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# Oxidative damage to DNA and single strand break repair capacity: Relationship to other measures of oxidative stress in a population cohort

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It is well accepted that oxidative DNA repair capacity, oxidative damage to DNA and oxidative stress play central roles in aging and disease development. However, the correlation between oxidative damage to DNA, markers of oxidant stress and DNA repair capacity is unclear. In addition, there is no universally accepted panel of markers to assess oxidative stress in humans. Our interest is oxidative damage to DNA and its correlation with DNA repair capacity and other markers of oxidative stress. We present preliminary data from a small comet study that attempts to correlate single strand break (SSB) level with single strand break repair capacity (SSB-RC) and markers of oxidant stress and inflammation. In this limited study of four very small age-matched 24-individual groups of male and female whites and African-Americans aged 30–64 years, we found that females have higher single strand break (SSB) levels than males ($p = 0.013$). There was a significant negative correlation between SSB-RC and SSB level ($p = 0.041$). There was a positive correlation between SSBs in African American males with both heme degradation products ($p = 0.008$) and high-sensitivity C-reactive protein (hs-CRP) ($p = 0.022$). We found a significant interaction between hs-CRP and sex in their effect on residual DNA damage ($p = 0.002$). Red blood cell reduced glutathione concentration was positively correlated with the levels of oxidized bases detected by endonuclease III ($p = 0.047$), heme degradation products ($p = 0.015$) and hs-CRP ($p = 0.020$). However, plasma carbonyl levels showed no significant correlation with other markers. The data from the literature and from our very limited study suggest a complex relationship between measures of oxidative stress and frequently used clinical parameters believed to reflect inflammation or oxidative stress.

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## 1. Introduction

DNA repair capacity in humans is an attractive biomarker for clinical investigations that focus on the relationship between the repair of DNA damage, overall health status, and disease susceptibility. Described as the repair or removal of many different types of lesions via several biochemical pathways, human DNA repair capacity has been shown to be affected by alterations in several DNA repair pathways and linked to both heritable and sporadically occurring age-associated diseases such as cancer [1,2]. Work by numerous investigators has documented the relationship between repair capacity and disease state. For example, Collins et al. have shown that the capacity to repair of oxidative damage to DNA, is associated with heart disease mortality rate [3]. Many studies have employed the alkaline comet assay and the host cell reactivation assay to assess the effect of age on, the repair of γ-radiation-, UV-induced and hydrogen peroxide-induced DNA damage [2,4–9]. In our earlier work, we demonstrated a set of DNA repair parameters that permitted us to examine both the fast and slow component of SSB repair [10]. Later work used the alkaline comet assay to examine whether demographic and epidemiologic factors, such as age, sex, race, BMI, hypertension, tobacco or alcohol use, affect the repair capacity of single strand breaks (SSB) in humans. We found

*Abbreviations:* AP sites, apurinic/apyrimidinic sites; BER, base excision repair; BMI, body mass index; DSB, double strand break; Endo III, endonuclease III (thymine glycol-DNA glycosylase); Fpg, formamidopyrimidine-DNA glycosylase; GSH, reduced glutathione; hs-CRP, high sensitivity C-reactive protein; PBMC, peripheral blood mononuclear cell; RBC, red blood cell; SSB, single strand break; SSB-RC, single strand break repair capacity.

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that age, race and sex affect the fast component of DNA repair, while the slow component was unaffected [11]. However, examining DNA repair capacity of various lesions is only a part of the work that needs to be done to understand the relationship of DNA repair and human health. While measuring DNA repair of various lesions is critically important, recent focus has partially shifted to investigations that assess levels of damage to DNA particularly oxidative damage to DNA and oxidative stress because of the importance of these lesions in aging and disease development.

The Harman Free Radical Theory states that accumulation of oxidative damage to DNA and other cellular components and tissues over the lifespan leads to aging, disease and death [12]. Oxidative damage to DNA includes single strand breaks (SSBs) and oxidative base damage. An increased baseline level of oxidative damage to DNA is associated with several age-related diseases including: cardiovascular disease [3], diabetes mellitus [13], cancer [14], neurodegenerative disease [15], and end-stage renal disease [16]. The level of oxidative damage to DNA depends on a variety of factors. They may include age [14], environmental exposure to genotoxic factors [17], smoking [18], ethanol intake [19], and intracellular and extracellular metabolism [20]. The specific role of oxidative stress in aging and the development of age-related diseases is a subject of active investigation but the exact mechanisms that may define this relationship are unclear. The type of oxidative stress that is most relevant to aging and aging-related diseases has yet to be determined because many systems maintain the redox status in cells and tissues [21]. Understanding this complex relationship may provide potential biomarkers of oxidative stress, which can be objectively measured as indicators of normal and pathologic processes that result in age-related disease and decrement in cellular function associated with aging.

Cellular defenses have evolved that combat oxidative stress-induced DNA damage. They include antioxidants, which either scavenge free radicals or prevent their formation. One component, the glutathione system, is central to the defense against oxidative stress. Reduced glutathione (GSH), a non-enzymatic antioxidant, plays a central role in eliminating peroxides and thus helping to maintain the correct thiol/disulfide redox state of proteins for proper biological function. Several diseases are associated with decreased blood GSH concentration including rheumatoid arthritis, coronary artery disease, AIDS, Alzheimer's disease, and Werner syndrome (reviewed in [22]) and reports show that reduced glutathione levels decline with age (reviewed in [21]). These associations indicate that measuring GSH levels could aid in earlier detection of increased accumulation of oxidative damage to DNA and associated diseases.

Protein oxidation is a consequence of oxidative stress, and assaying for protein carbonyls is one of the most frequently used tools to investigate protein oxidation levels. Protein carbonyls are formed as a consequence of oxidation of lysine, arginine, proline and threonine residues, and fragmentation products of peptide bond cleavage reactions. Higher levels of protein carbonyls are observed with increased age in healthy human subjects [21] and in Alzheimer's disease, Werner syndrome, chronic inflammation and rheumatoid arthritis [23,24].

A second measure of protein oxidation is the presence of fluorescent heme degradation products, the end products formed from autoxidation of hemoglobin [25]. When oxyhemoglobin is oxidized, superoxide anions are produced and then converted to hydrogen peroxide by dismutase [26,27]. The oxidized hemoglobin proteins can interact with this cellular hydrogen peroxide, resulting in degradation of the heme moiety. The heme degradation products auto-fluoresce, and in fresh blood samples suggest that reactive oxygen species (ROS) resulting from hemoglobin autoxidation reaction escape the red blood cell antioxidant system. Since red cells have no enzymatic system in place to remove these degradation products, it is believed that they may be a relevant biomarker of oxidative stress originating from the red cell [28].

Recently, inflammation has been assessed in clinical settings by measuring levels of high-sensitivity C-reactive protein (hs-CRP). CRP is an acute phase protein that is associated with an increased risk of coronary artery disease, peripheral arterial disease, stroke, diabetes and metabolic syndrome [29]. hs-CRP has been linked to chronic inflammation that is a consequence of oxidative stress; therefore it is relevant to examine the importance of hs-CRP as a biomarker of disease states related to oxidative stress.

The aim of this work was to assess whether demographic factors, such as age, sex and race affect the baseline level of DNA oxidation and to examine whether there were consistent relationships between frequently studied markers of oxidative stress and DNA repair parameters in a small bi-racial cohort. Based on recent studies in the literature, we have chosen to examine a diverse panel of markers representative of the antioxidant system, systemic inflammation, and oxidative damage to DNA for this study. Using the single cell gel electrophoresis (comet) assay, we examined the level of single strand breaks and oxidized bases detected by endonuclease III and Fpg in cryopreserved PBMCs to examine whether there is a correlation between the level of DNA oxidation in lymphocytes and other oxidative stress-related measures including: red blood cell glutathione (RBC GSH), heme degradation products, and protein carbonyls in plasma. The use of high-sensitivity C-reactive protein (hs-CRP) as a measure of systemic inflammation is of particular relevance because it is regularly used in the everyday clinical practice of physicians. Additionally, we examined the SSB-RC for each of the participants to determine if we are able to use DNA repair techniques as an indicator of oxidatively induced DNA damage. Currently it is unknown how various demographic parameters affect and are affected by oxidative stress, and what the effect on repair of the damage caused by oxidative stress will be. In this study we have examined different oxidative stress parameters in order to gain a better understanding of the relationship between biological mechanisms associated with oxidative stress.

## 2. Material and methods

### 2.1. Study design

Blood donors were participants in the Healthy Aging in Neighborhoods of Diversity across the Life Span (HANDLS) study of the National Institute on Aging Intramural Research Program (NIA IRP). The purpose of this study is to unravel the effects of race and socioeconomic status on the development of age-associated health disparities among African-Americans and whites. HANDLS is a prospective population-based interdisciplinary, longitudinal study. Our baseline is representative of working-age African Americans and whites between 30 and 64 years old recruited as a fixed cohort of participants by household screenings from an area probability sample of twelve neighborhoods (contiguous census tracts) in Baltimore City and one trial neighborhood. Power analyses for longitudinal analyses after twenty years of follow-up with repeated assessments every three years specify at least 80% power for a minimum sample size of 30 participants per cell defined by race (African American, white), socioeconomic status (self-reported household income based on 125% of the 2004 Health and Human Services Poverty Guidelines), age (seven 5-year age groups 30–64 years old), and sex [30].

The study has undergone IRB review and informed consent was obtained from all participants. The HANDLS sub-cohort used in this pilot study consists of 24 African-American females, 24 African-American males, 24 white females and 24 white males ranging in age from 30 to 64 years. The characteristics of the study population presented in Table 1 were collected from the self-reported medical history for each participant obtained by a health care professional as part of a complete physical examination and collection of blood and urine for clinical laboratory analysis.

### 2.2. Peripheral blood mononuclear cells (PBMCs) isolation and cryopreservation

Fasting blood samples were collected in 8-ml Vacutainer® heparinized vials (BD, Franklin Lakes, NJ). Blood was transported at room temperature to the lab and the isolation procedure of peripheral blood mononuclear cells (PBMCs), red blood cells (RBCs) and plasma was performed within 3 h of phlebotomy.

