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Intra- and inter-individual variability in measurements of biomarkers for oxidative damage *in vivo*: Nutrition and Breast Health Study

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Abstract

Oxidative stress has been implicated in the pathogenesis of various chronic diseases, such as cancer, cardiovascular disease and inflammatory conditions, as well as in ageing. Although a number of markers are now available, little is known about the reliability of single measurements of such markers in healthy individuals. The study examined the distribution of variance for three oxidative stress markers, 8-oxo-2′-deoxyguanosine (8-oxodG), 5-hydroxymethyl-2′-deoxyuridine (5-OHmdU) and total 8-isoprostane-F2α, which were measured every 3–6 months over 1 year in blood and breast nipple aspirate fluid (NAF) for 103 premenopausal women. For both plasma and NAF, the between-subject variances of 8-isoprostane-F2α were consistently greater than the within-subject variances. Consequently, their reliability coefficients were close to the level of those for cholesterol. On the other hand, the within-subject variances were much greater than the between-subjects variances for blood 5-OHmdU, resulting in low reliability coefficients, i.e. <0.3. Overall, the reliability coefficients for blood 8-oxodG were between those of 8-isoprostane-F2α and 5-OHmdU, but closer to those of 8-isoprostane-F2α. The results suggest that the reliability of oxidative stress markers may vary considerably depending on the type of marker. Caution should be exercised in selecting markers as well as in determining the number of study subjects or the number of samples per subject in a study. There also may be ample room to optimize laboratory techniques to quantify markers of oxidative DNA damage.

Keywords: *Biomarkers, variability, oxidative stress*

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Introduction

Oxidative stress has been implicated in the pathogenesis of various chronic diseases, such as cancer, cardiovascular disease and inflammatory conditions, as well as ageing (Moller et al. 1998, Giammarioli et al. 1999, Cooke et al. 2003). Substantial effort has been made in the past few decades to develop methods to quantify oxidative damage on important biomolecules (e.g. DNAs, lipids and proteins) *in vivo* (Giammarioli et al. 1999). A number of markers are now available and have been applied to clinical studies, prevention trials and epidemiological research. Those studies have demon-

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strated that such measurements are potential markers for disease severity (Farinati et al. 1988, Montuschi et al. 2004) and for environmental exposures (Frenkel et al. 1994, Helmersson et al. 2005).

Oxidative damage to DNA has received much attention in cancer research because it can be mutagenic if not repaired (Halliwell 1998, Moller et al. 1998, Cooke et al. 2003). Among various types of oxidative DNA damage products, 8-oxo-2′-deoxyguanosine (8-oxodG) and 5-hydroxymethyl-2′-deoxyuridine (5-OHmdU) are two major products that have been extensively studied because of their mutagenic potential, chemical stability and sensitivity in assays (Djuric et al. 1991, Halliwell 1998, Moller et al. 1998). Quantitation of these oxidized DNA bases typically involves HPLC or gas chromatography-mass spectrometry (GC-MS) techniques (Halliwell 1998, Moller et al. 1998, Giammarioli et al. 1999). There have also been a number of markers of lipid oxidative damage proposed to reflect oxidative stress levels (Giammarioli et al. 1999, Halliwell 2000, Montuschi et al. 2004). Lipid peroxidation not only plays an important role in the development of atherosclerosis (Halliwell 2000, Montuschi et al. 2004), but also is a source of free radicals that can cause DNA damage (Giammarioli et al. 1999). Among such markers, isoprostanes are specific products arising from the peroxidation of unsaturated fatty acid residues in lipids and currently measurement of isoprostanes is the most widely used marker of lipid peroxidation *in vivo* (Halliwell 2000, Montuschi et al. 2004). Moreover, commercial kits based on enzyme immunoassays are available for 8-iso-PGF2α.

Despite successful application of these markers in some studies, laboratory variability has been suggested to be a serious concern in evaluating those results (Lunec 1998, Huang et al. 2001). In addition, little information is available on the intra-individual variability of these markers in healthy individuals. The goal of this study was to assess the intra-individual variability of three measures of oxidative stress: 8-oxodG, 5-OHmdU and total 8-isoprostane-F2α. These data impact on how liable single measurements are to misclassification, and they provide basic information about how many repeated measurements are required to obtain reliable levels of the markers among healthy individuals. Note that the term of 'reliability' in this study is used purely as a statistical term (as defined in the statistical analysis) and that the study was not intended to validate specific assay techniques.

