# Serum Thiols as a Surrogate Estimate of DNA Repair Correlates to Mammalian Life Span

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## ABSTRACT

Biologically occurring thiols are a sensitive estimate of the reduction/oxidation balance of cells, being easily and reversibly converted from sulfhydryl to disulfide structures in proteins and amino acids. Thiols are also known to regulate DNA repair, especially via the influence on poly (adenosine diphosphate–ribose) polymerase activity. Here the thiol content of saturated ammonium sulphate-precipitated proteins from sera was correlated to a mammalian life span of 17 species. A close correlation was established between the thiol-rich proteins and the life span of the mammals ($r = 0.841$, $p < 0.001$). These data provide a strong scientific connection between mechanisms of DNA repair and oxidative stress leading to DNA damage accumulation and mutation, which may be important to the aging process.

## INTRODUCTION

DNA DAMAGE AS THE SOURCE OF cellular mutations and one of the primary causes of chronic diseases in humans is now one of the oldest and best substantiated medical hypotheses.[1,2] Originally, the evidence was based on inherited conditions in which individuals were genetically predisposed to cancer, which is a well recognized mutagenic disease in which defects in DNA repair could often be shown.[3,4] However, a purely inherited DNA repair defective enzyme could not explain the much higher incidence of cancer, nor the fact that epidemiological evidence supported an environmental component as responsible for most cancers.[5] Oxidative stress relating to the metabolic rate of oxygen consumption, dietary factors, and exposures was soon identified as an environmental factor capable of predisposing individuals to elevated levels of DNA damage.[1,6] Oxidative stress could be genetically inherited, such as familial polyposis and ulcerative colitis, or acquired, such as human immunodeficiency virus (HIV) infections or oxygen-radical generated genotoxic exposures. Viewed from this historical perspective, not only have the effects of oxidative stress on cellular DNA damage accumulation been more recently confirmed, but its contribution to the downregulation of DNA repair has also been documented.[2,7,8] Together, the data reviewed here clearly justify the use of DNA repair estimating as a surrogate biomarker of longevity and as a potential epidemiological indicator for measuring risk caused by the susceptibility of humans to DNA damage associated diseases.

Our laboratory (at the University of Lund, Lund, Sweden) has participated in the scientific development of the concept of environmentally regulated DNA repair. Over the years, using several different biochemical techniques for estimating DNA repair, such as unscheduled DNA synthesis, nucleoid sedimen-

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tation, DNA strand breaks, and PARP (poly ADP [adenosine diphosphate]–ribose polymerase) activity, we have shown the potential for DNA damage accumulation from: (a) occupational exposures;[9–18] (b) genetic predisposition to cancer;[19–25] (c) drug resistance;[26] (d) aging;[17,27] (d) immune dysfunction;[28] and (e) for enhanced DNA damage removal from immune stimulation.[29] Other laboratories have also confirmed our observations of a downregulated DNA repair synthesis among individuals who are predisposed to cancer.[30–32]

In an effort to explain a broader-based regulation of DNA repair in the population than just that originating from inherited repair gene defects, researchers at our laboratory began an investigation of oxidative stress as a possible environmental source of downregulation of DNA repair synthesis. Our data clearly supported that DNA repair, and in particular PARP activity, was severely inhibited by natural occurring oxygen-centered radicals, especially hypochlorous acid. And, if cells were incubated with glutathione or other reducing agents, these cells were protected from DNA repair inhibition.[33–35] These results were taken as strong evidence for demonstrating the powerful influence cellular oxidation/reduction balance has on regulation of DNA repair.

Screening populations for risk to diseases requires easy access to individual biological samples. Tissue biopsies and blood are sometimes clinically available, but they are limited by the isolation and culture procedures of viable cell populations. So far, this has been a serious shortcoming in developing a DNA repair test for estimation of disease risk in human populations. However, serum/plasma samples are collected from patients as a routine procedure during the practice of clinical medicine. Availability and patient compliance with this type of sample collection are expected to be high. Therefore, our laboratory has investigated if the oxidation/reduction balance of serum could be used as a surrogate indicator of DNA repair capacity because of the strong control that oxidative stress has on the process of DNA repair. First, we have shown that plasma total thiol levels significantly correlated with the PARP levels estimated in the mononuclear leukocytes coming from the same blood sam-

