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Age-Related Influence on Thiol, Disulfide, and Protein-Mixed Disulfide Levels in Human Plasma

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In this study, plasma levels of both low-molecular-mass sulfhydryls/disulfides and mixed disulfides with proteins in 41 healthy humans aged 21–92 years were measured, with the aim of assessing whether there is a shift of the thiol/disulfide balance during aging and verifying some of the possible effects of the thiol imbalance. Our data suggest that aging is strictly correlated to a decrease in plasma glutathione and cysteinylglycine with the concomitant increase of most oxidized forms of thiols and a parallel increase in total cysteine and total homocysteine, probably due to an augmented efflux of these amino acids from various organs. The occurrence of two distinct regulatory systems for plasmatic pools of glutathione/cysteinylglycine on the one hand and cysteine/homocysteine on the other hand is hypothesized.

THE mechanisms responsible for the onset of senescence are still unknown. Many theories have been advanced to account for the aging process, among which the ''free radical theory,'' which proposes that the increase in oxidative stress with aging is the primary cause of age-related declines in cellular function, has received the most attention [reviewed in (1)]. However, relevant indications able to clarify whether oxidative stress has an important role in the aging process or is simply a consequence of it are still lacking.

Much support has been obtained for the presence of oxidative/nitrosative stress in aging (2–5). Aging tissue shows a progressive accumulation of oxidatively modified biomolecules such as carbonylated proteins (6), advanced glycation end products (7), lipid peroxidation products (8), and lipofuscin (9). As a consequence, the progressive accumulation of damaged biomolecules could be a factor in the adverse physiological modifications occurring with aging. Additionally, some clinical studies on humans have revealed an age-related shift of plasma thiol/disulfide redox couples (10,11), which could be a contributing factor to the pathogenesis of some age-related diseases including cardiovascular diseases, diabetes, rheumatoid arthritis, and neurological disorders (10,12–15). The cause of the age-related shift of the thiol/disulfide balance is poorly understood; it could be simply due to a higher reactive oxygen and/or nitrogen species burden in the hematic compartment. Alternatively, a decreased supply of the reduced forms through diet may be hypothesized.

Cysteine (Cys), homocysteine (Hcys), glutathione (GSH), and cysteinylglycine (CysGly) are the most abundant low-molecular-mass sulfhydryls (LMM-SH) occurring in the extracellular milieu and, together with albumin, represent almost all the thiols in plasma. All these molecules are metabolically interrelated and are important in determining the redox environment and free radical interactions (16,17). The turnover of plasma thiols is complex in that multiple organs may contribute to various extents to their delivery and uptake. In particular, GSH seems to be released mainly by the liver and, to a minor extent, by muscle, where dietary Cys (or Cys derived from methionine, via transsulfuration pathway [TSP]) is packaged into the tripeptide (18,19). Plasmatic GSH is hydrolyzed at tissue sites rich in the ectoenzyme γ–glutamyltranspeptidase (γGT) and dipeptidases (mainly kidney and lung) to CysGly and Cys, respectively, which may be taken up locally and/or mixed in the plasma pool (20). Usually, extracellular GSH is cleaved by γGT into CysGly, which is immediately taken up locally after its scission into the two amino acids by the action of membrane dipeptidases. However, it is not well established if and to what extent some amount of CysGly may escape the action of dipeptidases and thereby remain in the plasma (20,21). Cys is unstable and toxic at high concentration; however, cells and tissues have an absolute requirement for it, and plasma GSH seems to be a critical source for maintaining steady Cys availability. Cys may also derive from intracellular stores of various tissues (e.g., skeletal muscle during starvation) that can deliver it to the plasma; the other main source is diet (19). Methionine can also serve as a source of Cys because it can be converted to this amino acid through the TSP. Hcys does not derive directly from GSH but is an intermediate in the transformation of methionine into Cys, and is probably delivered by all those cells capable of performing the TSP reactions (22). (For a summary, see Figure 1).

Human plasma contains GSH, Cys, CysGly, and Hcys in the 0.1- to 20-μM range (16). These thiols can also be found in disulfide forms, both as low-molecular-mass disulfides (LMM-SS) and as protein/LMM-SH mixed disulfides (Table 1). Usually, disulfide forms (with the exception of glutathione disulfide [GSSG]) are more concentrated than the respective thiol. Considering the total concentration for each thiol species (i.e., reduced + disulfide forms),

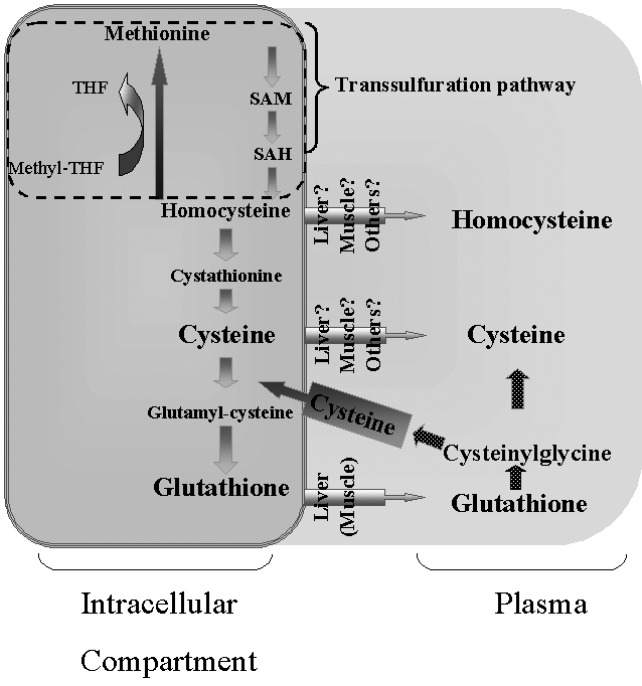

Figure 1. Schematic representation of the main biochemical pathways that regulate the production and the intra-/extracellular transfer of low-molecular-mass sulfhydryls (LMM-SHs). THF, tetrahydrofolate; SAM, *S*-adenosylmethionine; SAH, *S*-adenosyl-homocysteine.

Cys is usually present at the highest concentration, with Cys > CysGly > Hcys > GSH (13,16). Additionally, the ratio Cys/cystine is significantly shifted to the oxidized species with respect to the ratio GSH/GSSG, suggesting that these may represent two pools with distinct regulation and significance (17).

The attempt to increase LMM-SH availability in plasma by treatment with thiol compounds such as *N*-acetylcysteine (NAC), to hamper age-related damages and increase life span, has been carried out in various experimental models (10,23). Nevertheless, apart from a few promising results, most of these studies have been inconclusive (24). A number of studies have looked at GSH and GSSG levels and Cys/cystine ratio modulation during aging [for a recent review, see (11)]; however, a complete study on all plasma thiols and their disulfides including low-molecular-mass protein-mixed disulfides in human plasma during aging is lacking.