PBMCs were separated by centrifugation of blood collected in heparinized vials and diluted with RPMI-1640 medium over Histopaque 1077 (Sigma–Aldrich, St.

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**Table 1**
Clinical characteristics of the cohort.

| | Whites | | African-Americans | |
|---|---|---|---|---|
| | Females ($n = 24$) | Males ($n = 24$) | Females ($n = 24$) | Males ($n = 24$) |
| Age (in years; mean ± SD) | 45.5 ± 11.0 (32.7–64.7) | 47.7 ± 10.4 (32.1–64.7) | 47.3 ± 10.4 (30.5–64.8) | 48.5 ± 10.4 (31.3–64.5) |
| Hypertension (%) | 26.3 | 43.5 | 36.8 | 42.9 |
| Coronary atherosclerotic heart disease (%) | 5.3 | 4.3 | 0.0 | 23.8 |
| Diabetes mellitus, type 2 (%) | 10.5 | 13.0 | 10.5 | 23.8 |
| Current tobacco use (%) | 58.3 | 22.0 | 50.0 | 66.7 |
| Pack-years (mean ± SD) | 14.8 ± 14.9 (0–50) | 6.5 ± 12.0 (0–39) | 8.9 ± 12.1 (0–40) | 12.6 ± 13.0 (0–40) |
| $\log_{10}$ (hs-CRP in serum [pg/ml]) | 6.73 ± 0.88 (5.55–8.84) | 6.60. ± 0.87 (5.15–8.19) | 7.10 ± 0.87 (5.33–8.19) | 7.34 ± 0.51 (6.53–8.09) |
| HBsAG positive (%) | 0.0 | 0.0 | 0.0 | 16.7 |
| HCV positive (%) | 18.2 | 15.4 | 40.0 | 62.5 |
| History of cancer (%) | 10.5 | 8.7 | 10.5 | 0.0 |

Louis, MO) at 200 × *g* for 15 min. The interphase layer was collected by pipette in a separate tube and was washed in RPMI-1640 medium. The pellet was resuspended to a final volume of 1.5 ml with freezing medium that consisted of 40% RPMI-1640 and 50% FBS and 10% DMSO. Aliquoted vials were frozen in isopropanol-containing containers placed at −80 °C.

### 2.3. Culture and cryopreservation of AG10097 cells

The non-malignant lymphoblastoid cell line AG10097 was obtained from the Aging Cell Repository (National Institute of General Medical Sciences, Coriell Repository) and was grown at 37 °C in 5% $CO_2$ environment in RPMI-1640 supplemented with 10% FBS (Invitrogen). For cryopreservation, cells in the exponential phase of growth were collected, suspended in freezing medium (40% RPMI-1640, 50% FBS, 10% dimethyl sulfoxide) and aliquoted to obtain samples containing $10^6$ cells in 200 μl of freezing medium. Vials were frozen overnight in isopropanol-containing freezing containers (Nalgene, Rochester, NY) placed at −80 °C. Samples were subsequently stored at −140 °C.

### 2.4. Examination of the baseline level of DNA oxidation in cryopreserved PBMCs using alkaline comet assay

We analyzed the baseline level of single strand breaks (SSBs), thymine glycol-DNA glycosylase (endonuclease III, Endo III)-labile sites and formamidopyrimidine-DNA glycosylase (Fpg)-labile sites using the alkaline comet assay [31,32]. All samples were analyzed in 3 repeats for Endo III-labile sites and Fpg-labile sites and in 6 repeats for SSBs.

Frozen PBMC samples were thawed by submersion in a 37 °C water bath. PBMCs were suspended in tubes containing 10 ml of cold complete RPMI-1640 medium supplemented with 20% FBS. Cells were then centrifuged at 200 × *g* at 4 °C for 15 min. After washing, cells were suspended in 0.5% low melting point agarose (Invitrogen, Carlsbad, CA) in PBS and spread on fully frosted microscope slides (A. Daigger & Company, Wheeling, IL) precoated with 0.5% normal agarose (Invitrogen). After a 20-min gel solidification period at 4 °C, slides were lysed overnight at 4 °C in cold lysis buffer (10 mM Tris–HCl, pH 10, 2.5 M NaCl, 100 mM EDTA, 5% DMSO, 1% Triton X-100). DNA on slides was then treated with bacterial DNA glycosylases, endonuclease III or Fpg. Each set of samples used for studying base lesions detected by a particular DNA glycosylase contained enzyme-treated and buffer-treated (control) samples. The enzyme treatment of gels was performed for 2 h at 37 °C in moist chambers using 1250×-diluted endonuclease III (obtained from Dr. Andrew R. Collins, University of Oslo, Oslo, Norway) or 2 U of Fpg per gel (New England Biolabs, Inc., Ipswich, MA) in enzyme buffer (0.1 M KCl, 0.5 mM EDTA, 40 mM HEPES, 0.2 mg/ml bovine serum albumin, pH 8.0). The unwinding step was performed for 40 min at 4 °C in unwinding/electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13). Electrophoresis was performed at 8 °C for 30 min in unwinding/electrophoresis buffer at electric field strength 0.73 V/cm and current 343 mA. The slides were then neutralized (0.4 M Tris–HCl, pH 7.5), rinsed with distilled water, air dried, stained with 2 μg/ml ethidium bromide and covered with standard cover slips. Comets were scored using an Eclipse E-400 fluorescence microscope (Nikon, Japan) attached to a Pulnix video camera (Kinetic Imaging LTD, Liverpool, UK) connected to the image analysis system Komet version 5.5 (Kinetic Imaging LTD). Olive tail moment was used here as a measure of DNA damage level [33,34]. As we have chosen to report our data with OTM values, for reference purposes we have shown the data for SSB, Endo III labile sites and Fpg labile sites as both OTM and tail DNA (Figs. S1, S2 and 1 and 2).

Reference standard samples were prepared from a single vial of cryopreserved AG10097 cells. After thawing, cryopreserved cells were washed in complete RPMI-1640 medium and embedded in 0.5% low melting point agarose onto fully frosted microscope slides. After a 20-min gel solidification period at 4 °C, slides with embedded cells were placed in cold lysis buffer, treated with enzymes and processed through the alkaline comet assay in parallel with the PBMC samples. Eight gels with AG10097 cells were arranged on four slides for a single endonuclease or Fpg experiment containing samples for twelve individuals. Six gels treated with enzyme were served as positive reference standards in the experiment. The other two gels treated only with enzyme-free buffer were used as negative reference standards.

### 2.5. Examination of SSB repair capacity in cryopreserved PBMCs using alkaline comet assay

The alkaline comet assay was also used to study repair capacity of single strand breaks (SSBs) and other types of DNA damage detected by this assay in cryopreserved PBMCs. Four replicates of SSB removal kinetic data were obtained for each individual. Each replicate contained PBMCs irradiated with dose of 6.3 Gy and unirradiated PBMCs subjected to 0, 5, 15, 30 and 60 min repair incubation. After slide preparation, the procedure included the following steps: lysis, unwinding, electrophoresis, neutralization and microscope image analysis. Positive (irradiated) and negative (unirradiated) internal standards were also prepared and they were processed with PBMC samples [10,11]. The data from this repair assay for this cohort are historical data from our previous publication on this group of individuals [11].

Since the DNA repair data shown in this work are derived using OTM, we have shown for reference purposes the relationships between age, sex and race on SSB-RC derived both from data expressed as standardized OTM (Fig. S3a and b) and standardized tail DNA (Fig. S3c and d). Furthermore, our earlier work shows more comparisons of DNA repair data obtained using both OTM and tail DNA [10].

### 2.6. Measurement of RBC GSH

HANDLS blood samples were shipped to our laboratory on ice. Upon receipt, plasma was separated, the white buffy coat was carefully removed and red blood cells (RBCs) were washed 3 times by resuspension and centrifugation (1500 × *g*, 10 min) with phosphate buffered saline (PBS) containing 100 μM $Na_2$EDTA. The final RBC pellet was mixed with an equal volume of PBS–EDTA resulting in a 50% hematocrit. 0.2 ml of 50% hematocrit RBC was mixed with 1 ml of precipitating solution (1.67 g of glacial metaphosphoric acid, 0.2 g of disodium EDTA, 30 g of NaCl) to precipitate proteins. Proteins were removed by centrifugation on a table top microfuge (∼14,000 × *g*) and supernatants collected for glutathione (GSH) assay. GSH standard solutions were prepared in the precipitating solution mentioned above. Samples and GSH standard dilutions (0.16 ml) were neutralized by the addition of 0.3 M $Na_2HPO_4$ solution (0.7 ml) and 0.2 ml aliquots of the mix were placed in triplicates on a polystyrene microtiter plate. 0.05 ml of 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) solution (20 mg of DTNB in 100 ml of 1% sodium citrate) was added per well and the plate was read at 410 nm within 10 min using a microplate reader (Biorad). The average absorbance of sample wells at 410 nm was used to determine the concentration of GSH in RBCs based on the GSH standard curve generated.

### 2.7. Measurement of basal RBC fluorescent heme degradation products

Washed RBCs (100 μl) were lysed in 10 ml deionized double distilled water. The Hb spectrum of the hemolysate was recorded from 490 nm to 640 nm using a Perkin Elmer lambda 6 spectrophotometer. The concentrations of oxyHb and metHb were determined by a least square-fitting program using spectra of oxyHb and metHb at known concentrations. The Hb concentration of the hemolysate was then adjusted to 50 μM. The emission (em) fluorescence spectrum was measured from 400 nm to 600 nm at an excitation (ex) wavelength of 321 nm using a Perkin Elmer model LS 50B spectrofluorimeter. The ex and em slit widths were kept at 10 nm. The relative measure of fluorescent heme degradation products was determined by subtracting the emission at 400 nm from the emission at 465 nm.