ples.[36] Second, we have confirmed that an abnormal oxidative stress–exposed population of individuals who are HIV positive patients with autoimmune deficiency syndrome[37] had reduced levels of plasma thiols in accordance with their chances of survival.[38] The theoretical hypothesis behind this relationship is that the PARP enzyme has two cysteine-rich zinc fingers in the DNA binding domain of the enzyme[39] and, if the cysteine residues are oxidized by oxidative stress, the enzyme cannot bind zinc nor DNA and subsequently participate in repair. Here, we report on a more extended definition of the thiol test as a surrogate DNA repair measure, in which only thiols present in ammonium sulfate precipitated serum proteins are shown to predict the life span of mammalian species. Life span has, in turn, already been strongly correlated to DNA repair capacity.[27,40,41]

## MATERIALS AND METHODS

### Sample collection

Fresh blood from only mature animals was collected from various sources including zoos, slaughterhouses, veterinarian practices, and university experimental animal housing quarters. The blood samples were allowed to clot and the sera were harvested by centrifugation at $1000 \times G$ within 5 hours of collection. The mammalian sera were dialyzed (exclusion 12,000 MW) overnight at room temperature in 2 liters of physiological saline in order to avoid interference from low-molecular components when determining the colorimetric bioassays. Sera were stored at $-20°C$ until they were analyzed for thiol content.

### Separation of protein-containing thiols from sera

To achieve a 60% saturated $(NH_4)_2SO_4$ solution 300 $\mu$l room-temperature saturated $(NH_4)_2SO_4$ was added to 200 $\mu$l serum. The resulting solution was gently vortex-mixed, incubated for 15 minutes on ice, and subsequently centrifuged at $12,000 \times g$ for 10 minutes in order to retrieve the 0–60% $(NH_4)_2SO_4$ precipitated protein fraction. The supernatant was transferred to a new tube,

the volume was adjusted to compensate for the volume changes occurring from a 60% $(NH_4)_2SO_4$ solution, and then the appropriate amount of saturated $(NH_4)_2SO_4$ was added to raise the concentration to 80%. The 60–80% $(NH_4)_2SO_4$ precipitated protein fraction was gently vortex-mixed, incubated for 15 minutes on ice, and then centrifuged at 12,000 × g for 15 minutes to retrieve the 60–80% precipitated fraction. The supernatant containing all the low-molecular, nonprecipitated components was discarded.

The precipitated fractions were dissolved in 300 $\mu l$ saline.

## Quantitative determination of protein-containing thiols in sera

The protein pellets precipitated with 0–60% and 60–80% $(NH_4)_2SO_4$ were dissolved in 300 $\mu l$ physiological saline and the final volume expansions arising from the residual $(NH_4)_2SO_4$ solution were measured for correction when calculating the thiols present per 200 $\mu l$ serum sample. Then, 50 $\mu l$ aliquots of the $(NH_4)_2SO_4$ redissolved protein fractions were mixed with 200 $\mu l$ physiological saline-dinitrothiobenzoic acid (DTNB) solution (10 mL of saline containing 200 $\mu l$ stock DTNB solution, which, in turn contains 9.5 mg DNTB/mL in 0.1 M $K_2HPO_4$-17.5 mM ethylenediaminetetraacetic acid, pH 7.5), gently vortex-mixed, and then incubated for 1 hour in the dark at room temperature. An appropriate blank was also prepared by replacing the 50 $\mu l$ $(NH_4)_2SO_4$ protein fraction with 50 $\mu l$ saline. The yellow color absorbance was read at 412 nm in a spectrophotometer against the blank (50 $\mu l$ saline + 200 $\mu l$ saline-DTNB solution). A thiol standard curve using 0–9 nmol cysteine per 50 $\mu l$ saline was used as a reference to quantify the thiol content of the $(NH_4)_2SO_4$ precipitated protein fractions. The following calculation was used:

$(NH_4)_2SO_4$ precipitated thiols present in 200 $\mu l$ serum = 300 $\mu l$ + volume change × nmol of cysteine/50 $\mu l$ sample volume.

