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Age-associated analysis of oxidative stress parameters in human plasma and erythrocytes

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Abstract

Oxidative damage accumulation in macromolecules has been considered as a cause of cellular damage and pathology. Rarely, the oxidative stress parameters in healthy humans related to the individual age have been reported. The purpose of this study was to examine the redox status in plasma and erythrocytes of healthy individuals and determine correlations between these parameters and the aging process. The following parameters were used: malondialdehyde (MDA), protein carbonyls (PCO), 4-hydroxy-2,3-*trans*-nonenal (HNE), reduced glutathione (GSH), glutathione disulfide (GSSG) and uric acid (UA) in blood and plasma samples of 194 healthy women and men of ages ranging from 18 to 84 years. The results indicate that the balance of oxidant and antioxidant systems in plasma shifts in favor of accelerated oxidation during ageing. That is demonstrated by increases of MDA, HNE, GSSG and by the slight decrease of erythrocytic GSH with age. As the content of UA is more determined by metabolic and nutritional influences than by the balance between prooxidants and antioxidants there was no significant age-related change observed. For plasma concentrations of HNE the first time age-dependent reference values for healthy humans are presented.

Keywords: *Ageing, lipid peroxidation, 4-hydroxynonenal (HNE), malondialdehyde (MDA), protein carbonyls, glutathione*

Introduction

The ageing process includes the accumulation of changes with time and a decline of the organism response to these changes. A general feature of the ageing or senescence process is a progressive, physiological deterioration with time leading to an impairment of the homeostasis, vulnerability to diseases and ultimately to death of the organism [1]. There are numerous hypotheses trying to explain the aging process, considering the complex physiological alteration in the organisms including: mitochondrial changes, accumulation of aberrant proteins in the cytosol, chemical damage to macromolecules, and somatic mutations. No one of these theories has been generally accepted [2]. The oxidative stress hypothesis offers the best mechanistic elucidation of the ageing process and other age-related phenomena such as age-related diseases [3–7].

Aerobic cells through diverse metabolic pathways produce ROS constantly. ROS are able to inflict

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molecular, oxidative damage to lipids, proteins and DNA when their production overwhelms the capacity of antioxidant systems [8–10]. The efficiency of antioxidative and repair mechanism cannot avoid completely ROS reactions with lipids, proteins and nucleic acids. Therefore, cells also under normal physiological conditions manifest a certain level of oxidative damage [11–13].

Changes in oxidative damage and antioxidant capacity during ageing have been shown in several tissues in different species [14–19]. Different methods and parameters have been used producing a variety of sometimes contradictory data [20,21]. A large amount of data about oxidative stress implications in tissues damage, diseases, biological variables and life habits has been shown [22–26] but reports on changes of oxidative stress markers in blood and blood plasma of healthy populations during ageing are very rare. There exist reports on human glutathione (GSH) and glutathione disulfide (GSSG) values in 122 participants by Jones et al. [27]. In 2002, data of Turkish [28,29] and in 2004 data from Brazil [30] healthy populations have been published. These investigations include values of plasma malondialdehyde (MDA), blood GSH and blood enzyme activities, such as of superoxide dismutase and glutathione peroxidase. In those investigations [27–30], tendencies for some parameters and also significant correlations were established showing an increase of prooxidative capacities and a decrease of antioxidative capacities during aging. Further studies which were carried out previously present conflicting and contradictory results, e.g. concerning total antioxidative capacity of human blood plasma which increased in one study, but decreased with age in another study [31,32].

Consequently, the aim of this study was to determine a pattern of important prooxidative and antioxidative parameters in human plasma and red blood cells in healthy subjects of different ages. MDA and 4-hydroxynonenal (HNE) are major aldehydic products of lipid peroxidation, which can react very rapidly with different groups of biomolecules to amplify in that way the primary ROS reactions. Protein carbonyl (PCO) determination is reflecting the protein oxidation process. Uric acid (UA) as an important hydrophilic radical scavenger is present in high concentration both intra- and extra-cellular. MDA, HNE, PCO and UA were all measured in plasma.

GSH in its reduced form is accepted to be the most important representative of intra-cellular low molecular antioxidant. As in blood reduced GSH is preferentially located within the red blood cells while the concentration is low in extracellular fluids and the oxidized form is distributed equal within and without the cells we measured the GSH concentrations in whole blood. Since the total redox capacity of the plasma is important and not only the absolute amount of reduced GSH, we calculated the percentage of oxidized GSSG to the total amount of glutathione (GSH + 2 GSSG). Therefore, the six parameters were selected as they are easy to measure and they reflect different targets of oxidative damage like lipid peroxidation, protein damage and the antioxidative capacity at all.

Materials and methods

Subjects and blood collection

The study population included 194 apparently healthy individuals of different ages, 77 women and 117 men. The patients were recruited from two departments, the Department of Physical Medicine and Rehabilitation (Bad Harzburg) and from the Tinnitus Center of the Department of Otorhinolaryngology at Medical Faculty (Charité) of the Humboldt University Berlin during the period 1998–2005. The test persons recruited underwent a special physicians investigation. Any acute or chronic disease or treatment leads to the exclusion from this study. None of them showed any physical or mental abnormalities at the time of physical examination. The blood and blood plasma of these patients, therefore, could be called as blood or blood plasma of healthy control persons. All subjects gave informed consent. The selection excluded not only individuals with acute infections or chronic diseases, but excluded also healthy individuals undergoing supplementation with antioxidative substances.

