 nmol/min/mL respectively. This finding demonstrates for the first time that NP enhances 8-oxodG and 8-NO₂Gua levels in late pregnancy. Additionally, the association between NP level and 8-oxodG level was stronger than between NP level and 8-NO₂Gua, as evidenced by the fact that the adjusted OR of high 8-oxodG level exhibited a significant dose-response relationship. Thus, 8-oxodG appears to be a more sensitive and effective biomarker for DNA damage than is 8-NO₂Gua. Based on the present study, we propose that urinary 8-oxodG or/and 8-NO₂Gua may serve as surrogate biomarkers of oxidatively/nitratively damaged DNA following cumulative NP exposure. Further studies are necessary to shed light on the mechanism by which NP affects oxidative stress and the health of this vulnerable population.

### Table III. Adjusted odds ratios (95% confidence interval) between oxidative and nitrative stress biomarkers (cutoff 50th percentile) and urinary NP levels (in the 1st, 2nd, 3rd, and 4th quartile) calculated in an adjusted logistic regression model.

| Variables | 8-oxodG (μg/g creatinine) | 8-NO₂Gua (μg/g creatinine) | HNE-MA (μg/g creatinine) | 8-isoPF₂a (μg/g creatinine) | GPx (nmol/min/mL) | SOD (U/mL) |
|-----------|---------------------------|-----------------------------|--------------------------|-----------------------------|------------------|------------|
| 3rd trimester NP | | | | | | |
| 1st quartile (reference) | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| 2nd quartile | 3.83 (1.15, 12.74)* | 3.67 (1.20, 11.27)* | 1.07 (0.37, 3.05) | 1.13 (0.39, 3.25) | 0.52 (0.11, 2.35) | 0.84 (0.25, 2.76) |
| 3rd quartile | 5.93 (1.75, 20.24)* | 2.18 (0.71, 6.68) | 1.16 (0.40, 3.55) | 2.30 (0.77, 6.81) | 0.37 (0.07, 1.99) | 0.32 (0.08, 1.32) |
| 4th quartile | 13.30 (3.40, 52.06)* | 3.41 (1.05, 11.04)* | 0.91 (0.30, 2.75) | 1.42 (0.46, 4.37) | 0.24 (0.05, 1.26) | 1.23 (0.30, 5.05) |
| p value for trend | <0.0001* | 0.12 | 0.84 | 0.45 | 0.09 | 0.82 |

Adjusted covariates: maternal age, delivery BMI, regular exercise, and vitamin supplements

Quartiles: 1st: <25th percentile, 2nd: 25th–50th percentile, 3rd: 50th–75th percentile, 4th: ≥75th percentile

*p<0.05.

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### Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. The authors would also like to thank the National Science Council of the Republic of China, Taiwan (Contracts Nos. NSC 99-2314-B-010-018-MY3, NSC 102-2314-B-010-031-MY3) and Taipei City Government Department of Health (Contract No. 102TPECH11) for financially supporting this research.
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