In this study we measured plasma levels of both low-molecular-mass sulfhydryls/disulfides and protein/low-molecular-mass thiol mixed disulfides in healthy persons at different ages, with the aim of exploiting plasma thiols as biomarkers of oxidative stress and evaluating whether an increase in disulfides and a decrease in thiols take place during the aging process. The possibility that the regulation of the delivery of the different LMM-SH by tissues to plasma is impaired with age is also discussed.

Methods

Study Participants

The study group comprised 41 consenting individuals (17 men, 24 women; age range: 21–92 years). All of the participants reported that they were in good health, and none of them had any abnormality on physical examination or in routine laboratory blood tests (serum glutamic oxaloacetic transaminase [SGOT], serum glutamic pyruvate transaminase [SGPT], γGT, uremia, creatininemia, erythrocyte sedimentation rate (ESR), blood cell number, platelet number, mean cellular volume (MCV), hematocrit, hemoglobin concentration, plasma protein concentration, albumin concentration). In particular, none of the participants had any alterations in renal function (evaluated by the serum levels of creatinine and urea levels) or in hepatic function (evaluated by activity of γGT and transaminases). All participants declared not to have taken any medicine in the last 15 days, and gave their informed consent.

Plasma Thiol Measurements

Blood samples were drawn from the antecubital vein in the morning (7:30–8:30 AM) after 12 ± 2 hours of fasting, collected in evacuated plastic tubes containing K_3EDTA, acivicin (50 μg/mL; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and either monobromobimane (mBrB; Calbiochem, La Jolla, CA, for LMM-SH measurement) or *N*-ethylmaleimide (NEM; Sigma-Aldrich Chemie GmbH, for disulfide measurement) as previously described (16). Samples were then immediately centrifuged for 15 seconds at 12,000 *g* at 4°C for plasma separation. Time of centrifugation was shortened as much as possible to avoid the output of mBrB–GSH conjugate from erythrocytes that could

Table 1. Nomenclature of Different Redox Forms (Reduced, Disulfide, and Mixed Disulfide With Proteins) of Main Low-Molecular-Mass Thiols Found in Plasma

Low-Molecular-Mass Sulfhydryls (LMM-SH)	Low-Molecular-Mass Disulfides (LMM-SS)	Protein/LMM-SS-Mixed Disulfides	Total Thiols
Glutathione (GSH)	Glutathione disulfide (GSSG)	Protein/glutathione-mixed disulfides (GSSP)	Total glutathione (tGSH) GSH + 2×GSSG + GSSP
Cysteine (Cys)	Cystine (CySS)	Protein/cysteine-mixed disulfides (CySSP)	Total cysteine (tCys) Cys + 2×CySS + CySSP
Cysteinylglycine (CysGly)	Cystinylglycine (CySSGly)	Protein/cysteinylglycine-mixed disulfides (CysGlySSP)	Total cysteinylglycine (tCysGly) CysGly + 2×CySSGly + CysGlySSP
Homocysteine (Hcys)	Homocystine (HcySS)	Protein/homocysteine-mixed disulfides (HcySSP)	Total homocysteine (tHcys) Hcys + 2×HcySS + HcySSP

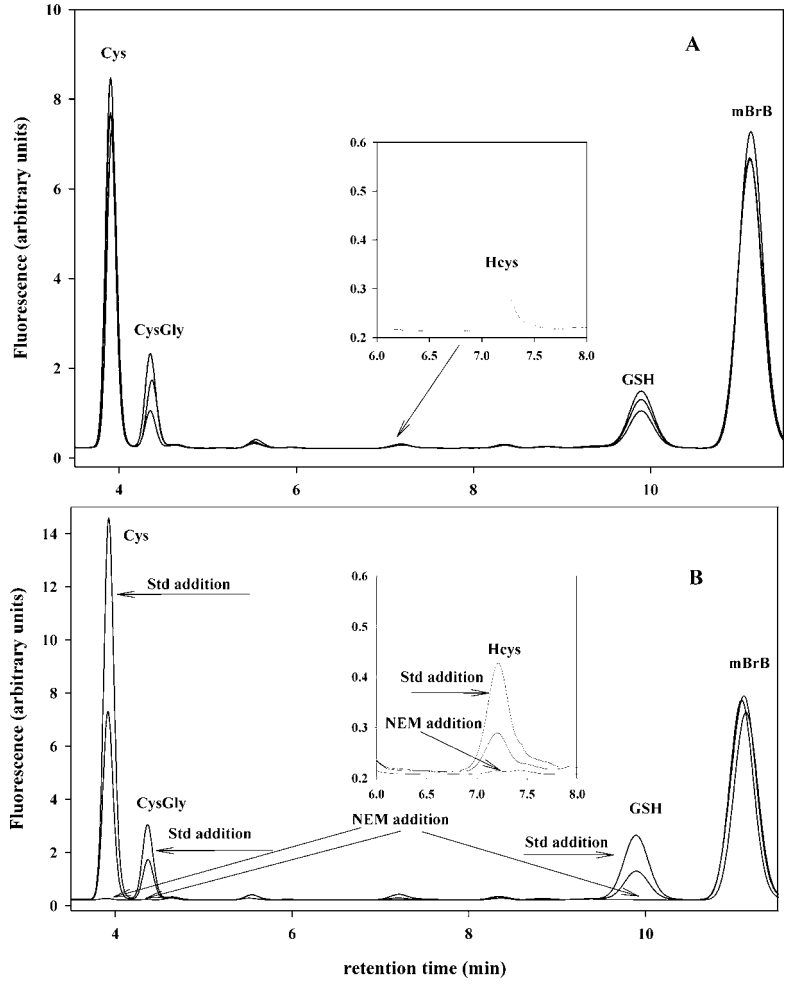

Figure 2. Typical chromatograms obtained from human plasma analysis after derivatization of low-molecular-mass sulfhydryls (LMM-SHs) with monobromobimane (mBrB) for detection. **A,** Chromatograms from three participants at different ages (22, 62, and 83 years). **B,** Same chromatogram shown in A for the 62-year-old participant in comparison with both the same sample to which a standard amount of thiols were added and the same sample pretreated with the thiol alkylating agent *N*-ethylmaleimide (NEM). Standard (Std) solutions of cysteine (Cys), cysteinylglycine (CysGly), homocysteine (Hcys), and glutathione (GSH; at final concentrations of 10, 2, 0.4, and 3 μM, respectively) were added to the plastic tubes together with K_3EDTA, acivicin, and mBrB, immediately after blood collection. Alternatively, NEM (50 mM final concentration) was added to the plastic tubes together with K_3EDTA, acivicin, and mBrB, immediately after blood collection. Samples were then treated as indicated in the Methods section.

artefactually increase the measured levels of plasmatic GSH (25). After plasma separation, thiols and disulfides were measured by high-performance liquid chromatography (HPLC) with fluorometric determination as previously described (13).