For the age-dependent investigation (Table II) the subjects were divided into six groups: group 1 ($n = 35$), 18–29 years of age, group 2 ($n = 28$), 30–39 years of age, group 3 ($n = 31$), 40–49 years of age, group 4 ($n = 35$), 50–59 years of age, group 5 ($n = 48$), 60–69 years of age and group 6 ($n = 17$), 71–84 years of age. As there were no significant differences between both genders within each the single groups for any of the parameters we divided them for the gender dependent relations (Table III) only into two age groups, below and over 50 years of age in order to reflect the postmenopausal influences in the female group. Therefore, it was possible to compare the investigated biomarkers between the genders of the same age and between the younger and older group of the same gender. Blood samples were collected into heparinized tubes. GSH and GSSG were measured in whole blood. As GSH is mainly abundant in the intra-cellular compartment while GSSG is distributed in the intra- and extra-cellular compartment, the values are corrected by hematocrite. For plasma collection, the samples were immediately after drawing centrifuged at 1500*g* for 5 min, the plasma was separated and stored at −80°C until the analyses were carried out.

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Table I. Pearson correlation coefficients of oxidative stress parameters.

	MDA ($\mu mol \times l^{-1}$)	HNE ($nmol \times l^{-1}$)	GSH ($mmol \times l^{-1}$)	GSSG ($\mu mol \times l^{-1}$)	2 GSSG/ (GSH+2 GSSG)(%)
MDA ($\mu mol \times mg$ $protein^{-1}$)					
HNE ($nmol \times l^{-1}$)	0.518 (<0.001)				
GSH ($mol \times l^{-1}$)	0.351 (<0.001)	0.508 (<0.001)			
GSSG ($\mu mol \times l^{-1}$)	0.187 (<0.001)	0.174 (<0.001)	0.106 (<0.001)		
2 GSSG/(GSH + 2 GSSG) (%)	0.326 (<0.001)	0.384 (<0.001)	0.401 (<0.001)	0.384 (<0.001)	
UA ($\mu mol \times l^{-1}$)	0.067 (0.003)	0.015 (0.182)	0.120 (<0.001)	0.039 (0.03)	0.004 (0.502)

The numbers are the correlation coefficients and the numbers in parentheses are the two sided *p*-values. MDA, malondialdehyde; HNE, hydroxynonenal; GSH, reduced glutathione; GSSG, glutathione disulfide; UA, uric acid.

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Biochemical measurements

4-Hydroxynonenal measurement. The measurement of HNE was performed according to Esterbauer et al. [33] with modifications of Grune et al. [34]. A mixture of 2.5 ml of plasma reacted for 2 h in the dark with 2.5 ml of dinitrophenylhydrazine (DNPH) solution (1.8 mM in 1 M HCl) at room temperature. The organic phase was extracted with dichloromethane before evaporating it to dryness. The residue was redissolved with 1.5 ml of dichloromethane and spotted on TLC plates, which were developed with dichloromethane. The zone containing the 4-hydroxyalkenals was scraped off, extracted with methanol, and evaporated to dryness. The residue was dissolved in 1 ml of methanol. Methanol/water (4:1, v:v) was used as eluent for the isocratic HPLC-analysis. The detection wavelength was 378 nm. Peak detection was performed by comparison of the retention time of peaks of biological extracts with the reference compounds, by comparison of the spectra, and by spiking. The HNE standard was produced from the diacetale, which was stored in a $CHCl_3$ solution at −20°C. The reproducibility of this method was ±8% and the median recovery was 33%.

Malondialdehyde measurement. MDA was determined according to Wong et al. [35] with modifications of Sommerburg et al. [36] as the thiobarbituric acid (TBA) derivative. Phosphoric acid (440 mM), sample or MDA standard, and TBA solution (42 mM) were incubated at 100°C for 60 min and then the samples and standards were cooled on ice. To neutralize the phosphoric acid and to precipitate the proteins before the sample was injected into the HPLC system, the samples and standards were diluted 1:1 (v:v) with NaOH (0.1 M) in methanol. Afterwards, all samples were centrifuged at 10,000*g* for 2 min. Aliquots of the derivatized samples were injected into the reversed phase HPLC and separated by isocratic elution with phosphatous buffer (50 mM, pH 6.8) containing 40% (v/v) methanol. TBA–MDA complex was detected by means of fluorescence using an excitation wavelength of 525 nm and emission of 550 nm.

Reduced glutathione and oxidized glutathione determination. For analysis of GSH and GSSG concentrations in red blood cells, plasma was removed and cells were diluted to equal volumes by 0.9% NaCl solution. Samples were drawn with ice-cold metaphosphoric acid and kept at 4°C. Samples were centrifuged for 10 min at 1200*g*. Supernatants were collected and separated into two aliquots for the GSH and GSSG measurements [37]. GSH was assayed by means of Ellman's reagent (DTNB) [38]. GSSG was determined fluorimetrically after addition of *o*-phthaldialdehyde [39]. GSH autoxidation was prevented by addition of 50 mM *N*-ethylmaleimide (NEM). GSH and GSSG were recalculated, by means of the hematocrite value, as intraerythrocytic concentrations. The half-cell reduction potential (E_{hc}) was calculated from the GSH and GSSG concentrations according to the method of Schafer and Buettner [40].

