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Dietary Intake of Proteins and Calories is Inversely Associated with the Oxidation State of Plasma Thiols in End-Stage Renal Disease Patients

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Abstract

Background—Oxidative stress contributes to the pathogenesis of protein-energy wasting (PEW) in maintenance hemodialysis (MHD) patients. It remains uncertain, however, whether and how food intake is involved in the causal relationship between oxidative stress and PEW.

Methods—71 adult MHD Patients and 24 healthy subjects (Control) were studied cross-sectionally with analyses of diet record and of oxidative stress, as measured by a battery of plasma

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Disclosures

The authors declare no conflict of interest.

Practical Application

The study contributes to clarify the intricate relationship among oxidative stress, inflammation and food intake, as putative contributors to PEW and poor outcome in in clinically stable hemodialysis patients. These finding support a role of dietary intervention as a means for reducing the oxidative burden in CKD patients.

thiols including the protein sulfhydryl (-SH) group levels (PSH, a marker of total protein –SH reducing capacity), the protein thiolation index (PTI, the ratio between disulfide, i.e. oxidized, and reduced –SH groups in proteins), low molecular mass (LMM) thiols, LMM disulfides and mixed LMM-protein disulfides. In addition, interleukin-6 (IL-6), albumin, C-reactive protein (CRP) and neutrophil gelatinase-associated lipocalin (NGAL) were measured as markers of inflammation.

Results—The Patients showed low energy (22.0±8.4 Kcal/kg/d) and adequate protein (1.0±0.4 g/kg/d) intakes, high levels of cystine [CySS; Patients vs. Control: 113.5 (90.9-132.8) vs. 68.2 (56.2-75.7) μM], cysteinylated proteins [CySSP; 216.0 (182.8-254.0) vs. 163.5 (150.0-195.5) μM] and high PTI [0.76 (0.61 – 0.88) vs. 0.43 (0.40 – 0.54); $p<0.001$ in all comparisons]. In Patients, variation of CySSP was explained by a standard regression model ($R=0.775$; $p=0.00001$) that included significant contributions of protein intake ($\beta=-0.361$), NGAL ($\beta=0.387$), age ($\beta=0.295$) and albumin ($\beta=0.457$). In the same model, variation of PTI ($R=0.624$; $p=0.01$) was explained by protein intake ($\beta=-0.384$) and age ($\beta=0.326$) and NGAL ($\beta=0.311$). However, when PSH was entered as dependent variable ($R=0.730$; $p=0.0001$) only serum albumin ($\beta=0.495$) and age ($\beta=-0.280$), but not dietary intake or NGAL, contributed to the model.

Conclusions—In MHD, markers of thiol oxidation including CySSP and PTI show independent association with dietary intake and NGAL, while PSH, a marker of thiol reducing capacity, did not associate with these same variables. The mechanism(s) responsible for inverse association between oxidative stress and food intake in MHD remain undefined.

Keywords

oxidative stress; acute-phase response; uremia; cysteine; glutathione; interleukin-6; neutrophil gelatinase-associated lipocalin; C-reactive protein

Introduction

Abnormalities of the thiol redox balance stand out as a prominent expression of oxidative stress in chronic kidney disease (CKD). CKD patients generally experience increased plasma levels of disulfides – the byproduct of thiol oxidation - and imbalance between disulfides and thiols in favor of the formers.[1-7] It is generally assumed that thiol abnormalities reflect extent and severity of disease in CKD patients. However, besides studies associating thiol abnormalities with body mass and composition in CKD [8] and demonstrating removal of plasma disulfides with hemodialysis in ESRD,[5] scarce evidence directly links thiol stress with specific clinical manifestations of renal failure.

Protein-energy wasting (PEW), a common secondary manifestation of CKD, is characterized by decreased body stores of somatic and visceral proteins, loss of muscle mass and low availability of energy fuels[9] and by association with high morbidity and mortality.[9-11] Knowledge about the pathogenesis of CKD-associated PEW, however, remains fragmented. It is established, for example, that oxidative stress contributes to PEW by directly inducing protein breakdown and atrophy of skeletal muscle [12-14] yet it is unknown whether anorexia and low food intake, which are common in CKD patients, may contribute to the association between oxidative stress and PEW. It was suggested recently that low intake of food may cause oxidative stress in CKD [15, 16] although the opposite is

also conceivable since oxidative stress is mechanistically linked to inflammation and the latter is a known suppressant of appetite.[15, 17]

In the present study, we used analysis of the plasma thiols and of spontaneous dietary intake to test the hypothesis that thiol redox correlates with food intake in CKD patients. Since plasma proteins are by far the major source of reactive -SH groups,[3, 18, 19] we analyzed both the plasma protein –SH groups (PSH) as a marker of plasma protein reducing capacity, and the protein thiolation index (PTI), i.e. the molar ratio between oxidized disulfide and reduced –SH groups in plasma proteins, as a marker of the plasma redox state. Also, to further capture the complexity of the plasma thiol system, we analyzed the five major low molecular mass (LMM) plasma thiols, i.e. cysteine (Cys), cysteinyl-glycine (CysGly), homocysteine (Hcys), γ-glutamyl-cysteine (γ-GluCys) and glutathione (GSH), each divided into its major redox fractions, i.e. LMM thiols, LMM disulfides and mixed LMM-protein disulfides (hereafter, thiolated proteins; see Table 1 for complete nomenclature) and we calculated the ratios between disulfides and reduced thiols.

Methods

Patient population and protocol

The study sample consisted of 71 subjects with ESRD on maintenance hemodialysis (Patients), ranging in age from 24 to 89 years old, and 24 aged-matched healthy subjects (Controls). Patients demographic, clinical and nutritional characteristics and the methods of collection of medical and dietary history were described in detail in a previous publication[20]. Briefly, Patients were recruited from a total pool of 132 hemodialysis patients, excluding subjects with ò 4% variation in end-of-dialysis weight during the preceding 3 months, hemodialysis initiation within 6 months, use of semi-permanent catheter as dialysis access, age >90 years, moderate or advance dementia, acute or chronic active infection, active malignancy or advanced liver cirrhosis. In addition, 13 subjects refused to participate and 3 transferred care around the time of enrollment. Controls were recruited from the general population using posted advertising and word of mouth. Blood specimens were collected before and at the end of the mid-week dialysis treatment and stored at −80°C until analysis. Measurement of nutrition status in the Patients included the body mass index (BMI), and spontaneous food intake which included a 1-day diet recall and a 2-day food record within 1 week and was analyzed with The Food Processor software program (ESHA Research, Salem, OR). [20] Adequacy of the dialysis treatment was captured by extracting the routine monthly Kt/V from the medical record. The study was conducted in accordance with the Helsinki Declaration of 1975 (as revised in 1983) and approved by The University of Texas Health Science Center San Antonio and the South Texas Veteran Health Care System (STVHCS) R&D Office IRB. Written informed consent was obtained from every subject.