## Determination of proteins in sera

5 $\mu l$ of the serum samples were diluted 1:15 with saline (75 $\mu l$), and then 5 $\mu l$ aliquots were assayed in duplicate in 96-well microtiter plates. 200 1 BCA/Cu reagent were added per well (10 mL bicinchoninic acid + 0.2 ml 4% $CuSO_4$ × $5H_2O$, prepared fresh before use), and then incubated 30 minutes at 37°C. The blue color absorbance was read at 540 nm in a spectrophotometer. For background calculations, 5 $\mu l$ saline/well replaced the protein fractions in the reaction mixture. Note that the serum samples are assayed as a 15-fold dilution compared to the thiol determination, which, in turn, represents only 1/40 (i.e., 5 $\mu l$ out of 200 $\mu l$ serum) of the thiol sample size (1/15 × 1/40 = 1/600 dilution factor correction). 5 $\mu l$ aliquots of dialyzed sera were quantified for protein by comparison to a standard curve of 0–10 $\mu g$ of bovine albumin in 5 $\mu l$ dissolved in saline. Alternatively, nondialyzed sera could also be quantified for protein by producing a standard curve of serum supplemented with 0–10 $\mu g$ of bovine albumin and analyzed in 5 $\mu l$ aliquots. Both techniques required calculation to the standardized sample volume of 200 $\mu l$ serum as described above.

## Statistics

There were 47 samples representing 17 species investigated for this study. The life spans used for calculation purposes were obtained from the literature.[42,43] The number of specimens and their estimated longevity were: mouse (n = 1; 2 years); rat (n = 1; 3.3 years); wolf (n = 1, 10 years); dog (n = 2; 12 years); goat (n = 1; 12 years); sheep (n = 2; 12 years); rabbit (n = 1; 13 years); bear (n = 1; 14 years); cat (n = 1, 15 years); lynx (n = 1; 15 years); musk ox (n = 1; 20 years); fallow deer (n = 1, 20 years); cow (n = 2; 30 years); gorilla (n = 2; >39.3 years); chimpanzee (n = 2; >46.6 years); horse (n = 2; 46 years); and human (n = 25; 95 years). The gorilla and chimpanzee were recorded as having minimal life spans in the cited references because of the relatively small number of specimens that were studied. Animals of both genders were used and all were judged as being adults based on body-weight measurements for females and males of each species. Estimated life spans for each species were used for statistical correlation analyses to serum thiols, and the actual age of each speci-

men was not recorded in this study. For analysis, replicate determinations were averaged for each specimen entered into the study (i.e., 47 samples of 17 species), and then the individual samples in different species were used for Pearson correlation analyses between thiols and life span. A two tailed $T$-test was used to compare the means between the determinations of thiols by two technicians. The statistically significant level was set at 0.05.

## RESULTS

### Intertechnician reproducibility

The biochemical reproducibility was evaluated by analysis of intertechnician variability in the determination of protein thiols and proteins coming from 30 individual human-serum samples. The analyses were performed in duplicate and the average for each individual serum was calculated for comparison of means for the 30 individual sera determined by technician #1 and technician #2. The data recorded in Figure 1 show that no statistically significant variations could be found between the data collected by technician #1 or technician #2. This was taken as evidence that the biochemical evaluation of $(NH_4)_2SO_4$ precipitated thiol-containing proteins or total proteins in sera were reproducible and, hence, this measure could be used to assess interspecies variations in relation to health risk such as longevity.