Uric acid quantification. UA was determined as described by Bergmeyer [41]. An enzymatic test using spectrophotometric detection with absorption maximum at 293 nm was carried out.

Protein carbonyl determination. For determination of PCO groups, the method of Buss et al. [42] with modifications of Sitte et al. [43] was used. After determination of the protein concentration, the samples were diluted to the same concentration of protein (1 mg/ml) and derivatized with DNPH solution. Sample loading and washing of ELISA plates was performed as described by Buss et al. [42]. Development was performed using a detection system described by Sitte et al. [43]. The absorbance of the derivatized samples at 492 was measured and the

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Table II. The effect of age on MDA, HNE, PCO, GSH, GSSG and UA concentrations in healthy subjects.

Age (years)	Gender (% male)	MDA ($\mu mol \times l^{-1}$)	PCO (nmol × mg Protein^{-1})	HNE (nmol × l^{-1})	GSH (mmol × l^{-1})	GSSG ($\mu mol \times l^{-1}$)	2 GSSG/ (GSH+2 GSSG) (%)	E_{hc} (mV)	UA ($\mu mol \times l^{-1}$)
Group 1 18–29 ($n = 35$)	60	0.44 ± 0.26	0.012 ± 0.003	68.9 ± 15.0	2.65 ± 0.30	44.9 ± 17.7	3.3 ± 1.4	− 315 ± 8	276 ± 34
Group 2 30–39 ($n = 28$)	46	0.45 ± 0.17	0.009 ± 0.001	70.3 ± 13.9	2.72 ± 0.28	51.2 ± 26.3	3.7 ± 1.7	− 314 ± 8	288 ± 40
Group 3 40–49 ($n = 31$)	68	0.51 ± 0.23	0.009 ± 0.001	72.0 ± 20.0	2.56 ± 0.35	51.1 ± 23.4	4.0 ± 1.5	− 312 ± 7	297 ± 57
Group 4 50–59 ($n = 35$)	54	0.62 ± 0.30†	0.013 ± 0.004	83.0 ± 20.2*,†	2.46 ± 0.39†	56.4 ± 17.4*	4.6 ± 1.9*	− 309 ± 7*,†	301 ± 50
Group 5 60–69 ($n = 48$)	58	0.89 ± 0.50*,†,‡,¶	0.012 ± 0.005	96.3 ± 21.6*,†,‡	2.38 ± 0.40*,†	57.2 ± 14.7*	4.7 ± 1.5*	− 308 ± 7*,†	292 ± 40
Group 6 70–84 ($n = 17$)	53	0.69 ± 0.24*,†,‡	0.009 ± 0.001	107.4 ± 27.3*,†,‡,¶,§	2.37 ± 0.62*,†	68.5 ± 9.4*,†,‡,¶,§	5.8 ± 1.5*,†,‡	− 305 ± 7*,†,‡	280 ± 64

MDA, malondialdehyde; HNE, hydroxynonenal; PCO, protein carbonyls; GSH, reduced glutathione; GSSG, glutathione disulfide; E_{hc}, redox potential; UA, uric acid. The values represent the mean ± SD. $^{\star}p < 0.05$ in comparison with the group 1. $^{\dagger}p < 0.05$ in comparison with the group 2. $^{\ddagger}p < 0.05$ in comparison with the group 3. $^{\P}p < 0.05$ in comparison with the group 4. $^{\S}p < 0.05$ in comparison with the group 5.

concentration of protein bound carbonyls was calculated by standards of oxidized bovine serum albumin (BSA). Blanks of PBS without protein were subtracted from standards and samples absorbencies. Oxidized BSA was prepared by modifying solved BSA with hypochlorite. The carbonyl content of the oxidized BSA was determined according to Buss et al. [42]. Reduced BSA was obtained as described by Buss et al. [42].

Statistics

Measured values were tested for normal distribution and statistical analyses were done using unpaired *t*-test (two-tailed) in the comparison of groups' means according to age and gender. In those cases, where the values were not normal distributed the Mann–Whitney rank sum test was used. Additional the Pearson correlation was used. The values were expressed as mean ± SD. Statistical significance was set at $p < 0.05$ [44,45].

Results

Test for normal distribution

In order to perform the statistical analysis of all measurements, we tested the data using the Kolmogorov–Smirnov test for normality to decide which statistic methods have to be used for the further analysis. Since not all data were normally distributed we used Mann–Whitney rank sum test for statistical analysis. The histograms of frequency distributions are shown in Figure 1. These data represent the range of the distribution of the measured values of individuals independent of the age. As shown also in a normal, healthy population the distribution of the levels of different parameters of oxidative stress is quite wide. It seems to be interesting to compare the correlation of these parameters with each other, in order to suggest that the "oxidative status" of an individual person is really reflected by the individual parameters.

