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Original Article

Age, Sex, and BMI Influence on Copper, Zinc, and Their Major Serum Carrier Proteins in a Large European Population Including Nonagenarian Offspring From MARK-AGE Study

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Abstract

The analysis of copper (Cu) and zinc (Zn) along with their major serum carriers, albumin (Alb) and ceruloplasmin (Cp), could provide information on the capacity of humans to maintain homeostasis of metals (metallostasis). However, their relationship with aging, sex, body mass index, as well as with nutritional and inflammatory markers was never investigated in a large-scale study. Here, we report results from the European large-scale cross-sectional study MARK-AGE in which Cu, Zn, Alb, Cp, as well as nutritional and inflammatory parameters were

determined in 2424 age-stratified participants (35–75 years), including the general population (RASIG), nonagenarian offspring (GO), a well-studied genetic model of longevity, and spouses of GO (SGO). In RASIG, Cu to Zn ratio and Cp to Alb ratio were higher in women than in men. Both ratios increased with aging because Cu and Cp increased and Alb and Zn decreased. Cu, Zn, Alb, and Cp were found associated with several inflammatory as well as nutritional biomarkers. GO showed higher Zn levels and higher Zn to Alb ratio compared to RASIG, but we did not observe significant differences with SGO, likely as a consequence of the low sample size of SGO and the shared environment. Our results show that aging, sex, body mass index, and GO status are characterized by different levels of Cu, Zn, and their serum carrier proteins. These data and their relationship with inflammatory biomarkers support the concept that loss of metallostasis is a characteristic of inflammaging.

Keywords: Albumin, Ceruloplasmin, Chronic inflammatory status, Homeostasis, Metallostasis

In the last decade, multiple attempts to identify reliable biomarkers of biological age, physical function decline, frailty, and age-related diseases have been made to help clinicians with the early detection of disability or incipient diseases (1,2). Among the entire panel of biomarkers, plasma copper (Cu) to zinc (Zn) ratio (CuZn) and the respective serum protein carriers, ceruloplasmin (Cp) and albumin (Alb), have been found associated with the aging process and age-related diseases (3–6). This is consistent with the presumed loss of metallostasis (homeostasis of trace elements) as an integrative hallmark of aging (7).

In humans, increased values of plasma CuZn have been reported in aging (3,8) as well as in several pathological conditions (9) and are associated with mortality risk in persons older than 65 years (3).

The serum concentrations of Cu and Zn are strictly regulated by compensatory homeostatic mechanisms that act to stabilize Cu and Zn concentrations in human plasma (10). However, during aging, the serum concentration of Zn gradually decreases (3–5,11) whereas Cu increases (3,8). Both nutritional and inflammatory factors are thought to contribute to these changes. The serum concentration of Cu is tightly related to the concentration of its major serum-binding protein, Cp, which is an important component of the acute phase (12). Serum Zn concentration, on the other hand, is related to serum levels and oxidative state of Alb which, in turn, are affected by aging as well as by nutritional and inflammatory status (13,14).

Regarding the various conditions that may lead to an increased CuZn with advancing age, the following hypotheses were posited: (a) a decrease of serum Zn as a consequence of hypalbuminaemia often present in older adults (14,15), (b) a displacement of Zn from the serum Alb pool to other tissues and compartments to sustain stress response (16), and (c) an increase of Cu–Ceruloplasmin to combat the increasing stress conditions occurring with advancing age and in the presence of age-related diseases (3). Hence, the association of CuZn with mortality in older persons could be the consequence of these physiopathological changes.

However, our knowledge on the relationship of CuZn with aging is derived from fragmented data, and there are no comprehensive aging studies evaluating this biomarker and the relevant major carriers (Alb and Cp) as well as nutritional and inflammatory markers.

Here, we report results obtained from the European large-scale cross-sectional study MARK-AGE, aimed at investigating biomarkers of aging in age-stratified individuals (age range 35–75 years), including nonagenarian offspring as human longevity model as well as their spouses as controls of the shared environment (17,18). The aims of this study were (a) to establish the sex-stratified age patterns of circulating metallostasis biomarkers in a wide European population, as well as the trajectories of these biomarkers in a model of healthy aging and potential longevity (nonagenarian's offspring); (b) to establish the impact of body mass index (BMI) and the self-rated health status (SRH) on these biomarkers; and (c) to investigate the relationship between these biomarkers with dietary habits as well as circulating lipids and inflammatory mediators.

Method

Study Population, Recruitment, Data, and Blood Collection

MARK-AGE is a European-wide cross-sectional population study aimed at the identification of biomarkers of aging (17,18). Two large groups of participants were recruited, that is, (a) randomly recruited age-stratified individuals from the general population covering the age range 35–75 years (RASIG group, $n = 2262$) and (b) participants born from a long-living parent (nonagenarian) belonging to a family with long-living sibling(s) already recruited in the framework of the EU GEHA project (Genetics of Healthy Aging (19); GO group, $n = 528$). For genetic reasons, such individuals ("GEHA offspring") are expected to age at a slower rate. They were recruited together with their spouses (SGO group, $n = 305$) as controls of the shared environment.

Prior to the recruitment of participants, each PI involved obtained the local Ethics Committee approval. Exclusion criteria were the following: (a) self-reported seropositivity for human immunodeficiency virus, for hepatitis B virus (except seropositivity by vaccination) or hepatitis C virus; (b) measured seropositivity for hepatitis B virus and hepatitis C virus; (c) presence of a diagnosed cancer disease and current use of anticancer drugs or glucocorticoids (chronic treatment); (d) less than 50% of lifetime spent in the country of residence; or (e) inability to give informed consent; or (f) any acute illness (eg, common cold) within 7 days preceding blood collection (17,18). Other details of the recruitment procedures and of the collection of anthropometric, clinical, and demographic data have been published elsewhere (20–24).

