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Comparison of three oxidative stress biomarkers in a sample of healthy adults

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Abstract

Oxidative stress is a potentially important aetiological factor for many chronic diseases, including cardiovascular disease, neurodegenerative disease and cancer, yet studies often find inconsistent results. The associations between three of the most widely used biomarkers of oxidative stress, i.e. F_2-isoprostanes for lipid peroxidation and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) and the comet assay with FPG for oxidative DNA damage, were compared in a sample of 135 healthy African-American and white adults. Modest associations were observed between F_2-isoprostanes and the comet assay ($r=0.22$, $p=0.01$), but there were no significant correlations between 8-oxo-dG and the comet assay ($r=-0.09$) or F_2-IsoP ($r=-0.04$). These results are informative for researchers seeking to compare results pertaining to oxidative stress across studies and/or assessment methods in healthy disease-free populations. The development and use of oxidative stress biomarkers is a promising field; however, additional validation studies are necessary to establish accuracy and comparability across oxidative stress biomarkers.

Keywords: *Oxidation DNA damage; lipid peroxidation; 8-hydroxy-2-deoxyguanosine; comet assay; isoprostanes*

Introduction

Oxidative stress is commonly described as the imbalance that occurs when reactive oxygen species (ROS) or radical-generating agent concentrations exceed the body's defence mechanisms (Sies 1991). Oxidative stress can be caused by exogenous factors, such as smoking, as well as endogenous processes during normal cell metabolism. Humans have well-developed defence systems that generally maintain homeostasis by detoxification of these oxidative products or by DNA repair; however, defences may be overwhelmed under conditions of elevated oxidative stress, leading to damage of lipids, proteins and DNA. Thus, oxidative stress is postulated to be a potentially important factor in the development of many chronic diseases, including cardiovascular disease, neurodegenerative disease and cancer (Ames 2001, Klaunig & Kamendulis 2004, Cooke et al. 2006, Dalle-Donne et al. 2006, Tsimikas 2006, Montuschi 2007, Musiek et al. 2007).

Although oxidative stress also affects proteins, this work focuses on oxidative DNA damage and lipid peroxidation because they are the forms of oxidative stress most frequently reported in the literature. The two most widely studied measures of oxidative DNA damage are the comet assay and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) (Gallo et al. 2008). The comet assay, also called single-cell gel electrophoresis, measures DNA

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strand breaks and alkali-labile sites within individual cells. Breaks in DNA allow supercoiled loops of DNA to relax and if damaged, appear like a comet with a tail under the conditions of the assay (Collins et al. 1996, Burlinson et al. 2007). Levels of 8-oxo-dG or 8-hydroxy-2'-deoxyguanosine (8-OHdG), which are formed by direct attack of ROS on DNA, can be measured using several methods, including liquid chromatography–mass spectrometry (LC/MS), enzyme-linked immunosorbent assay (ELISA) and high-performance LC (HPLC). Although 8-oxo-dG is a frequently used method, study results have been inconsistent due to variation in the quantification method used, sample preparation and DNA isolation, which may increase artifactual oxidation due to traces of transition metals and oxygen (Cadet et al. 1997, Halliwell 2000). However, recent refinements in methods using ultraperformance LC–heated electrospray ionization–tandem MS (UPLC-HESI-MS/MS) have greatly improved sensitivity and specificity (G. Boysen et al., unpublished data). F_2-IsoPs are prostaglandin-like compounds produced by the peroxidation of arachidonic acid independently of cyclooxygenase (COX) enzymes (Milne & Morrow 2006, Montuschi et al. 2007). Quantification of F_2-IsoPs in plasma or urine has recently been described as the gold standard for measuring lipid peroxidation (Kadiiska et al. 2005).

An increasing number of studies are examining associations of oxidative stress with chronic diseases (Klaunig & Kamendulis 2004, Tsimikas 2006, Montuschi et al. 2007, Musiek et al. 2007) and other disease risk factors, such as dietary exposures (Ames 2001, Collins 2005, Hwang & Bowen 2007, Montuschi et al. 2007, Watters et al. 2007). However, the biomarkers of oxidative stress and the methods used to measure them often vary widely across studies, making it difficult to put results from different studies into context. Recently, the European Standards Committee on Oxidative DNA Damage (ESCODD) compared results measuring oxidative DNA damage levels in healthy males using six different laboratories ($n=88$) and found no overall association between samples measured by the comet assay and 8-oxo-dG by HPLC methods (Gedik et al. 2005). Considering that the validity of many biomarkers remains to be established, the association between oxidative stress and disease *in vivo* in humans may be obscured due to noted inconsistencies among oxidative stress assays (Halliwell & Grootveld 1987, Dalle-Donne et al. 2006, Montuschi et al. 2007). Thus, further investigations into the comparability and validity of biomarkers used to assess oxidative stress are warranted.