Influence of age

Our main goal of this study was the determination of a possible age-dependency of the oxidative stress parameters. In Figure 2, the determined oxidative stress parameters are shown in dependence on the age of the blood donor. The results from our study show clearly an increase in oxidative stress during the aging process is taking place as measured by MDA, HNE, PCO and GSSG. The antioxidative capacity is decreasing during the aging process if measured by the GSH level. No age related tendency was measurable for the UA levels. To determine the anti-oxidative capacity of the GSH system the absolute

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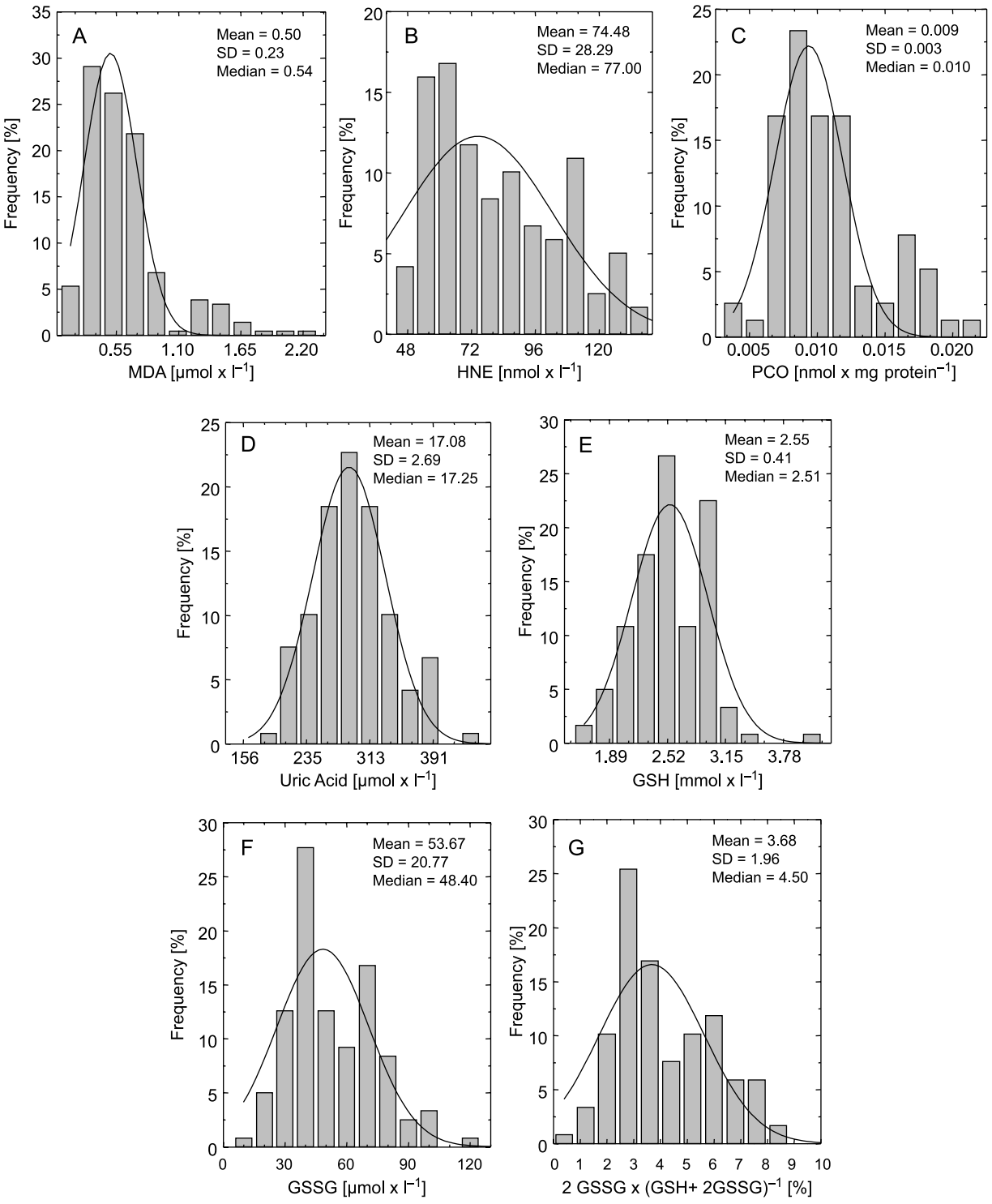

Figure 1. Histograms of frequency distribution. The panels demonstrate the histograms of frequency distribution of measured biomarkers indicated in the legend of the *x*-axis. The superimposed curve represents a normal distribution for the mean and standard deviation of the data. In each panel, the mean, the standard deviation (SD) as well as the median are indicated. MDA, malondialdehyde; HNE, hydroxynonenal; PCO, protein carbonyls; GSH, reduced glutathione; GSSG, glutathione disulfide.

concentrations of these compounds might be of lesser relevance, but the GSH ratio, calculated as the percentage ratio of oxidized GSH to the total GSH [$2GSSG \times (GSH + 2\,GSSG)^{-1}$], seems to be important. Therefore, we calculated this value and correlated it to the age (Figure 3A). The GSH-, GSSG-values as well as the ratio thereof are in accordance with literature data [46,47]. Clearly, the GSH ratio is age dependent with an increasing level over the age of the individual. In further studies it was tested whether