Analysis of plasma protein -SH groups (PSH), protein thiolation index (PTI), low molecular mass (LMM) thiols, LMM disulfides, thiolated proteins, and total thiols

Artifactual oxidation of plasma thiols during storage and sample manipulation was prevented by mixing immediately after collection 1 ml blood aliquots with stabilizing solution (110 µl of 0.5 M citrate, pH 4.3), followed by centrifugation at 10,000g for 30s,

separation of plasma, and storage at −80°C. [21] The PSH was quantified with the Ellman's reagent, based on established standard methods. [19, 22] The PTI was calculated as previously describe. [18] Briefly, (a) the plasma protein thiolated -SH groups were measured in 400 μl plasma samples that were mixed with 40 μl of stabilizing solution and 400 μl H_2O and then analyzed by HPLC following established methods, [23] while (b) the plasma protein reduced –SH groups were measured in 100 μl of stabilized plasma (see above) that were mixed with 800 μl 200 mM Na_2HPO_4, followed by addition of 10 μl of 20 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and continuous recording of the colorimetric reaction at 410 nm, until achievement of a plateau. [22] The ratio between plasma protein thiolated and reduced –SH groups was then calculated. [18] The LMM thiols, LMM disulfides and thiolated proteins were measured by HPLC, concurrent with the above measurements. [23]

Analysis of cytokines, neutrophil gelatinase-associated lipocalin (NGAL), high-sensitivity c-reactive protein (CRP), albumin, transferrin and blood urea nitrogen (BUN)

Serum Albumin, Transferrin, BUN and CRP were analyzed by the STVHCS centralized clinical laboratory using standardized methods. Serum interleukin-6 (IL-6) and NGAL were analyzed by ELISA with R&D Systems (Minneapolis, MN) Quantikine reagents and protocol. Sensitivity and detection range of the ELISA were respectively 0.7 pg/mL and 3.12-300 pg/mL for IL-6, 5.5 pg/mL and 40 pg/mL and 156-10,000 pg/mL for NGAL.

Statistical Analysis

Variables were expressed as mean ± SD or median with interquartile range (IQR), based on distribution. Normality was assessed with the skewness/kurtosis test and normal quantile plots, and homogeneity of variance with the Levene test. A standard log or Box-Cox transformation was used for non-normally distributed variables. Difference between groups was assessed with Chi-square, Mann-Whitney U, or two-tailed t-test for independent samples, as appropriate. Holm-Bonferroni was used to adjust for multiple comparisons. [24] Pearson's *r* and Spearman's rho were used to assess simple bivariate correlations with level of significance set at $p \leq 0.0005$ based on maximal size of the correlation matrix.[24] MANOVA was used to assess regression after appropriate transformation and tests of collinearity with eigenvalue and condition index procedures. To test the study hypothesis, regression models were built that included CySS, CySSP, PTI or PSH, i.e. the thiol parameters more strongly correlated with clinical and nutritional variables, as dependent variables and dietary calorie or protein intake as covariates. The latter two variables were tested independently in separate models due to evidence of strong collinearity. The models also included age, diabetes mellitus, BMI, Kt/V, CRP, IL-6 and NGAL as covariates, based on association of several of these with CySS, CySSP, PTI and PSH in simple correlation and on prior evidence of association of these parameters with oxidative stress and/or nutrition. [5, 8, 25-29] The participants' dialysis clinic was also selected as one of the covariates because of differences in IL-6 levels in group analysis (see reference [20]), while the dialysis access was not chosen as covariate because not a determinant of differences in group analysis or of associations in bivariate correlations. IBM SPSS Statistics 21 (IBM Corp., Armonk, NY) was used for statistical analysis.

Results

Subject characteristics

The baseline clinical characteristics of the 71 Patients and 24 Controls are shown in Table 2. The two groups were balanced for age, race, ethnicity, sex distribution and BMI. The Patients' age and dialysis vintage were consistent with national statistics, [30] but they departed from the latter for high prevalence of diabetes mellitus (73%) and Mexican–American ethnicity (82%). Dialysis access type consisted of an arteriovenous fistula or graft in respectively 83% and 17% of the subjects and the Kt/V was 1.9 ± 0.3 (range 1.25 – 2.49). Despite having been selected for stable clinical conditions, Patients had lower levels of the nutrition markers albumin and transferrin ($p<0.001$) and higher levels of the inflammation markers CRP, NGAL and IL-6, as compared to Controls ($p<0.001$). Dietary intake was collected in Patients only and, despite elevated BMI, the patients reported daily calorie and protein intakes that were 37% and 17% below national recommendations. [31] Daily intake of fat was modestly above recommendations and carbohydrate intake was clearly low.

Protein Sulfhydryl Groups (PSH) and Protein Thiolation Index (PTI)

The PSH concentration was 20% lower in Patients than Controls [355.0 (310.0 - 385.0) vs. 446.5 (416.2 – 468.5) M, $p<0.001$; Fig 1.a]. Conversely, the PTI was 77% higher in Patients than Controls [0.76 (0.61 – 0.88) vs. 0.43 (0.40 – 0.54); $p<0.001$; Fig 1.b]. These two parameters provide different but complementary information. Low PSH concentration indicates an absolute loss of protein –SH reducing power in the Patients but provides no information on concomitant changes in disulfide forms, while high PTI indicates a relative excess of oxidized vs. reduced proteins in Patients but, being a ratio, offers no indication about the absolute amounts of disulfide and reduced forms.

Plasma Concentration of Reduced LMM Thiols, LMM Disulfides and Thiolated Proteins and Calculated Disulfide-Thiol Ratios

Consistent with prior reports, [5] we found that the LMM thiols circulate primarily as disulfides rather than as reduced thiols and that all fractions were more concentrated in Patients than Controls. Figure 2 shows the plasma concentration of the LMM disulfides CySS and CySSP to be approximately 10- and 20-fold higher than that of reduced Cys and the concentration of each of these fractions to be higher in the Patients than Controls. Cys was 15.2 (12.1-18.3) vs. 11.6 (9.3-12.6) μM, CySS 113.5 (90.9-132.8) vs. 68.2 (56.2-75.7) μM, and CySSP 216.0 (182.8-254.0) vs. 163.5 (150.0-195.5) μM in Patients vs. Controls ($p<0.001$ in all comparisons). The CysGly, Hcys, γ-GluCys and GSH disulfide and thiol fractions were present at much lower concentrations as compared to Cys, but the disulfide-thiol proportions were similar to those of the Cys fractions (Appendix Table 1). Furthermore, the calculated disulfide-thiol ratios were higher in Patients than Controls with the notable exception of the CySSP/Cys and (CySS+CySSP)/Cys ratios which were not different between groups (Appendix, Table 2). Specifically, CySS/Cys was 6.93 (3.55-5.39) in Patients vs. 5.57 (4.97-6.80) in Controls ($p=0.003$), while the CySSP/Cys [14.0 (11.6-16.9) vs. 15.4 (11.8-18.1)] and (CySS+CySSP)/Cys [20.7 (18.1-24.6) vs. 20.9 (16.9-25.4)] were not different between groups.