### 2.8. ELISA assay of protein carbonyls

Measurements of protein carbonyls in human plasma were performed in triplicate using ELISA kits obtained from BioCell according to the company protocol. The assay was done using 5 μl (∼80 mg/ml) of each of the plasma samples. The samples are reacted with dinitrophenylhydrazine (DNP) and the protein is then adsorbed to an ELISA plate. Unconjugated DNP and non-protein constituents are

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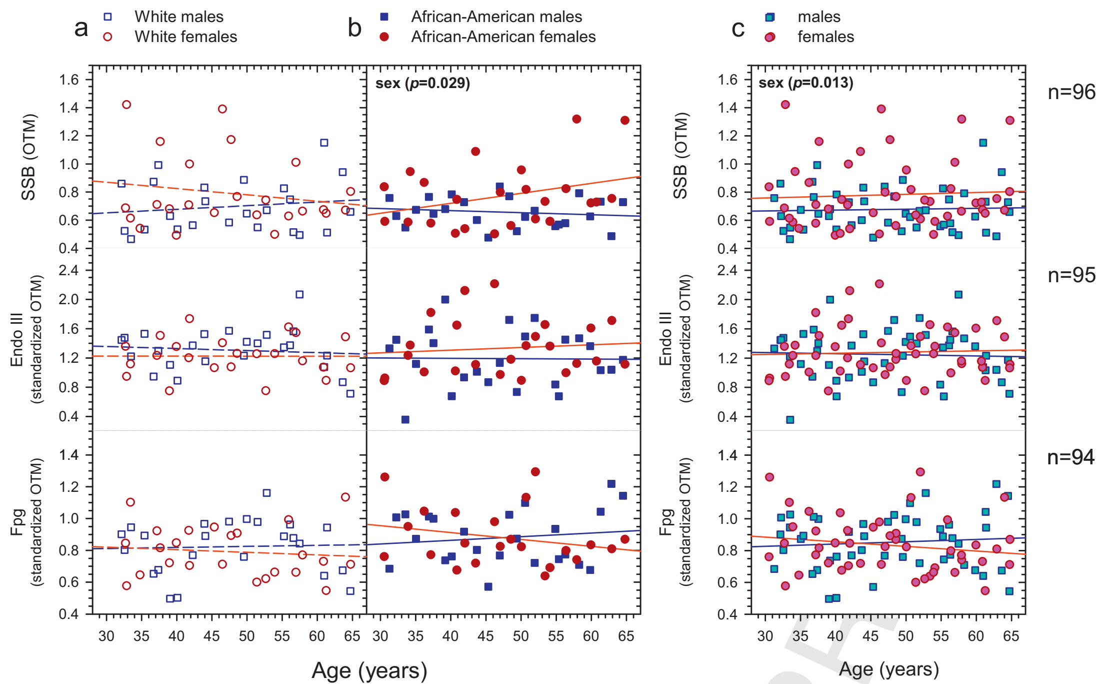


**Fig. 1.** Effect of age, sex and race on the baseline level of oxidative damage to DNA in white males, African-African males, white females and African-American females. Graphs in panels (a), (b) and (c) present the relationship between age ($X_1$), sex ($X_2$) on the level of oxidative damage to DNA ($Y$) in whites (a), African-Americans (b) and total cohort (c). All DNA damage data shown in this figure are expressed as OTM or derived from data expressed as OTM. Levels of SSB, Endo III- and Fpg-labile sites are shown. Independent variables significantly related to oxidative damage to DNA found from the multiple regression relationship $\widehat{Y}_i = b_0 + b_1 \cdot X_{1i} + b_2 \cdot X_{2i} + b_3 \cdot X_{1i} + b_{2i}$ or $\widehat{Y}_i = b_0 + b_1 \cdot X_{1i} + b_2 \cdot X_{2i}$ (if no interaction between $X_1$ and $X_2$ was found from the first relationship) in whites (a), African-Americans (b) and total population (c) are shown in the upper left hand corner of the graphs together with corresponding $p$ values in parentheses. Statistically significant ($p < 0.05$) variables are marked in bold, whereas the values that are close to significance are underlined. Results of multiple regression analyses are shown in details in Supplemental Table S1. Each point corresponds to a single individual. The number of participants analyzed for each relationship was indicated on the right side of each row. Dashed and continuous lines represent curves for simple linear regression describing the relationship between age and the level of oxidative damage to DNA and performed separately for each group of white males (blue dashed lines) and white females (red dashed lines) in panel (a), African-American males (blue continuous lines) and African-American females (red continuous lines) in panel (b), and males (blue continuous lines) and females (red continuous lines) in panel (c).

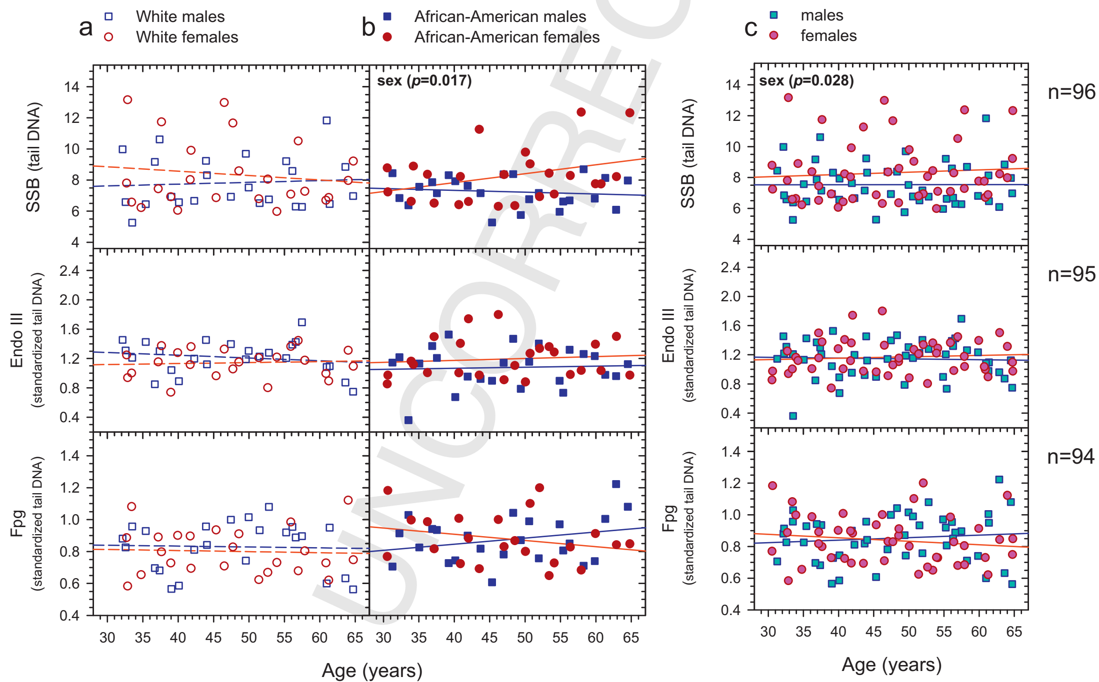


**Fig. 2.** Effect of age, sex and race on the baseline level of oxidative damage to DNA in white males, African-African males, white females and African-American females. Graphs in panels (a), (b) and (c) present the relationship between age ($X_1$), sex ($X_2$) on the level of oxidative damage to DNA ($Y$) in whites (a), African-Americans (b) and total cohort (c). All DNA damage data shown in this figure are expressed as tail DNA or derived from data expressed as tail DNA. Refer to Fig. 1 for more description.

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washed away. The adsorbed protein is probed with biotinylated anti-DNP antibody followed by streptavidin-linked horseradish peroxidase. Absorbances are related to a standard curve prepared from serum albumin containing increasing proportions of hypochlorous acid-oxidized protein.

*2.9. Measurement of serum hs-CRP*

The assessment of high-sensitivity C-reactive protein (hs-CRP) in serum was performed by Quest Diagnostics using nephelometric method utilizing latex particles coated with CRP monoclonal antibodies. The method was standardized against the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)/Bureau Communautaire de Référence (BCR)/College of American Pathologists (CAP) CRP reference preparation.

*2.10. Mathematical and statistical analysis*

*2.10.1. Assessment of level of single strand breaks (SSBs) and oxidized bases in PBMCs*

For each individual, three sets of cryopreserved PBMC samples were prepared for examination of Endo III- and Fpg-sensitive base lesions. One sample from each set was treated with enzyme, while the other sample was treated only with buffer. Each of three sets of PBMC samples was processed with six positive (treated) and two negative (untreated) reference standards through comet assay steps. Since conditions can differ slightly from electrophoresis to electrophoresis, the use of reference standards may minimize inter-electrophoresis and inter-experimental variation. Standardization was performed using the arithmetic mean values for the PBMC samples and reference standards. Negative and positive reference standards were used to standardize experimental values for enzyme- and buffer-treated PBMCs using the formula:

$$y_s = \frac{y}{S_p - S_n}$$

where $y_s$ is the standardized value for the level of DNA oxidation in PBMCs, $y$ is the experimental value for the level of DNA oxidation in PBMCs, $s_n$ is the value for negative reference standard, and $s_p$ is the value for positive reference standard.

Standardized values obtained from the three data sets were taken together to calculate the means for the level of DNA damage in samples treated with Endo III or Fpg, or the level of SSBs in PBMCs for each individual. Since, the level of DNA damage in enzyme-treated samples correspond to the sum of the levels of enzyme-detected base lesions and SSBs, we calculated the level of enzyme-detected base lesions by subtracting the standardized values for SSBs from the standardized values for the samples treated with a particular enzyme. Finally, the standardized levels of oxidized bases detected by Endo III and Fpg as well as the means for unstandardized (experimental) levels of SSBs were used to express the level of oxidative DNA lesions in this work.

*2.10.2. Assessment of SSB repair capacity (SSB-RC) in PBMCs*

DNA repair parameters were assessed based on four repeats of SSB removal kinetics obtained for PBMCs irradiated with dose of 6.3 Gy and subjected to 0, 5, 15, 30 and 60 min repair incubation. The detailed procedure was described in our previous publication [10]. The data referred to in this paper were published in our previous analysis of this cohort. These experiments were not repeated for this preliminary study.