Materials and methods

Study population

Details concerning the study design, characteristics of study participants, and data and biological specimen collection have been published elsewhere (Djuric et al. 2002, 2005). Briefly, the Nutrition and Breast Health Study was designed as a two-by-two (four groups) factorial randomized intervention trial of low fat and high vegetable/fruits diets over 1 year. With this study design, approximately one-quarter of the participants were assigned to the control arm (usual diet). Eligible participants were healthy premenopausal non-smoking women aged 21–50 years who had at least one first-degree relative with breast cancer. Written informed consent was obtained from each subject before participation.

Fasting venous blood specimens were obtained at months 0, 3, 6 and 12. Nipple aspirate fluid (NAF) was collected at 0, 6 and 12 months using the self-expression method as described (Djuric et al. 2005) to accumulate fluid for 5 days.

Approximately 60% of the participants provided NAF specimens. In addition to dietary assessments every 3 months, basic demographics and reproductive and medical history were obtained by structured questionnaires.

Laboratory methods

Fasting blood samples were collected in heparinized tubes and processed immediately after drawing. All samples from a given individual were assayed in the same laboratory batch of 24–36 samples. Each blood sample was coded by number, and the diet arm assignment was blinded until after the analyses were completed and the results calculated. Furthermore, to control laboratory batch variability, the values of 8-oxodG, 5-OHmdU and total 8-isoprostane-F2α were standardized with the measurements of quality control samples.

Samples were spun to prepare plasma, and plasma aliquots were stored under argon at −70°C, but BHT was not added. Nuclei were prepared from the buffy coat by mixing it with 12-ml cold sucrose buffer (320 mM sucrose, 10 mM TRIS, pH 7.4, 5 mM $MgCl_2$, 10 ml Triton, 50 mM mannitol) according to the method of Ciulla et al. (1988). Centrifugation was used to isolate nuclei. Nuclei were then suspended in 1 ml of 1% SDS, 1 mM EDTA, 20 mM TRIS pH 7.4, 50 mM mannitol and stored at −70°C.

For isolation of DNA, the nuclei in the SDS solution were treated with RNases and protease as described (Djuric et al. 2004). DNA was isolated after precipitation of proteins with NaCl and one extraction each with chloroform/isoamyl alcohol (48:2) and *n*-butanol. The DNA was precipitated with ethanol and dissolved in 200-µl water (Djuric et al. 2004). The DNA samples were hydrolysed enzymatically at 37°C using sequential enzyme addition: 50 units DNase I for 2 h, 3 units nuclease P1 for 1 h, 2.5 units alkaline phosphatase for 30 min and a cocktail of DNase I, 0.015 unit phosphodiesterase I and 1 unit phosphodiesterase II, 0.1 unit acid phosphatase and 1 unit alkaline phosphatase overnight. Internal standards were then added (8-oxodG-$^{13}C_4$; Wako Pure Chemical Industries Ltd, Osaka, Japan), 5-OHmdU-$^{13}C_2d_2$ (synthesized as described by Djuric et al. 1991) and dThd-d_4 (MDS Isotiopes, St Louis, MO, USA) and nucleosides recovered with Sep-Paks as previously (Yu & Djuric 1999).

The eluate was dried and redissolved in 50 µl HPLC mobile phase. Levels of 8-oxodGuo and dGuo were determined by HPLC-MS-MS using standard curves constructed from commercial dGuo (Sigma Chemical Co., St Louis, MO, USA) and 8-oxodGuo (Wako Pure Chemicals). HPLC-MS/MS multiple reaction monitoring (MRM) analysis was carried out with Micromass Quattro triple quadrupole mass spectrometer (Manchester, UK) equipped with an electrospray ionization source. Nitrogen was used as the drying and nebulizing gas at 690 and 157 l h^{-1}. Argon was used as the collision gas for collision-induced dissociation (CID). The capillary voltage was set at 3.16 kV. The source and nebulizing temperatures were maintained at 110 and 400°C, respectively. The HPLC system consisted of Waters Alliance (Milford, MA, USA) model 2690 pump and auto-injector. The MD150 C18 column (2.0 × 150 mm, 3 µm) was used at a flow rate of 200 µl min^{-1}. A gradient solvent system from methanol (solvent A) to 5% methanol/10 mM ammonium acetate, pH 5.3 (solvent B) was employed for the separation of samples. The inter-assay coefficient of variation was 25%.