Correlation of thiol concentrations and ratios with dietary intake, laboratory markers of inflammation and clinical parameters in ESRD patients

In the Patients, we explored association of the above thiol concentrations and ratios with markers of nutrition and inflammation as well as standard clinical parameters. The Cys fractions stood out among the individual thiol forms for multiple correlations with dietary parameters (see Table 3). In particular, the CySSP fraction correlated inversely with dietary intake of calories ($r = -0.406$; $p \leq 0.005$; Table 3) and proteins ($r = -0.461$; $p \leq 0.0005$) and directly with serum albumin levels ($r = 0.363$; $p \leq 0.005$). CySSP also showed a weaker correlation with NGAL ($r = 0.301$; $p = 0.008$). The CySS fraction showed a similar inverse correlation with calorie and protein intake and direct with age. The PSH levels correlated inversely with presence of diabetes ($r = -0.355$; $p \leq 0.005$) and age ($r = -0.390$; $p \leq 0.005$) and directly with albumin ($r = 0.526$; $p \leq 0.0005$). Among the thiol ratios, PTI stood out for inverse correlation with the dietary intake of proteins ($r = -0.444$; $p \leq 0.0005$) and calories ($r = -0.388$; $p \leq 0.005$) and direct with age ($r = 0.370$; $p \leq 0.005$; see Table 3). In addition, the CySS/Cys ratio correlated inversely with age ($r = -0.388$; $p \leq 0.005$; see Table 3). The CysGly, Hcys, γ-GluCys and GSH fractions showed no correlation with dietary proteins and calories and more sporadic associations with age, albumin and IL-6 (Appendix Table 3.a and 3.b.).

Independent association of cysteine fractions and PTI with dietary intake, albumin, NGAL and age in ESRD patients

Based on these findings, we used multiple regression to further analyze in Patients the relationship of CySS, CySSP, PTI and PSH with the covariates dietary calorie or protein intake as well as dialysis clinic, Kt/V, age, BMI, diabetes mellitus, albumin, CRP, NGAL, and IL-6 (see Statistical Analysis, Methods section for the rationale leading to selection of these covariates). As shown in Table 4.A, 60% variation of $_{Log}$CySSP levels was explained by a standard regression model ($R = 0.775$; $p = 0.00001$) that included inverse correlation with protein intake ($\beta = -0.361$; $p = 0.001$; Figure 4.a) and direct with NGAL ($\beta = 0.387$; $p = 0.0008$; Figure 4.b), age ($\beta = 0.295$; $p = 0.006$) and albumin ($\beta = 0.457$; $p = 0.0002$). Somewhat weaker associations were found when CySS was the dependent variable instead of CySSP (not shown). Regarding the disulfide-thiol ratios (Table 4.B), 39% of $_{Log}$PTI variation was explained by the same model ($R = 0.624$; $p = 0.01$) including inverse correlation with protein intake ($\beta = -0.384$; $p = 0.004$; Figure 4.c) and direct with age ($\beta = 0.326$; $p = 0.014$; Figure 4.d) and NGAL ($\beta = 0.311$; $p = 0.03$). Finally (Table 4.C), 53% of PSH variation was explained by the same model ($R = 0.730$; $p = 0.0001$), although only serum albumin ($\beta = 0.495$; $p = 0.0002$) and age ($\beta = -0.280$; $p = 0.02$) contributed to the model while protein and calorie intakes or presence of diabetes did not contribute. As expected based on the mentioned collinearity between protein and calorie intake (see Statistical Analysis in the Methods section), similar results were obtained when the calorie substituted protein intake in the regression model (Appendix Table 4.a through c).

Discussion

The major novel observation of this study is that markers of uremia-related thiol stress, including the plasma levels of the disulfides CySS and CySSP and the PTI are inversely associated with spontaneous intake of calories and protein in medically stable well-dialyzed

hemodialysis patients. Furthermore, CySSP is directly associated with the marker of inflammation NGAL. The study also confirms presence of high levels of most plasma thiol forms and high disulfide-thiol ratios in ESRD patients.[5, 6]

The inverse association of CySSP, CySS and PTI with protein and calorie intakes persists after adjustment for age, BMI, diabetes, Kt/V, dialysis clinic, albumin, CRP, IL-6 and NGAL and it is, to our knowledge, the first evidence of a link between spontaneous food intake and oxidative stress in ESRD patients. Our observation is in apparent conflict with previous studies in patients with advanced CKD[32] and in experimental animals with 5/6 nephrectomy[33] which found a direct - rather than inverse - correlation between protein intake and oxidative stress and concluded that excessive protein intake generates free radicals that accelerate the progression of renal disease. This discordance is possibly explained by differences in study design, since our patients had on average nearly optimal dietary protein intake but clearly low calories, while the latter studies manipulated the protein intake while keeping adequate the calories [32, 33]. These differences are relevant since excess dietary amino acids are expected to undergo degradation and to generate reactive oxidants when the calorie intake is adequate[34-36]; conversely, with low calorie intake, adaptation to starvation diverts dietary amino acids toward production of energy to prevent metabolic breakdown of tissues and haphazard formation of reactive radicals. The latter scenario is applicable to our study cohort and to the general ESRD population that often has low food and energy intake.[9-11, 37-39]

We also report direct associations of CySSP, CySS and PTI with NGAL, suggesting functional coupling of thiol stress and acute-phase response in uremia. NGAL, a lipocalin protein produced by several tissues including leukocytes, kidney and liver, was initially described in renal literature as a marker of acute kidney injury although high blood levels of NGAL have been reported more recently in CKD and ESRD.[40-42] High circulating levels of NGAL are only in part the consequence of impaired renal elimination of the protein as they also result from increased systemic production in response to CKD-related inflammation and possibly to iron supplementation.[28, 29, 43] Interestingly, NGAL binds with high affinity to endocytic receptor and it activates signaling cascades that affect inflammation and oxidation pathways in complex manners[28, 44] and could therefore participate in the pathogenesis of inflammation and thiol stress. However, having been discovered only recently, its specific mechanisms linking NGAL with CKD, thiols and oxidative stress are unexplored and at the moment just the object of speculation.

The observed associations of CySS, CySSP, PTI and PSH with age confirm prior similar observations in the general population.[25, 45] Furthermore, direct associations of the disulfide CySSP and of PSH with albumin were expected since albumin is the protein constituent of most plasma CySSP's and it contributes the large majority of –SH that are measured by the PSH assay. These observations also confirm prior similar reports on tCys and albumin.[46]

Our work introduces the PTI for assessment of thiol redox state in renal failure patients,[18] We find this test to perform comparably to CySS and CySSP in regression analysis against a panel of clinical variables. Notably, we did not find association of plasma Cys or PSH with the tested nutritional variables, suggesting that disulfides and disulfide-thiol ratios rather

than the reduced thiols are more informative markers of nutrition in CKD and ESRD. It remains to be seen whether PTI, CySS and CySSP are as informative when tested against other clinical variables or against hard endpoints of morbidity or mortality.

The study has several limitations including the relatively small study cohort with diverse primary etiologies of ESRD. The latter, however, did not seem to affect our findings since the regression analyses remained highly significant irrespective whether ESRD etiology was entered or not into the statistical model. Second, we did not collect information about diet in the healthy Control group; this omission prevents dietary comparison between the two groups although it does not affect the validity of our associative findings in the Patient cohort. Third, we did not analyze other byproducts of oxidative stress besides the thiols which would have allowed further characterization of the significance of thiol redox in our study subjects. Lastly, we did not collect information on iron supplementation which may have affected the results pertaining to redox balance and NGAL levels. [43] Despite these limitations, the study contributes innovative observations that will warrant further investigation.

In conclusion, we describe inverse association of extracellular thiol oxidation with food intake and direct association with markers of inflammation in clinically stable hemodialysis patients. These findings fall short from establishing a cause and effect relationship between diet, redox balance and inflammation but contribute to clarify the intricate relationship among these variables, as putative contributors to PEW and poor outcome in CKD.[9-11, 47] Dietary intervention could be used as a means for reducing the oxidative burden in CKD patients and to try tease out the mechanisms responsible for the above newly discovered associations.