*2.10.3. Analysis of the baseline level of DNA oxidation and SSB repair capacity (SSB-RC) in PBMCs in human population*

We analyzed the strength of effect of demographic and oxidative stress-related variables on the level of DNA oxidation and SSB-RC. Each relationship was graphed using SigmaPlot 8.0 and statistical analyses were performed using Statistica 7.1 (Statsoft Inc., Tulsa, OK). Simple linear regression analyses were applied to investigate relationships between continuous independent variable (age, RBC GSH concentration, heme degradation products, the logarithm of hs-CRP concentration) and dependent variable (SSB-RC or intercellular variability in DNA damage). Simple regression analyses were performed in 96-individual cohort (Figs. 3 and S4) or subsets of this cohort characterized by sex or race or both sex and race (Figs. 1, 2, 4–6, S5).

Multiple regression analyses were done separately for males, females, whites and African-Americans (Figs. 1, 2, 4–6, S5). In multiple regression analyses, we tested simultaneously the effect of single discrete (sex or race) and single continuous (age, RBC GSH concentration, heme degradation products, the logarithm of hs-CRP concentration, protein plasma carbonyls) independent variables on dependent variables (the level of DNA oxidation or SSB-RC). When necessary, interaction of sex or race with age, RBC GSH concentration, heme degradation products, the logarithm of hs-CRP concentration, plasma protein carbonyls and other continuous variables was included in the regression. A significant test for the interaction coefficient indicates that the relationship between the dependent variable and one of the independent variables in the interaction is related to the value of the other variable in the product. Residual analysis was performed during all regression analyses to identify outliers. The residual analysis involved computing raw residuals, deleted residuals and Cook's distances (Statistica).

## 3. Results

We employed the alkaline comet assay to measure the baseline level of SSB, endonuclease III (Endo III) and Fpg labile sites (oxidized bases recognized by these enzymes) in cryopreserved PBMCs from four age-matched 24-individual groups of male and female whites and African-Americans. We performed correlation analyses between the levels of DNA oxidative lesions and other measures related to oxidative stress including SSB repair capacity, the levels of blood antioxidants (reduced glutathione in red blood cells, inflammation markers (high sensitivity C-reactive protein (hs-CRP) in serum) and oxidative protein lesions (protein carbonyls in plasma, heme degradation products). We also correlated findings with participants' self-report of tobacco and alcohol use. We found that a positive history of smoking, positive history of ethanol consumption, and protein carbonyls in plasma in serum were not associated with the level of oxidative damage to DNA or SSB-RC in PBMCs as measured by comet assay. However, sex and selected oxidative stress measures, RBC GSH concentration, the level of heme degradation products and the level of serum hs-CRP appeared to influence the level of SSB, Endo III labile sites and/or SSB-RC.

The effect of age, sex and race on the baseline level of DNA oxidative lesions in PBMCs is shown in Fig. 1. Analysis of the SSB levels by sex revealed a difference in the SSB level between females and males ($p = 0.013$) (Fig. 1c). This sex related difference persists when analyzed among is African-American study participants ($p = 0.029$) (Fig. 1b) whereas it is not observed among whites ($p = 0.164$) (Fig. 1a). Age, sex and race do not affect the level of oxidized bases recognized by endonuclease III and Fpg (Fig. 1) as analyzed by multiple regression analysis. There is also a significant positive correlation between the baseline level of Fpg III-labile sites and SSB in PBMCs ($p = 0.021$) (Fig. S4). Interestingly, no significant relationships are found between Endo III-labile sites and SSB and between Endo III-labile sites and Fpg-labile sites (Fig. S4). We also found significant relationships between SSB-RC and SSB level with a more efficient repair mechanism resulting in fewer single strand breaks. This is observed by the significant positive correlations between the logarithm of the half-time of DNA repair and SSB (Fig. 3b) ($p = 0.041$), and between the residual DNA damage after 30 min and SSB ($p = 0.047$) (Fig. 3c). In addition, there is a close to significant negative relationship between the logarithm of the initial rate of DNA repair and SSB ($p = 0.056$) (Fig. 3a), and close to significant positive relationship is between the residual DNA damage after 60 min and SSB ($p = 0.056$) (Fig. 3d). The logarithm of the initial rate of DNA repair is directly proportional to SSB repair capacity, whereas other DNA parameters are inversely proportional to SSB repair capacity. Thus, in all four relationships shown in Fig. 3, negative correlations between SSB repair capacity and SSB level are observed.

In addition, we observed that there are similarities in the effect of different oxidative stress measures on both SSB level and SSB-RC (Figs. 4–6). Fig. 4 presents the effect of GSH concentration in RBCs on the level of DNA oxidative lesions and SSB repair. We found that the RBC GSH concentration is positively correlated with the level of SSB in white females ($p = 0.004$) (Fig. 4a). The RBC GSH concentration in white females is negatively correlated with SSB repair capacity as measured by two parameters: the logarithm of the initial rate of DNA repair ($p = 0.012$) and the logarithm of the half-time in SSB repair ($p = 0.033$) (Fig. 4d). There is no effect of RBC GSH concentration on the SSB level or SSB-RC in white males (Fig. 4a and d) or in African-American males and females (Fig. 4b and e). In addition, RBC GSH concentration is found to be significantly correlated to the level of Endo III-labile sites in all study participants ($p = 0.047$) (Fig. 4c). This effect is very significant in white males and females ($p = 0.003$) (Fig. 4a), but not in African Americans (Fig. 4b).

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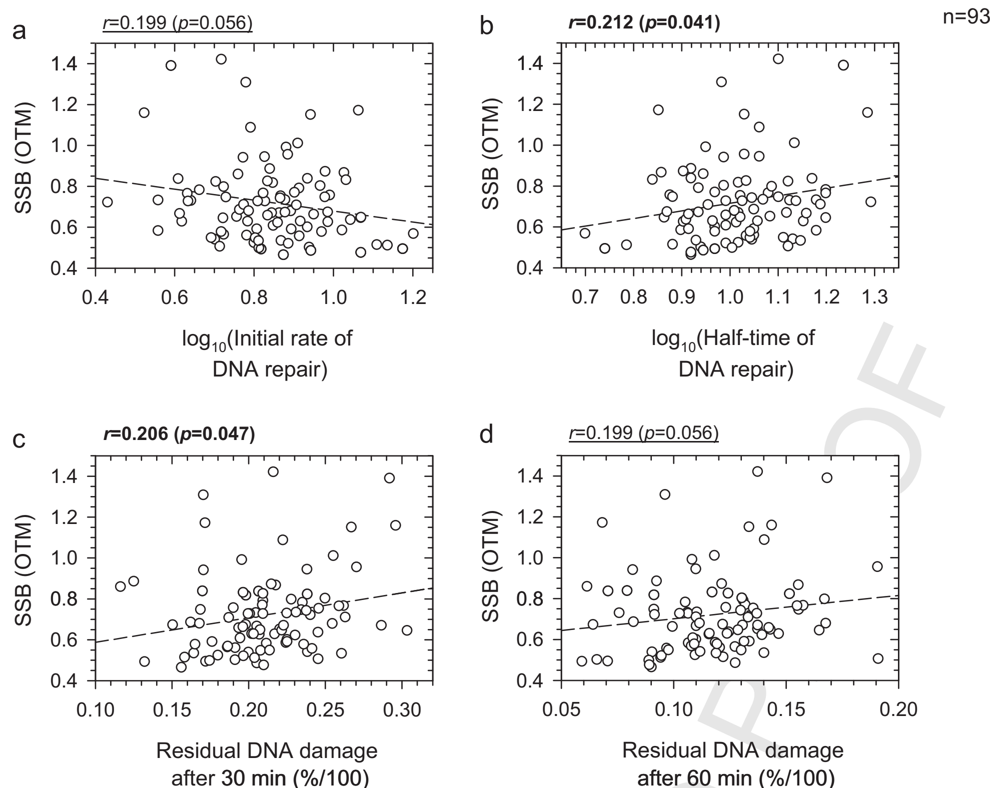


**Fig. 3.** Effect of SSB repair capacity (SSB-RC) on the baseline level of SSBs. SSB repair capacity was measured using the parameters related to the fast component of SSB-RC (the logarithm of the initial rate of DNA repair and the logarithm of the half-time of DNA repair) and describing the slow component of SSB-RC (the residual DNA damage after 30 min or 60 min). All DNA damage and DNA repair data shown in this figure are expressed as OTM or derived from data expressed as OTM. Correlation coefficient $r$ with its corresponding statistical significance in parenthesis is indicated on each graph. Statistically significant ($p < 0.05$) variables are marked in bold, whereas the values that are close to significance are underlined. Each point corresponds to a single individual. The number of participants analyzed for each relationship was indicated on the right side of the figure.

The relationship between the level of heme degradation products and SSB level and SSB-RC is shown in Fig. 5, SSBs levels increase with increasing heme degradation product levels in African-American males ($p = 0.008$), while for African-American females there is a decrease in SSBs with levels of heme degradation products ($p = 0.006$) (Fig. 5b). This opposite relationship between heme degradation and SSB level in African-American males and females can also be described by the observed interaction between sex and the level of heme degradation products in their effect on SSB level ($p = 0.001$) (Fig. 5b). In addition, a negative correlation is present between the level of heme degradation products and SSB-RC as measured by the residual DNA damage after 60 min in all participants ($p = 0.014$) (Fig. 5f).

We also examined the relationship between the clinically relevant inflammation marker hs-CRP comparing the logarithm of hs-CRP concentration the level of oxidative DNA lesions and SSB-RC. There is a statistically significant increase in SSB level with increases in the logarithm of hs-CRP concentration in African-American males ($p = 0.022$) (Fig. 6b). The overall analysis of the cohort revealed an interaction between sex and the logarithm of hs-CRP concentration in their effect on SSB-RC as measured by the residual DNA damage after 30 min ($p = 0.002$) and the residual DNA damage after 60 min ($p = 0.038$) (Fig. 6f). The interaction is still present when analyses were performed in whites ($p = 0.018$) (Fig. 6d) and African-Americans ($p = 0.025$) (Fig. 6e). There were no statistically significant relationships between hs CRP concentration and the level of oxidized bases (Fig. 6a–c). There was also no significant relationship between the levels of protein carbonyls in plasma and oxidative DNA lesions (Fig. S5).

