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RESEARCH ARTICLE

Plasma copper/zinc ratio: an inflammatory/nutritional biomarker as predictor of all-cause mortality in elderly population

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Abstract Associations have been reported between plasma Cu and Zn levels and the incidence of the most important age-related diseases. Previously proposed methods of using plasma Cu/Zn as a predictor of all-cause mortality have been derived from populations in which old and very old subjects were underrepresented. The purpose of this paper is to estimate the usefulness of plasma Cu/Zn as a sensitive biomarker of harmful inflammatory or nutritional changes in the elderly and its incremental prognostic utility as a predictor of all-cause mortality in a functionally independent elderly Italian cohort. The association between plasma Cu/Zn and inflammatory (CRP, ESR, IL-6) or nutritional (albumin, BMI) markers was studied in 498 elderly subjects. Blood samples were taken from 164 healthy 20- to 60-year-old volunteer controls. A 3.5 years prospective follow-up study of mortality by age-related diseases was performed in $n = 218$ over 70-year-olds. Plasma Cu/Zn ratio was associated with all the inflammatory markers studied, as well as with serum albumin, and predicted 3.5 years mortality in subjects over 70. Plasma Cu/Zn was higher in women than men and increased with advancing age. Subjects with stable cardiovascular disease (CVD) displayed higher plasma Cu/Zn than those without, due mainly to increased plasma Cu. However, most of the age-related changes of Cu/Zn resulted from a progressive decline of plasma Zn. Cu/Zn ratio may be considered an important clinical inflammatory-nutritional biomarker as well as a significant predictor of all-cause mortality in over 70-year-olds.

Keywords Zinc · Copper · Inflammation · Biomarker · Mortality · Elderly

Abbreviation

CVD	Cardiovascular disease
CRP	C-reactive protein
ESR	Erythrocytes sedimentation rate
IL-6	Interleukin-6
BMI	Body mass index
NSAIDs	Non-steroidal anti-inflammatory drugs
ACE	Angiotensin converting enzyme
ICP-MS	Induction coupled plasma mass spectrometer
IRP	Immune risk phenotype
CMV	Cytomegalovirus

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Introduction

The rapid increase in the proportion of older individuals in the general population has been accompanied by substantial interest in identifying those biomarkers able to predict functional decline and mortality in the elderly (Simm et al. 2008; Goldman et al. 2006).

Among the circulating biomarkers which predict mortality in old and very old subjects, those related to inflammatory status, such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and Interleukin-6 (IL-6), as well as a laboratory parameter commonly used to assess nutritional status, serum albumin, seem to play a major role (Alley et al. 2007; De Martinis et al. 2006; Campbell et al. 1985; Corti et al. 1994; Djoussé et al. 2002).

Incremental changes of these biomarkers have been associated with augmented plasma Cu (Bo et al. 2008) and/or decreased plasma Zn (Mariani et al. 2006) concentrations, but relatively little attention has been paid to the potential use of plasma Cu/Zn as a predictor itself of mortality in the old or oldest subjects.

Cu and Zn have a significant influence on immune functions (Maggini et al. 2007) and play a central or putative role in the development of important age-related diseases, including CVD (Leone et al. 2006), cancer (Zuo et al. 2006), type 2 diabetes (Mocchegiani et al. 2008; Aguilar et al. 2007) and Alzheimer's disease (Barnham and Bush 2008). Plasma concentration of these trace elements is affected by physiological conditions such as age, gender and nutritional status, as well as by pathophysiological conditions, like inflammation and the presence of cardiovascular risk factors (Ghayour-Mobarhan et al. 2005). In particular, ageing has been associated with a general decrease of plasma Zn, especially in elderly subjects with a sub-optimal nutritional status (Prasad et al. 1993). Conversely, increased plasma levels of Cu have been more specifically associated with cardiovascular risk factors (Ford 2000) and cancer (Zowczak et al. 2001).

It has been proposed that increased mortality in subjects with high serum Cu is unlikely to result from dietary imbalances, but rather from secondary compartmentalisation in the body caused by inflammatory or injurious processes (Reunanen et al. 1996). This fact suggests that various immunological and inflammatory changes associated with physiological and pathological conditions can affect trace element distribution in the body. Thus, plasma Cu/Zn could potentially represent one of the most sensitive clinical markers of these changes. Indeed, plasma Cu/Zn was found to be higher in hospitalised elderly subjects than in their healthy counterparts (Belbraouet et al. 2007) and has been associated with the risk of CVD death (Leone et al. 2006; Reunanen et al. 1996) and malignancy (Cunzhi et al. 2003; Dìez et al. 1989). But, proposed methods of using Cu/Zn as a predictor of all-cause mortality have largely been derived from populations in which old and, especially, very old subjects were underrepresented.

We set out to estimate the usefulness of plasma Cu/Zn as a sensitive biomarker of harmful inflammatory or nutritional changes in the elderly along with its prognostic utility as a predictor of all-cause mortality in an Italian population which also comprises old and very old subjects.

Methods

Subjects

The present study includes a group of 498 non-institutionalised elderly subjects (age range 61–100 years) born and living in east central Italy (the Marche Region). A pre-requisite for the study required subjects to be wholly independent and not on vitamin supplements.

The exclusion criteria included diabetes, autoimmune diseases, neurodegenerative diseases, severe or unstable CVD, infections, cancer, Crohn's disease and acrodermatitis enteropathica, kidney disease, liver disease, sickle cell anaemia, chronic skin ulcerations and endocrine disorders. Subjects also had to be free of anticonvulsants, anti-depressives drug, antibiotics, anti-diabetic and hypoglycemic medications. A standardised questionnaire was administered to collect data on medical history, anti-inflammatory medication (acetylsalicylic acid, corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs), statins, ACE-inhibitors and fibrates) and personal habits, such as smoking. To ensure validity, the family doctor was requested to check the completed questionnaire.