All HPLC analyses were performed using a Spherisorb C18 column (Varian Inc, Palo Alto, CA) with an Agilent series 1100 HPLC system equipped with a fluorometric detector. For determination of protein SH groups, aliquots of blood samples were collected in evacuated plastic tubes containing only K_3EDTA and acivicin (50 μg/mL) and then immediately centrifuged for 15 seconds at 12,000 *g* at 4°C. When separated, the plasma was kept at 0°C. Protein SH determination (carried out within 1 hour) was performed by spectrophotometry with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) as titrating agent (13,26). Spectrophotometric analyses were performed using a Jasco V/530 instrument (Jasco Europe s.r.l. Cremella, Como, Italy).

Statistical Analysis

The degree of correlation between the biochemical parameters and aging was measured by calculating the Spearman's correlation coefficient.

Results

HPLC Analysis of Plasma Thiols

Fluorometric thiol derivatization with mBrB combined with the HPLC separation allows the simultaneous analysis of all SH-containing molecules occurring in plasma within a single run. We applied this method to measure the levels of the reduced, disulfide, and protein-mixed disulfide forms of GSH, Cys, CysGly, and Hcys in plasma from 41 participants aged 21–92 years. In Figure 2A, the typical HPLC tracings for plasma LMM-SH in participants of different ages (22, 62, and 83 years) are shown. Additional

Table 2. Plasma Values (μM) for Each Measured Thiol, Disulfide, and Protein-Mixed Disulfide in the Study Participant

		Thiols					Disulfides				Protein-Mixed Disulfides			
Age (y)	Gender	PSH	Cys	CysGly	Hcys	GSH	CySS	CySSGly	HcySS	GSSG	CySSP	CysGlySSP	HcySSP	GSSP
21	M	530	11.7	3.07	0.445	5.75	53.2	5.77	1.70	1.85	140	12.8	16.7	1.66
21	M	445	11.5	3.06	0.223	3.45	58.9	6.42	1.31	1.55	132	10.3	7.83	1.88
22	F	454	10.8	3.11	0.453	3.46	61.0	4.89	1.41	1.47	140	9.77	9.43	1.91
23	F	442	11.3	3.57	0.130	3.06	70.0	6.11	1.02	1.47	149	11.3	10.3	1.70
25	M	489	13.8	3.04	0.429	3.78	81.8	6.75	1.28	1.20	159	15.2	8.30	1.85
26	M	424	8.50	2.92	0.594	3.78	59.1	5.75	1.35	1.65	145	12.2	9.29	1.91
27	F	483	12.4	3.37	0.184	3.62	51.4	4.43	0.91	1.22	131	8.38	9.46	1.30
29	F	474	12.4	3.10	0.242	4.15	53.0	4.66	1.05	1.52	127	8.84	9.51	2.03
33	F	450	9.13	3.15	0.175	2.27	59.8	7.65	1.73	1.37	152	23.9	4.95	5.79
35	M	433	10.3	2.99	0.150	3.51	61.3	6.10	1.20	1.20	141	10.7	7.40	1.80
36	F	476	10.2	1.57	0.410	2.06	54.7	5.40	0.90	1.43	151	14.4	5.10	2.50
37	F	398	8.55	2.75	0.130	2.99	69.5	5.90	1.30	0.98	138	12.1	8.20	3.40
44	M	450	9.65	2.48	0.350	2.54	53.3	7.00	1.41	1.70	155	12.3	8.80	2.40
46	F	486	9.31	1.94	0.070	1.82	57.3	5.21	0.74	1.20	131	10.5	5.05	1.39
48	F	449	8.55	1.41	0.040	1.84	68.5	5.04	0.83	1.10	160	10.9	5.67	1.80
49	F	434	9.26	2.43	0.170	2.31	55.7	4.65	0.57	0.88	161	13.2	7.77	2.22
50	M	399	9.53	1.72	0.100	1.44	57.8	4.06	0.48	0.58	125	11.0	3.36	1.89
52	F	387	9.02	2.51	0.180	2.74	60.2	6.47	0.79	1.58	135	13.2	5.59	1.73
53	F	435	9.91	1.96	0.320	3.51	76.5	6.14	2.37	0.78	104	7.35	3.74	0.75
54	M	449	10.3	2.05	0.240	2.19	64.1	5.37	0.99	1.80	161	12.3	7.44	1.97
57	F	452	8.88	2.28	0.250	2.13	48.5	4.67	0.71	1.54	145	10.7	6.48	1.62
59	M	402	8.18	2.57	0.480	2.26	66.8	7.31	1.25	1.70	124	11.5	9.18	3.05
62	F	304	7.63	1.88	0.350	1.32	77.1	7.85	1.59	1.21	160	12.1	11.7	2.31
62	M	422	10.1	2.44	0.351	2.92	64.6	6.65	1.49	0.98	175	10.4	8.30	3.12
62	M	472	12.2	2.18	0.140	2.04	55.6	3.70	0.64	0.55	113	7.76	4.31	1.10
64	F	445	9.93	1.84	0.250	1.77	57.7	4.62	1.23	1.14	188	12.3	12.4	1.84
64	M	367	11.3	2.02	0.192	2.68	83.2	9.36	2.59	1.26	170	18.4	7.94	5.17
66	F	401	11.0	2.02	0.123	2.34	72.2	6.23	3.40	1.10	161	12.1	6.80	2.45
67	F	428	9.61	1.32	0.220	1.92	62.4	5.46	1.13	2.12	175	12.3	9.85	1.74
68	F	383	9.42	1.51	0.160	1.44	52.3	4.21	0.91	0.93	154	10.4	10.6	1.38
68	M	388	9.90	1.87	0.144	2.32	65.0	4.20	2.80	1.10	158	11.2	10.3	1.10
72	F	343	8.66	1.55	0.270	0.86	79.2	5.12	1.68	0.97	168	10.3	10.4	1.65
73	M	376	9.50	1.43	0.233	2.01	86.0	4.50	3.20	1.20	194	13.2	12.3	1.50
77	F	380	8.06	0.96	0.137	1.25	96.2	7.08	2.53	1.00	236	13.6	19.2	2.30
81	M	376	10.2	0.92	0.178	1.73	97.1	5.77	2.86	0.96	199	9.54	18.2	1.71
81	F	438	11.8	0.81	0.278	2.31	88.4	6.35	3.28	0.91	195	12.7	16.0	4.38
82	F	418	10.2	1.28	0.341	1.98	81.9	5.85	2.88	1.22	184	12.0	11.4	3.06
83	M	401	10.2	1.23	0.143	1.43	86.7	6.50	3.80	1.40	201	11.3	14.1	1.90
84	M	365	9.54	1.02	0.165	1.69	84.8	7.30	4.00	0.98	172	13.7	12.0	2.20
91	F	406	11.9	1.57	0.232	2.33	92.3	9.30	3.71	1.35	178	17.4	13.3	5.58
92	F	389	9.36	1.12	0.165	1.65	81.2	6.80	3.33	1.51	195	14.5	15.5	2.10