Finally, we found in the entire population that there is a positive correlation between the levels of RBC GSH and heme degradation products ($p = 0.015$) (Fig. 7a) as well between the logarithm of hs-CRP concentration in serum and RBC GSH concentration ($p = 0.020$) (Fig. 7b). However, we did not observe a significant relationship between the levels of hs-CRP and heme degradation products.

## 4. Discussion

In order for us to understand the relationship between oxidative damage to DNA and DNA repair capacity and their roles in human health and disease we must first begin to examine traditional and novel measures of oxidative stress and oxidative damage to DNA in the context of DNA repair. In this pilot study we examined the effect of sex, race and age on the baseline level of traditional and novel measures of oxidative stress: SSBs and oxidized bases detected by the bacterial DNA glycosylases, Endo III and Fpg (Fig. 1). We found that there is a higher level of SSBs in females as compared to males in this cohort (Fig. 1c). This difference remains significant when African-American males and females are compared (Fig. 1b); however, there is no significant difference between white males and females (Fig. 1a).

Despite the widely held notion and some evidence in the literature that forms of DNA damage increase with age, we did not observe an increase in SSBs with age in this cohort (Fig. 1). We believe that a cohort whose age range is 30–64 years with a median age of 47.6 years may not provide a broad enough age span to delineate differences with age if they exist. Other studies which have included individuals across the entire life span (0–82 years) have shown an increase in SSBs with age [35,36]. However, our study

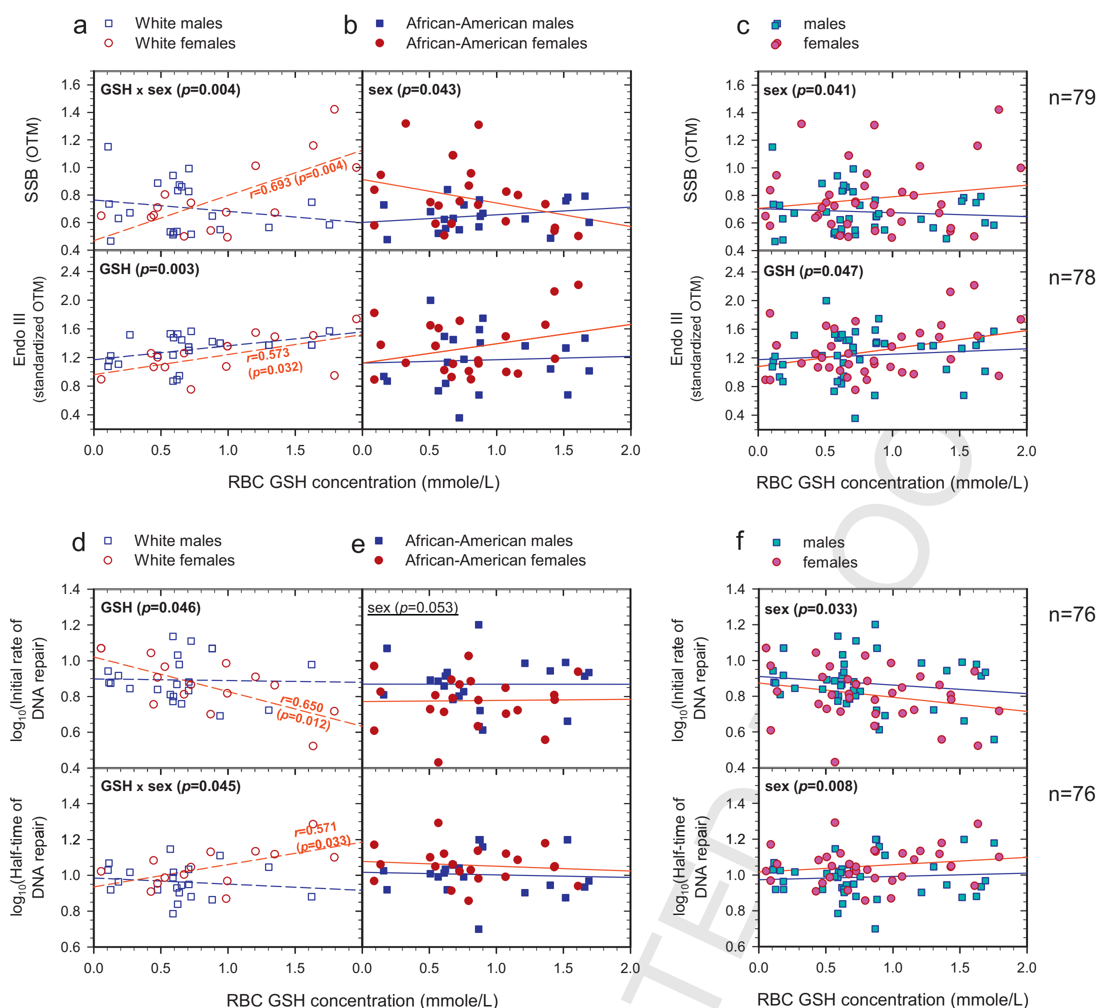


**Fig. 4.** Effect of RBC GSH concentration, sex and race on the baseline level of oxidative damage to DNA and SSB-RC. Graphs in panels (a), (b) and (c) present the relationship between RBC GSH concentration ($X_1$), sex ($X_2$) on the level of oxidative damage to DNA ($Y$) in whites (a), African-Americans (b) and total cohort (c). Graphs in panels (d), (e) and (f) present the relationship between RBC GSH concentration ($X_1$), sex ($X_2$) on the level of the fast component of SSB repair ($Y$) in whites (d), African-Americans (e) and total cohort (f). All DNA damage and DNA repair data shown in this figure are expressed as OTM or derived from data expressed as OTM. The level of following oxidative DNA lesions was measured and shown in the figure as the level of SSB and Endo III-labile sites. The fast component of SSB repair was expressed using the logarithm of the initial rate of DNA repair and the logarithm of the half-time of DNA repair. Independent variables significantly related to oxidative damage to DNA or SSB-RC found from the multiple regression relationship $\widehat{Y}_i = b_0 + b_1 \cdot X_{1i} + b_2 \cdot X_{2i} + b_3 \cdot X_{1i} + b_{2i}$ or $\widehat{Y}_i = b_0 + b_1 \cdot X_{1i} + b_2 \cdot X_{2i}$ (if no interaction between $X_1$ and $X_2$ was found from the first relationship) in whites (a, d), African-Americans (b, e) and total population (c, f) are shown in black on graphs together with corresponding $p$ values in parentheses. Results of multiple regression analyses are shown in details in Supplemental Table S2. Independent variable ($X_1$) significantly related to oxidative damage to DNA or SSB-RC found from the single regression relationship $\widehat{Y}_i = b_0 + b_1 \cdot X_{1i}$ in white males (a, d) and in African-American males (b, e) is indicated by corresponding correlation coefficient $r$ and $p$ value (in parenthesis) in blue, whereas in white females (a, d) and in African-American females (b, e) is marked by correlation coefficient $r$ and $p$ value (in parenthesis) in red. Statistically significant ($p < 0.05$) variables are marked in bold, whereas the values that are close to significance are underlined. Refer to Fig. 1 for more description.

findings are consistent with data from studies which included middle aged individuals and showed no change in SSB levels with age [36–38].

There are no significant changes in the levels of Endo III- and Fpg-sensitive sites related to age, sex or race (Fig. 1). This was also the case for reports on European populations [36,38,39]. However, Humphreys et al. found that 75–82 year-old individuals had elevated levels of Fpg-labile sites as compared with younger participants from two age groups: 20–35 and 63–70 years [35]. We did not observe an increase in Fpg-labile sites with age, likely because our population sample lacked individuals older than 64 to provide a robust comparison of damage levels. In contrast to our results, Walters et al. found a lower level of Fpg-labile sites in 20–45 years-old African-Americans as compared to whites from the same age group using comet assay [40].

We tested the relationship between markers related to oxidative stress in RBCs and the level of DNA oxidative lesions and repair in PBMCs. Autoxidation of oxyhemoglobin is a major source of ROS in RBCs [41] and it is a potential source of damage to PBMCs representing another source of systemic oxidative stress. We analyzed two markers of oxidative stress in RBCs: RBC GSH concentration and the level of heme degradation products. The RBC GSH concentration may fluctuate and depends on the oxidative stress level at a given time. In contrast, heme degradation products accumulate during the life span of the RBCs, because the cells do not seem to have a system to remove these degradation products [28]. Therefore, their concentration may serve as a longer-term measure of oxidative stress. Since RBC GSH concentration and the level of heme degradation products depend on the level of oxidative stress in RBCs, it could be expected that there is a significant correlation between both measures. In fact, we found a significant positive correlation between these parameters (Fig. 7a). While GSH utilization in erythrocytes will increase during oxidative stress, the other pathways involved in GSH synthesis, uptake