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Appendix

Appendix Table 1

Plasma Concentration of Reduced Thiols and of LMM and LMM-Protein Mixed Disulfides (μM)

a. Reduced Thiols (μM)				b. LMM Disulfides (μM)				c. LMM-Protein Mixed Disulfides (μM)			
Thiol	**C**	**MHD**	***p***	**Thiol**	**C**	**MHD**	***p***	**Thiol**	**C**	**MHD**	***p***
Cys	**11.5** (9.3-12.9)	**15.2** (12.1-18.3)	*<0.001*	CySS	**68.2** (56.2-75.6)	**113** (90.9-132)	<0.001	CySSP	**163** (150-195)	**216** (182-254)	*<0.001*
CysGly	**1.79** (1.50-2.20)	**1.73** (1.41-2.24)	ns	CySSGly	**5.50** (4.52-6.46)	**7.75** (6.03-10.1)	<0.001	CySSGlyP	**15.0** (13.3-18.1)	**21.0** (16.5-25.5)	*<0.001*
Hcys	**0.15** (0.12-0.21)	**0.21** (0.17-0.26)	*0.04*	HcySS	**1.27** (0.96-1.39)	**3.10** (2.36-4.09)	<0.001	HcySSP	**8.23** (6.45-9.96)	**18.5** (14.9-23.4)	*0.001*
γ-GluCys	**0.06** (0.05-0.09)	**0.06** (0.05-0.08)	ns	γ-GluCySS	**0.99** (0.86-1.07)	**1.36** (1.03-1.68)	<0.001	γ-GluCySSP	**1.55** (1.31-1.71)	**1.51** (1.22-1.78)	ns
GSH	**2.50** (1.94-2.77)	**1.58** (1.31-2.13)	*<0.001*	GSSG	**1.07** 0.93-1.17)	**0.94** (0.66-1.39)	ns	GSSP	**3.11** (2.66-3.38)	**3.01** (2.07-3.91)	ns

MHD, Maintenance Hemodialysis Patients; Control, Healthy Subjects.

Values indicate **median** (IQR); Statistical comparison with Kruskal-Wallis 1 Way ANOVA; p, level of significance

Appendix Table 2

Ratio of Disulfide-Reduced Thiol Forms in Plasma

a. LMM Disulfide / Reduced Thiol				b. LMM-protein Disulfide / Reduced Thiol				c. (LMM + LMM-protein Disulfide) / Reduced Thiol			
Ratio	**C**	**MHD**	***p***	**Ratio**	**C**	**MHD**	***p***	**Ratio**	**C**	**MHD**	***p***
CySS / Cys	**557** (4.97-6.80)	**6.93** (3.55-5.39)	*0.003*	CySSP / Cys	**15.4** (11.8-18.1)	**14.0** (11.6-16.9)	ns	(CySS + CySSP) /Cys	**20.9** (16.9-25.4)	**20.7** (18.1-24.6)	ns

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Appendix Table 3

Spearman's Correlation Matrix of Plasma CysGly and Hcys Forms and Ratios with Clinical Parameters and Dietary Intake

CysSGly / CysGly	**3.08** (2.48-3.90)	**4.52** (3.55-5.39)	*<0.001*	CysSGlyP / CysGly	**8.88** (7.46-10.8)	**11.9** (10.4-13.6)	*<0.001*	(CysSGly + CysSGlyP) / CysGly	**11.9** (10.1-14.6)	**16.7** (14.2-18.4)	*<0.001*
HcySS / Hcys	**7.42** (5.84-8.62)	**14.6** (12.1-17.3)	*<0.001*	HcySSP/Hcys	**49.8** (42.2-62.7)	**87.6** (70.2-107)	*<0.001*	(HcySS + HcySSP) /Hcys	**58.1** (48.5-69.3)	**102** (80.3-123)	*<0.001*
γ-GluCySS / γ-GluCys	**13.7** (11.1-19.5)	**21.1** (13.5-27.6)	*0.001*	γ-GluCySSP / γ-GluCys	**23.0** (16.3-29.6)	**24.3** (19.1-30.2)	ns	(γ-GluCySS + γ-GluCySSP)/ γ-GluCys	**37.1** (27.4-49.6)	**45.0** (33.0-58.3)	*0.039*
GSSG / GSH	**0.43** (0.34-0.57)	**0.54** (0.42-0.76)	*0.016*	GSSP / GSH	**1.22** (0.91-1.50)	**1.79** (1.12-2.67)	*0.010*	(GSSG+GSSP) / GSH	**1.60** (1.26-2.18)	**2.43** (1.72-3.40)	*0.003*

a. Cysteinylglycine (CysGly)

Variable	CysGly	CySSGly	CySSGlyP	PSH	PTI	CySSGly/ CysGly	CySSGlyP/ CysGly	(CySSGly+CySSGlyP) / CysGly
Protein	.260	.158	.119	.236	−.444$^{\psi}$	−.020	−.025	−.003
Calories	.302*	.069	.142	.187	−.388$^{\zeta}$	−.166	−.057	−.087
Age	−.335	−.020	−.119	−.357$^{\zeta}$	.370$^{\zeta}$	**.346**$^{\zeta}$	.232	.266*
BMI	.101	.053	.086	.019	.130	.009	.026	.044
DM	−.172	−.166	−.313*	−.205	−.005	−.283*	−.194	−.231
Albumin	−.012	.167	.191	.478$^{\psi}$	.029	.197	.208	.224
CRP	.042	−.065	−.008	−.062	.126	−.125	.015	−.008
NGAL	−.102	.064	.038	.148	.193	.174	.169	.200
IL-6	.122	.116	.134	.181	−.047	.016	−.031	−.005
(CySSGly+CySSGlyP) / CysGly	−.371$^{\zeta}$	.401$^{\zeta}$	.403$^{\zeta}$	−.162	**.430**$^{\psi}$	**.812**$^{\psi}$	**.972**$^{\psi}$	
CySSGlyP/ CysGly	−.390$^{\zeta}$	.288	.398$^{\zeta}$	−.160	**.443**$^{\psi}$	**.682**$^{\psi}$		
CySSGly/ CysGly	−.299	**.594**$^{\psi}$	.289*	−.170	**.347**$^{\zeta}$			
PTI	**−.590**$^{\psi}$	−.138	−.179	**−.641**$^{\psi}$				
PSH	**.499**$^{\psi}$	.257	**.404**$^{\zeta}$					
CySSGlyP	**.627**$^{\psi}$	**.772**$^{\psi}$						
CySSGly	**.534**$^{\psi}$							

b. Homocysteine (Hcys)