Participants were asked to rate their physical health using a standard 5-point scale with the responses excellent, very good, good, fair, or poor (25). In order to obtain comparable group size, those answering excellent and very good and those answering fair and poor were grouped together, respectively.

Plasma isolation procedure has been described by Moreno-Villanueva et al. (20). Briefly, plasma was isolated from LiHep-whole blood (obtained by phlebotomy after overnight fasting), by centrifugation at 300 *g*, 15 minutes, room temperature (RT).

Samples were then shipped from the various recruitment centers to the MARK-AGE Biobank located at the University of Hohenheim, Stuttgart, Germany. From the Biobank, coded samples were subsequently sent to the Sapienza University of Rome on dry ice to be stored in liquid nitrogen until analysis of CuZn levels.

Copper and Zinc Determination

Plasma Zn and Cu were determined by a Thermo XII Series ICP-MS (Thermo Electron Corporation, Waltham, MA) by adapting methods used for the measurement of trace elements in human plasma with slight modifications (3). Samples were thawed, centrifuged (2000 *g*, 10 minutes at RT), and diluted 1:20 with a diluent containing 0.1% Triton X-100 (BDH Chemicals), 0.1% Trace

Select Ultra HNO_3 (Sigma–Aldrich, Steinheim, Germany), and 10 ppb Rh (Merck, Darmstadt, Germany) as internal standard. External multielement calibration solutions containing Zn and Cu (blank to 100 ppb) were prepared by serial dilution of a parent multielement solution (Inorganic Ventures, Christiansburg, VA), using the same diluent used for the samples. Data were acquired for ^{64}Zn and ^{63}Cu. The instrument was operated with a MicroMist nebulizer (Glass Expansion, Melbourne, Australia), a Cinnabar Spray chamber with helix (Glass Expansion), a single piece quartz torch (1.5-mm i.d. injector) together with Xi interface cones and a Cetac ASX 100 autosampler (CETAC Technologies, Omaha, NE). The instrument was operated in collision cell technology with kinetic energy discrimination mode by introducing a mixture of 8% hydrogen in helium into the octapole cell. The gases were introduced into the cell under mass flow control through stainless steel lines. The ICP-MS was operated using 1250 W of RF power, 0.79 L/minute of nebulizer gas flow, 1.15 L/minute of auxiliary gas flow, 16.0 L/minute of cool gas flow, 20-ms dwell time, 90-second sample uptake, and 60-second wash time (3 repeats per sample). The instrument was calibrated daily with oxide and doubly charged ions set below 0.03%. The accuracy of the system was routinely checked with quality control samples prepared from Seronorm Trace Element Serum level 1 and level 2 (Sero AS, Billingstad, Norway).

Batch effect correction

The measurements of Cu and Zn were performed over 3 years in a small amount of plasma (triplicate measurements, each 50 µL). Such a long period for the analysis of thousands of samples exposes to potential batch effects. Correction of batch effects was performed for all the assessments when data of external reference samples (Seronorm L1 and Seronorm L2, measured within each batch) were outside of 5% from the range of the certified data. Corrections were made by normalization using the reference samples (corrected value = observed value × % mean difference of Seronorm values from certified data). Measurements that were outside 15% from both reference values were repeated or dismissed in the case of the second failure.

Ceruloplasmin and Albumin Determination

Cp and Alb were measured on an LX20 autoanalyzer from Beckman Coulter (Woerden, the Netherlands) with turbidimetric and colorimetric endpoints, respectively. Cp (assay nr A26842) was determined in serum and expressed in mg/dL. Alb (assay nr 442765) was determined in EDTA-plasma and expressed in g/L. Both assays were obtained from Beckman Coulter. In all measurement sessions (usually 100 samples per day), 3 quality control samples were included. The measurements were performed in 5 batches with a mean interassay coefficient of variation of 1.0% for Alb and 4.5% for Cp.

Lipid and Systemic Inflammation Parameters Determination

Lipoprotein determination has been previously reported (20,23,26). The measurements of HS-CRP and fibrinogen were performed on an LX20 autoanalyzer (Beckman Coulter), as described previously (26,27).

Statistical Analysis

Characteristics of the population studied were described using means and *SD* for continuous variables (ie, age, BMI) and frequencies (%) for the categorical variables (sex, BMI classes, age class, groups, country, and health status). The normality of the variables included in the study was tested by both the Kolgomorov Smirnov test and the Shapiro–Wilk test.

The panel of circulating metallostasis biomarkers including Cu, Zn, Alb, Cp, CuZn, Ceruloplasmin to Albumin ratio (CpAlb), Zn to Albumin ratio (ZnAlb), and Cu to Ceruloplasmin ratio (CuCp) were compared among sex, age class, BMI, recruitment center, SRH, and participant groups (GO, SGO, and RASIG) through a Generalized Linear Model (GLM) including age, sex, BMI, and recruitment center as main effects. Pairwise comparisons (Bonferroni's methods for GLM tests) were used to identify significant differences between each categorized variable. The association analysis between metallostasis biomarkers and dietary habits as well as inflammatory and nutritional variables was performed by automatic linear regression (ALR) to show the *importance* of parameters in relation to each metal biomarker. The correlation coefficient of the strongest relationships was estimated by univariate analysis. As previously demonstrated (4), ALR is useful to estimate the importance of biomarkers and other factors included in the analysis on the prediction of the target variables. Only those parameters showing significant association with the dependent variable in both ALR and univariate analysis were shown. All statistical analyses (excluding analysis of batch effects) were carried out using SPSS software (SPSS Inc., Chicago, IL; version 20.0).