### Variability of $(NH_4)_2SO_4$ precipitated protein yield and total protein from sera

In order to study interspecies variation in serum protein thiols, it was important to study whether the percentage yield of proteins from $(NH_4)_2SO_4$ precipitation, or the total proteins present in the sera coming from different mammalian species, could in themselves explain any statistically significant associations, especially in relation to life span. In general, although there was a great interspecies variation in the amount of $(NH_4)_2SO_4$ precipitated protein from the mammalian sera studied, in all cases, most of the protein precipitated in the
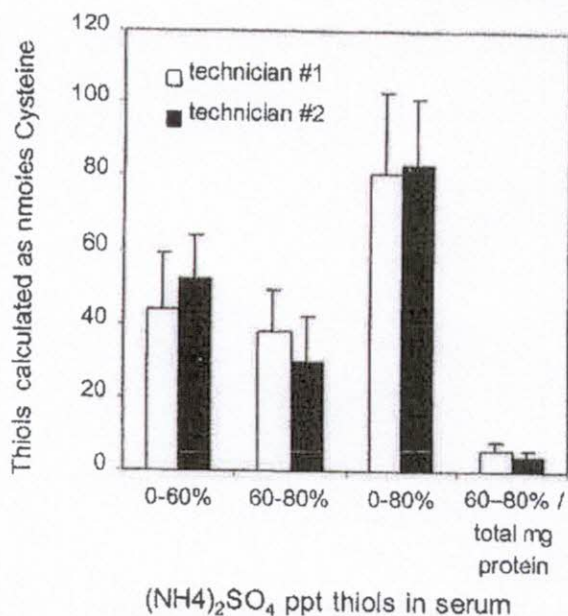


**FIG. 1.** The reproducibility of serum thiol determinations in protein fractions precipitated with 0–80% saturated ammonium sulphate determined by comparison of intertechnician variability. Thirty human sera were analyzed and then technician #1 values were compared to technician #2 values by $t$-test. The data are standardized for quantification of protein thiols and total protein content for 200 $\mu l$ serum samples.