In this report, we examine associations among three of the most widely used biomarkers of oxidative stress, F_2-isoprostanes (lipid peroxidation) and 8-oxo-dG and the comet assay with FPG (oxidative DNA damage), as well as associations with participant characteristics, in a sample of 135 healthy African-American and white adults. Results from this study provide insight into the extent to which there is agreement among the three methods and inform on the appropriateness of comparing results across studies that have employed different biomarkers of oxidative stress.

Materials and methods

Study population

Data reported here are from the Diet, Supplements and Health (DISH) study, a cross-sectional study of dietary antioxidants and oxidative stress in healthy African-American and white adults in North Carolina. Details on the DISH study design and methods have been published elsewhere (Watters et al. 2007). Briefly, participants were recruited between March and December 2005 via flyers displayed in public venues throughout the Research Triangle area in North Carolina, including churches, gyms and campus buildings, and by university-wide emails. Eligible persons were aged 20–45 years, generally healthy, free of diseases related to oxidative stress (i.e. cancer, diabetes, heart disease or Alzheimer's disease), and fluent in written and spoken English. Persons likely to have high levels of oxidative stress, such as current smokers and those with a self-reported body mass index (BMI) of 30 or greater, were ineligible. Other exclusion criteria included anorexia or bulimia nervosa, large weight change (more than 15 lbs or 6.8 kg) in the past year, inability to fast for 6 h, and pregnancy. Of the 191 respondents deemed eligible during the screening interview, 168 (88.0%) were enrolled and 164 (85.9%) completed all aspects of the study. Data for nine participants were excluded because of serum cotinine levels that were consistent with active smoking (≥ 15 ng ml^{-1}) and all three oxidative stress assays were not available for 20 participants, leaving a total of 135 participants for these analyses (65 African-Americans, 70 whites).

Data collection

Participants completed a self-administered 12-page demographic, health and dietary questionnaire which included 37 questions pertaining to general health, demographic, behaviour and lifestyle characteristics. During a one-time visit to the University of North Carolina (UNC) General Clinical Research Center (GCRC), participants had height, weight and waist circumference measured, provided semifasting (≥ 6 h) blood and urine samples, participated in a dietary supplement inventory and answered questions about the use of non-steroidal anti-inflammatory drugs and lipid-lowering drugs, current occupation, outdoor exposure and last menstrual

cycle (women only). Blood samples that were protected from heat and light were analysed for oxidative DNA damage, lipid peroxidation, plasma antioxidant nutrient concentrations, cholesterol, haemoglobin A1c (to confirm self-reported absence of diabetes) and serum cotinine (to validate self-reported smoking status). Plasma concentrations of retinol, tocopherols, vitamin C and carotenoids (lutein, zeaxanthin, α-cryptoxanthin, β-cryptoxanthin, lycopene, α-carotene, β-carotene) were measured using HPLC with multi-wavelength photodiode-array absorbance detection (Craft 1996). Each participant received US$100 compensation for his/her time upon completion of all study activities. This study was approved by the Institutional Review Board at UNC and written (signed) informed consent was obtained from all participants.

Measures of oxidative stress

F_2-Isoprostanes

F_2-IsoPs, also called 8-iso-$PGF_2\alpha$, are prostaglandin-like compounds produced by the peroxidation of arachidonic acid, independently of COX enzymes, and have been described as the 'gold standard' *in vivo* measure of lipid peroxidation (Milne & Morrow 2006, Montuschi et al. 2007). Urinary F_2-IsoPs represent a biomarker for systemic or 'whole body' oxidative stress over time (Milne et al. 2007) and are very stable because artifactual F_2-IsoPs are not formed by auto-oxidation due to the low lipid content of urine (Montuschi et al. 2007). Urinary F_2-IsoP levels were measured according to previously published methods (Milne et al. 2007) using spot urine samples that had been stored at –80°C, as recommended. Briefly, urinary F_2-IsoPs were quantified by gas chromatography with negative ion chemical ionization MS using $[^2H_4]$-15-F_{2t}-IsoP as an internal standard (Milne et al. 2007). Compounds were analysed as pentafluorobenzyl ester and trimethylsilyl ether derivatives by measuring the *m/z* 569 ion for endogenous F2-isoPs and *m/z* 573 ion for $[^2H_4]$-15-F_{2t}-IsoP. All urinary F_2-IsoPs levels were normalized to creatinine clearance and expressed as ng mg^{-1} creatinine.