Results

Characteristics of the Population

MARK-AGE participants were stratified into four 10-year age groups (35–44, 45–54, 55–64, 65–75 years old). The age distribution of RASIG individuals was almost homogeneous, while GO and SGO individuals fell into age ranges older than 54 years (Supplementary Table S1). The composition of male and female individuals was mostly comparable in all age groups (Supplementary Table S1). In agreement with literature data studying participants in the age range 35–75 years, the BMI increased with age, SRH declined with age, indicating that the analyzed population was effectively representative of a physiological aging process (Supplementary Table S1).

Effect of Aging, Sex, SRH, and BMI on Metal Biomarkers

Identification of outliers and distribution tests

The analysis of metallostasis biomarkers was carried out on plasma from blood samples obtained from $n = 3007$ individuals. Due to positively skewed values, all the variables failed to pass the Kolmogorov–Smirnov test for the normal distribution. For this reason, all the values outside 3-fold the *SD* (for each variable) were excluded from the analysis. This procedure was performed independently for each of the following categories: sex, recruitment center, and age class. After the removal of outliers, all the variables passed the Kolmogorov–Smirnov test for the normal distribution. The resulting population ($n = 2424$) including 445 GO, 1736 RASIG, and 243 SGO is described in Supplementary Table S1.

Main effect of country, sex, age, BMI, and SRH in RASIG

All the main results are reported in Figures 1 and 2. The country has been always considered as a confounding factor in all analyses because all the parameters were different between EU countries ($p < .001$). Cu and Cp as well as CuZn and CpAlb were higher in women than in men ($p < .001$), whereas the opposite was found for Zn and Alb ($p <$

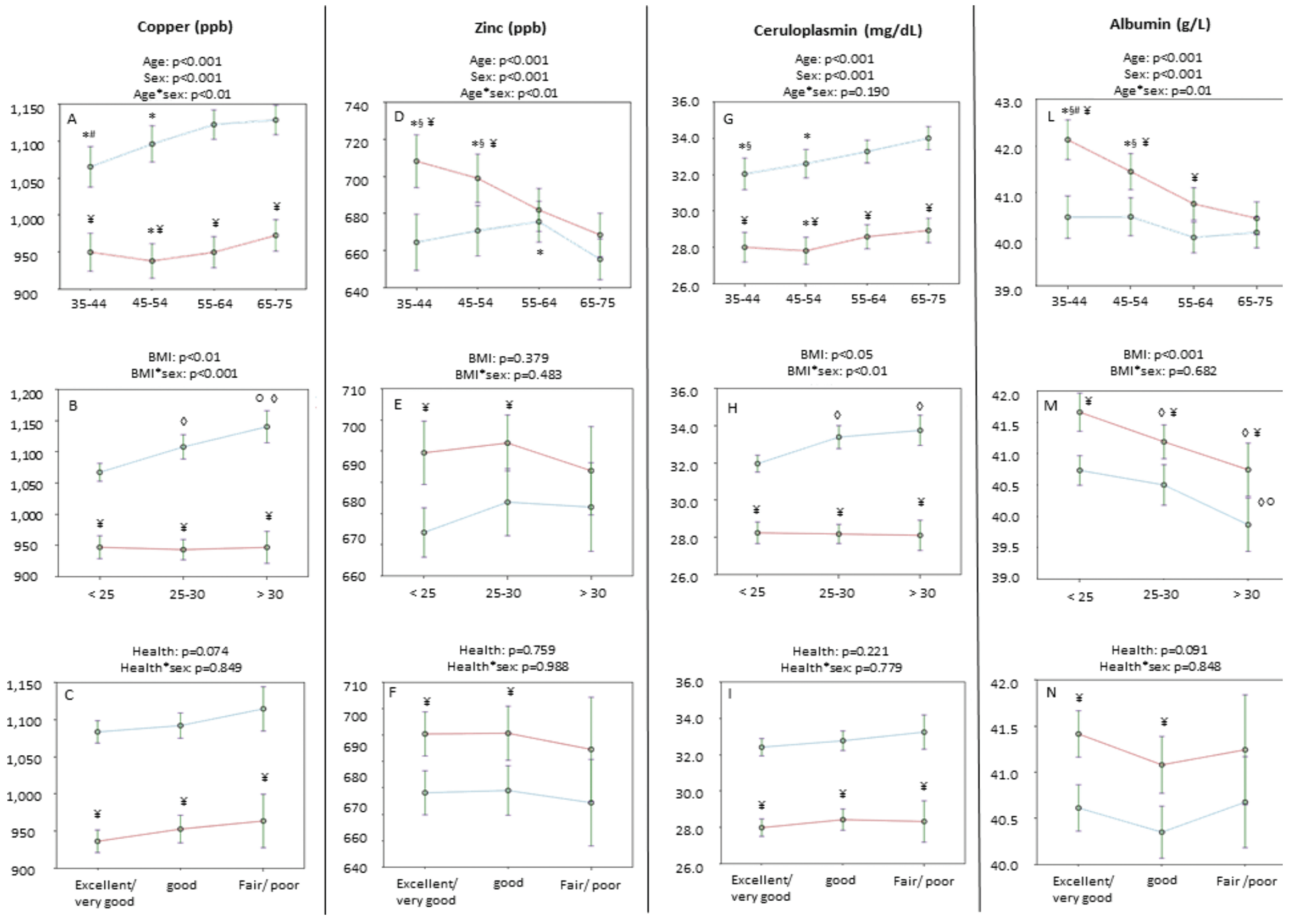

Figure 1. Copper (boxes A, B, C), zinc (boxes D, E, F), ceruloplasmin (boxes G, H, I), and albumin (boxes L, M, N) levels in 4 age classes (35–44, 45–54, 55–64, and 65–75), in 3 BMI classes (<25, 25–30, and >30), and in 3 selfrated health categories (excellent/very good, good, and fair/poor) in RASIG population. Men in grey line and women in black line. Comparative analyses were performed by GLM considering the effects of gender, BMI, recruitment center, and age. *$p < .05$ with respect to age class 65–75; §$p < .05$ with respect to age class 55–64; #$p < .05$ with respect to age class 45–54; ◊$p < .05$ with respect to BMI class less than 25; ○$p < .05$ with respect to BMI class 25–30; ¥$p < .05$ with respect to the value of females of the same category.