Note: PSH = protein sulfhydryl groups; Cys = cysteine; CysGly = cysteinylglycine; Hcys = homocysteine; GSH = glutathione; CySS = cystine; CySSGly = cystinylglycine; HcySS = homocystine; GSSG = glutathione disulfide; CySSP = protein/cysteine-mixed disulfides; CysGlySSP = protein/cysteinylglycine-mixed disulfides; HcySSP = protein/homocysteine-mixed disulfides; GSSP = protein/glutathione-mixed disulfides; M = male; F = female.

runs performed in the presence of standard solutions for each tested thiol allowed the peak identification (Figure 2B). Finally, in some blood samples, thiols were alkylated by NEM before the addition of mBrB. The chromatogram obtained by HPLC analysis of these samples was devoid of the fluorescent peaks corresponding to different plasma thiols (Figure 2B). This finding confirmed both that the peaks obtained by HPLC analysis of plasma samples from our study group were due to the conjugation of mBrB with thiols, and that no additional peaks deriving from mBrB hydrolysis interfered with our measurements.

Thiol/Disulfide Balance Is Influenced by Aging

In Table 1, the list of the plasmatic thiols and their redox forms measured in our study group is shown. The primary data, including age and gender, are shown in Table 2. Correlation coefficients and rank of significance with aging for every measured parameter are reported in Table 3. Some of the significant correlations found are graphically shown in Figures 3 and 4.

Among thiols, the concentration of GSH and CysGly is strongly inversely correlated with age, whereas Cys and Hcys did not show any significant correlation. Conversely, the oxidized forms of Hcys (i.e., HcySS and HcySSP) and Cys (i.e., CySS and CySSP) showed a significant direct correlation with aging, evidencing an increase in the elderly participants (Figure 3). Interestingly, residuals on the linear regressions of HcySS (and, particularly, HcySSP) do not appear to have a random distribution with respect to age; in fact, the data seem to possess a biphasic distribution, with an evident increase in their concentration only after the sixth decade of life. Upon analyzing the ratios between thiols and

their disulfide forms (e.g., GSH/GSSG), some highly significant correlations were found, with a decrease in all thiol/disulfide ratios analyzed being a general feature (Figure 4). Total Cys (tCys) and total Hcys (tHcys), but not total CysGly (tCysGly) or total GSH (tGSH) concentrations increased significantly with aging. Protein thiols were found to significantly decrease in elderly participants, probably due to their increased transformation into mixed disulfides with LMM-SH.

Taking into consideration data from Figures 3 and 4 and Table 3, it is evident that: (a) the oxidative burden increases with aging; (b) GSH, CysGly, and their disulfides behave differently with aging with respect to Cys and Hcys; and (c) an increased delivery of Cys and Hcys, probably by TSP, but not of GSH efflux, by liver and/or other organs occurs with increasing age. This latter phenomenon is evidenced by the fact that neither tGSH nor tCysGly (which can derive exclusively from GSH) was found to increase. Moreover, as indicated in Figure 4, a highly significant inverse correlation with age was found for the ratios (CysGly+GSH)/LMM-SH and (tGSH+tCysGly)/(tCys+tHcys). Consequently, the percentage (to total thiols) of plasmatic thiols deriving exclusively from GSH load in plasma tends to decrease with aging, both as free thiols and as total forms. In summary, our data suggest that the oxidative burden increases with aging, as indicated by the decrease in thiol/disulfide ratios in parallel with (or as a consequence of) an increased delivery to the plasma of Hcys and Cys.

Table 3. List of Measured Parameters and Statistical Analysis of Their Correlation With Aging

Parameters	*r*	*p*
Thiols		
Cys	−0.1708	.281
CysGly	*−0.8577*	*<.0001*
Hcys	−0.1885	.2333
GSH	*−0.6844*	*<.0001*
PSH	*−0.6708*	*<.0001*
Disulfides		
CySS	*0.618*	*<.0001*
CySSGly	0.1653	.2959
HcySS	*0.5563*	*<.001*
GSSG	−0.3325	.05345
Protein-mixed disulfides		
CySSP	*0.7172*	*<.0001*
CysGlySSP	0.1937	.2206
HcySSP	*0.4986*	*<.01*
GSSP	0.1338	.3974
Thiol/disulfide ratio		
Cys/tCys	*−0.7045*	*<.0001*
CysGly/tCysGly	*−0.8074*	*<.0001*
Hcys/tHcys	*−0.5068*	*<.01*
GSH/tGSH	*−0.608*	*<.001*
Other ratios		
(CysGly+GSH)/LMM-SH	*−0.8252*	*<.0001*
(tCysGly+tGSH)/(tCys+tHcys)	*−0.6759*	*<.0001*

Notes: Italicized items are statistically significant correlations.

Cys = cysteine; CysGly = cysteinylglycine; Hcys = homocysteine; GSH = glutathione; PSH = protein sulfhydryl groups; CySS = cystine; CySSGly = cystinylglycine; HcySS = homocystine; GSSG = glutathione disulfide; CySSP = protein/cysteine-mixed disulfides; CysGlySSP = protein/cysteinylglycine-mixed disulfides; HcySSP = protein/homocysteine-mixed disulfides; GSSP = protein/glutathione-mixed disulfides; t = total; LMM-SH = low-molecular-mass sulfhydryl.

Discussion

In the present study we have focused our attention on plasma thiols with the aim of: (a) assessing whether during aging there is a shift in the thiol/disulfide balance, and (b) finding evidence for the age-related variation in the relative percentages of all thiol forms (i.e., reduced, disulfide, and protein-mixed disulfides).

To clarify these aspects, the age-derived changes of the main thiols present in plasma have been analyzed, i.e., protein thiols and LMM-SH (Cys, CysGly, Hcys, and GSH). Additionally, redox forms of LMM-SH have been measured, i.e., low-molecular-mass disulfides and mixed disulfides with proteins. This is, to our knowledge, the first study in which all redox forms of plasma thiols have been investigated in relation to aging in the age range of 21–92 years. Our data (Tables 2 and 3, Figures 3 and 4) show that a strong inverse correlation exists between aging and levels of CysGly and GSH, whereas neither their disulfides nor tGSH and tCysGly changed. Conversely, direct correlations were found between the disulfide forms of Cys and Hcys and age, whereas Cys and Hcys did not show any correlation with age. Interestingly, tCys and tHcys were positively correlated with age. In addition, the ratios between reduced and oxidized forms decreased for all LMM-SHs considered. As expected, the concentration of protein SH groups decreased with age, as a consequence of the increased levels of mixed disulfides with Cys and Hcys, and the decline of the plasma concentration of albumin that takes place in elderly persons (10). All these facts, as a whole, suggest that a higher oxidative burden in elderly persons shifts the redox state of plasma thiols and induces an increased delivery of some LMM-SHs to the hematic compartment.