8-Oxo-7,8-dihydro-2′-deoxyguanosine

The quantitative analysis of 8-oxo-dG was performed by UPLC-HESI-MS/MS (personal communication with G. Boysen). In brief, DNA was isolated from tissues using the Puregene® (Qiagen, Valencia, CA, USA) procedure with modifications. Tissues were lysed with Puregene® Cell Lysis Solution® in the presence of 20 mM 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO) (Acros, Fair Lawn, NJ, USA). Proteins were precipitated by addition of Protein Precipitation Solution® containing 20 mM TEMPO. RNA was hydrolysed with RNase A (Sigma-Aldrich, St Louis, MO, USA). DNA was precipitated by addition of isopropanol containing 20 mM TEMPO. Purity and amount of DNA was determined by ultraviolet photospectrometry Abs 260/280 > 0.8. DNA solutions of 0.5–1 μg μl^{-1} were prepared in Tris-HCl, 20 mM $MgCl_2$, pH 7.0 and 20 mM TEMPO. Stable isotope-labelled internal standard (500 fmol) $[^{15}N_5]$8-oxo-dG (Cambridge Isotope Laboratories, Andover, MA, USA) was added for accurate quantitation. DNA (50 μg) was digested to single nucleotides with DNase I, Type II (Sigma-Aldrich), phosphodiesterase I and alkaline phosphatase. Proteins were removed by Centricon YM-10 cellulose centrifuge filters (Millipore, Bedford, MA, USA) according to the manufacturer's specifications. 8-Oxo-dG and $[^{15}N_5]$8-oxo-dG were separated from normal nucleotides by reverse-phase HPLC using a Beckman ODS C_{18} 4.6 × 250 mm column, and 7% methanol–10 mM ammonium formate (pH 4.3) in HPLC-grade water as the mobile phase. The fraction containing 8-oxo-dG and $[^{15}N_5]$8-oxo-dG was collected into a collection tube containing 300 μl 75 mM TEMPO and the solvent was removed in speed vac. Samples were reconstituted in 20 μl water and measured with an UPLC (Waters, Milford, MA, USA) coupled to a TSQ-Quantum Ultra triple-quadrupole mass analyser (ThermoFinnigan, San Jose, CA, USA) using a HESI source. A 2.1 × 100 mm HSS T3 C_{18}, 1.8 μm column (Waters) was operated with a linear gradient of 1% methanol–0.1% acetic acid in water to 5% methanol–0.1% acetic acid and then held for 10 min, followed by increase to 50% methanol–0.1% acetic acid in 2 min, at a flow rate of 200 μl min^{-1}. The analyte and internal standard were detected in selected reaction monitoring (SRM) mode, monitoring the transitions of the *m/z* 284.1 to 168.05 and *m/z* 289.1 to 173.05 for 8-oxo-dG and $[^{15}N_5]$8-oxo-dG, respectively. The electrospray conditions were as follows: spray voltage of 3000 V, vaporizer (HESI) temperature of 250°C, sheath gas pressure 30, auxillary gas pressure 35, capillary temperature of 285°C and collision energy of 12 eV.

Comet assay with FPG

The single-cell gel electrophoresis or comet assay, measures DNA strand breaks and alkali-labile sites at the level of a single cell in which lymphocytes are digested with lesion-specific repair endonucleases (Collins 2005). The comet assay used here was a slightly modified version in which formamidopyrimidine DNA glycosylase (FPG) (kindly provided by Dr A. R. Collins, Oslo, Norway) was added to convert oxidized purines into strand breaks (Collins 2005, Gedik et al. 2005). FPG (5 μl) was diluted into 0.5 ml reaction buffer containing 40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg ml^{-1} BSA, pH 8.0, and 10% glycerol and stored as 10 μl aliquots at –80°C until used. Peripheral whole blood lymphocytes were washed in PBS, counted using a haemacytometer and cryopreserved in 1 ml RPMI-