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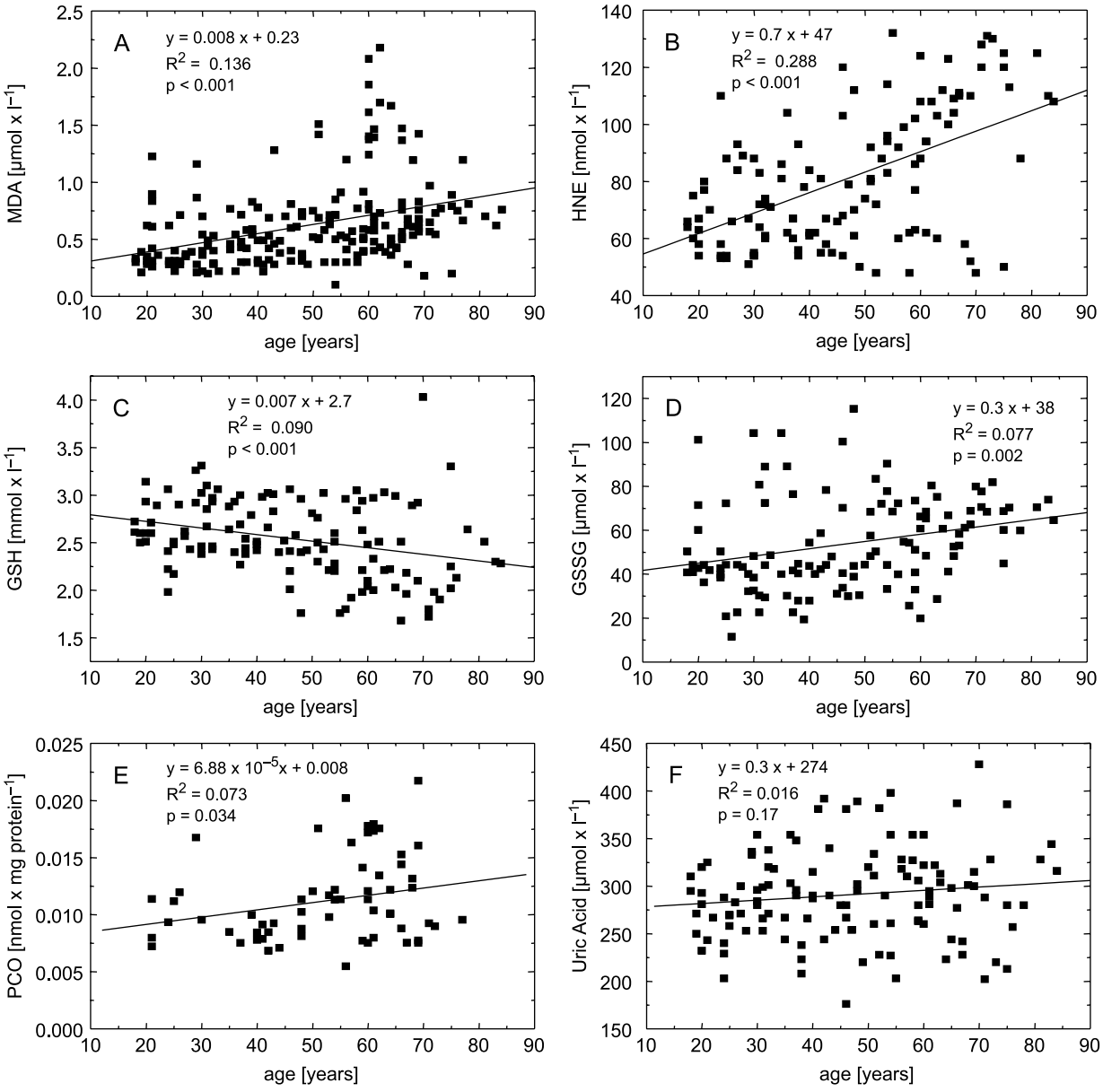

Figure 2. Pearson correlation of the measured biomarkers in relation to age. Each dot reflects the measurement of an individual person of the parameter indicated in the *y*-axis. The Pearson correlations between the parameter in relation to age are shown. Within the panels, the equation of the linear fit, the correlation coefficient and the *p*-value are indicated. Abbreviations as listed in the legend to Figure 1.

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GSH ratio is correlated to the components of the GSH system (GSH and GSSG). As expected, there is a highly significant negative correlation with the reduced GSH, whereas, the correlation with the oxidized GSH is positive (Figure 3C and D). Further, we calculated the E_{hc} for the GSH/GSSG-balance on the basis of our values according to the method of Schafer and Buettner [40] (Figure 3B). The values ranking from −320 to −280 mV fit quite well with the literature data. The redox potential is declining with age like it could be seen in the increasing percentage of GSSG (Figure 3A and B).

On the other hand, it seems to be interesting whether the GSH ratio is correlating also with other parameters of oxidative damage. Therefore, we demonstrated in Figure 3E and F the correlation with the lipid peroxidation products MDA and HNE. Highly significant positive correlations could be demonstrated. This raises the question whether all oxidative stress parameters are correlating significantly. In Table I, the calculated Pearson correlation coefficients are shown. As demonstrated in Table I most of the parameters of oxidative stress are correlated with the exception, perhaps, of UA. In Figure 4, we demonstrated the correlation of HNE and MDA; MDA and GSH, and finally also HNE and GSH. These correlations have the highest correlation coefficient of the directly measured parameters. If the