Some of the data we obtained in our study agree with some previous results. Most of the data available in the literature on this issue derive from analyses of plasma total thiols. In particular, tCys increase has previously been well evidenced (10,27,28). Additionally, the increase in tHcys plasma levels with aging has also been recently described (27,29), and this observation may be of particular interest, because hyperhomocysteinemia is widely considered an independent risk for vascular disease (30,31). In contrast to our results, some existing data also indicate a possible decrease in tGSH levels with aging (29); it is possible that differences in sample storage before analyses (our measurements were taken within a few hours from blood collection) may have influenced the results. Finally, tCysGly levels were not found to change with aging (27).

Fewer data from the literature describing the possible modulation in elderly persons of the different redox forms of thiols normally occurring in plasma are available. Among reduced thiols, the age-related decline in GSH levels that we found has been previously observed in a number of senescent organisms, including humans, in plasma and/or various organs (14,17,23,32,33). Additionally, in contrast to our data, Cys was also shown to decrease with aging (but

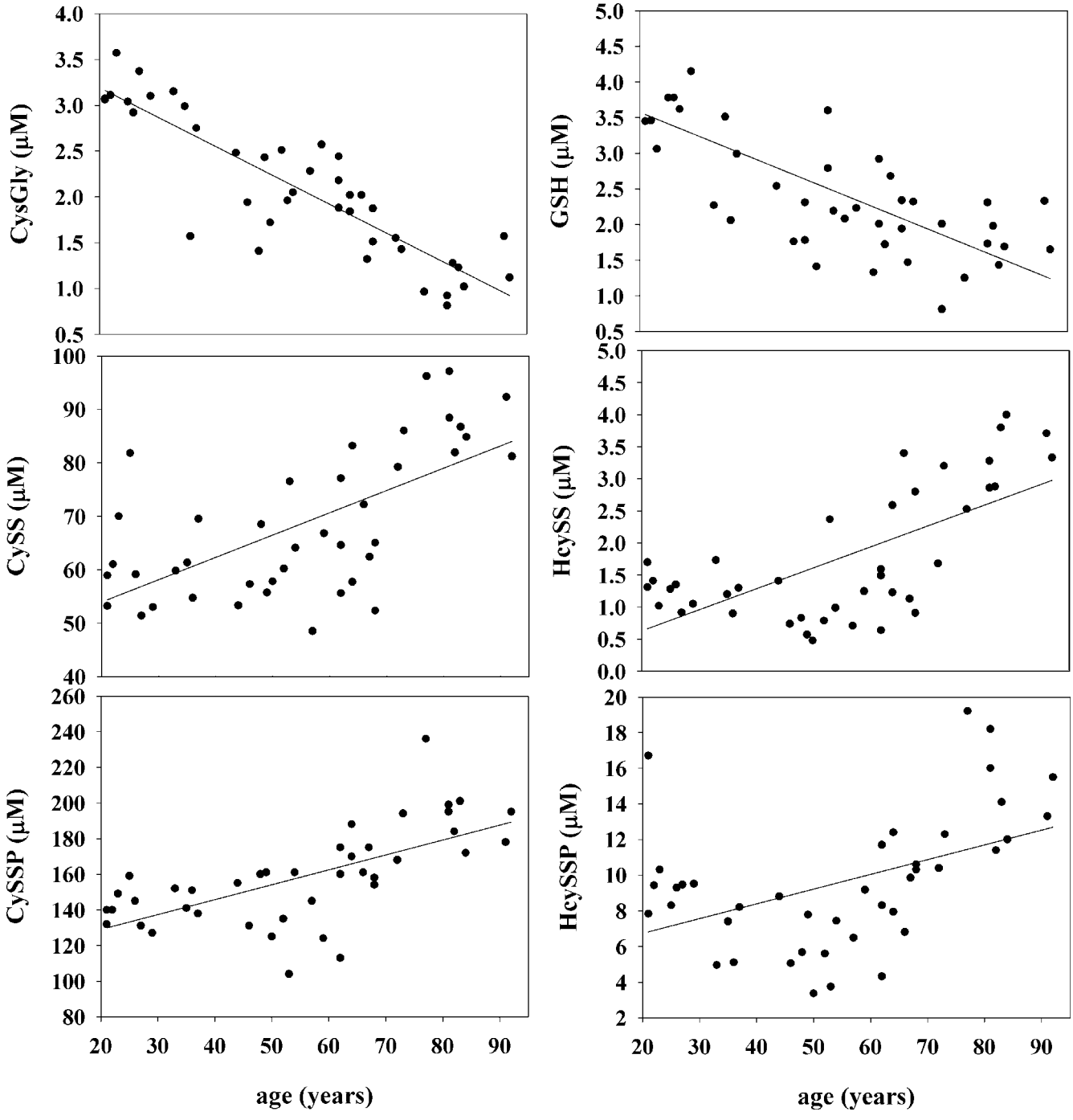

Figure 3. Scatter diagrams relating measured plasma values for cysteinylglycine (CysGly), glutathione (GSH), cystine (CySS), homocystine (HcySS), protein/cysteine mixed disulfides (CySSP), and protein/homocysteine mixed disulfides (HcySSP) with aging. Regression line, calculated by applying the Spearman's correlation rank, is shown. Thiols and disulfides were measured in plasma samples by using high-performance liquid chromatography fluorescence detection immediately after blood derivation with monobromobimane (for thiol measurements) or *N*-ethylmaleimide (for both disulfide and protein-mixed disulfide analyses).

this phenomenon was less evident) and seems to significantly decrease only after the fifth decade of life (17). To our knowledge, no data on Hcys and CysGly levels in relation to aging are available from the literature.

It has also been observed in previous work that an oxidative shift of Cys and GSH towards disulfide forms occurs in human plasma in elderly persons (10,19,34). Specifically, a linear oxidation for Cys/CySS was found to increase throughout the entire life span, whereas the GSH/GSSG ratio was found to increase only after the fourth decade of life. These data have suggested a possible occurrence of two different pools regulated separately in vivo (19).