1640 containing 15% BSA and 10% DMSO. All samples were processed within 2 h of collection and stored at –80°C until assays were performed, as recommended. Lymphocytes were sandwiched between 0.5% agarose and 0.5% low-melting-point (37°C) agarose (Fisher, Fair Lawn, NJ, USA). The resulting slides were placed into cold, freshly made lysis solution (10 mM Tris (pH 10), 2.5 M NaCl, 100 mM EDTA, 10% DMSO and 1% Triton X-100) at 4°C for 1 h. Fifty microlitres of FPG (10 µl aliquot diluted into 0.3 ml reaction buffer with no glycerol) was added to each slide for digestion at 37°C for 30 min. After lysis, slides were washed three times in buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg ml^{-1} bovine serum albumin, pH 8.0). Fifty microlitres of FPG in this buffer was added to each slide and then treated for 20 min in electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH 13) (Gedik et al. 2005, da Costa et al. 2006). After electrophoresis was performed at 25 V and 300 mA for 20 min, slides were incubated three times for 5 min in neutralization buffer (0.4 M Tris, pH 7.5), washed with methanol and stained with ethidium bromide. Multiple levels of quality control samples (e.g. 10% blinded duplicate samples) were included in each batch and all assays were performed by the University of North Carolina Clinical Nutrition Research Unit. Comet tail length (the distance of DNA migration from the body of the nuclear core) was visualized by using a fluorescence microscope (typically 100 cells per sample) and SCION IMAGE software (da Costa et al. 2006). The comet tail moment (defined as the integrated density in the comet tail multiplied by the distance from the centre of the nucleus to the centre of mass of the tail) was calculated by using the NIH Image Analysis Macro software (http://dir.nhlbi.nih.gov/labs/ldn/macroanalysis.asp). The comet tail moment reported here was calculated using FPG-sensitive sites only, but was also assessed for strand breaks without the addition of FPG.

Statistical analysis

Data analyses were performed using Stata (version SE 8.2, STATACorp, College Station, TX, USA). Descriptive statistics (means, standard deviations, medians and percentages) were calculated for all variables. Twenty participants with one or more missing values for the oxidative stress measures were excluded. Log transformations were applied to the three oxidative stress measures: lipid peroxidation (F_2-isoprostanes) and oxidative DNA damage (8-oxo-dG and the comet tail moment) to help meet normality distribution assumptions, as each was right-skewed. Furthermore, oxidative stress distributions were normalized by z score transformation to account for differences in units among the three methods and used in all correlation analyses. Differences between participant characteristics and categories of each oxidative stress measure were evaluated using analysis of variance. The demographic and lifestyle characteristics of participants analysed included sex, race, age, BMI, education, income, dietary supplement use, physical activity, alcohol consumption, passive smoke exposure, outdoor exposure, self-rated health status and county of residence. To examine associations among the three oxidative stress measures, Pearson correlations, crude and partialled for relevant covariates, were computed between each pair of methods in the total sample and separately by race and gender. Age, sex, race, BMI (calculated as weight in kilograms divided by height in metres squared), physical activity, education, days since last menses (for women only), alcohol consumption and serum cotinine, a metabolite of tobacco exposure have been found to be associated with oxidative stress (Hercberg et al. 1994, Willett 1998, Galan et al. 2005) and thus, were evaluated as potential confounders and included in all models as each affected point estimates by at least 10%. All statistical tests were two-sided and p-values ≤ 0.05 were considered statistically significant.

Results

The mean oxidative stress values for each of the three methods, stratified by participant (demographic, lifestyle and behavioural) characteristics, are given in Table 1. The mean age of participants was 31.6 years (8.2 SD), 50% were female, 48% were African-American and 69% had a college degree. F_2-IsoP levels were statistically significantly lower in men those who exercised more than twice a week, and among those with more than 30 h of outdoor exposure per month. As with F2-IsoP, 8-oxo-dG levels were also lower among those with higher levels of physical activity ($p = 0.02$), as well as among males and whites. In contrast, mean comet tail moment indicated significantly higher oxidative DNA damage levels in whites and respondents who did not live with smoker(s), $p < 0.05$. There were no differences among the three oxidative stress measures within categories based on age, BMI, education, income, dietary supplement use, alcohol consumption, self-rated health status and county of residence.