Variable	Hcys	HcySS	HcySSP	PSH	PTI	HcySS / Hcys	HcySSP / Hcys	(HcySS+HcySSP) / Hcys
Protein	−.082	−.160	−.218	.236	−.444$^{\psi}$	−.194	−.149	−.171
Calories	−.108	−.224	−.190	.187	−.388$^{\zeta}$	−.244	−.130	−.153
Age	.038	.300	.193	−.357$^{\zeta}$	.370$^{\zeta}$	**.428**$^{\psi}$	.164	.186
BMI	.022	.006	−.107	.019	.130	−.005	−.167	−.145
DM	−.081	−.166	−.154	−.205	−.005	−.068	.004	−.007
Albumin	.083	.140	.156	.478$^{\psi}$	.029	.173	.037	.044
CRP	−.061	−.047	.005	−.062	.126	.034	.080	.093
NGAL	.123	.206	.217	.148	.193	.225	.135	.148
IL-6	.317	.146	.012	.181	−.047	−.095	−.269	−.260
(HcySS+HcySSP) / Hcys	**−.501**$^{\psi}$	.187	**.532**$^{\psi}$	−.308	**.532**$^{\psi}$	**.756**$^{\psi}$	**.995**$^{\psi}$	
HcySSP / Hcys	**−.530**$^{\psi}$	.130	**.509**$^{\psi}$	−.278	**.500**$^{\psi}$	**.708**$^{\psi}$		
HcySS / Hcys	−.215	**.558**$^{\psi}$	**.557**$^{\psi}$	**−.420**$^{\psi}$	**.626**$^{\psi}$			
PTI	.035	**.499**$^{\psi}$	**.578**$^{\psi}$	**−.641**$^{\psi}$				
PSH	.161	−.192	−.133					
HcySSP	.373$^{\zeta}$	**.777**$^{\psi}$						
HcySS	**.632**$^{\psi}$							

MHD, Maintenance Hemodialysis Patients; Control, Healthy Subjects.

Values indicate **median** (IQR); Statistical comparison with Kruskal-Wallis 1 Way ANOVA; p, level of significance

Protein and calorie intakes are respectively g/kg/day and Kcal/kg/day. DM, diabetes mellitus; CRP, C-reactive protein; BMI, body mass index; CHO, carbohydrate; NGAL, neutrophil gelatinase-associated lipocalin; IL-6, interleukin-6.

Significance levels:

$^{\zeta}$p<0.005

$^{\psi}$p<0.0005.

Appendix Table 4

Spearman's Correlation Matrix of Plasma γ-GluCys and GSH Forms and Ratios with Clinical Parameters and Dietary Intake

a.	γ-Glutamylcysteine (γ-GluCys)								b.	Glutathione (GSH)							
Variable	**γ-GluCys**	**γ-GluCySS**	**γ-GluCySSP**	**PSH**	**PTI**	**γ-GluCySS / γ-GluCys**	**γ-GluCySSP / γ-GluCys**	**(γ-GluCySS + γ-GluCySSP) / γ-GluCys**	**Variable**	**GSH**	**GSSG**	**GSSP**	**PSH**	**PTI**	**GSSG / GSH**	**GSSP / GSH**	**(GSSG+GSSP) / GSH**
Protein	−.067	−.223	−.191	.236	−.444ψ	−.152	−.086	−.151	Protein	.133	.091	−.178	.236	−.444ψ	.004	−.272	−.256
Calories	.068	−.307	−.105	.187	−.388ζ	−.307	−.170	−.295	Calories	.224	.163	−.035	.187	−.388ζ	−.001	−.209	−.194
Age	.120	.370ζ	.090	−.357ζ	.370ζ	.242	−.027	.094	Age	−.312	−.097	−.074	−.357ζ	.370ζ	.160	.052	.057
BMI	.044	.060	.001	.019	.130	−.007	−.065	−.035	BMI	.050	−.070	.094	.019	.130	−.090	.035	−.006
DM	−.110	−.179	−.068	−.205	−.005	−.083	.038	−.036	DM	−.081	.043	−.138	−.205	−.005	.093	−.094	−.020
Albumin	−.060	.305	.124	.478ψ	.029	**.420ψ**	.183	.322ζ	Albumin	.250	−.096	.244*	.478ψ	.029	−.343	−.015	−.142
CRP	.030	−.089	−.066	−.062	.126	−.135	−.063	−.116	CRP	.055	.098	.069	−.062	.126	.053	.042	.098
NGAL	−.146	.182	−.056	.148	.193	.371ζ	.077	.249	NGAL	.106	−.155	.061	.148	.193	−.207	−.014	−.044
IL-6	−.287	.033	−.236	.181	−.047	.269	.103	.217	IL-6	−.258	−.480ψ	.081	.181	−.047	−.257	.261	.114
(γ-GluCySS+γ-GluCySSP) / γ-GluCys	**−.705ψ**	.221	.163	.099	.196	**.859ψ**	**.879ψ**		(GSSG + GSSP) / GSH	**−.553ψ**	.233	**.703ψ**	−.227	.281*	**.596ψ**	**.929ψ**	
γ-GluCySSP / γ-GluCys	**−.702ψ**	−.096	.328	.127	.117	**.533ψ**			GSSP / GSH	**−.540ψ**	−.014	**.795ψ**	−.116	.257*	.332		
γ-GluCySS / γ-GluCys	**−.502ψ**	**.536ψ**	−.026	.033	.273				GSSG / GSH	−.326	**.732ψ**	.176	**−.426ψ**	.120			
PTI	.249	**.495ψ**	**.421ψ**	**.641ψ**					PTI	−.110	−.008	.228	**−.641ψ**				
PSH	−.254	−.194	−.135						PSH	.238	−.211	.021					
γ-GluCySSP	.377ζ	.334ζ							GSSP	−.014	.217						
γ-GluCySS	.383ζ								GSSG	.342							

Protein and calorie intakes are respectively g/kg/day and Kcal/kg/day. DM, diabetes mellitus; CRP, C-reactive protein; BMI, body mass index; CHO, carbohydrate; NGAL, neutrophil gelatinase-associated lipocalin; IL-6, interleukin-6.

Significance levels:

ζ p<0.005

ψ p<0.0005.

Appendix Table 5

Multiple regression models for CySSP, PTI and PSH

A **Dependent variable: CySSP[a]**
Model summary: standard; R^2 = 0.587, p=0.00001

Predictor	B	*p*	95% CI (low)	95% CI (high)
Clinic	0.192	*ns*	−0.018	0.098
Kt/V	−0.023	*ns*	−0.048	0.038
Age	0.238	0.03	0.000	0.004
BMI	0.112	*ns*	−0.002	0.006
DM	0.018	*ns*	−0.045	0.053
Albumin	0.516	0.00004	0.079	0.202
CRP[a]	0.127	*ns*	−0.020	0.071
IL-6[a]	−0.057	*ns*	−0.092	0.059
NGAL[a]	0.341	0.003	0.048	0.218
Calorie/kg/d[a]	−0.340	0.002	−0.330	−0.078

B **Dependent variable: PTI[a]**
Model summary: standard; R^2 = 0.418, p=0.010

Predictor	B	*p*	95% CI (low)	95% CI (high)
Clinic	0.261	*ns*	−0.020	0.141
Kt/V	0.047	*ns*	−0.049	0.070
Age	0.272	0.04	0.000	0.006
BMI	0.120	*ns*	−0.004	0.009
DM	0.008	*ns*	−0.066	0.070
Albumin	0.150	*ns*	−0.040	0.130
CRP[a]	0.074	*ns*	−0.047	0.080
IL-6[a]	−0.053	*ns*	−0.122	0.088
NGAL[a]	0.262	0.05	−0.004	0.231
Calorie/kg/d[a]	−0.332	0.02	−0.395	−0.046

C **Dependent variable: PTI[a]**
Model summary: standard; R^2 = 0.659, p=<0.001

Predictor	B	*p*	95% CI (low)	95% CI (high)
Clinic	−0.268	*ns*	−58.805	3.785
Kt/V	−0.026	*ns*	−25.797	20.522
Age	−0.268	0.03	−2.443	−0.162
BMI	−0.113	*ns*	−3.401	1.324
DM	−0.118	*ns*	−39.312	13.482
Albumin	0.428	0.001	24.220	90.130
CRP[a]	0.069	*ns*	−17.685	31.304
IL-6[a]	0.047	*ns*	−34.141	47.206
NGAL[a]	−0.064	*ns*	−57.848	33.372
Calorie/kg/d[a]	0.114	*ns*	−34.009	101.282

CySSP, cysteinylated proteins; PTI, protein thiolation index; PSH, protein –SH groups; BMI, body mass index; CRP, C-reactive protein; NGAL, neutrophil gelatinase-associated lipocalin; IL-6, interleukin-6; CHO, carbohydrate; IBW, ideal body weight; B, coefficient B; p, significance level; CI, confidence interval.