It should be emphasized that plasma thiol redox state cannot be simply considered as an indicator of oxidative

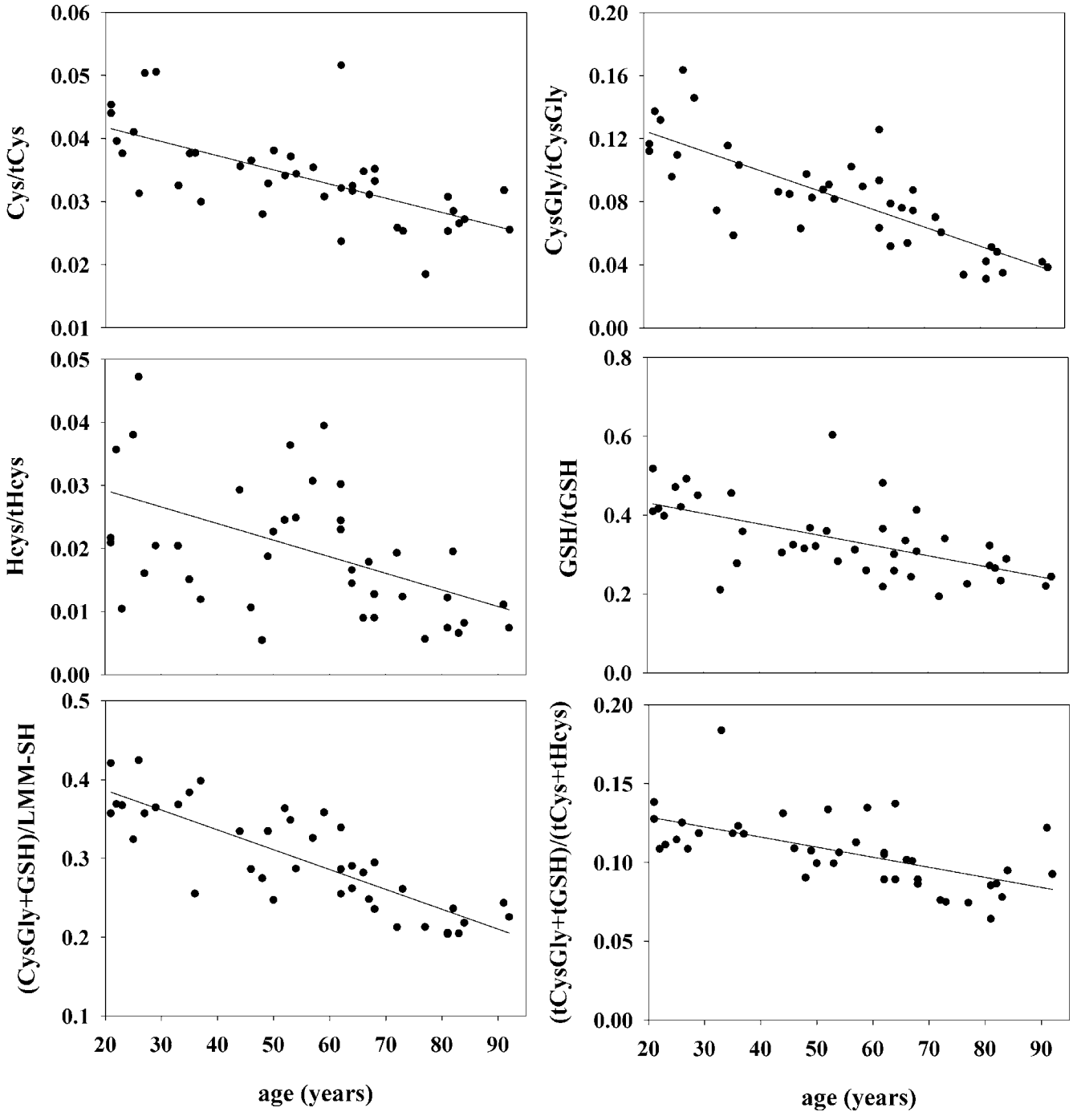

Figure 4. Scatter diagrams relating calculated plasma values for cysteine/total cysteine (Cys/tCys), cysteinylglycine/total cysteinylglycine (CysGly/tCysGly), homocysteine/total homocysteine (Hcys/tHcys), glutathione/total glutathione (GSH/tGSH), (CysGly+GSH)/low-molecular-mass sulfhydryls (LMM-SH), and (tCysGly/tGSH)/(tCys+tHcys) with aging. Regression line, calculated by applying the Spearman's correlation rank, is shown. Thiols and disulfides were measured in plasma samples by using high-performance liquid chromatography fluorescence detection immediately after the blood derivation with monobromobimane (for thiol measurements) or *N*-ethylmaleimide (for both disulfide and protein-mixed disulfide analyses).

status, as it has been previously observed that even minimal oxidation shifts of the Cys/CySS or GSH/GSSG redox couple (e.g., 21 and 9 mV changes, respectively, observed in aging) are sufficient to cause a large increase in the oxidized forms of intracellular proteins bearing vicinal thiols (17), and thus are able to influence specific signaling pathways. Such proteins include the *N*-methyl-D-aspartate (NMDA) receptor, protein disulfide isomerase, the epidermal growth factor receptor, the extracellular signal-regulated kinase, and many others (3,35–37). Moreover, the Cys/CySS ratio seems to strictly regulate many adhesion factors of endothelial cells and to have a role in the atherogenetic process (12).

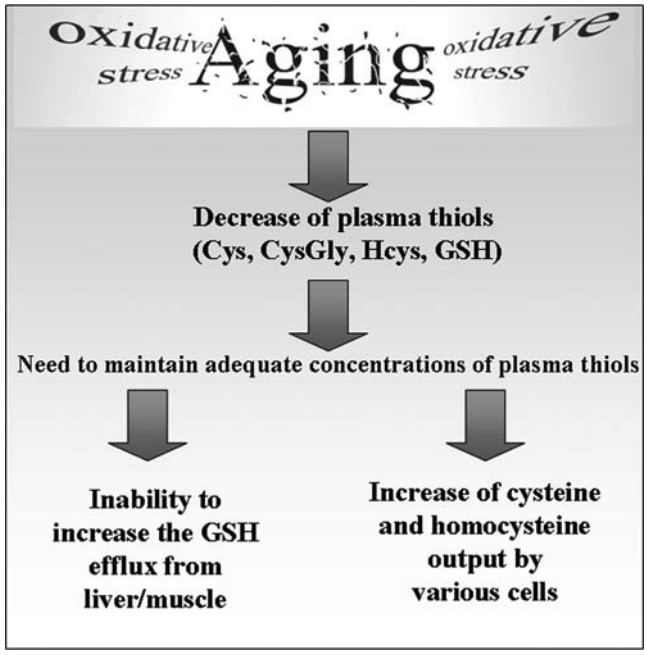

Figure 5. Schematic representation of age-derived oxidative stress and its supposed effects on plasma low-molecular-mass sulfhydryls. Cys, cysteine; CysGly, cysteinylglycine; Hcys, homocysteine; GSH, glutathione.