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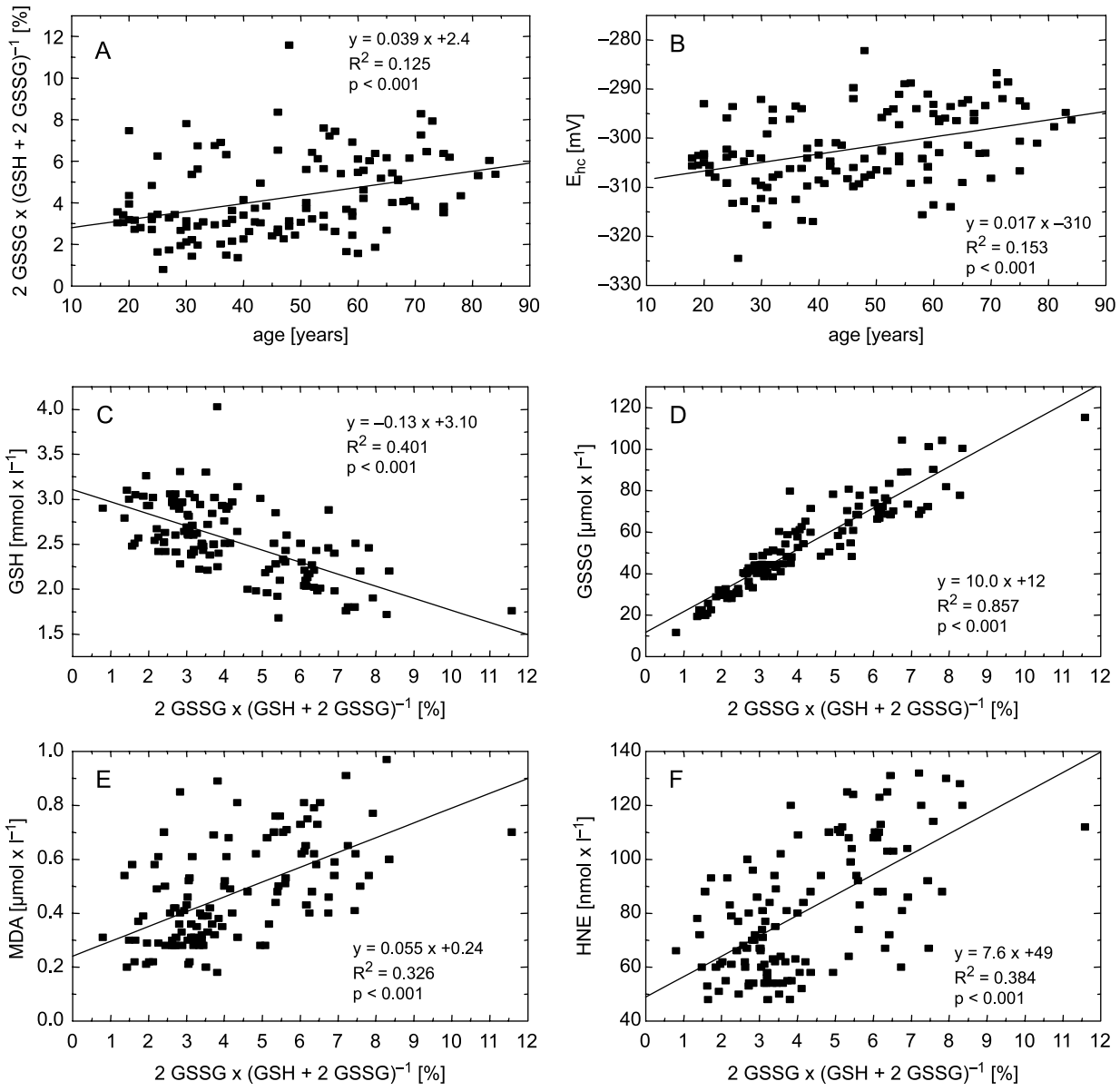

Figure 3. Correlations of the glutathione ratio. The Pearson correlation of the relation of the glutathione ratio [2GSSG × (GSH + 2GSSG)$^{-1}$] with age is shown in Panel A. Panel B shows the calculated redox potenials E_{hc} according to Schafer and Buettner [40]. Panels C and D show the correlation of the reduced (GSH) and the oxidized glutathione (GSSG) with the glutathione ratio. Panels E and F demonstrate the dependence of the lipid peroxidation products HNE and MDA from glutathione ratio. Within the panels are the equation of the linear fit, the correlation coefficient and the *p*-value indicated. Abbreviations as listed in the legend to Figure 1.

level of reduced GSH is high, the oxidative stress is low and the level of lipid peroxidation is low, but with age the level of GSH is falling down and this is seen as raising amounts of lipid peroxidation products HNE and MDA.

In order to test whether the age-dependent change of oxidation parameters takes place gradually or seems to be a continuous process, we divided the tested group of human individuals into decades of live and tested whether there are differences between individual groups. As shown in Table II, the most significant differences to the 20–30 year old people start at the age of 50–60. Rarely, there is a significant increase in the oxidation parameters from one decade to the following.