[a] Log_{10} transformed variable

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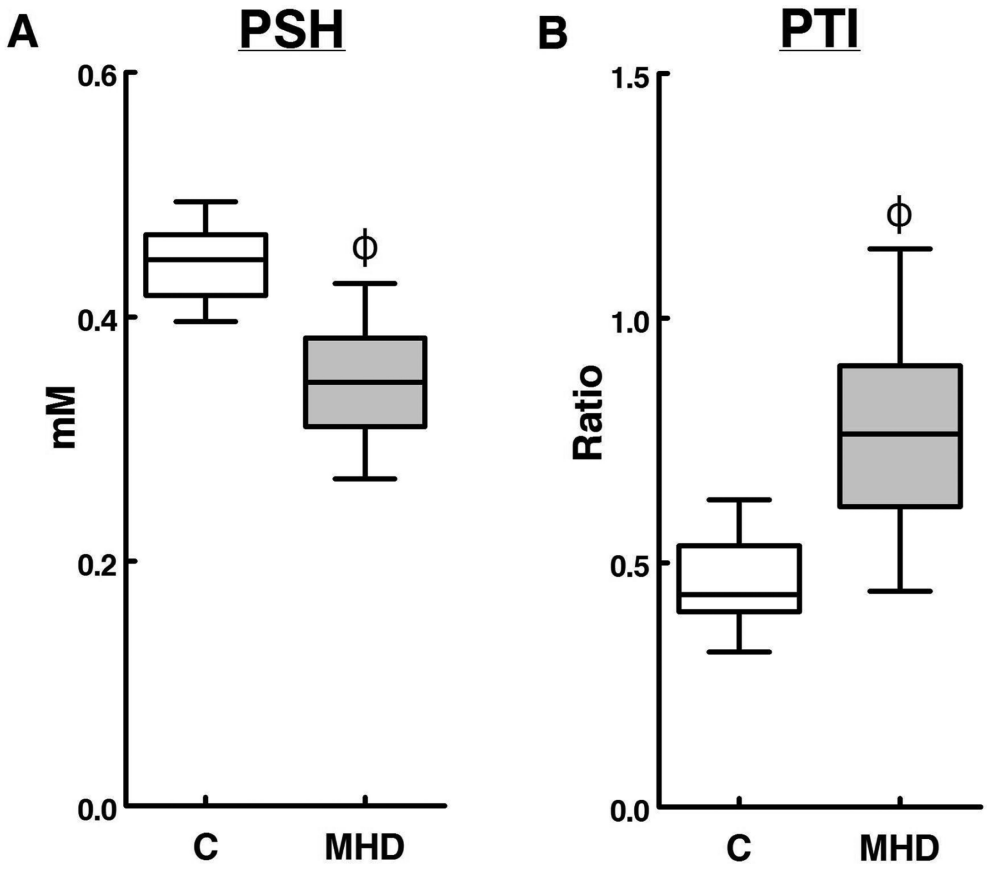

Figure 1.

Plasma levels (median [IQR]) of reduced and disulfide fractions of cysteine in 24 healthy subjects (C) and 71 hemodialysis (MHD) patients. **Panel A**: cysteine (Cys); **Panel B:** cystine (CySS); **Panel C:** mixed cystine-protein disulfide (CySSP). Statistical comparison of C and MHD for each cysteine fraction with the Mann-Whitney U; Φ indicates $p<0.001$.

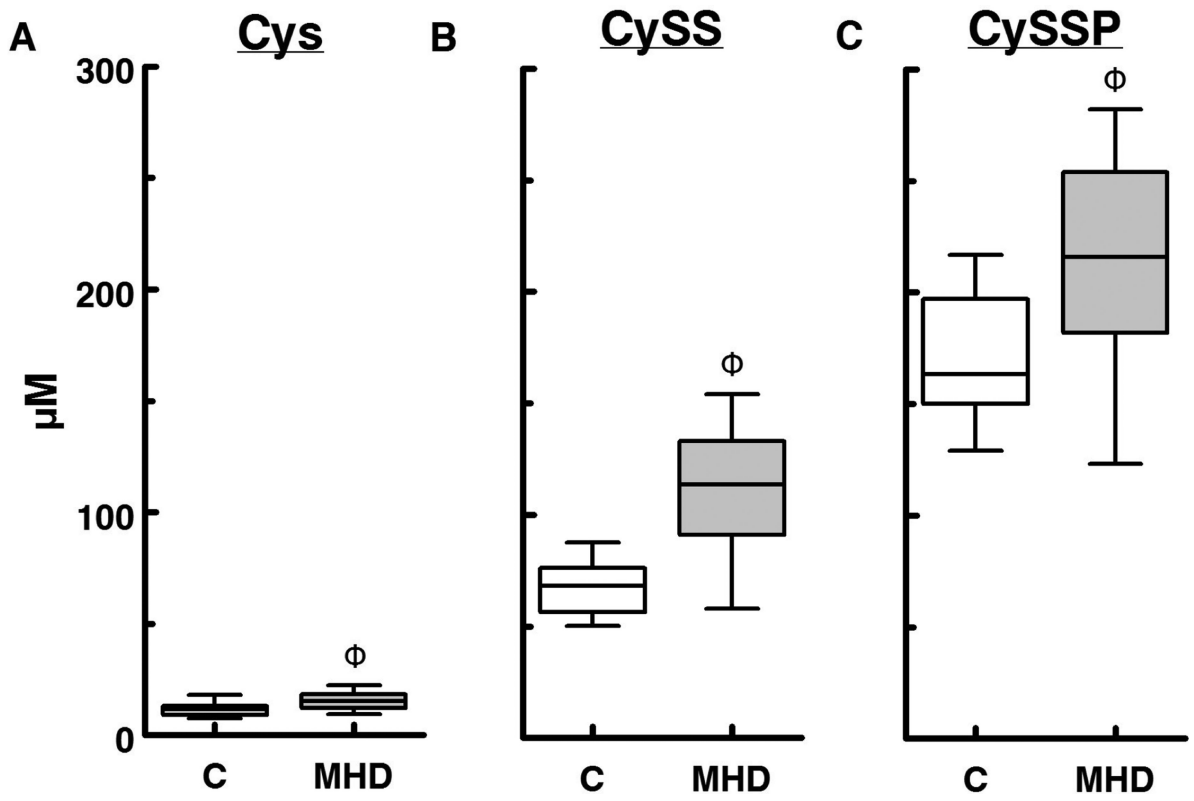

Figure 2.