.001). No significant sex differences were found for ZnAlb and CuCp. All parameters except CuCp were different among age groups ($p < .05$ for ZnAlb; $p < .001$ for the others). All parameters except Zn and CuCp were different among BMI categories ($p < .05$ for CuZn and Cp; $p < .01$ for Cu and ZnAlb; $p < .001$ for Alb and CpAlb). Only CuZn was significantly different among the SRH categories ($p < .05$).

Sex-stratified comparisons within age, BMI, and SRH categories

Copper.—Copper was higher in women than in men, independent of age, BMI, and health status (Figure 1A–C). Men in the age class 45–54 years showed lower Cu than men in the age class 65–75 (Figure 1A). Women in the 35–44 and 45–54 age classes showed lower Cu values than women in the age class 65–75 (Figure 1A). Cu was also lower in women aged 35–44 years compared to those aged 45–54 years (Figure 1A). Regarding BMI, we found that Cu progressively increases from the lowest to the highest BMI class only in women, while no significant difference was observed in men (Figure 1B).

Zinc.—Zinc was higher in men than in women only within age classes 35–44 and 45–54 (Figure 1D). Zinc in males was also significantly different from zinc in females in the stratified categories of BMI and SRH, except BMI more than 30 (Figure 1E) and "fair/poor" (Figure 1F), respectively. Men in the age classes 55–64 and 65–75 years showed lower Zn levels than those in the age classes 35–44 and 45–54 years (Figure 1D). Women aged 55–64 years showed higher Zn values than those in the age class 65–75 (Figure 1D).

Ceruloplasmin.—Ceruloplasmin was higher in women than in men, independent of age, BMI, and SRH (Figure 1G–I). Men aged 45–54 years showed lower Cp values than those aged 65–75 years (Figure 1G). Women in the age class 65–75 years showed higher Cp values compared to those aged 35–44 and 45–54 years (Figure 1G). Moreover, women in the age class 35–44 years had reduced Cp values when compared with those in the age class 55–64 (Figure 1G). Cp in women was lower in the lowest BMI class (<25 kg/m^2) compared to the other BMI classes (Figure 1H).

Albumin.—Albumin was higher in men than in women with the exception of the oldest age class and the worst SRH class (Figure 1L–N). In men, the youngest age class showed higher Alb values with respect to the other age classes (Figure 1L). Alb was also higher in men aged 45–54 years compared to men aged 65–75 years (Figure 1L). Alb levels in males were higher in the lowest BMI class (<25 kg/m^2) compared to the other BMI classes (Figure 1M). Alb levels in females were lower in the highest BMI class (>30 kg/m^2) compared to the other 2 classes (Figure 1M).