Associations among the three oxidative stress measures, based on crude and adjusted Pearson's correlations are given in Table 2. Adjusted correlations were partialled for age, BMI, cotinine levels, alcohol intake, physical activity level, education, days since last menses (for women only) and where appropriate, sex and race. The strongest associations were observed between F_2-isoprostanes and the comet tail moment, with correlations ranging from 0.09 to 0.40 (crude) and –0.09 to 0.55

Table 1. F_2-isoprostanes, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) and comet tail moment by demographic and behavioural characteristics ($n = 135$).

Characteristic	Distribution *n* (%)	F_2-isoprostanes Mean (SD)	8-oxo-dG 10^{-6} dG Mean (SD)	Mean comet tail moment Mean (SD)
Sex				
Male	67 (50)	1.34 (0.74)	1.54 (0.85)	1.45 (0.32)
Female	68 (50)	2.06 (1.42)	1.69 (0.94)	1.47 (0.30)
p-Value[a]		0.0009	0.27	0.66
Race				
African-American	65 (48)	1.67 (1.39)	1.74 (0.90)	1.37 (0.28)
White	70 (52)	1.74 (0.97)	1.50 (0.88)	1.55 (0.32)
p-Value		0.27	0.05	0.0003
Age (years)				
20–28	56 (41)	1.51 (0.76)	1.67 (0.93)	1.43 (0.31)
29–37	38 (28)	1.60 (0.84)	1.45 (0.66)	1.41 (0.21)
38–45	41 (30)	2.05 (1.75)	1.69 (1.03)	1.57 (0.37)
p-Value		0.67	0.46	0.09
BMI (kg m^{-2})				
Normal (18.5–24.9)	80 (59)	1.78 (1.35)	1.57 (0.88)	1.48 (0.35)
Overweight (25–29.9)	50 (37)	1.59 (0.92)	1.63 (0.72)	1.42 (0.24)
Obese (≥ 30)	5 (4)	1.64 (0.49)	2.16 (2.19)	1.63 (0.22)
p-Value		0.92	0.70	0.33
Education				
Some college or less	42 (31)	1.71 (1.22)	1.77 (0.98)	1.44 (0.33)
College graduate	55 (41)	1.66 (1.03)	1.46 (0.69)	1.49 (0.29)
Advanced degree	38 (28)	1.77 (1.37)	1.68 (1.04)	1.45 (0.33)
p-Value		0.95	0.17	0.57
Income ($)				
< 20 000	25 (20)	1.58 (0.90)	1.76 (0.96)	1.43 (0.31)
20 000–39 000	32 (25)	1.77 (1.26)	1.59 (1.00)	1.50 (0.33)
40 000–59 000	40 (32)	1.73 (1.33)	1.66 (0.94)	1.47 (0.32)
≥ 60 000	29 (23)	1.71 (1.27)	1.52 (0.78)	1.46 (0.31)
p-Value		0.97	0.80	0.84
Dietary supplement use				
Non-users	79 (59)	1.65 (1.07)	1.72 (0.99)	1.46 (0.31)
Users	54 (41)	1.78 (1.35)	1.45 (0.71)	1.47 (0.31)
p-Value		0.51	0.33	0.19
Physical activity				
≤ 2 times per week	43 (35)	2.18 (1.58)	1.72 (0.76)	1.49 (0.30)
3–4 times per week	48 (39)	1.38 (0.92)	1.69 (1.02)	1.46 (0.33)
≥ 5 times per week	32 (26)	1.56 (0.78)	1.30 (0.71)	1.46 (0.30)
p-Value		0.01	0.02	0.76
Alcohol consumption				
Never	8 (6)	1.06 (0.33)	1.62 (0.71)	1.64 (0.37)
< 1 weekly	48 (36)	1.66 (0.92)	1.56 (1.00)	1.52 (0.29)
1–6 times weekly	37 (27)	1.57 (1.00)	1.57 (1.00)	1.41 (0.31)
≥ 1 daily	42 (31)	1.99 (1.60)	1.71 (0.71)	1.42 (0.30)
p-Value		0.22	0.33	0.08
Passive smoke exposure				
Lives with a smoker	8 (6)	1.81 (0.93)	1.53 (1.08)	1.23 (0.26)
No one at home smokes	126 (94)	1.69 (1.21)	1.60 (0.87)	1.48 (0.31)
p-Value		0.52	0.74	0.02
Outdoor exposure				
< 30 h monthly	15 (11)	2.54 (2.12)	1.42 (0.50)	1.41 (0.26)
30–59 h monthly	41 (30)	1.90 (1.24)	1.65 (0.90)	1.46 (0.29)
60–89 h monthly	36 (27)	1.41 (0.76)	1.85 (1.20)	1.54 (0.35)

Table 1. continued on next page.