Plasma levels (median [IQR]) of (**A)** protein sulfhydryl groups (PSH) and (**B)** protein thiolation index (PTI) in 24 healthy subjects (C) and 71 hemodialysis (MHD) patients. Statistical comparison of C and MHD for each parameter with the Mann-Whitney U; Φ indicates $p<0.001$.

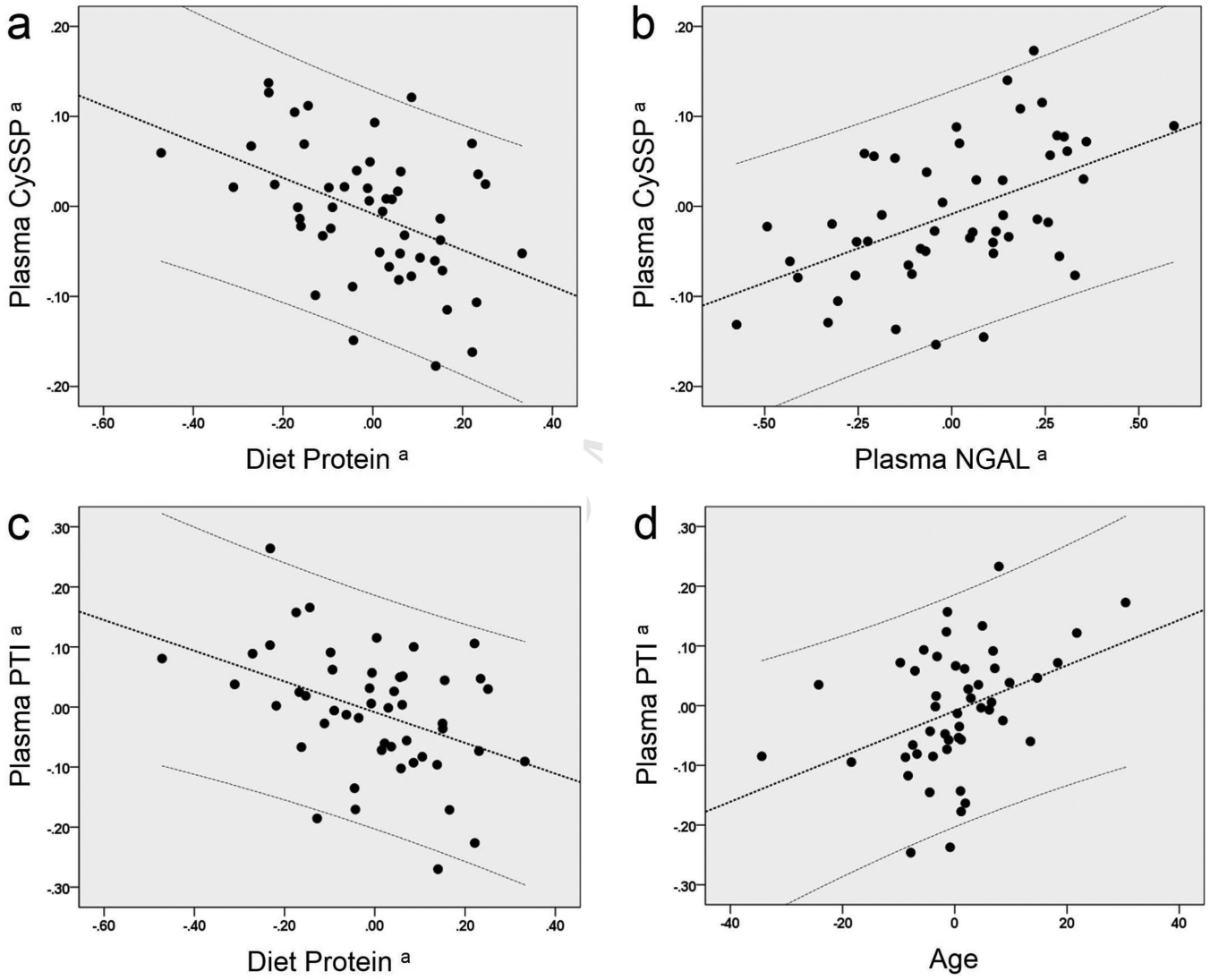

Figure 3.

Partial correlation plots from the standard model multiple regression analysis of dependent variables CySSP and PTI against covariates dietary calorie and protein intake, dialysis clinic, Kt/V, age, BMI, CCI, albumin, CRP, NGAL, and IL-6. Dotted lines indicate linear fit and 95% individual confidence interval. [(a)] $\log_{10}$ transformed variables.

Table 1

Plasma Thiols Nomenclature

Full Name	Abbreviation
Protein Sulfhydryl Groups	PSH
Protein Thiolation Index [disulfide/reduced protein thiols][18]	PTI
Cysteine	Cys
Cystine	CySS
Cysteinylated Protein	CySSP
Total Cysteine (Cys +2x CySS + CySSP)	tCys
Homocysteine	Hcys
Homocystine	HcySS
Homocysteinylated Protein	HcySSP
Total Homocysteine (Hcys + 2x HcySS + HcySSP)	tHcys
Cysteinyl-Glycine	CysGly
Cystinyl-Glycine	CySSGly
Cysteinyl-Glycinated Protein	CySSGlyP
Total Cysteinyl-glycine (CysGly +2x CySSGly + CySSGlyP)	tCysGly
γ-Glutamyl-Cysteine	γ-GluCys
γ-Glutamyl-Cystine	γ-GluCySS
γ-Glutamyl-Cysteinylated Protein	γ-GluCySSP
Total γ-Glutamyl-Cysteine (γ-GluCys + 2x γ-GluCySS + γ-GluCySSP)	tγ-GluCys
Glutathione	GSH
Glutathione disulfide	GSSG
Glutathionylated Protein	GSSP
Total Glutathione (GSH + 2x GSSG + GSSP)	tGSH

Table 2

Clinical characteristics

Characteristic	Reference Range	MHD (n=71)	Control (n=24)	*p*
General				
Age	-	60.0 ± 10.4	57 ± 8.7	*ns*
Gender (M/F)	-	52/19	19/5	*ns*
Ethnicity (MA/NHW/AA/As)	-	58/2/11/0	12/7/0/2	nd
Body Mass Index	-	28.9 ± 6.2	28.9 ± 0.9	*ns*
Diabetes Mellitus (DM/other)	-	52/19	0/0	nd
Dialysis Vintage	-	53 ± 34	-	nd
Dialysis access (AVF/AVG)	-	59/12	-	nd
Dialysis treatment adequacy (Kt/V)	≥1.2	1.9 ± 0.3	-	nd
Diet Record				
Energy (Kcal/kg/day)	35*(d)*	21.5 ± 8.2	nd	nd
Protein (g/kg/day)	1.2*(d)*	1.0 ± 0.4	nd	nd
Fat (% Kcal/day)	25 - 35*(d)*	37.8 ± 6.7	nd	nd
Carb (% Kcal/day)	50 - 60*(d)*	43.5 ± 8.3	nd	nd
Nutrition				
Albumin (g/dL)	3.4 – 4.6*(b)*	3.49 ± 0.38	4.23 ± 0.31	*<.001*
Transferrin (mg/dL)	185 - 350*(b)*	171.9 ± 27.8	252.5 ± 34.1	*<.001*
Blood Urea Nitrogen (mg/dL)	6- 23*(b)*	51.1 ± 16.3	14.7 ± 5.5	*<.001*
Creatinine (mg/dL)	0.7 – 1.2*(b)*	8.8 ± 2.6	0.9 ± 0.1	*<.001*
Inflammation				
CRP (mg/L)*(a)*	0 –3.0*(b)*	2.9 (1.4-6.5)	0.25 (0.20-0.90)	*<.001*
NGAL (ng/mL)*(a)*	0 – 245*(d)*	729 (552-1256)	74 (70.7-78.8)	*<.001*
IL-6 (pg/mL)*(a)*	0 – 6.2[48]	8.3 (4.2-17.9)	3.6 (2.7-7.1)	*<.001*

MHD, maintenance hemodialysis; Control, Healthy Subjects; MA, Mexican American; NHW, Non-Hispanic White; AA, African American; As, Asian; Kt/V, Dialyzer clearance*time/Volume of distribution; AVF, arteriovenous fistula; AVG, arteriovenous graft; nd, not determined; p, level of significance.