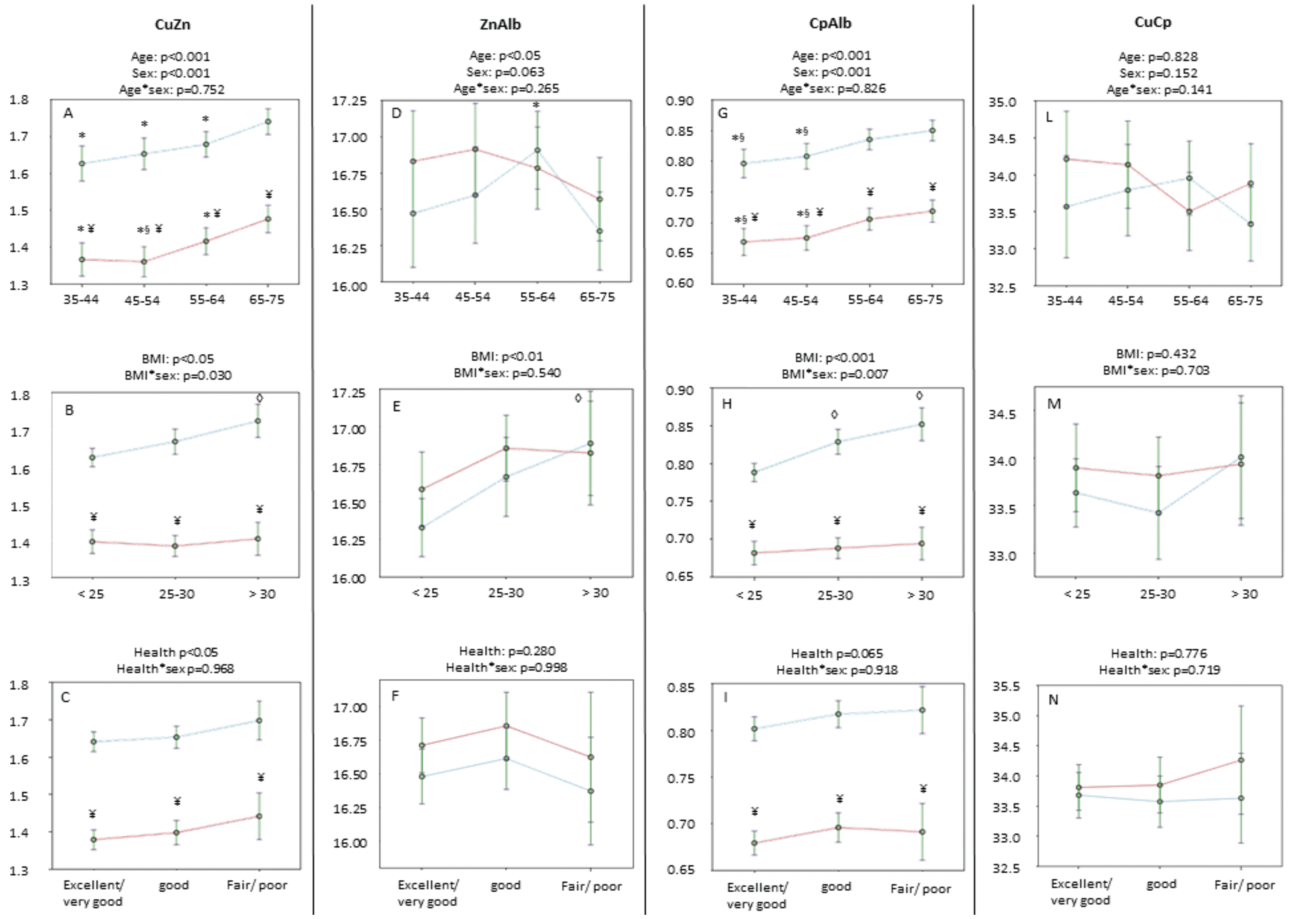

Figure 2. Copper to zinc ratio (boxes A, B, C), zinc to albumin ratio (boxes D, E, F), ceruloplasmin to albumin ratio (boxes G, H, I), copper to ceruloplasmin ratio (boxes L, M, N) levels in 4 age classes (35–44, 45–54, 55–64, and 65–75), in 3 BMI classes (<25, 25–30, and >30), and in 3 self-rated health categories (excellent/very good, good, and fair/poor) in RASIG population. Men in grey line and women in black line. Comparative analyses were performed by GLM considering the effects of gender, BMI, recruitment center, and age. *$p < .05$ with respect to age class 65–75; §$p < .05$ with respect to age class 55–64; #$p < .05$ with respect to age class 45–54; ◊$p < .05$ with respect to BMI class less than 25; ¥$p < .05$ with respect to the value of females of the same category.

Copper to zinc ratio.—CuZn was higher in women than in men, independent of age, BMI, and SRH (Figure 2A–C). Independent of sex, CuZn was higher in the age class 65–75 years compared to all the other age classes (Figure 2A). Men aged 45–54 years showed also lower CuZn values than those aged 55–64 years (Figure 2A). CuZn in females was higher in the highest BMI class (≥30 kg/m^2) compared to the lowest (<25 kg/m^2; Figure 2B). CuZn levels were different between health status categories ($p < .05$) independent of sex (p for sex interaction = .968; Figure 2C). In particular, participants in excellent/very good status displayed lower levels of CuZn than participants in fair/poor status in nonstratified analyses ($p < .05$). However, significant pairwise differences in the population stratified by sex were not detected (Figure 2C), likely due to the reduction in sample size in the stratified analysis.

Zinc to albumin ratio.—Women aged 55–64 years showed higher ZnAlb values than those aged 65–75 years (Figure 2D). ZnAlb in females was lower in the lowest BMI class compared to the highest (Figure 2E).

Ceruloplasmin to albumin ratio.—CpAlb was higher in women than in men, independent of age, BMI, and SRH (Figure 2G–I). Independent of sex, CpAlb was lower in the age classes 35–44 and 45–54 years compared to the age classes 55–64 and 65–75 years (Figure 2G). CpAlb in females was lower in the lowest BMI class compared to the other BMI classes (Figure 2H).

Copper to ceruloplasmin ratio.—No differences were observed in relation to sex, age, BMI classes, or SRH (Figure 2L–N).

Association of Metallostasis Biomarkers With Nutritional and Inflammatory Parameters

From all participants enrolled in the MARK-AGE project, a large number of biomarkers, as well as anthropometric and questionnaire data were collected. As previously described (4), we performed an ALR model to identify the most important predictors of our panel of metallostasis biomarkers among dietary habits (consumption of vegetables, dairy products, fruit, brown bread, white bread, whole-grain bread, eggs, fish, French fries potatoes, meat, vitamins, and alcohol) as well as nutritional, lipid, and inflammatory biomarkers (low-density lipoprotein [LDL], high-density lipoprotein [HDL], total cholesterol, free fatty acid [FFA], triglycerides, albumin, fibrinogen, alpha 2 macroglobulin [A2M], ceruloplasmin, and C-reactive protein [CRP]). We also analyzed each model by univariate analysis to show the coefficient of correlation of each parameter with the dependent variable. Only those parameters that

showed significant associations with both analyses are shown. The results for Cu, Zn, Cp, and Alb are described in Table 1 and for CuZn, CpAlb, ZnAlb, and CuCp in Table 2.

Copper

The most important predictor of Cu was ceruloplasmin (importance = 0.912, $p < .01$), followed by sex (importance = 0.035, $p < .01$), triglycerides (importance = 0.028, $p < .01$), country (importance = 0.007, $p < .01$), CRP (importance = 0.006, $p < .01$), and fibrinogen (importance = 0.002, $p < .01$; Table 1).

Zinc

The most important predictor of Zn was albumin (importance = 0.424, $p < .01$), followed by country (importance = 0.286, $p < .01$), triglycerides (importance = 0.067, $p < .01$), ceruloplasmin (importance = 0.061, $p < .01$), age (importance = 0.041, $p < .01$), and sex (importance = 0.039, $p < .01$; Table 1).

Albumin

The most important predictor of Alb was country (importance = 0.391, $p < .01$), followed by sex (importance = 0.182, $p < .01$), age (importance = 0.173, $p < .01$), cholesterol (importance = 0.074, $p < .01$), CRP (importance = 0.059, $p < .01$), fibrinogen (importance = 0.042, $p < .01$), and vegetables consumption (importance = 0.022, $p < .05$; Table 1).

Ceruloplasmin

The most important predictor of Cp was sex (importance = 0.387, $p < .01$), followed by CRP (importance = 0.352, $p < .01$), fibrinogen (importance = 0.098, $p < .01$), country (importance = 0.055, $p < .01$), A2M (importance = 0.027, $p < .01$), triglycerides (importance = 0.025, $p < .01$), HDL (importance = 0.023, $p < .01$), and LDL (importance = 0.014, $p < .05$; Table 1).