0–60% fraction being $71.4 \pm 9.6\%$ compared to the 60–80% that was $28.6 \pm 9.6\%$ ($p < 0.05$). Because the thiol content present in the 0–60% protein fraction was similar to the 60–80% protein fraction as shown in Figures 1–2, and yet the amount of thiol-containing protein in the 0–60% was on average 3-fold increased compared to the 60–80% fraction, it was concluded that the 0–60% fraction contained thiol-poor proteins whereas the thiol-rich proteins were in the 60–80% fraction. Moreover, the statistics summarized in Table 1 provide analyses for the influence of total protein and yields originating from the various mammalian sera. Although the recovery of proteins from sera using saturated ammonium sulfate precipitation was incomplete being $76.7 \pm 18.4\%$ for the various mammalian species ($n = 17$), there was no relationship to the thiol levels detected in the precipitated protein fractions nor to the life span of the mammals. These data support that nonrecovered serum proteins do not predict the thiol levels in the recovered protein frac-
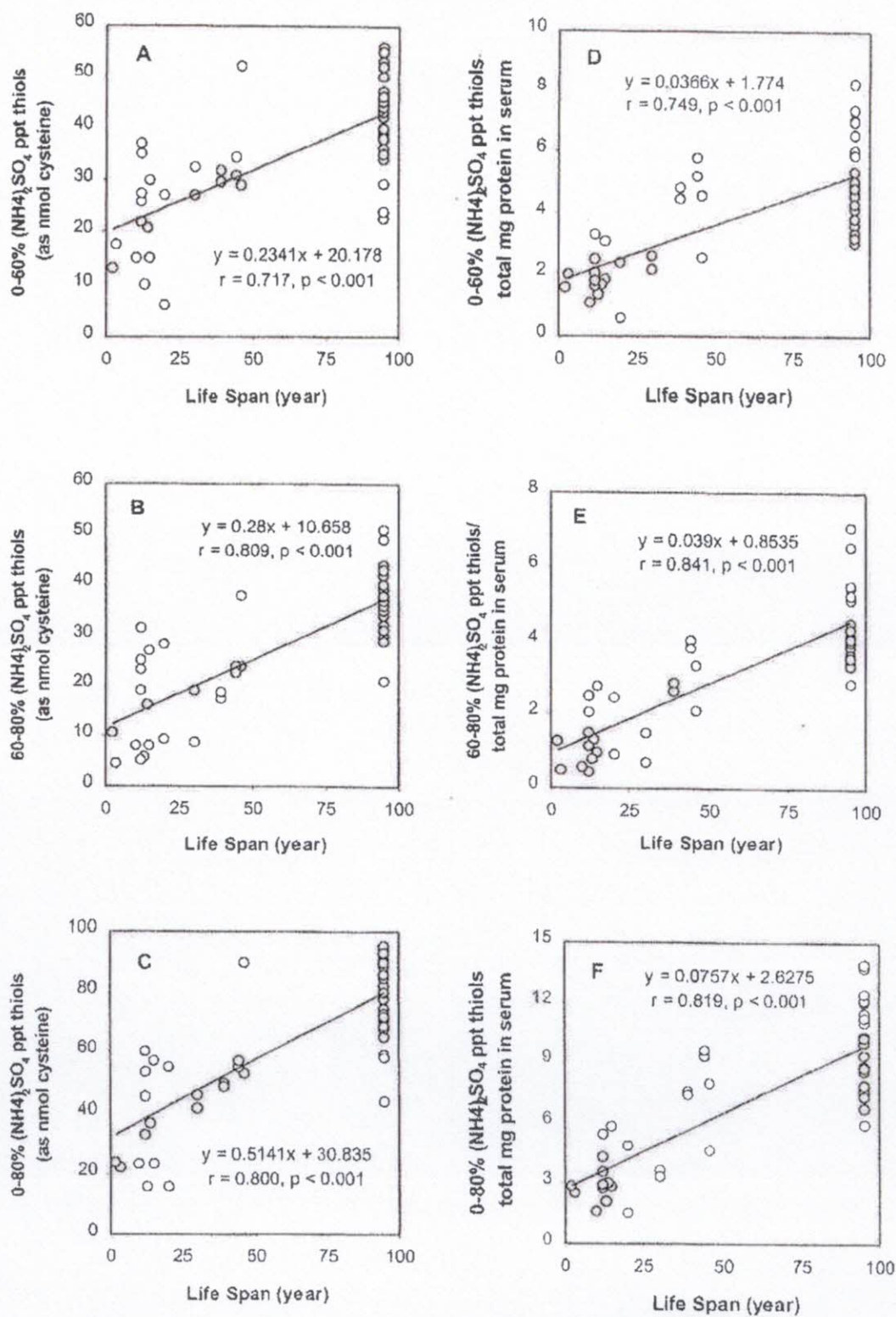
FIG. 2. Pearson correlation between serum protein thiols precipitating 0–60% (top panel, A and D), 60–80% (middle panel, B and E), 0–80% (bottom panel, C and F) saturated ammonium sulphate and the life spans of 47 samples from 17 mammalian species before (left panel A–C) and after (right panel D–F) the total serum protein correction. Thiol proteins are calculated as present in 200 $\mu l$ serum.