Values are presented as mean ± SD unless otherwise indicated. Statistical comparison between MHD and Control groups with Kruskal-Wallis 1-Way ANOVA.

(c) investigator laboratory.

(a) Values presented as median (IQR).

(b) STVHCS centralized laboratory reference range.

(d) Recommended intake for 60 y.o. man with ESRD.[49]

Table 3

Spearman's Correlation Matrix of Plasma Thiols, Thiol Ratios, Clinical Parameters and Dietary Intake.

		Plasma Thiol Concentrations					Plasma Thiol Ratios			Clinical Parameters							Diet
	Variable	**Cys**	**CySS**	**CySSP**	**PSH**	**PTI**	**CySS / Cys**	**CySSP / Cys**	**(CySS+CySSP)/Cys**	**IL-6**	**NGAL**	**CRP**	**Albumin**	**DM**	**BMI**	**Age**	**Calorie**
Diet	Protein	−.190	−.363$^{\zeta}$	**−.461$^{\psi}$**	.236	**−.444$^{\psi}$**	−.164	−.242	−.232	.026	.020	.026	−.072	−.115	−.145	−.156	**.799$^{\psi}$**
	Calorie	−.135	−.433$^{\zeta}$	−.406$^{\zeta}$	.187	−.388$^{\zeta}$	−.307	−.210	−.259	−.064	−.006	.082	.003	−.180	−.052	−.194	
Clinical Parameters	Age	.143	**.456$^{\psi}$**	.279	−.357$^{\zeta}$	.370$^{\zeta}$	−.388$^{\zeta}$	.022	.149	.046	−.053	−.011	−.142	.234	.065		
	BMI	.276	.269	.186	.019	.130	.032	−.207	−.135	.266	−.094	.403$^{\zeta}$	.057	.088			
	DM	.020	.107	.112	−.355$^{\zeta}$	.172	.068	−.026	.004	−.176	−.077	−.051	−.129				
	Albumin	.203	.282	.363$^{\zeta}$	**.478$^{\psi}$**	.029	.236	.146	.178	−.084	.248	−.268					
	CRP	.042	−.039	.073	−.062	.126	−.083	.002	−.015	.126	.033						
	NGAL	.166	.272	.301	.148	.193	.251	.118	.187	.176							
	IL-6	.132	.185	−.038	.181	−.047	.062	−.158	−.066								
Thiol Ratios	(CySS+CySSP) / Cys	**−.567$^{\psi}$**	.061	.379$^{\zeta}$	−.167	.412$^{\zeta}$	**.799$^{\psi}$**	**.959$^{\psi}$**									
	CySSP / Cys	**−.654$^{\psi}$**	−.127	.313	−.116	.333$^{\zeta}$	**.620$^{\psi}$**										
	CySS / Cys	−.258	**.452$^{\psi}$**	.397$^{\zeta}$	−.228	**.440$^{\psi}$**											
	PTI	.342$^{\zeta}$	**.647$^{\psi}$**	**.857$^{\psi}$**	**−.641$^{\psi}$**												
PI. Thiols	PSH	−.070	−.255	−.236													
	CySSP	**.451$^{\psi}$**	**.714$^{\psi}$**														
	CySS	**.678$^{\psi}$**															

Protein and calorie intakes are respectively g/kg/day and Kcal/kg/day. DM, diabetes mellitus; CRP, C-reactive protein; BMI, body mass index; CHO, carbohydrate.

Significance levels:

$^{\zeta}$p≤0.005;

$^{\psi}$p≤0.0005;

Table 4

Multiple regression models for CySSP, PTI and PSH

A

Dependent variable: CySSP[a]
Model summary: standard; R^2 = 0.601, p=0.00001

Predictor	B	*p*	95% CI (low)	95% CI (high)
Clinic	0.135	*ns*	−0.029	0.085
Kt/V	−0.053	*ns*	−0.054	0.032
Age	0.295	0.006	0.001	0.005
BMI	0.096	*ns*	−0.003	0.006
DM	0.001	*ns*	−0.048	0.049
Albumin	0.457	0.0002	0.063	0.185
CRP[a]	0.100	*ns*	−0.025	0.065
IL-6[a]	−0.080	*ns*	−0.097	0.052
NGAL[a]	0.387	0.0008	0.067	0.235
Protein/kg/d[a]	−0.361	0.001	−0.339	−0.092

B

Dependent variable: PTI[a]
Model summary: standard; R^2 = 0.390, p=0.01

Predictor	B	*p*	95% CI (low)	95% CI (high)
Clinic	0.207	*ns*	−0.030	0.126
Kt/V	0.012	*ns*	−0.056	0.061
Age	0.326	0.014	0.001	0.006
BMI	0.099	*ns*	−0.004	0.008
DM	−0.013	*ns*	−0.070	0.063
Albumin	0.088	*ns*	−0.057	0.110
CRP[a]	0.047	*ns*	−0.051	0.072
IL-6[a]	−0.075	*ns*	−0.126	0.078
NGAL[a]	0.311	0.03	0.019	0.250
Protein/kg/d[a]	−0.384	0.004	−0.423	−0.085

C

Dependent variable: PSH
Model summary: standard; R^2 = 0.533, p=0.0001

Predictor	B	*p*	95% CI (low)	95% CI (high)
Clinic	−0.228	*ns*	−0.067	0.008
Kt/V	0.018	*ns*	−0.026	0.031
Age	−0.280	0.02	−0.003	0.000
BMI	−0.098	*ns*	−0.004	0.002
DM	−0.085	*ns*	−0.044	0.021
Albumin	0.495	0.0002	0.042	0.124
CRP[a]	0.091	*ns*	−0.019	0.041

C **Dependent variable: PSH**
Model summary: standard; R^2 = 0.533, p=0.0001

Predictor	B	*p*	95% CI (low)	95% CI (high)
IL-6[a]	0.083	*ns*	−0.035	0.064
NGAL[a]	−0.108	*ns*	−0.082	0.030
Protein/kg/d[a]	0.183	*ns*	−0.015	0.150

CySSP, cysteinylated proteins; PTI, protein thiolation index; PSH, protein –SH groups; BMI, body mass index; CRP, C-reactive protein; NGAL, neutrophil gelatinase-associated lipocalin; IL-6, interleukin-6; CHO, carbohydrate; IBW, ideal body weight; B, coefficient B; p, significance level; CI, confidence interval.

[a] Log_{10} transformed variable