Copper to zinc ratio

The most important predictor of CuZn was Cp (importance = 0.701, $p < .01$), followed by albumin (importance = 0.089, $p < .01$), country (importance = 0.088, $p < .01$), sex (importance = 0.074, $p < .01$), CRP (importance = 0.016, $p < .01$), age (importance = 0.014, $p < .01$), and FFA (importance, $p < .01$; Table 2).

Zn to Alb ratio

The most important predictor of ZnAlb was Alb (importance = 0.364, $p < .01$), followed by country (importance = 0.326,

Table 1. Association of Cu, Zn, Cp, Alb With Dietary, Nutritional, Lipid, and Inflammatory Parameters

		ALR		Univariate	
		Importance	p^*	Coefficient	$p^\dagger$
Copper	Ceruloplasmin	0.912	<.01	24.369	<.01
	Sex	0.035	<.01	—	—
	Triglycerides	0.028	<.01	15.015	<.01
	Country	0.007	<.01	—	—
	C-reactive protein	0.006	<.01	15.839	<.01
	Fibrinogen	0.002	<.05	28.014	<.01
Zinc	Albumin	0.424	<.01	8.624	<.01
	Country	0.286	<.01	—	—
	Triglycerides	0.067	<.01	14.668	<.01
	Ceruloplasmin	0.061	<.01	1.603	<.01
	Age	0.041	<.01	—	—
	Sex	0.039	<.01	—	—
Albumin	Country	0.391	<.01	—	—
	Sex	0.182	<.01	—	—
	Age	0.173	<.01	—	—
	Cholesterol	0.074	<.01	0.277	<.01
	C-reactive protein	0.059	<.01	−0.139	<.01
	Fibrinogen	0.042	<.01	−0.231	<.01
	Vegetables consumption	0.022	<.01	0.252	<.05
Ceruloplasmin	Sex	0.387	<.01	—	—
	C-reactive protein	0.352	<.01	0.482	<.01
	Fibrinogen	0.098	<.01	0.836	<.01
	Country	0.055	<.01	—	—
	Alpha 2 macroglobulin	0.027	<.01	0.012	<.01
	Triglycerides	0.025	<.01	−0.771	<.01
	HDL	0.023	<.01	1284	<.01
	LDL	0.014	<.05	0.637	<.01

Notes: ALR = automatic linear regression; LDL = low-density lipoprotein; HDL = high-density lipoprotein. Association of Cu, Zn, Alb, Cp with dietary habits (consumption of vegetables, dairy products, fruit, brown bread, white bread, whole-grain bread, eggs, fish, French fries' potatoes, meat, vitamins, and alcohol) as well as nutritional and inflammatory biomarkers (LDL, HDL, total cholesterol, free fatty acid, triglycerides, albumin, fibrinogen, alpha 2 macroglobulin, Cp, and C-reactive protein) considering the effect of age, country, and sex as factors. "Importance" represents the significance of each variable as a predictor of the related parameter. The coefficient of correlation was computed by univariate analysis only for continuous variables. Only those parameters showing significant associations by both ALR and univariate analysis are shown.

$^*p < .05$ by the ALR model.

$^\dagger p < .05$ by univariate analysis.

Table 2. Association of CuZn, ZnAlb, CuCp, CpAlb With Dietary, Nutritional, Lipid, and Inflammatory Parameters

		ALR		Univariate	
		Importance	p*	Coefficient	p†
CuZn	Ceruloplasmin	0.701	<.01	0.033	<.01
	Albumin	0.089	<.01	−0.022	<.01
	Country	0.088	<.01	—	—
	Sex	0.074	<.01	—	—
	C-reactive protein	0.016	<.01	0.027	<.01
	Age	0.014	<.01	—	—
	FFA	0.009	<.01	0.065	<.01
ZnAlb	Albumin	0.364	<.01	−0.204	<.01
	Country	0.326	<.01	—	—
	Triglycerides	0.082	<.01	0.381	<.05
	Ceruloplasmin	0.078	<.01	0.036	<.01
	Sex	0.039	<.05	—	—
	Age	0.034	<.05	—	—
CuCp	Ceruloplasmin	0.666	<.01	−0.341	<.01
	Triglycerides	0.125	<.01	1.584	<.01
	Country	0.123	<.01	—	—
CpAlb	Ceruloplasmin	0.861	<.01	0.025	<.01
	Albumin	0.139	<.01	−0.019	<.01
	C-reactive protein	0.001	<.01	0.015	<.01

Notes: ALR = automatic linear regression; FFA = free fatty acid. Association of CuZn, ZnAlb, CuCp, CpAlb with dietary habits (consumption of vegetables, dairy products, fruit, brown bread, white bread, whole-grain bread, eggs, fish, French fries' potatoes, meat, vitamins, and alcohol) as well as nutritional and inflammatory biomarkers (low-density lipoprotein, high-density lipoprotein, total cholesterol, FFA, triglycerides, albumin, fibrinogen, alpha 2 macroglobulin, Cp, and C-reactive protein) considering the effect of age, country, and sex as factors. "Importance" represents the significance of each variable as a predictor of the related parameter. The coefficient of correlation was computed by univariate analysis only for continuous variables. Only those parameters showing significant associations by both ALR and univariate analysis are shown.

*$p < .05$ by the ALR model.

†$p < .05$ by univariate analysis.

$p < .01$), triglycerides (importance = 0.082, $p < .05$), Cp (importance = 0.078, $p < .01$), sex (importance = 0.039, $p < .05$), and age (importance = 0.034, $p < .05$; Table 2).

Copper to ceruloplasmin ratio

The most important predictor of CuCp was Cp (importance = 0.666, $p < .01$), followed by triglycerides (importance = 0.125, $p < .01$) and country (importance = 0.123, $p < .01$; Table 2).