The complete analysis of LMM-SH performed on all redox forms allows us to infer some hypotheses about the age-derived impact on the levels of thiols and disulfides. From data reported in Tables 2 and 3 and Figures 3 and 4, it can be presumed that: (a) there is a constant decrease in GSH and CysGly levels, and an increase in tCys and tHcys over the life span; (b) the increase in tCys and tHcys is not due to an enhanced concentration of reduced forms, but to the increase in their disulfides; and (c) tGSH and tCysGly remained unchanged. These findings and the turnover of plasma thiols suggest that an increased production of reactive oxygen/nitrogen species in aging tends to decrease all plasma thiols, and that this can be balanced by an increased delivery of thiols from other tissues. In all probability, GSH delivery cannot be increased (nor, consequently, can CysGly) and, thereby, there is a decrease in GSH and CysGly levels, whereas an increased output of Cys takes place (Figure 5). Thus, the increased oxidative stress and Cys load are mirrored by a stable level of Cys (the oxidative stress is balanced by its increased output) and a huge increase in its disulfide forms (CySS and CySSP). The increase in tHcys could be a simple consequence of the activation of the TSP, due to the need to increase the delivery of Cys to the plasma.

The relative percentage of each LMM-SH measured in our study was shown to change over the entire life span. In particular, GSH and CysGly percentages decreased linearly at about 2% per year (Figure 3). Due to its intrinsic chemical feature, each individual thiol may have a different behavior and role in the redox homeostasis of the plasma. For example, it has been recently shown that NAC treatment is able to increase the plasmatic levels of Cys, but not of GSH, in patients with age-related macular degeneration (38). Additionally, the reactivity of the thiol group of CysGly is known to be largely greater than that of other LMM-SHs (39). Probably because of its high reactivity, CysGly can be considered a ''Janus'' molecule because, on one hand, it is particularly efficient in capturing electrophiles and potentially dangerous thiol-reactive species and, on the other hand, in the presence of metal ions and other particular conditions, it is able to generate free radicals by acting as a prooxidant (40). Consequently, it is not known if a decrease in CysGly levels during aging could be a useful adaptive condition or a potentially dangerous situation. For instance, it is widely accepted that LMM-SH may have a pivotal role in activating some nitric oxide donors such as *S*-nitrosoproteins (41–47) via transnitrosation reactions.

Several studies are now available showing that the plasma thiol/disulfide redox state becomes oxidized with aging [for a review, see (19)]. Among the well-documented changes, there is a decrease in GSH and an increase in both CySS and tHcys (34). In particular, a linear decrease of 0.2 mV/year for the Cys/CySS redox couple and a linear decrease of 0.7 mV/year for the GSH/GSSG couple were depicted (17). More generally, humans show, on the average, a significant oxidative shift between the 3rd and 10th decade of life (48). It is possible that, because a group of redox-regulated biological signaling pathways responds to changes in the thiol/disulfide redox state (12,49), this variation in the plasmatic redox state can be expected to contribute to aging-related diseases. One interesting example of this is represented by the findings of Go and Jones (12), which showed that the oxidation of the thiol/disulfide redox state over the range found in vivo in human plasma is a key determinant of early events in vascular disease development. Moreover, the authors suggested that the plasma thiol/disulfide state may provide a useful and early marker of atherosclerosis development, and that nutritional and therapeutic means to normalize the oxidized redox state could provide a strategy for protecting against disease development. However, available evidence for the specific contribution of each extracellular thiol/disulfide redox couple has not yet been established.

Here we have provided a view of the levels of the different main thiols in all redox forms in human plasma and the effect of aging on their levels. Our data suggest that aging is correlated to a decrease in plasma GSH and CysGly, with a concomitant rise in most oxidized forms of LMM-SH and a parallel increase in tCys and tHcys, probably due to an augmented afflux of these amino acids from various organs. The diminished levels of CysGly may have both useful and detrimental effects. Therapeutic interventions designed to increase one single plasmatic LMM-SH rather than all of them could be an interesting approach to evaluate the contribution of each LMM-SH in many possible pathological events.