Influence of age and gender

Since, the most dramatic changes are occurring in the age over 50 this raises the question whether the well-known postmenopausal changes in the antioxidative capacity of woman have any influence. Therefore, we decided to test whether the gender has any influence of the analyzed stress parameters. The influence of the gender on the biomarkers is shown in Table III. Only HNE, GSH and UA gave significant different values

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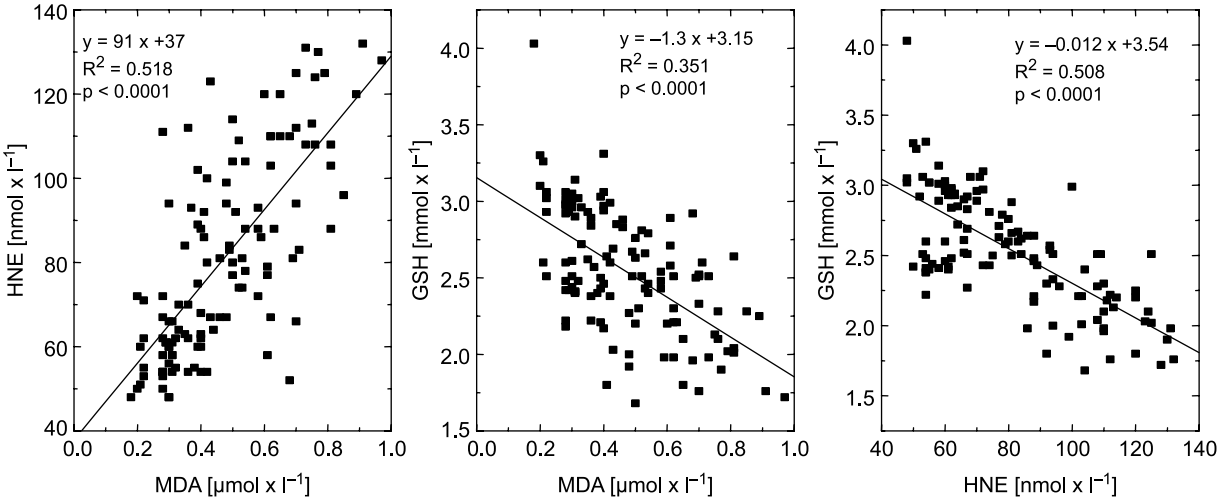

Figure 4. Correlations between HNE, MDA and GSH. The Pearson correlations between HNE, MDA and GSH are shown. Within the panels the equations of the linear fit, the correlation coefficient and the *p*-value are indicated. Abbreviations as listed in the legend to Figure 1.

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for men and women. The fact that male have a higher content of GSH is in accordance with the fact that they have lower HNE levels. To elucidate the influence of the menopause on the differences between the genders we divided the individuals into two groups. Group 1 consists of persons that were under 50 years and the other group consists out of the persons over 50 years of age. Except for UA and PCO, all values of group 2 were significantly different from those of group 1. As already seen in Tables I and II and Figure 2 higher levels of oxidation products are found in the older group while the antioxidative status is going down. This is demonstrated by significantly raised levels of MDA, HNE and GSSG and lowered amounts of GSH in group 2. Within group 1 only the GSH and UA levels were different between the genders, whereas, in group 2 significantly different values between the genders could be detected by measuring HNE, GSH and UA. The HNE value is going up with age for both man and women but for the female the increase is much stronger than for man, resulting in a significant higher level of this oxidation product in females.

Discussion

Since the share of elderly people within the population is constantly rising, it is important to investigate and understand the inevitable physiological changes accompanying the aging process [48]. The functional consequences of the age-related changes in the metabolism which are connected with physiological deterioration of defense systems have been by far incomplete studied and remain, therefore, largely unknown [49]. One of these changes might be located within the oxidative stress status and is frequently evaluated by several biochemical parameters [9,50]. There are some reports on detailed aspects of age-related changes of oxidative stress parameters in animals and some aspects of such changes in humans [14,51,52]. Unfortunately, there exist only a limited number of studies showing age-related changes of oxidative stress related parameters in a healthy population [27–32]. Taking into account the importance of ROS-initiated reactions in so many age-related diseases it seems necessary to establish age-dependent reference values of the most prominent parameters of oxidative stress. Lipid peroxidation represents an important part of ROS-initiated reactions. Among others, it can be characterized by the measurements of major aldehydic lipid peroxidation products MDA and HNE [18]. The PCO concentration is a useful, easy to perform and representative parameter for evaluation of protein oxidation processes [53–55]. As representative parts of the antioxidative cascade, which can be easily measured in blood and blood plasma we selected GSH, with its reduced (GSH) and oxidized (GSSG, glutathione disulfide) form, and UA. Reduced GSH is accepted as the most important intra-cellular hydrophilic antioxidant [14]. The age-dependent decline in antioxidant capacity measured as decline in GSH concentration is in accordance with the decline of the albumin concentration, which is the major thiol source in the plasma. Due to the high concentrations of UA in the human serum it is an important non-enzymatically acting antioxidant [29].

To our knowledge, serum HNE concentrations of healthy people were not published previously so this article is the first analysis measuring HNE concentrations showing a significant increase with ageing. In contrast to MDA and UA, HNE mean values increase from decennium to decennium to a top in the 8th decennium with 0.1 μM or higher. Also, the level of MDA increased with the age of the test person. Such

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Table III. The effect of age on MDA, HNE, PCO, GSH, GSSG and UA concentrations according to gender in healthy subjects.