The samples were analysed in positive-ion MS/MS MRM mode for the $[M+H]^+$ ion-to-base ($[B+H_2]^+$) transitions of 8-oxodG (*m/z* 265 to 165), dGuo (*m/z* 268 to 152), dThd (*m/z* 265 to 149), 8-oxodG-$^{13}C_4$ (*m/z* 288 to 172) and dThd-d_4 (*m/z* 269 to 153). The extraction cone at 19 V and collision at 13 eV were applied for the transitions of dThd, dG and dThd-d4. The cone at 7 V and collision at 11 eV were used for 8-oxodG and 8-oxodG-$^{13}C_4$. The levels of dGuo were determined using dThd-d_4 as the internal standard; since levels of dG were high and quantitation with dThd-d_4 gave excellent calibration curves for dG. Calibration curves were run before and after sample analysis. Sensitivity was not sufficient for analysis of endogenous levels of 5-OHmdU by HPLC-MS-MS, perhaps because ionization electrospray was not very efficient for this molecule.

The samples remaining after HPLC-MS-MS analysis were analysed for levels of 5-OHmdU. Nuleosides were derivatized at room temperature before analysis by GC-MS, as described (Yu & Djuric 1999). Briefly, samples were dried and derivatized with 70 ml N,O-*bis*(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% trimethyl-chlorosilane:acetonitrile, 2:1. GC-MS was conducted with a Hewlett-Packard (Palo Alto, CA, USA) Ultra 2 column (25 m $\times$ 0.2 mm $\times$ 0.011 mm film thickness), helium as the carrier gas, and a Hewlett-Packard 5971A mass-selective detector. The levels of 5-OHmdU and thymidine in each sample were calculated from standard curves. The inter-assay coefficient of variation was 33%.

Levels of total 8-isoprostane (F2α) in plasma were determined using a kit from Cayman Chemical Co. (Ann Arbor, MI, USA) following the manufacturer's recommendation, except that a modified Sep-Pak procedure was used as described (Djuric et al. 2005). Total 8-isoprostane $F_2\alpha$ levels represent both the free form and the esterified form since the samples were hydrolysed with KOH for 1 h before analysis. For analysis of 8-isoprostane (F2α) in NAF, a weighed aliquot of approximately 1 mg fluid was diluted to 200 µl with 5% USP dextrose, 50 mM mannitol, 10 mM Tris, pH 7.4, and the same procedure recommended for total 8-isoprostanes in plasma was followed, assaying two dilutions, each in duplicate. Levels of cholesterol were determined in a spectroscopic assay with Sigma Infinity Cholesterol Reagent using a standard curve constructed with plasma calibrators. Approximately 2 mg fluid were diluted with 10 µl dextrose solution, and 2.5-µl of the diluted fluid were assayed with 250 µl of the cholesterol reagent in 96-well plates in triplicate. The inter-assay coefficient of variation was 29% in plasma and 38% in a human milk sample.

Statistical analysis

Of 122 subjects randomized, 104 subjects who had measurements of at least one marker described above for at least two time points were included in the initial analysis. One subject was subsequently excluded due to a missing value for the covariates selected. Among the remaining 103 subjects, 25 were in the control arm. Estimates of the variance components associated with the three oxidative stress markers and with cholesterol as a reference were obtained using restricted maximum likelihood estimation in SAS. The intraclass correlation coefficient (ICC) was calculated as the reliability coefficient (Fleiss 1986). ICC describes the percentage of the total variance due to between-subject variation and indicates the ability of a measurement technique to distinguish between individuals' true marker values,

ranging from 0 to 1 (100%). Approximate 95% confidence intervals were calculated as described by Donner and Wells (1986). The estimates were calculated for two sets of the data: one limited to the control group and the other for the entire study cohort with adjustment for intervention related factors, i.e. number of vegetable/fruit servings per day, total fat intake (g day^{-1}) and body mass index (weight (kg)/height $(m)^2$). The analyses for the latter group were purposed to improve the precision of the ICC, because the numbers of available subjects in the control arm were fairly small for some markers. Although cholesterol itself is not an oxidative stress marker, it was included to aid interpretation of the data on other markers.