Ceruloplasmin to albumin ratio

The most important predictor of CpAlb was Cp (importance = 0.861, $p < .01$), followed by Alb (importance = 0.139, $p < .01$) and CRP (importance = 0.001, $p < .01$; Table 2).

Metallostasis Biomarkers in GO, SGO, and RASIG Population

Zn, Cu, Alb, Cp, CuZn, ZnAlb, CuCp, and CpAlb levels were compared between GO, SGO, and RASIG by a GLM including age, sex, BMI, and recruitment center effects, in order to investigate the influence of genetic or environmental factors on their plasma levels (Table 3). The analysis was performed in participants aged 55 years or older to reduce bias due to the low number of GO and SGO in the lower age classes. Sex, age, and recruitment center were always considered as independent variables in the analysis.

Table 3. Zinc, Copper, Ceruloplasmin, Albumin, Copper to Zinc Ratio, Ceruloplasmin to Albumin Ratio, Zinc to Albumin Ratio, and Copper to Ceruloplasmin Ratio Comparison Among GO, RASIG, and SGO

Variable	Group	Mean	St. Err	95% CI	
Zn (ppb)	GO (a)	694.6[b]	5.46	683.9	705.4
	RASIG (b)	670.9[a]	3.02	664.9	676.8
	SGO (c)	684.4	10.06	664.7	704.2
Cu (ppb)	GO (a)	1030.7	9.7	1011.6	1049.9
	RASIG (b)	1043.9	5.4	1033.3	1054.5
	SGO (c)	1060.5	17.9	1025.2	1095.7
Cp (mg/dL)	GO (a)	31.3	0.29	30.7	31.9
	RASIG (b)	31.2	0.16	30.9	31.6
	SGO (c)	32.3	0.54	31.3	33.4
Alb (g/L)	GO (a)	40.1	0.16	39.8	40.4
	RASIG (b)	40.4	0.09	40.2	40.6
	SGO (c)	40.5	0.30	39.9	41.0
CuZn	GO (a)	1.51[b]	0.02	1.50	1.55
	RASIG (b)	1.58[a]	0.01	1.56	1.60
	SGO (c)	1.57	0.03	1.50	1.63
CpAlb	GO (a)	0.79	0.01	0.77	0.81
	RASIG (b)	0.77	0.01	0.76	0.78
	SGO (c)	0.81	0.02	0.78	0.85
CuCp	GO (a)	33.1	0.24	32.7	33.6
	RASIG (b)	33.6	0.13	33.4	33.9
	SGO (c)	32.8	0.48	31.9	33.7
ZnAlb	GO (a)	17.3[b]	0.14	17.1	17.6
	RASIG (b)	16.6[a]	0.07	16.5	16.8
	SGO (c)	16.9	0.25	16.4	17.4

Notes: Alb = albumin; Cp = ceruloplasmin; CuZn = Cu to Zn ratio; CpAlb = Cp to Alb ratio; ZnAlb = Zn to Alb ratio; CuCp = Cu to Cp ratio. GO (n = 425), SGO (n = 223), and RASIG (n = 963) values are represented as estimated means, standard error (Std. Err.), and 95% confidence interval (CI) obtained by generalized linear models. All models included the effects of sex, recruitment center, BMI, and age. Bonferroni was used as post hoc test. Significant differences ($p < .05$) among groups are marked with the associated superscript.

As given in Table 3, Zn and ZnAlb were higher in GO with respect to RASIG. Conversely, CuZn was lower in GO with respect to RASIG (Table 3). These differences were confirmed also when including the SRH as a confounding variable (data not shown). It should be noted that SRH was not significantly different between GO, RASIG, and SGO after adjusting for age, sex, and country (Supplementary Table S2).

Discussion

This work shows how Cu, Zn, Cp, Alb as well as their ratios are influenced by age, sex, environmental factors, and the particular genetic background of a longevity model.

Concerning sex, all parameters involving Cu or Cp (Cu, Cp, CuZn, and CpAlb) were higher in women compared to men whereas the opposite was found for Zn and Alb (Figures 1 and 2). The causes of a sex effect on CuZn have been previously attributed by others to an increased Cu dietary intake or to the use of Cu-containing supplements (28,29). However, another study that strictly monitored Cu intake in mice, did not find any evidence that the difference in CuZn between males and females was due to nutritional intake (30). An

alternative explanation is offered by the hormonal influence on Cu and Cp levels (31). This hypothesis is in line with the finding in this study by which almost 74% of Cu outliers (values outside 3 times *SD*) were represented by women who made use of hormones and that most of them were below menopausal age (Supplementary Figure S1). The same phenomenon was observed for Cp (data not shown). These observations are also in agreement with a previous study reporting an influence of the menstrual cycle on Cu plasma levels (32). It is important to note that the SRH was not significantly different between women with "normal Cu levels" and women considered "Cu outliers," thus confirming that these outliers are not affected by pathological conditions (Supplementary Table S3). Interestingly, we have not observed any difference for ZnAlb and CuCp between men and women, suggesting that this aspect of metallostasis is similarly regulated in both sexes (Figure 1).

Concerning aging, this study confirms that CuZn and CpAlb increase with advancing age both in women and in men. As expected, CuZn and CpAlb displayed a parallel slope with aging (Supplementary Figure S2). The linear increase in the levels of Cu and Cp with aging is the major factor contributing to the age-related increase of CuZn and CpAlb in women. Conversely, the linear decrease of Zn and Alb was the major contributing factor in men (Figure 1). This finding is in line with a previous study showing that until the age of 65 years a decrease in serum Zn with aging is observed only in men (33). Because skeletal muscle is the largest pool of body Zn, this phenomenon may be related to the different pattern of muscle loss during aging in males (1.9 kg/decade) and in females (1.1 kg/decade) within the age range considered (34). Another study also observed a plateau of muscle loss in women from 50s to 60s before starting to decline (35). However, at a more advanced age, it is well established that plasma Zn declines in both sexes (3,8).