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References

1. Beckman KB, Ames BN. The free radical theory of ageing matures. *Physiol Rev*. 1998;78:547–581.
2. Mecocci C, Fanò G, Fulle S, et al. Age-dependent increases in oxidative damage to DNA, lipids and proteins in human skeletal muscle. *Free Radic Biol Med*. 1999;26:303–308.
3. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature*. 2000;408:239–247.
4. Hekimi S, Guarente L. Genetics and the specificity of the ageing process. *Science*. 2003;299:1351–1354.
5. Schoneich C. Mass spectrometry in ageing research. *Mass Spectrom Rev*. 2005;24:701–718.
6. Nystrom T. Role of oxidative carbonylation in protein quality control and senescence. *EMBO J*. 2005;24:1311–1317.
7. Singh R, Barden A, Mori T, Beilin L. Advanced glycation end-products: a review. *Diabetologia*. 2001;44:129–146.
8. Ward WF, Qi W, Van Remmen H, Zackert WE, Roberts LJ 2nd, Richardson A. Effects of age and caloric restriction on lipid peroxidation: measurement of oxidative stress by F2-isoprostane levels. *J Gerontol A Biol Sci Med Sci*. 2005;60A:847–851.
9. Terman A, Brunk UT. Aging as a catabolic malfunction. *Int J Biochem Cell Biol*. 2004;36:2365–2375.
10. Droge W. Aging-related changes in the thiol/disulfide redox state: implications for the use of thiol antioxidants. *Exp Gerontol*. 2002;37:1333–1345.
11. Maher P. The effects of stress and ageing on glutathione metabolism. *Ageing Res Rev*. 2005;4:288–314.
12. Go YM, Jones DP. Intracellular proatherogenic events and cell adhesion modulated by extracellular thiol/disulfide redox state. *Circulation*. 2005;111:2973–2980.
13. Giustarini D, Lorenzini S, Rossi R, Chindamo D, Di Simplicio P, Marcolongo R. Altered thiol pattern in plasma of subjects affected by rheumatoid arthritis. *Clin Exp Rheumatol*. 2005;23:205–212.
14. Samiec PS, Drews-Botsch C, Flagg EW, et al. Glutathione in human plasma: decline in association with aging, age-related macular degeneration, and diabetes. *Free Radic Biol Med*. 1998;24:699–704.
15. Polidori MC, Mecocci P. Plasma susceptibility to free radical-induced antioxidant consumption and lipid peroxidation is increased in very old subjects with Alzheimer disease. *J Alzheimer Dis*. 2002;4:513–518.
16. Mansoor MA, Svardal AM, Ueland PM. Determination of the in vivo redox status of cysteine, cysteinylglycine, homocysteine, and glutathione in human plasma. *Anal Biochem*. 1992;200:218–229.
17. Jones DP, Mody VC, Carlson JL, Lynn MJ, Sternberg P. Redox analysis of human plasma allows separation of pro-oxidant events of aging from decline in antioxidant defenses. *Free Radic Biol Med*. 2002;33:1290–1300.
18. Ookhtens M, Mittur AV, Erhart NA. Changes in plasma glutathione concentrations, turnover, and disposal in developing rats. *Am J Physiol*. 1994;266:R979–R988.
19. Moriarty-Craige SE, Jones DP. Extracellular thiols and thiol/disulfide redox in metabolism. *Annu Rev Nutr*. 2004;24:481–509.
20. Ookhtens M, Kaplowitz N. Role of the liver in interorgan homeostasis of glutathione and cyst(e)ine. *Semin Liver Dis*. 1998;18:313–329.
21. Anderson ME, Meister A. Transport and direct utilization of γ-glutamylcyst(e)ine for glutathione synthesis. *Proc Natl Acad Sci U S A*. 1983;80:707–711.
22. Finkelstein JD. Pathways and regulation of homocysteine metabolism in mammals. *Semin Thromb Hemost*. 2000;26:219–225.
23. Sohal RS. Role of oxidative stress and protein oxidation in the aging process. *Free Radic Biol Med*. 2002;33:37–44.
24. Junqueira VBC, Barros SBM, Chan SS, et al. Ageing and oxidative stress. *Mol Aspects Med*. 2004;25:5–16.
25. Rossi R, Milzani A, Dalle Donne I, et al. Different metabolizing ability of thiol reactants in human and rat blood: biochemical and pharmacological implications. *J Biol Chem*. 2001;276:7004–7010.
26. Ellman GL. Tissue sulfhydryl goups. *Arch Biochem Biophys*. 1959;82:70–77.
27. Bates CJ, Mansoor MA, Gregory J, Pentieva K, Prentice A. Correlates of plasma homocysteine, cysteine, and cysteinyl-glycine in respondents in the British National Diet and Nutrition Survey of young people aged 4-18 years, and a comparison with the survey of people aged 65 years and over. *Br J Nutr*. 2002;87:71–79.
28. El-Khairy L, Vollset SE, Refsum H, Ueland PM. Predictors of change in plasma total cysteine: longitudinal findings from the Hordaland Homocysteine study. *Clin Chem*. 2003;49:113–120.
29. Salazar JF, Schorr H, Herrmann W, Herbeth B, Siest G, Leroy P. Measurement of thiols in human plasma using liquid chromatography with precolumn derivatization and fluorescence detection. *J Chromatogr Sci*. 1999;37:469–476.
30. Nygard O, Nordrehaug JE, Refsum H, Ueland PM, Farstad M, Vollset SE. Plasma homocysteine levels and mortality in patients with coronary artery disease. *N Engl J Med*. 1997;337:230–236.
31. Chambers JC, Obeid OA, Refsum H, et al. Plasma homocysteine concentrations and risk of coronary heart disease in UK Indian Asian and European men. *Lancet*. 2000;355:523–527.
32. Sohal RS, Weindruch R. Oxidative stress, caloric restriction, and ageing. *Science*. 1996;273:59–63.
33. Michelet F, Gueguen R, Leroy P, Wellman M, Nicolas A, Siest G. Blood and plasma glutathione measured in healthy subjects by HPLC: relation to sex, aging, biological variables, and life habits. *Clin Chem*. 1995;41:1509–1517.
34. Hildebrandt W, Kinscherf R, Hauer K, Holm E, Droge W. Plasma cystine concentration and redox state in aging and physical exercise. *Mech Ageing Dev*. 2002;123:1269–1281.
35. Choi YB, Lipton SA. Redox modulation of the NMDA receptor. *Cell Mol Life Sci*. 2000;57:1535–1541.
36. Noiva R. Protein disulfide isomerase: the multifunctional redox chaperone of the endoplasmic reticulum. *Semin Cell Dev Biol*. 1999;10:481–493.
37. Kamata H, Shibukawa Y, Oka SI, Hirata H. Epidermal growth factor receptor is modulated by redox through multiple mechanisms. Effects of reductants and H_2O_2. *Eur J Biochem*. 2000;267:1933–1944.
38. Moriarty-Craige SE, Adkison J, Lynn M, et al. Antioxidant supplements prevent oxidation of cysteine/cystine redox in patients with age-related macular degeneration. *Am J Ophthalmol*. 2005;140:1020–1026.
39. Daubeuf S, Leroy P, Paolicchi A, et al. Enhanced resistance of HeLa cells to cisplatin by overexpression of gamma-glutamyltransferase. *Biochem Pharmacol*. 2002;64:207–216.
40. Maellaro E, Dominici S, Del Bello B, et al. Membrane gamma-glutamyl transpeptidase activity of melanoma cells: effects on cellular H(2)O(2) production, cell surface protein thiol oxidation and NF-kappa B activation status. *J Cell Sci*. 2000;113:2671–2678.
41. Orie NN, Vallance P, Jones DP, Moore DP. S-nitroso-albumin carries a thiol-labile pool of nitric oxide, which causes venodilation in the rat. *Am J Physiol Heart Circ Physiol*. 2005;289:H916–H923.
42. Giustarini D, Milzani A, Colombo R, Dalle Donne I, Rossi R. Nitric oxide, S-nitrosothiols and hemoglobin: is methodology the key? *Trends Pharmacol Sci*. 2004;25:311–316.
43. Scharfstein JS, Keaney JF Jr, Slivka A, et al. In vivo transfer of nitric oxide between a plasma protein-bound reservoir and low molecular mass thiols. *J Clin Invest*. 1994;94:1432–1439.
44. Singh RJ, Hogg N, Joseph J, Kalyanaraman B. Mechanism of nitric oxide release from S-nitrosothiols. *J Biol Chem*. 1996;271:18596–18603.
45. Tsikas D, Sandmann J, Rossa S, Gutzki FM, Frolich JC. Investigations of S-transnitrosylation reactions between low- and high-molecular-weight S-nitroso compounds and their thiols by high-performance liquid chromatography and gas chromatography-mass spectrometry. *Anal Biochem*. 1999;270:231–241.
46. Wolzt M, MacAllister RJ, Davis D, et al. Biochemical characterization of S-nitrosohemoglobin. Mechanisms underlying synthesis, no release, and biological activity. *J Biol Chem*. 1999;274:28983–28990.
47. Rafikova O, Rafikov R, Nudler E. Catalysis of S-nitrosothiols formation by serum albumin: the mechanism and implication in vascular control. *Proc Natl Acad Sci U S A*. 2002;99:5913–5918.
48. Hack V, Breitkreutz R, Kinscherf R, et al. The redox state as a correlate of senescence and wasting and as a target for therapeutic intervention. *Blood*. 1998;92:59–67.
49. Andrews NP, Prasad A, Quyyumi AA. N-acetylcysteine improves coronary and peripheral vascular function. *J Am Coll Cardiol*. 2001;37:117–123.

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