In both cases, basic demographic variables were considered as potential covariates by fitting a general liner model. Within this limited age range (21–50), age was not associated with any of the three oxidative stress markers, while major racial groups (white versus non-white) was marginally associated with at least one of the markers. In addition, seasons at the time of specimen collection were significantly or marginally associated with one or more markers. Thus, theses two variables were included as an indicator variable in both sets of analysis. Experimental batches were not included in the model because the measurements were adjusted for the value of quality control samples in each batch. Continuous independent and dependent variables were log-transformed if the data better approximated a Gaussian or normal distribution. As a result, all continuous variables except for plasma cholesterol were log-transformed in this analysis. However, anti-log values were presented in descriptive analyses.

Results

The distributions and number of measurements available for each marker are shown in Table I. While the maximum number of measurements (four for blood and three for nipple aspirate) were available for 8-isoprostane-F2α in most subjects (85–96% for blood and 72–73% for nipple aspirate), the number of measurements for oxidative DNA markers tended to be limited, particularly for 5-OHmdU. In the control arm less than half the subjects had full measurements of 5-OHmdU. These differences were

Table I. Number of subjects with repeated measurements for oxidative stress markers in blood and nipple aspirate fluid (NAF) in the Nutrition and Breast Health Study.

Markers	Control arm only				All subjects			
	Number of measurements				Number of measurements			
	Total	2	3	4	Total	2	3	4
Blood:								
8-Isoprostane-F2α	25	–	1	24	103	3	12	88
5-OHmdU	24	2	13	9	96	8	37	51
8-OxodG	24	1	8	15	100	6	23	71
Cholesterol	25	–	1	24	103	3	12	88
NAF:								
8-Isoprostane-F2α	18	4	14	–	57	15	42	–
Cholesterol	18	4	14	–	57	15	42	–

Table II. Mean (SD) level of oxidative stress markers in blood and nipple aspirate fluid (NAF) at baseline, 3, 6 and 12 months among subjects with repeated measurements in the Nutrition and Breast Health Study.

Markers[a]	Control arm only				All subjects			
	Months				Months			
	0	3	6	12	0	3	6	12
Blood:								
8-Isoprostane-F2α	110 (34.9)	118 (47.7)	127 (49.1)	120 (40.6)	116 (43.9)	110 (38.2)	115 (43.5)	110 (39.7)
5-OHmdU	187 (167)	136 (120)	228 (247)	166 (127)	171 (136)	149 (131)	164 (154)	158 (130)
8-OxodG	217 (278)	250 (259)	339 (356)	252 (209)	159 (230)	178 (233)	213 (264)	222 (239)
Cholesterol	172 (28.4)	170 (25.0)	178 (27.9)	180 (31.9)	175 (33.5)	171 (31.1)	175 (36.2)	176 (36.4)
NAF:								
8-Isoprostane-F2α	11372 (11129)	–	17757 (13106)	10651 (10760)	19348 (26754)	–	18049 (18572)	15636 (17676)
Cholesterol	2586 (2545)	–	2392 (2772)	1452 (2033)	2648 (2603)	–	2453 (2633)	2483 (2830)

[a]Units are pg ml^{-1} for blood total 8-isoprostane-F2α, fg ng^{-1} dThd for 5-OHmdU, fg ng^{-1} dG for 8-oxodG, mg ml^{-1} for blood cholesterol, pg g^{-1} for NAF total 8-isoprostane-F2α, and mg g^{-1} for NAF cholesterol.

mainly due to the volume of sample available and the fact that 5-OHmdU assays were done with hydrolysate that was available after the 8-oxodG assays were done.