TABLE 1. THE INFLUENCE OF TOTAL PROTEIN AND PERCENT OF PROTEIN RECOVERY
FROM SERA ON THIOL DETERMINATIONS AND CORRELATION TO LIFE SPAN

| Serum protein parameter[a] | Correlation coefficient (n = 15) | p |
|---|---|---|
| 0–60% ppt. thiols versus % protein recovery | $r = 0.03$ | >0.05 |
| 60–80% ppt. thiols versus % protein recovery | $r = 0.05$ | >0.05 |
| 0–80% ppt. thiols versus % protein recovery | $r = 0.01$ | >0.05 |
| % protein recovery versus life span | $r = 0.27$ | >0.05 |
| Mg total serum protein versus life span | $r = 0.29$ | >0.05 |

[a]Proteins were precipitated from sera with 0–60% or 60–80% saturated ammonium sulfate and total serum proteins were determined by bicinchoninic acid/copper sulfate reagent.

tions nor do they correlate to the longevity of mammals. In addition, the total protein in sera also did not give a significant correlation to the lifespan of mammals (Table 1; $r = 0.29$, $p > 0.05$).

*Serum thiols and mammalian life span*

Serum proteins are primary informational and nutritional molecules, which are in direct daily contact with the tissues of the body. Any metabolic disturbance in oxidation/reduction balance influencing health and survival, could then be very well-reflected by the thiol/oxidized thiol content of serum. In order to test this hypothesis, the thiol content of 0–60% and 60–80% $(NH_4)_2SO_4$ precipitated proteins from sera have been compared in relation to the life span of 17 mammals. The data presented in Figure 2A–F establish that both the thiol-poor (0–60%) and thiol-rich (60–80%) protein fractions from sera significantly correlated ($r = 0.717$, $p < 0.001$; $r = 0.809$, $p < 0.001$) to the life spans of the 17 mammalian species that supplied the serum samples. Interestingly, a strong correlation to life span was determined for the 60–80% $(NH_4)_2SO_4$-precipitated class of serum proteins ($r = 0.841$, $p < 0.001$), which, although it represented only approximately 29% of the total recoverable protein, it in turn was the most sensitive to oxidation because of its rich thiol content. Moreover, when the thiol-poor (0–60%) and thiol-rich (60–80%) protein fractions were pooled together, there still remained a highly significant correlation to life span (Fig. 2C; $r = 0.819$, $p < 0.001$). It was concluded from these data that a general reduction in thiol content of serum protein was strongly associated with a shorter life span, which, in turn, is consistent with oxidative stress being a key factor in limiting survival. An additional analysis of the thiol content of $(NH_4)_2SO_4$-precipitated fractions was carried out to correct for any differences in protein content in the various sera for each of the 17 mammals, that could have been in turn, be a potential variable in the final statistical consideration. It can be seen easily that, when protein content as a potential variable was taken into consideration, there was an improvement in the strength of the correlation coefficient between serum thiols and life span (Fig. 2, right panel D–F). It was concluded that, although the amount of serum protein might influence thiol content, the thiols themselves in the $(NH_4)_2SO_4$ precipitated fractions independently and strongly correlated to life span, because a significant correlation was still present even in the absence of correction for protein content (Fig. 2, left panel A–C).

## CONCLUSION

The importance of this study lies in the scientific links it provides surrounding the major hypothesis of aging. Mutations resulting from an age-related increase in DNA damage has been one of the most dominant explanations of aging.[1,2] Another explanation for increased age-related accumulations of DNA damage has been proposed as being caused by cellular generated oxygen-centered radicals (i.e., oxidative stress) or deficient DNA repair.[1,2,6–8] Here, we

report that increased serum protein thiols correlate to increased mammalian life span (Fig. 2). Under these conditions, oxidative stress would oxidize thiols, thus, reducing both thiols and life span. At least one thiol-containing protein that would be sensitive to redox imbalance is PARP, and when PARP is oxidized, it would inhibit DNA repair and predict life span and aging.[17,20,27,33,35,42] In addition, oxidized thiols would also reflect oxidized DNA damage and inhibited DNA repair, both of which would increase mutation and thereby reduce life span. Hence, in conclusion, we view our data as supplying a causative interaction between the best supported scientific theories of aging; namely mutation, DNA repair deficiency, and oxidative stress.

Clearly, the key discovery in this study is linking aging, oxidative stress, and DNA repair via serum thiols (Fig. 2)[33-36] and PARP.[27,39,43] The rationale for showing the dependence of DNA damage and mutation on oxidative stress has already been made,[1,2,6-8,37] but the relative contribution of oxidative stress to inhibited DNA repair as an individual health-risk factor has been appreciated only partially.[20,23-25,38] The hypothesis explored here is that the correlation between serum thiols and lifespan (Fig. 2) reflects the antioxidant status of an individual, which, in turn, indirectly translates into protecting PARP from oxidation, inactivation, and inhibited DNA repair capacity. There are no simple cost-effective ways to screen for either mutation, DNA damage, or survival, and, hence, identifying new endpoints as a means of estimating health risk in populations for the purpose of developing diagnostic and treatment strategies has not been forthcoming. Now that serum thiols have been shown to be closely associated to life span (Fig. 2), there seems to be a way to study a parameter associated with DNA repair and linked to oxidative stress and DNA damage (mutation), which may predict the health risk from aging in populations.

It is also of interest that the most thiol-rich serum protein fraction (i.e., 60–80% saturated ammonium sulphate precipitated, Fig. 2, B and E) yielded the strongest correlation to life span. This result is theoretically comforting because endogenous oxidative stress would be expected to alter the most redox-sensitive serum protein components, which in this case was the 60–80% fraction, even though it represented only 28.6 ± 9.6% of the total ammonium sulfate precipitated serum proteins. Because proteins are more signal-transducing molecules than antioxidants, such as cysteine or glutathione, the data reported in this study may provide an opportunity to identify serum proteins of particular interest to the aging process.

## ACKNOWLEDGMENTS

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