Table 1. Continued.

Characteristic	Distribution *n* (%)	F_2-isoprostanes Mean (SD)	8-oxo-dG 10^{-6} dG Mean (SD)	Mean comet tail moment Mean (SD)
≥90 h monthly	43 (32)	1.47 (0.79)	1.45 (0.63)	1.43 (0.31)
p-Value		0.05	0.54	0.46
Self-rated health status				
Excellent	31 (23)	2.13 (1.59)	1.64 (0.59)	1.47 (0.34)
Very good	66 (49)	1.48 (0.79)	1.73 (1.11)	1.45 (0.30)
Good, fair or poor	38 (28)	1.75 (1.31)	1.39 (0.62)	1.49 (0.31)
p-Value		0.09	0.20	0.76
County of residence				
Urban	115 (85)	1.68 (1.22)	1.61 (0.85)	1.47 (0.31)
Rural	13 (10)	1.80 (0.92)	1.72 (1.35)	1.42 (0.29)
None of the above	7 (5)	1.87 (1.08)	1.54 (0.78)	1.53 (0.30)
p-Value		0.65	0.98	0.69

Percentages may not add up to 100% because of rounding and missing data.

[a]Tests for differences between categories were calculated by ANOVA using log-transformed oxidative stress measures

Table 2. Correlations[a] of oxidative stress, measured by F_2-isoprostanes, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) and comet tail moment, by race and sex (*n* = 135)

	8-Oxo-dG and comet tail moment				8-Oxo-dG and F_2-isoprostanes				F_2-isoprostanes and comet tail moment			
	Crude		Adjusted[b]		Crude		Adjusted		Crude		Adjusted	
	Corr.	*p*-Value	Corr.	*p*-Value	Corr.	*p*-Value	Corr.	*p*-Value	Corr.	*p*-Value	Corr.	*p*-Value
All (*n* = 135)	−0.09	0.36	−0.07	0.47	−0.04	0.64	−0.09	0.34	0.22	0.01	0.14	0.16
Male (*n* = 67)	−0.02	0.85	−0.17	0.26	0.01	0.91	0.01	0.96	0.20	0.11	0.09	0.52
Female(*n* = 68)	−0.14	0.25	−0.04	0.79	−0.11	0.36	−0.11	0.48	0.26	0.03	0.16	0.33
Whites (*n* = 70)	−0.01	0.96	−0.07	0.63	−0.02	0.85	−0.16	0.25	0.10	0.40	0.08	0.74
Male (*n* = 36)	−0.01	0.93	−0.04	0.87	0.12	0.49	0.04	0.86	0.09	0.62	0.20	0.42
Female(*n* = 34)	0.003	0.99	−0.18	0.45	−0.22	0.21	−0.38	0.10	0.12	0.50	0.08	0.58
African-Americans (*n* = 65)	−0.08	0.51	0.01	0.95	−0.05	0.71	−0.19	0.25	0.34	0.005	0.27	0.01
Male (*n* = 31)	0.06	0.76	0.02	0.96	−0.23	0.22	−0.39	0.13	0.31	0.09	−0.09	0.74
Female (*n* = 34)	−0.17	0.34	−0.02	0.97	−0.08	0.64	0.11	0.77	0.40	0.02	0.55	0.01

[a]Crude and adjusted correlations were calculated using *z*-transformed variables to account for differences in units among the three methods.

[b]Partial Pearson's correlations were calculated using *z*-transformed variables and adjusted for age, body mass index, cotinine levels, alcohol intake, physical activity level, education, days since last menses (for women only) and where appropriate, sex and race.