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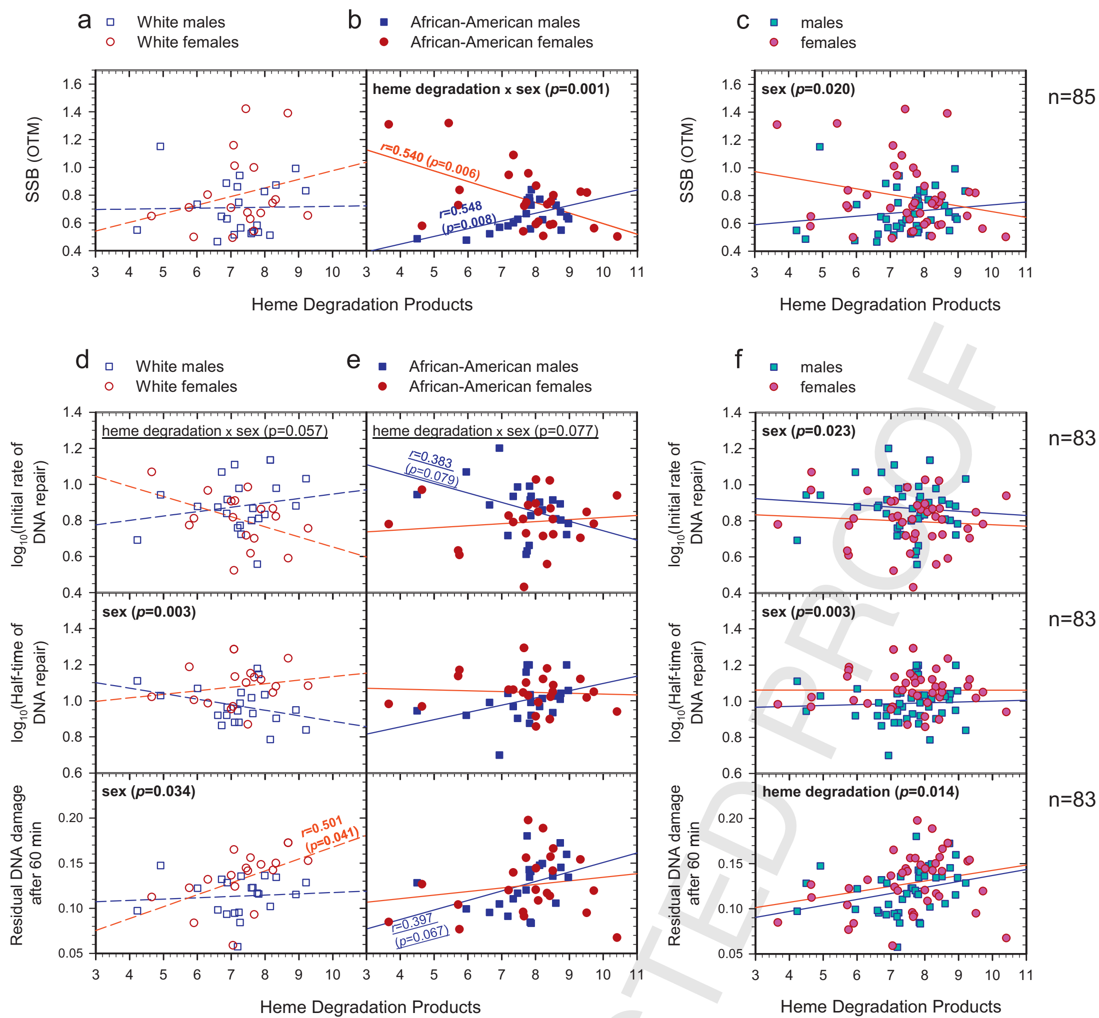


**Fig. 5.** Effect of the level of heme degradation products, sex and race on the baseline level of SSBs and SSB-RC. Graphs in panels (a), (b) and (c) present the relationship between the level of heme degradation products ($X_1$), sex ($X_2$) on the level of SSB ($Y$) in whites (a), African-Americans (b) and total cohort (c). Graphs in panels (d), (e) and (f) present the relationship between the level of heme degradation products ($X_1$), sex ($X_2$) on the SSB-RC ($Y$) in whites (d), African-Americans (e) and total cohort (f). All DNA damage and DNA repair data shown in this figure are expressed as OTM or derived from data expressed as OTM. The fast component of SSB repair was expressed using the logarithm of the initial rate of DNA repair and the logarithm of the half-time of DNA repair, whereas the slow component of SSB repair was measured as the residual DNA damage after 60 min. Results of multiple regression analyses are shown in details in Supplemental Table S3. Refer to Fig. 4 for more description.

and reduction (glutathione reductase) can possibly be stimulated by oxidative stress. An example in brains of young and old mice [42] indicates a greater increase in γ-glutamyl cysteine synthetase activity in old mice then young mice during oxidative stress. Similar findings were obtained using bronchial epithelium NCI-H292 cells. Exposure to menadione decreased total cellular GSH content immediately after exposure and increased intracellular GSH concentration and γ-glutamyl cysteine synthetase activity 6–12 h after exposure [43].

Our examinations of these markers of oxidative stress revealed significant correlations with DNA damage. We observed a positive relationship in all studied participants between RBC GSH and Endo III-labile sites (Fig. 4c). When the results were separated according to race, a significant increase in the relationship between Endo III and GSH was found for whites, (Fig. 4a) but not for African Americans (Fig. 4b). The increase in SSBs with RBC GSH concentration could be related to an increase in the systemic oxidative stress in blood. The increase in oxidative base damage with increasing RBC GSH concentration suggests a role for increased oxidative stress present in the circulation.

The level of heme degradation products was negatively correlated with the rate of SSB repair as measured by the residual DNA damage after 60 min (Fig. 5f). The link between the level of heme degradation products and SSB-RC is unclear. However, it is possible that SSB repair is negatively affected by this type of oxidative stress in the circulation.

Unlike the effect on SSB repair, which was found for the entire population, we found that SSBs increased with increasing levels of hemoglobin degradation products only in African-American males (Fig. 5b). In fact for African-American females, SSB levels declined with increases in heme degradation product level (Fig. 5b). We have no definitive explanation for these differences among African-American males and females and the unexpected decrease in SSB for African American females. This difference can be perhaps partially attributed to differences in the effects of heme degradation on SSB repair in African American males and females (Fig. 5e), which contribute to the observed levels of SSB. Furthermore, a review of the clinical characteristics of the population shows that African-American males in this cohort are more likely to have coronary atherosclerotic heart disease, diabetes mellitus type 2, HBV- and HCV-positivity, and higher rate of current tobacco use than African-American females (Table 1). These characteristics may in some way influence the level of DNA oxidation and the level of heme degradation products.

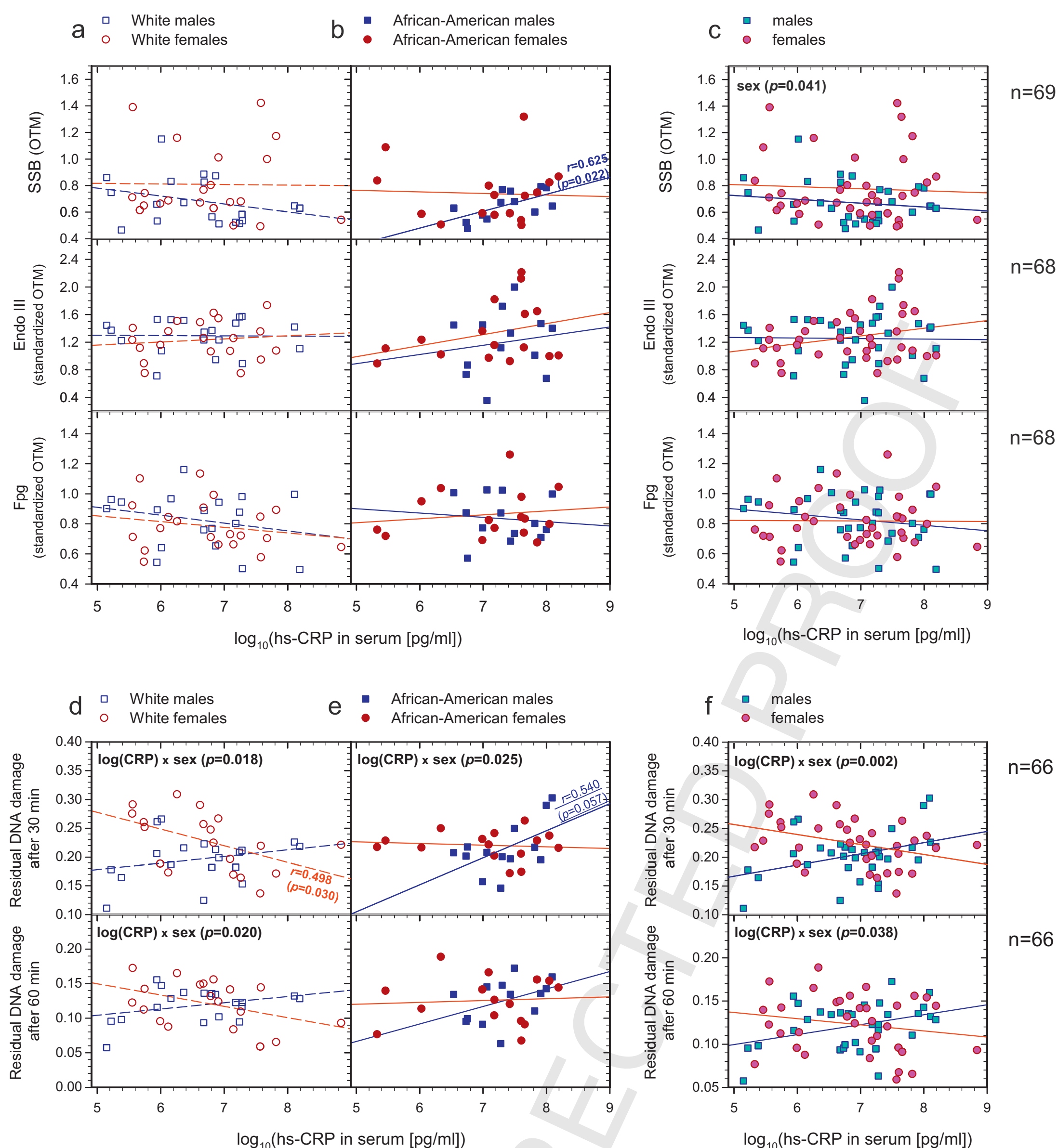


**Fig. 6.** Effect of the hs-CRP concentration, sex and race on the baseline level of oxidative damage to DNA and SSB-RC. Graphs in panels (a), (b) and (c) present the relationship between hs-CRP concentration ($X_1$), sex ($X_2$) on the level of oxidative damage to DNA ($Y$) in whites (a), African-Americans (b) and total cohort (c). Graphs in panels (d), (e) and (f) present the relationship between hs-CRP concentration ($X_1$), sex ($X_2$) on the level of the slow component of SSB repair ($Y$) in whites (d), African-Americans (e) and total cohort (f). All DNA damage and DNA repair data shown in this figure are expressed as OTM or derived from data expressed as OTM. The slow component of SSB repair was expressed using the residual DNA damage after 30 min and 60 min. Results of multiple regression analyses are shown in details in Supplemental Table S4. Refer to Fig. 4 for further description.