Previous studies in humans and animal models have clearly demonstrated that inflammatory stimuli modify serum concentration of Cu and Cp as well as Zn and Alb by increasing the former 2 (36–38) and decreasing the latter 2 (39) through changes in the liver metabolism during inflammation (5,40,41). Increased circulating levels of IL-6 are an established component of *inflammaging* (42). However, IL-6 can promote the synthesis of Cp mediated by the transcription factor, FOXO1 (38). In parallel, proinflammatory cytokines can also upregulate the Zn-importer Zip-14 in the liver contributing to decrease serum Zn during the inflammatory response (43). It is not surprising, in fact, to observe that the highest levels of CuZn were found in the highest age class for both sexes (Figure 2A). Indeed, the oldest class is likely to display a higher accumulation of senescent cells which, in turn, have been considered a major contribution to the "inflammaging" through their secretory phenotype (44). This phenomenon suggests that treatments aimed at removing the excessive accumulation of senescent cells (eg, senolytics) (45) might be potentially able to correct at least in part the excessively high levels of CuZn in aging or in pathological conditions associated with aging. Specific studies in this area would be helpful to clarify this hypothesis. CuZn was also the only parameter associated with SRH, thus confirming that it can be considered a sensitive biomarker of health status.

Previous studies have shown that an increase of plasma Cu and hypoalbuminemia are related to fat accumulation (46) and excessive adiposity (47), respectively. In this article, we show that high Cu and low Alb are distinctive traits of obesity (BMI >30) only in women. However, it is surprising that this phenomenon was not observed in men where only a linear decrease of Alb with BMI was found. This intersex dichotomy of Cu levels in obesity has been observed by some authors (48) but not others (49). These findings may indicate that men and women tune metallostasis to the increased inflammatory state of obesity in a different way.

Inflammatory parameters were the recurrent predictors of our metallostasis biomarkers. CRP and fibrinogen, 2 of the most important inflammatory acute-phase proteins of hepatic origin, were among the most important predictors of 5 and 3 of 8 biomarkers, respectively. CRP, as expected, was found positively associated with Cp and negatively with Alb. Considering that Alb is the major carrier and predictor of Zn, it was paradoxical to observe that Cp positively correlated to Zn. However, it has been demonstrated that intraperitoneal injection of Zn in rats increases serum Cp levels (50). Hence, feedback mechanisms regulating these 2 biomarkers make the interpretation of their age-related pattern more complex than expected.

The other correlations among inflammatory and metallostasis biomarkers were as expected. The proinflammatory mediators FFA and the acute-phase response protein A2M were found associated with CuZn and Cp, respectively.

Triglycerides were also found to be important predictors of metallostasis (present in 5–8 biomarkers). Triglycerides were positively correlated to Cu, confirming previous observations (51), but surprisingly inversely correlated to Cp. We further observed that this phenomenon was distinctive of men (data not shown) confirming the intersex variability previously discussed on the relationship between metallostasis and BMI. Given the relevance of Cu metabolism in obesity, these findings warrant further investigations.

Interestingly, total, HDL, and LDL cholesterol were not among the main predictors of Zn and Cu but were found positively correlated with Alb or Cp, thus suggesting that the carriers may be more related to cholesterol metabolism than their metal ligands.

Vegetable consumption was positively associated with Alb and was the only dietary parameter identified among the metallostasis predictors. This could be due to the protection of plant proteins against urinary Alb excretion (52) or to the anti-inflammatory and antioxidant properties of phytochemicals found in vegetables.

Concerning our human longevity model, GO showed lower CuZn compared to RASIG (Table 2). This finding is in line with the lower inflammation score observed in centenarian offspring compared to age-matched controls in another study (53). However, the lack of a significant difference between GO and SGO suggests that their shared environmental and lifestyle factors could be the predominant component driving this difference. However, SGO display also a higher variability as a consequence of their reduced sample size. Hence, the lack of significant differences between GO and SGO should be interpreted with caution. The higher Zn values of GO are responsible for their lower CuZn levels compared to RASIG, also because no differences between the 2 groups were found for Cu (Table 3). Surprisingly, no difference was observed between GO, SGO, and RASIG for CpAlb. Thus, the levels of trace elements rather than their carriers can better characterize GO. Furthermore, the difference observed in ZnAlb between GO and RASIG suggests a higher degree of saturation of Alb and a distinctive Zn homeostasis in GO (Table 3). It is also possible that the increased Zn levels of GO may be related to the increased circulating levels of cysteine. Indeed, the thiol group of cysteine governs its ability to bind metals, including Zn (54), and increased levels of cysteine have been previously found in GO compared to RASIG (55). This is consistent also with our previous finding of tighter regulation of intracellular Zn in peripheral blood mononuclear cells from GO compared to RASIG (56).

Disentangling ZnAlb from the individual contribution of Zn and Alb is critical for a correct interpretation of paradoxical data, such as the presence of high levels of ZnAlb both in the longevity model of GO (Table 3) and in participants with high BMI (Figure 2). In fact, while increased Zn explains the difference of ZnAlb between GO and RASIG, a decreased Alb explains the positive association of ZnAlb with BMI. This finding is in line with that observed in the RASIG population by which reduced Alb has been found associated with unfavorable metabolic profile characterized by increased adipose tissue and inflammation and adiposity.

In summary, we found that different levels of Cu, Zn, and their respective serum carrier proteins are distinctive of sex, BMI, and GO status, a widely studied longevity model (nonagenarian's offspring). We additionally confirmed that CuZn is reduced in nonagenarian's offspring and can be considered an important marker to monitor the chronic inflammatory status associated with aging.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of Interest

None declared.

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