(adjusted). These associations were generally stronger in African-Americans than in whites: the most robust association was in African-American women ($r = 0.55$) and the weakest in the combined sample of whites and white female subjects ($r = 0.08$). Correlations were also stronger for women ($r = 0.26$, $p = 0.03$) relative to men ($r = 0.20$, $p = 0.11$). There were no significant correlations between 8-oxo-dG and the comet assay or F_2-IsoP; the crude correlations ranged from –0.23 to 0.12, whereas the adjusted correlations ranged from –0.39 to 0.11. Correlations between the 8-oxo-dG and the F_2-IsoP measures were also relatively weak. The correlations did not differ when the comet tail moment was assessed using net FPG-sensitive sites or strand breaks plus FPG-sensitive sites. For 8-oxo-dG and the comet tail moment, the correlations for strand breaks plus FPG-sensitive sites ($r = -0.01$, $p = 0.90$) were similar to those for net FPG-sensitive sites ($r = -0.07$, $p = 0.47$), as well as correlations with F_2-IsoPs for strand breaks plus FPG-sensitive sites ($r = 0.03$, $p = 0.79$) and net FPG-sensitive sites ($r = 0.14$, $p = 0.16$) (data for strand breaks plus FPG-sensitive sites not shown).

Discussion

This study examined associations among three of the most commonly used measures of oxidative stress (i.e. urinary F_2-isoprostanes, 8-oxo-dG and the comet assay) in a sample of healthy African-American and white adults aged 20–45 years in North Carolina. We found modest associations between F_2-IsoPs and the comet tail moment, but weak associations of 8-oxo-dG with both F_2-IsoPs and the comet tail moment. The results of our study are informative, particularly in light of the fact that researchers often compare results pertaining to oxidative stress across studies and/or assessment methods. While the low levels of associations observed here do not

necessarily minimize the internal validity of any measure within a study, they certainly raise concerns about the legitimacy of comparing different oxidative stress measures or assuming that the measures are equivalent, particularly in healthy populations.

Although these measures are widely used, few studies have directly compared oxidative stress measures using the same human samples (Gallo et al. 2008). ESCODD analysed oxidative DNA damage data (8-oxo-dG and the comet with FPG) from healthy males ($n=88$) using six different laboratories, and similar to our results, found no overall association between methods (Gedik et al. 2005). One laboratory found a strong, statistically significant correlation ($r=0.93$, $p<0.0001$); however, the correlations from the remaining five labs were not significant and ranged from –0.41 to 0.59 (Gedik et al. 2005). To our knowledge, no study has directly compared urinary F_2-IsoPs with 8-oxo-dG and the comet assay in human lymphocytes. Several studies have assessed oxidative stress using at least two of these assays in an attempt to capture a more complete estimate; however, the different methods often produced conflicting results (Sorensen et al. 2005, Kato et al. 2006, Kuhnt et al. 2006, Rossner et al. 2006, Chia et al. 2008). For example, in a case–control study in Long Island, NY, there was an observed association between F_2-IsoPs and breast cancer risk, but no such association was seen for 8-oxo-dG and breast cancer risk in the same study (Rossner et al. 2006). While it is possible that lipid peroxidation, and not oxidative DNA damage, is the relevant oxidative stress pathway for breast cancer or that urinary levels did not reflect levels in the target tissue (i.e. breast), the absence of an association may also be due to measurement error. Similar inconsistent results were observed in other studies that included both 8-oxo-dG and isoprostane assays (Kato et al. 2006, Kuhnt et al. 2006, Rossner et al. 2006), as well as in two other studies that investigated oxidative DNA damage via both 8-oxo-dG and the comet assay (Sorensen et al. 2005, Chia et al. 2008).

The differences seen between the lipid peroxidation and oxidative DNA damage assays are not entirely unexpected because they reflect different pathways of oxidative stress (Bartsch & Nair 2006) and were also assayed using different biological specimens (urine vs lymphocytes). However, we are less clear about the differences between 8-oxo-dG and the mean comet tail moment. Regardless of whether the comet assay measured overall strand breaks or FPG-sensitive sites only, there was no correlation between 8-oxo-dG and the comet tail moment. The addition of FPG to the comet assay ensures the measurement of oxidative DNA damage from oxidized purines, but is not specific to 8-oxo-dG. Additional modified purines, such as 4,6-diamino-5-formamidopyrimidine (FapyAde) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), which are produced from a common precursor to 8-oxo-dG by one-electron reduction, are recognized by the FPG protein and may partly account for differences seen here (Dizdaroglu et al. 2008). Furthermore, we cannot rule out that these measures may reflect still unknown pathways or aspects of oxidative stress.