Consistent with other studies [44], we found that hs-CRP levels were higher in African-Americans than whites (Table 1). Furthermore, there is a strong significant positive correlation between the levels of hs-CRP and RBC GSH in the population overall (Fig. 7b). The relationship between the levels of hs-CRP and heme degradation products is not significant (Fig. 7c). We found that there was a significant positive relationship between the levels of hs-CRP in serum and SSBs in African-American males (Fig. 6b). This increase in SSB level may, therefore, be attributed to the increase in ongoing chronic inflammatory processes and free radical production taking place in the vascular system [45]. Interestingly, we did not observe significant relationships between hs-CRP and SSBs in African-American females (Fig. 6b) or in the white study participants (Fig. 6a). We did find that levels of residual DNA damage after 30 min and 60 min decline with increasing levels of hs-CRP in females when compared to males (Fig. 6f).

Previous studies have shown a weak positive correlation between hs-CRP values and SSB levels in patients with acute coronary syndrome [46]. Studies of community dwelling healthy participants and patients with metabolic syndrome, however, showed no significant correlation between hs-CRP and SSB level [47,48]. The statistically significant relationship between hs-CRP and SSB in African-American males may relate to the high disease burden present in these individuals. We did not find significant relationships between hs-CRP concentration and oxidized bases detected by Endo III and Fpg (Fig. 6a–c). This is in contrast to a published study that showed a weak but significant positive correlation between serum 8-oxo-dG levels and serum hs-CRP measures in hemodialysis patients [49].

The lack of a strong overall relationship between hs-CRP, a marker of systemic inflammation in clinical use, and markers of DNA oxidation may suggest that each of these markers represents

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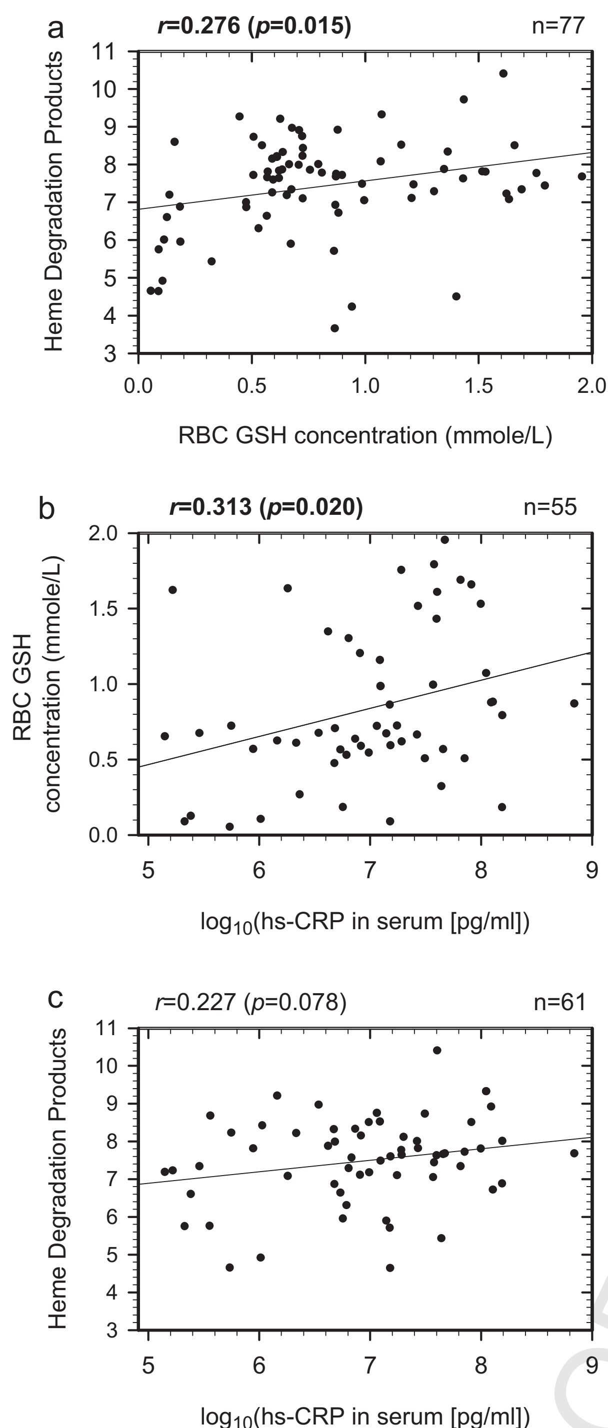


**Fig. 7.** The relationship between the levels of RBC GSH, heme degradation products (panel a) and between the levels of hs-CRP in serum and RBC GSH (panel b), and hs-CRP and heme degradation products (panel c). Correlation coefficient *r* with its corresponding statistical significance in parenthesis is indicated on each graph. Statistically significant ($p < 0.05$) variables are marked in bold. Each point corresponds to a single individual. The number of participants analyzed for each relationship was indicated on the top side of each graph.

a different pathway through which inflammation and oxidative stress occur and are monitored and/or modulated. This highlights the gaps in our knowledge about the relationships between the various oxidative stress markers. Several prominent hypotheses suggest that oxidative stress and oxidative damage to DNA and other cellular macromolecules promote aging and the development of age associated diseases. Therefore, it might be reasonable to expect that correlations might exist between various commonly used markers of oxidative stress, inflammation and DNA damage. However, the lack of consistent correlation between frequently measured markers of oxidative stress in our hands may suggest that each of these markers measure only a single, non-correlative element of the multiple additive factors that comprise what we refer to as oxidant stress. For example, plasma protein carbonyls are frequently used as a marker of oxidant stress; however in our hands their levels did not correlate with any other marker studied. Although smoking and ethanol consumption may be associated with oxidative stress, neither correlated with SSBs and oxidized bases in this population. There is also no significant relationship between plasma protein carbonyls and the level of DNA oxidation (Fig. S5). Consistent with this, Humphreys et al. found that α-tocopherol did not influence the levels of SSBs, Fpg-labile sites, or the capacity of lymphocyte extracts to remove 8-oxoguanine [35]. However, we have determined that RBC GSH levels positively correlate with Endo III-sensitive sites in total population, and with SSB level in white females, that heme degradation products and hs-CRP correlate positively with SSBs in African-American males, and heme degradation products correlate negatively with SSBs in African-American females. Furthermore, RBC GSH correlates with the levels of heme degradation products and hs-CRP in the total population.

This pilot study suggests some measures of oxidative damage to DNA correlate with traditional and novel markers of oxidative stress and inflammation. However, limitations of our pilot study include the small size and the narrow age-range of the cohort studied. The size of the pilot cohort precluded our ability to assess important confounders such as lifestyle, diet and other socioeconomic factors. Future work will focus on enlarging the cohort to provide the opportunity to examine relevant confounders.

The data in this pilot study clearly highlight the need for further examination to clarify the relationship as well as the assessment of the relationship between oxidant stress, inflammation, and DNA damage, and human DNA repair capacity in diverse population cohorts. Although several of the markers currently in use appear to have consensus results in population studies, others are still noticeably variable. Great strides have been made by both the ESCODD and ESCULA groups to provide methodologies and establish references values for the measurement of 8-oxodG levels. With these in place, larger population studies with more consistent results may be on the horizon. Further investigations are still needed for many of the markers of oxidant stress to establish background levels and consensus results. Once these are achieved, their use in larger studies will be possible. With additional studies, utilizing more diverse cohorts, we will be able to begin correlating and understanding the relationships between, the various markers of oxidant stress, DNA damage, and human DNA repair capacity. The verification of each of these markers is essential to our understanding of how to accurately assess the potential clinical applications of these measures or to identify other more relevant and correlative markers.

## Conflict of interest statement

None declared.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mrfmmm.2012.01.002.

## References

[1] C. Alapetite, A. Benoit, E. Moustacchi, A. Sarasin, The comet assay as a repair test for prenatal diagnosis of *Xeroderma pigmentosum* and trichothiodystrophy, J. Invest. Dermatol. 108 (1997) 154–159.

[2] T.R. Smith, M.S. Miller, K.K. Lohman, L.D. Case, J.J. Hu, DNA damage and breast cancer risk, Carcinogenesis 24 (2003) 883–889.

[3] A.R. Collins, C.M. Gedik, B. Olmedilla, S. Southon, M. Bellizzi, Oxidative DNA damage measured in human lymphocytes: large differences between sexes and between countries, and correlations with heart disease mortality rates, FASEB J. 12 (1998) 1397–1400.

[4] W.U. Muller, T. Bauch, C. Streffer, D. von Mallek, Does radiotherapy affect the outcome of the comet assay? Br. J. Radiol. 75 (2002) 608–614.

[5] F. Marcon, C. Andreoli, S. Rossi, A. Verdina, R. Galati, R. Crebelli, Assessment of individual sensitivity to ionizing radiation and DNA repair efficiency in a healthy population, Mutat. Res. 541 (2003) 1–8.

[6] M. Zana, A. Szecsenyi, A. Czibula, A. Bjelik, A. Juhasz, A. Rimanoczy, K. Szabo, A. Vetro, P. Szucs, A. Varkonyi, M. Pakaski, K. Boda, I. Rasko, Z. Janka, J. Kalman, Age-dependent oxidative stress-induced DNA damage in Down's lymphocytes, Biochem. Biophys. Res. Commun. 345 (2006) 726–733.

[7] N. Rajaee-Behbahani, P. Schmezer, A. Risch, W. Rittgen, K.W. Kayser, H. Dienemann, V. Schulz, P. Drings, S. Thiel, H. Bartsch, Altered DNA repair capacity and bleomycin sensitivity as risk markers for non-small cell lung cancer, Int. J. Cancer 95 (2001) 86–91.