Means and standard deviations (SD) of each measurement at months 0, 3, 6 and 12 are shown in Table II. There was limited variability by time in the means of markers studied in comparison with their SDs at each time point. The SDs tended to be larger for the entire study population than in the control arm only. It was also noted that the concentrations were much higher in NAF than in blood. There were no statistically significant correlations among the three blood oxidative stress markers and of 8-isoprostane-F2α between plasma and NAF at any time points, except between the two oxidative DNA markers at months 6 and 12.

Table III shows the between- and within-subject variances and resulting reliability coefficients. For both plasma and NAF, the between-subject variances of total 8-isoprostane-F2α were consistently greater than the within-subject variances. Consequently, their reliability coefficients were close to the level of those for cholesterol. On the other hand, the within-subject variances were much greater than the between-subjects variances for blood 5-OHmdU, resulting in low reliability coefficients, i.e. <0.3. Overall, the reliability coefficients for blood 8-oxodG were between those of total 8-isoprostane-F2α and 5-OHmdU, but closer to those of 8-isoprostane-F2α. Generally, the point estimates based on the control arm only and those based on the entire study population were very similar and the latter was accompanied with much narrower 95% confidence intervals. However, there was a modest difference in these estimates for 8-oxodG. The reliability coefficients were higher for NAF markers compared with those for corresponding blood markers. In addition, these statistical models for the entire study population indicated total fat intake and BMI had

Table III. Between- and within-subject variance and reliability coefficient for oxidative stress markers in blood and nipple aspirate fluid (NAF) in the Nutrition and Breast Health Study.

Markers[c]	Control arm only[a]			All subjects[b]		
	Variance components		Reliability coefficients	Variance components		Reliability coefficients
	Between	Within	(95% CI[d])	Between	Within	(95% CI)
Blood:						
8-Isoprostane-F2α	0.0754	0.0593	0.560 (0.367–0.752)	0.0700	0.0575	0.549 (0.453–0.645)
5-OHmdU	0.2082	0.5553	0.273 (0.025–0.520)	0.1909	0.4490	0.298 (0.180–0.417)
8-OxodG	0.6290	0.4863	0.564 (0.362–0.766)	0.8082	0.8384	0.491 (0.386–0.596)
Cholesterol	527.26	300.10	0.637 (0.465–0.809)	770.23	358.58	0.682 (0.605–0.760)
NAF:						
8-Isoprostane-F2α	0.4413	0.3125	0.585 (0.332–0.839)	0.9627	0.4992	0.659 (0.534–0.783)
Cholesterol	0.7236	0.4141	0.636 (0.404–0.868)	0.7314	0.4147	0.638 (0.509–0.768)

[a]Adjusted for race (white versus non-white) and seasons.
[b]Adjusted for race (white versus non-white), seasons, fat intake (g day^{-1}), number of fruit/vegetable servings day^{-1} and body mass index.
[c]Except for blood cholesterol, measurements of all other markers were log-transformed.
[d]Confidence intervals.

significant effects on variabilities of blood 8-isoprostane-F2α and 8-oxodG levels, respectively.

Discussion

The long-term (over at least 1 year) reliability of biomarkers is of research interest particularly for markers associated with chronic conditions, such as cancer (Zeleniuch-Jacquotte et al. 1998). The Nutrition and Breast Health Study provided a unique opportunity to analyse the repeated measurements of multiple oxidative stress markers in two different types for biological specimens over 1 year. In most of the earlier studies, the comparison of these markers between different groups of subjects or different intervention treatments was based on single measurements. However, the ability of such studies to estimate true differences depends on the reliability of the measurements. In other words, greater intra-individual variability will reduce the power to detect the association. Because the measurements of oxidative stress markers were standardized for the values of the quality control specimens, the within-subject variability observed in the present study more likely reflects changes within individuals over time rather than laboratory batch variability in principle. On the other hand, it is possible that the inter-individual variability observed in the study was underestimated because the study population was limited to only non-smoking premenopausal women.

The results demonstrate that the reliability of oxidative stress markers may vary considerably with the type of marker. Interestingly, some of the markers studied were as reliable as plasma cholesterol, whose clinical value has long been established. Particularly, 8-isoprostane-F2α yielded the best result, while DNA markers were more likely prone to measurement errors. The latter may explain inconsistent results on DNA markers reported in earlier publications, e.g. concerning associations with cigarette smoking (Nakajima et al. 1996, Wako et al. 2001). Several factors must be taken into account when evaluating the results of theses two types of markers.