Methodological differences may also affect results. ESCODD noted that results from enzymatic methods, e.g. the comet assay with FPG, were several times lower and also more precise than those from chromatography (Gedik et al. 2005). Similarly, when electrospray tandem MS and ELISA were used to quantify 8-oxo-dG in 140 workers exposed to polyaromatic hydrocarbons, there was only a significant difference between cases and controls using the LC/MS/MS data, even though the association between methods was significant (Hu et al. 2004). A multilaboratory validation study of oxidative stress biomarkers using an acute CCl_4 rat model, sponsored by the National Institute of Environmental Health Sciences (NIEHS), concluded that the best candidate biomarkers were plasma malondialdehyde (MDA), urinary 8-oxo-dG, and plasma or urinary isoprostanes (Kadiiska et al. 2005).

Our results suggest that there were significant differences by sex, race, physical activity and passive cigarette smoke exposure according to the oxidative stress assessment method used. For example, African-Americans had lower oxidative stress levels when measured by the comet assay ($p=0.0003$) and F_2-IsoPs ($p=0.27$), but higher 8-oxo-dG ($p=0.05$) than whites. Studies examining oxidative stress with intermediate markers, such as dietary antioxidants or disease endpoints, have often found conflicting results. These differences may in part, explain conflicting results seen across oxidative stress measures.

Considering that there are few intermediate measures of chronic disease risk, the identification and validation of such markers is crucial. The criteria necessary for optimal biomarkers of oxidative stress have been extensively discussed (Mayne 2003, Blumberg 2004, Dalle-Donne et al. 2006, Swenberg et al. 2008) and include specificity, accuracy, precision and reliability. Artifactual oxidation during collection and storage remains a constant challenge (Mayne 2003, Blumberg 2004, Gedik et al. 2005, Dalle-Donne et al. 2006). Adding antioxidant agents, such as TEMPO, ensuring proper handling techniques and storage conditions should be considered to minimize potential artifact. ESCODD identified a reasonable range of 0.3–4.2 8-oxo-dG 10^{-6} guanines (Gedik et al. 2005, Milne et al. 2007) and 1.6 ± 0.6 ng mg^{-1} creatinine has been defined as the normal range of F_2-IsoPs in healthy adults (Gedik et al. 2005, Milne et al. 2007). Almost of all values measured here were within the documented ranges of acceptable values for healthy people,

yet there was still only modest agreement between methods used.

We acknowledge some limitations in this analysis. Due to the cross-sectional nature of the study, there were no repeated measures and thus, seasonal and diurnal variability could not be assessed. In addition, there was little variation in oxidative stress levels because the original study examined healthy, non-obese, non-smokers 20–45 years of age. The values presented here for all three biomarkers represent a relatively narrow range of natural variability in normal values. Thus, potential correlations may be hard to detect without greater sample sizes or without widening the distribution of values by inclusion of subjects with known oxidative stress (e.g. smokers or diabetics). Furthermore, we cannot exclude the possibility that correlations were not seen because these methods were not sensitive enough to detect differences within healthy ranges of oxidative stress. These analyses were limited to only three measures of oxidative stress; however, there are other viable methods that are worth examining. We expressed the results of the comet assay using tail moment; however, some studies that suggest percentage tail DNA better captures damage, especially at low levels (Collins et al. 2008), yet others support tail moment as the most stable estimate of DNA damage (Lee et al. 2004). Also, as these estimates reflect DNA damage in lymphocytes and lipid peroxidation in urine, they may not represent the status of oxidative stress in other tissues and cells within the body. However, both biospecimens represent 'whole body' exposure as lymphocytes circulate throughout the body and urine undergoes metabolism in the excretion process. Furthermore, because urine has a low lipid content, F_2-IsoPs are very stable and not subject to auto-oxidation formation (Morrow et al. 1990, Dalle-Donne et al. 2006). There are also notable strengths to this study. All assays were performed by experienced laboratories with a demonstrated record of successful use of these techniques. Samples were processed within 2 h of collection and proper storage techniques were employed. In addition, the study population included equal numbers of African-Americans and whites and had sufficient power to examine associations by race and sex.

Although the development and use of oxidative stress biomarkers is an exciting and promising field, additional validation studies are necessary to assess their accuracy and the information they contribute to various disease-related endpoints. We compared three commonly used measures of oxidative stress, F_2-isoprostanes, 8-oxo-dG, and the comet assay, in healthy adults and found modest correlations between F_2-IsoPs and the comet assay, but little association between 8-oxo-dG and either F_2-IsoPs or the comet assay. This work highlights the limitations of comparing results of oxidative stress across studies or measurement tools in healthy disease-free populations.

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We are saddened to report that Dr. Morrow has passed away.

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