Age (years)	MDA ($\mu mol \times l^{-1}$)	PCO (nmol × mg Protein^{-1})	HNE ($nmol \times l^{-1}$)	GSH ($mmol \times l^{-1}$)	GSSG ($\mu mol \times l^{-1}$)	2 GSSG/(GSH+2 GSSG) (%)	E_{hc}(mV)	UA ($\mu mol \times l^{-1}$)
Female ($n = 77$)	0.65 ± 0.41	0.011 ± 0.003	86.1 ± 23.6	2.39 ± 0.32	50.9 ± 20.8	4.2 ± 1.9	−311 ± 9	263 ± 43
Male ($n = 117$)	0.60 ± 0.34	0.011 ± 0.004	76.8 ± 22.8*	2.65 ± 0.43*	55.7 ± 20.7	4.2 ± 2.0	−311 ± 8	310 ± 41*
Group 1 18–49								
Female ($n = 39$)	0.45 ± 0.17	0.009 ± 0.001	72.9 ± 18.2	2.52 ± 0.24	46.2 ± 22.4	3.6 ± 1.8	−314 ± 8	259 ± 40
Male ($n = 56$)	0.48 ± 0.26	0.010 ± 0.003	68.2 ± 14.3	2.75 ± 0.34*	51.0 ± 23.6	3.7 ± 2.1	−314 ± 7	306 ± 37*
Group 2 50–84								
Female ($n = 38$)	0.86 ± 0.47†	0.013 ± 0.003	104.3 ± 17.3†	2.22 ± 0.33†	57.4 ± 16.6†	5.1 ± 1.8†	−306 ± 7†	267 ± 47
Male ($n = 61$)	0.70 ± 0.38†	0.012 ± 0.005	86.8 ± 26.7*,†	2.53 ± 0.51*,†	61.1 ± 15.4†	4.8 ± 1.6†	−309 ± 7†	316 ± 45*

MDA, malondialdehyde; HNE, hydroxynonenal; PCO, protein carbonyls; GSH, reduced glutathione; GSSG, glutathione disulfide; E_{hc}, redox potential; UA, uric acid. The values are mean ± SD.
*$p < 0.05$ male persons vs female persons of the same age group. †$p < 0.05$ group 1 vs group 2 of the same gender.

changes are confirming earlier results showing also a significant positive correlation between MDA plasma level and age [28,30]. Age-related significant changes in the PCO concentration were already reported earlier [53,55,56]. Largely these changes are referring to changes in tissues like heart, muscle or brain. However, in our study, we could demonstrate only a minor age-related increase in PCO concentration in blood plasma. Oxidized proteins in tissues very often reflect the accumulated, non-degradable cross-linked protein material accumulating during lifetime [56,57]. On the other hand, newly synthesized plasma proteins are turned over and PCO formed in this compartment reflect only the oxidative stress occurring within this compartment. This is in clear contrast to the lipid peroxidation products diffusing from the tissue into the plasma and contributing to the level measured in clinical trials. This might be a reason for finding only a weak age-dependency for PCO. On the other hand, we were able to find a slow, but significant decline of the intraerythrocytic GSH concentration with ageing. This last observation is in accordance with the report of Erden-Inal et al. [29].

Other authors reported an augmentation of UA levels with age in human plasma [58,59]. Since the UA levels contribute to the antioxidant capacity of plasma this might be important. On the other hand, it is well known that UA concentrations are largely influenced by nutrition and, therefore, oxidative stress might have a minor influence. This results in no-significant age related change of UA plasma levels.

In the study, we demonstrated significant correlations between oxidative stress parameters. On the background of the correlation of parameters of the GSH system and of lipid peroxidation parameters it is clearly important to note that in a healthy human population these parameters are highly correlative.

We could confirm in this study earlier results demonstrating that UA serum concentrations are higher in a male population than in females [58]. Other parameters, like GSH and HNE, were also significantly different between genders. The high GSH levels and the lower HNE concentrations in the male group in comparison with the female group might be the result of the protective hydroxyl radical scavenging action by increased UA. This observation is in accordance with a previous study that found significant difference in GSH values between genders in a population of age media of 42 years [60]. The gender related significant differences found in our study are in contrast to other studies in healthy individuals [28,29,61] finding no-significant gender specific differences or even protective effects in female due to the up-regulation of some antioxidative active enzymes like manganese-superoxide dismutase and glutathione peroxidase by oestrogen [62,63]. Interestingly in the population of the over 50 year old persons this situation is much more pronounced, since

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more parameters are significantly different between genders. In the female population older than 50 the oxidative stress growth worse in comparison to the age-matched male group. Recent work from Block et al. [64] evaluated plasma MDA in healthy people using commercial kit (Oxis International Inc., Portland, OR). This author found that MDA values are strongly related to gender as women have significantly higher MDA than man. In our study, only women older than 50 years did show higher MDA levels compared to the men but this difference was not significant. The significant higher levels of oxidation products in the females of the elderly group are in contrast to the investigations of Vina et al. [63] who reported that different oestrogen levels are responsible for a higher radical production in males than in females [62,63].

In this study, we were able to demonstrate significant age related changes of important biomarkers of oxidative stress in human plasma. The increasing oxidative stress during aging is reflected in blood plasma of healthy persons by several parameters. This conclusion does not only underline the free radical hypothesis of aging, but is also methodologically important for the evaluation of further clinical studies with regard to basic "normal" values and with regard to the usage of age matched healthy control groups.

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