A major biological difference between oxidative damage to lipids and DNA is the presence of specific repair mechanisms for oxidative DNA damage, but not for lipids (Giammarioli et al. 1999, Cooke et al. 2003). This makes oxidative DNA markers attractive as they reflect steady-state levels of DNA damage taking into account not only exposure to oxidants, but also DNA repair capacity. Multiple DNA repair systems are known to exist including enzymes of OGG1, MTH1, SMUG1, 5-OHMCyt glycosylase and 5-OHmdU DNA glycosylase (Demple & Harrison 1994, Cooke et al. 2003). Because each enzymatic activity displays intra-individual variability, the net result is likely to be more susceptible to variability. For total plasma 8-isoprostane-F2α, enzymic activity of lipases is a potential source of variability because it plays a major role in releasing isoprostanes from cells and lipoproteins into plasma.

In addition, it should be noted that different fractions of blood were used to determine lipid and DNA markers, i.e. plasma for lipid and peripheral white blood cells for DNA. Plasma may be a relatively more homogeneous biomaterial than white blood cells, which are comprised of heterogeneous cell types whose functions and life times are considerably different, and their relative levels in an individual will depend at least in part on transient changes in immune status. There is some evidence to suggest that oxidized DNA levels are higher in polymorphonuclear cells than in mononuclear cells or lymphocytes among the white blood cell populations (Bashir et al. 1993, Nakajima et al. 1996). On the other hand, plasma 8-isoprostane-F2α levels reflect the amount of isoprostanes released from all tissues as well as formed in plasma.

A more critical issue may be the differences in laboratory techniques to quantify these two types of markers. There have been several publications concerning technical problems in assays for oxidative DNA markers (Halliwell 1998, Lunec 1998, Moller et al. 1998, Giammarioli et al. 1999, Huang et al. 2001). It has been suggested that there are several possibilities to introduce laboratory variability, which include both over- and underestimation. The most serious concern with common laboratory techniques, HPLC and GC-MS, are artefactual formation of oxidized bases during sample preparation and derivatization processes (Halliwell 1998), while underestimation due to incomplete DNA digestion has also been acknowledged as an important source of laboratory variability (Huang et al. 2001). Consequently, it has been reported that discrepancies in measurements of 8-OHdG occur over a range of two orders of magnitude (Lunec 1998). With regard to EIA, an inherent limitation is in antibody specificity, i.e. cross-reactivity with other isoprostane species in the present case. However, with prior purification of plasma specimens, the kit applied in the study has been proven to produce excellent agreement with GC-MS measurements, which is considered the gold standard (Wang et al. 1995). Consistent with the present findings on oxidized DNA markers, Pilger et al. (2001) found that intra-individual variability in urinary 8-oxodG concentrations quantified by HPLC was greater than

inter-individual variability. On the other hand, another marker for an oxidized DNA base determined by ELISA showed excellent reliability of measurements (ICC = 0.99), although it was an indirect marker based on antibodies measured in serum (Kato et al. 1998).

Another important finding in the present study is that the reliability coefficients were generally higher for NAF markers than for corresponding blood markers. At least two factors may account for these differences. First, the turnover rate of breast fluids is slower than of blood. Second, and more importantly, collection schemes were different between both sample types, single venipuncture for blood versus pooled samples for 5 days for NAF. The latter indicates that repeated sampling and averaging in fact reduces intra-individual variability. It was also noticed that 8-isoprostane-F2α levels were much higher in NAF than in plasma. Higher lipid contents would not necessarily lead to higher 8-isoprostane-F2α, but its correlation with cholesterol concentrations has also been reported (Davi et al. 1997).

In conclusion, the results of the present study suggest that increases in the number of study subjects or in the number of samples per subject may be helpful in maintaining adequate statistical power, in some instances, as described by McKeown-Eyssen and Tibshirani (1994). For studies involving oxidized DNA base markers, there may be ample room to optimize laboratory techniques to quantify those markers.

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