[8] Q. Wei, G.M. Matanoski, E.R. Farmer, M.A. Hedayati, L. Grossman, DNA repair and aging in basal cell carcinoma: a molecular epidemiology study, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 1614–1618.

[9] N.P. Singh, D.B. Danner, R.R. Tice, L. Brant, E.L. Schneider, DNA damage and repair with age in individual human lymphocytes, Mutat. Res. 237 (1990) 123–130.

[10] A.R. Trzeciak, J. Barnes, M.K. Evans, A modified alkaline comet assay for measuring DNA repair capacity in human populations, Radiat. Res. 169 (2008) 110–121.

[11] A.R. Trzeciak, J. Barnes, N. Ejiogu, K. Foster, L.J. Brant, A.B. Zonderman, M.K. Evans, Age, sex, and race influence single-strand break repair capacity in a human population, Free Radic. Biol. Med. 45 (2008) 1631–1641.

[12] D. Harman, Aging: a theory based on free radical and radiation chemistry, J. Gerontol. 11 (1956) 298–300.

[13] M.P. Hannon-Fletcher, M.J. O'Kane, K.W. Moles, C. Weatherup, C.R. Barnett, Y.A. Barnett, Levels of peripheral blood cell DNA damage in insulin dependent diabetes mellitus human subjects, Mutat. Res. 460 (2000) 53–60.

[14] D.C. Malins, P.M. Johnson, T.M. Wheeler, E.A. Barker, N.L. Polissar, M.A. Vinson, Age-related radical-induced DNA damage is linked to prostate cancer, Cancer Res. 61 (2001) 6025–6028.

[15] M. Morocz, J. Kalman, A. Juhasz, I. Sinko, A.P. McGlynn, C.S. Downes, Z. Janka, I. Rasko, Elevated levels of oxidative DNA damage in lymphocytes from patients with Alzheimer's disease, Neurobiol. Aging 23 (2002) 47–53.

[16] F.A. Domenici, M.T. Vannucchi, A.A. Jordao Jr., M.S. Meirelles, H. Vannucchi, DNA oxidative damage in patients with dialysis treatment, Ren. Fail. 27 (2005) 689–694.

[17] A. Trzeciak, J. Kowalik, E. Malecka-Panas, J. Drzewoski, M. Wojewodzka, T. Iwanenko, J. Blasiak, Genotoxicity of chromium in human gastric mucosa cells and peripheral blood lymphocytes evaluated by the single cell gel electrophoresis (comet assay), Med. Sci. Monit. 6 (2000) 24–29.

[18] S.M. Piperakis, E.E. Visvardis, M. Sagnou, A.M. Tassiou, Effects of smoking and aging on oxidative DNA damage of human lymphocytes, Carcinogenesis 19 (1998) 695–698.

[19] J. Blasiak, A. Trzeciak, E. Malecka-Panas, J. Drzewoski, M. Wojewodzka, In vitro genotoxicity of ethanol and acetaldehyde in human lymphocytes and the gastrointestinal tract mucosa cells, Toxicol. In Vitro 14 (2000) 287–295.

[20] A.M. Knaapen, R.P. Schins, D. Polat, A. Becker, P.J. Borm, Mechanisms of neutrophil-induced DNA damage in respiratory tract epithelial cells, Mol. Cell. Biochem. 234–235 (2002) 143–151.

[21] P. Voss, W. Siems, Clinical oxidation parameters of aging, Free Radic. Res. 40 (2006) 1339–1349.

[22] A. Pastore, G. Federici, E. Bertini, F. Piemonte, Analysis of glutathione: implication in redox and detoxification, Clin. Chim. Acta 333 (2003) 19–39.

[23] E.R. Stadtman, Protein oxidation in aging and age-related diseases, Ann. N. Y. Acad. Sci. 928 (2001) 22–38.

[24] I. Dalle-Donne, R. Rossi, R. Colombo, D. Giustarini, A. Milzani, Biomarkers of oxidative damage in human disease, Clin. Chem. 52 (2006) 601–623.

[25] E. Nagababu, J.M. Rifkind, Formation of fluorescent heme degradation products during the oxidation of hemoglobin by hydrogen peroxide, Biochem. Biophys. Res. Commun. 247 (1998) 592–596.

[26] R.W. Carrell, C.C. Winterbourn, E.A. Rachmilewitz, Activated oxygen and haemolysis, Br. J. Haematol. 30 (1975) 259–264.

[27] C. Giulivi, P. Hochstein, K.J. Davies, Hydrogen peroxide production by red blood cells, Free Radic. Biol. Med. 16 (1994) 123–129.

[28] E. Nagababu, J.M. Rifkind, Heme degradation by reactive oxygen species, Antioxid. Redox Signal. 6 (2004) 967–978.

[29] P.M. Ridker, Inflammatory biomarkers and risks of myocardial infarction, stroke, diabetes, and total mortality: implications for longevity, Nutr. Rev. 65 (2007) S253–S259.

[30] M.K. Evans, J.M. Lepkowski, N.R. Powe, T. LaVeist, M.F. Kuczmarski, A.B. Zonderman, Healthy aging in neighborhoods of diversity across the life span (HANDLS): overcoming barriers to implementing a longitudinal, epidemiologic, urban study of health, race, and socioeconomic status, Ethn. Dis. 20 (2011) 267–275. Q2

[31] A.R. Collins, S.J. Duthie, V.L. Dobson, Direct enzymic detection of endogenous oxidative base damage in human lymphocyte DNA, Carcinogenesis 14 (1993) 1733–1735.

[32] A.R. Collins, M. Dusinska, C.M. Gedik, R. Stetina, Oxidative damage to DNA: do we have a reliable biomarker? Environ. Health Perspect. 104 (Suppl. 3) (1996) 465–469.

[33] T.S. Kumaravel, A.N. Jha, Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals, Mutat. Res. 605 (2006) 7–16.

[34] P.L. Olive, J.P. Banath, R.E. Durand, Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the comet assay, Radiat. Res. 122 (1990) 86–94.

[35] V. Humphreys, R.M. Martin, B. Ratcliffe, S. Duthie, S. Wood, D. Gunnell, A.R. Collins, Age-related increases in DNA repair and antioxidant protection: a comparison of the Boyd Orr Cohort of elderly subjects with a younger population sample, Age Ageing 36 (2007) 521–526.

[36] P. Moller, Assessment of reference values for DNA damage detected by the comet assay in human blood cell DNA, Mutat. Res. 612 (2006) 84–104.

[37] M. Wojewodzka, M. Kruszewski, T. Iwanenko, A.R. Collins, I. Szumiel, Application of the comet assay for monitoring DNA damage in workers exposed to chronic low-dose irradiation. I. Strand breakage, Mutat. Res. 416 (1998) 21–35.

[38] M. Dusinska, Z. Dzupinkova, L. Wsolova, V. Harrington, A.R. Collins, Possible involvement of XPA in repair of oxidative DNA damage deduced from analysis of damage, repair and genotype in a human population study, Mutagenesis 21 (2006) 205–211.

[39] M. Kruszewski, M. Wojewodzka, T. Iwanenko, A.R. Collins, I. Szumiel, Application of the comet assay for monitoring DNA damage in workers exposed to chronic low-dose irradiation. II. Base damage, Mutat. Res. 416 (1998) 37–57.

[40] J.L. Watters, J.A. Satia, L.L. Kupper, Correlates of antioxidant nutrients and oxidative DNA damage differ by race in a cross-sectional study of healthy African American and white adults, Nutr. Res. 28 (2008) 565–576.

[41] E. Nagababu, F.J. Chrest, J.M. Rifkind, Hydrogen-peroxide-induced heme degradation in red blood cells: the protective roles of catalase and glutathione peroxidase, Biochim. Biophys. Acta 1620 (2003) 211–217.

[42] M.L. Chang, L.K. Klaidman, J.D. Adams Jr., The effects of oxidative stress on in vivo brain GSH turnover in young and mature mice, Mol. Chem. Neuropathol. 30 (1997) 187–197.

[43] S. Ray, D.N. Watkins, N.L. Misso, P.J. Thompson, Oxidant stress induces gamma-glutamylcysteine synthetase and glutathione synthesis in human bronchial epithelial NCI-H292 cells, Clin. Exp. Allergy 32 (2002) 571–577.

[44] M.H. Wener, P.R. Daum, G.M. McQuillan, The influence of age, sex, and race on the upper reference limit of serum C-reactive protein concentration, J. Rheumatol. 27 (2000) 2351–2359.

[45] L. Ferder, F. Inserra, M. Martinez-Maldonado, Inflammation and the metabolic syndrome: role of angiotensin II and oxidative stress, Curr. Hypertens. Rep. 8 (2006) 191–198.

[46] R. Demirbag, R. Yilmaz, M. Gur, A. Kocyigit, H. Celik, S. Guzel, S. Selek, Lymphocyte DNA damage in patients with acute coronary syndrome and its relationship with severity of acute coronary syndrome, Mutat. Res. 578 (2005) 298–307.

[47] R. Demirbag, R. Yilmaz, M. Gur, H. Celik, S. Guzel, S. Selek, A. Kocyigit, DNA damage in metabolic syndrome and its association with antioxidative and oxidative measurements, Int. J. Clin. Pract. 60 (2006) 1187–1193.

[48] M. Gur, A. Yildiz, R. Demirbag, R. Yilmaz, A. Kocyigit, H. Celik, N. Aksoy, Increased lymphocyte deoxyribonucleic acid damage in patients with cardiac syndrome X, Mutat. Res. 617 (2007) 8–15.

[49] S. Haghdoost, Y. Maruyama, R. Pecoits-Filho, O. Heimburger, A. Seeberger, B. Anderstam, M.E. Suliman, S. Czene, B. Lindholm, P. Stenvinkel, M. Harms-Ringdahl, Elevated serum 8-oxo-dG in hemodialysis patients: a marker of systemic inflammation? Antioxid. Redox Signal. 8 (2006) 2169–2173.

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