Subjects with CVD (angina, arrhythmia, compensated heart failure) were included in the study if these conditions did not compromise the individual's independence.

Hypertension was defined as seated systolic blood pressure ≥160 mmHg, diastolic pressure ≥95 mmHg or both, or self-reported hypertension and if the subject was already on use of anti-hypertensive medication.

Subjects were classified as hyperlipidemic if was defined as lipid-lowering medication use or as LDL cholesterol above 160 mg/dl or as triglycerides above 150 mg/dl.

Blood samples from one-hundred and sixty-four 20- to 60-year-old healthy volunteers (23 males and 33 females aged 20–40 years and, 36 males and 62 females aged 41–60 years) were collected over the same time span and served as controls.

All subjects signed an informed consent, which was approved by the Ethical Committee of INRCA.

Follow-up study

At the beginning of the study, 218 subjects (65 female and 59 male aged 71–80 years; 56 female and 38 male aged 81–100 years) agreed to participate in a prospective follow-up study of mortality from age-related diseases. The cohort was followed up for 3.5 years using mortality records held by the General Register Office in the subject's place of residence. Death certificates were examined by a medical doctor to determine the main cause of death.

Laboratory measurements

Peripheral blood samples, collected after an overnight fast, were tested using basal biochemical laboratory determinations and immunological studies were performed to exclude the presence of pathologies included in the exclusion criteria.

CRP values were detected by CardioPhase hsCRP (Dade Behring Inc., USA).

IL-6 was assayed in duplicate using a commercially available bead-based immunoassay kit (Bio-Rad Laboratories, Hercules, CA, USA) as previously described (Mariani et al. 2006).

Plasma zinc and copper measurement

Plasma Zn and Cu were determined by a Thermo XII Series ICP-MS (Thermo Electron Corporation, Waltham, MA, USA) following the manufacturer's application note (AN_EO604) which was slightly modified in the manner previously described (Giacconi et al. 2007).

Statistical analysis

The cohort of elderly subjects was subdivided into three age groups: 61–70 years (144: 74 female, 70 male), 71–80 years (199: 110 female 89 male) and 80–100 years (155: 92 female, 63 male). Due to skewed distribution, all variables had to be log transformed for statistical analysis. Any significant differences within the individual groups were detected by analysis of variance.

Within each age group, elderly males and females were separately regrouped into three tertiles of increasing Cu/Zn (Low Cu/Zn, Med Cu/Zn and High Cu/Zn). Correlations between Cu/Zn and other inflammatory parameters were studied on the log transformed parameters.

Survival curves were constructed using the Kaplan–Meier method. Cox regression models were used to investigate the effects of plasma Cu/Zn on all-cause mortality after adjustments of potential confounders (age, gender, smoking, BMI, presence of stable CVD, hyperlipidemia, hypertension and anti-inflammatory medication). Linear regression was used to estimate age-related trends of plasma Cu, Zn and Cu/Zn in elderly subjects with and without CVD. Univariate analysis using age as a covariate was used to estimate significant differences among subjects with and without stable CVD.

Results

Influence of age and gender on Cu/Zn

Plasma Cu/Zn levels were closely related to gender, with higher levels in females than in males (Fig. 1) ($P < 0.001$ for each comparison); it was therefore decided to proceed with separate investigations for male and female subjects. An age-related increase of Cu/Zn was occurred both in elderly females and males (Fig. 1) with the highest levels detected in octa and nonagenarians. Cu/Zn levels in all elderly groups were significantly higher than those in the young-adult groups (Fig. 1).

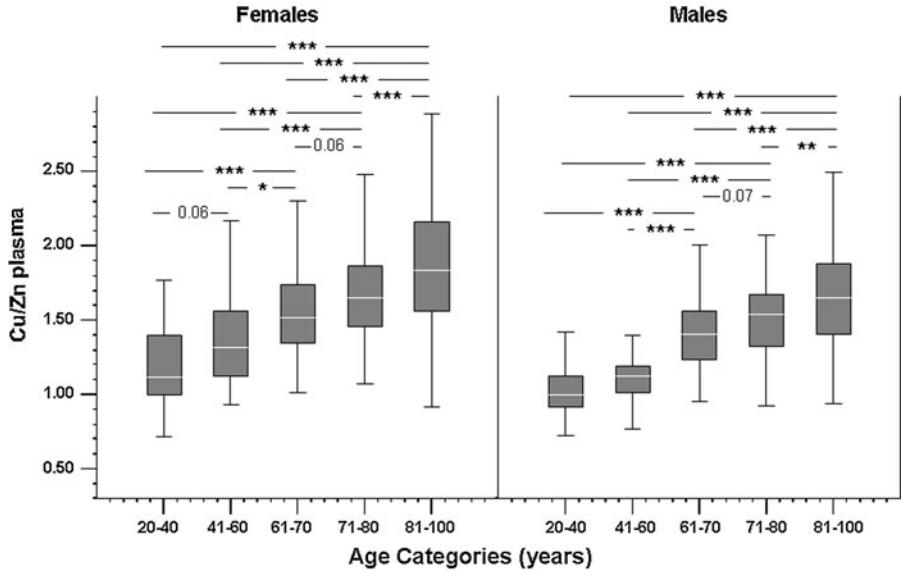

Fig. 1 Plasma Cu/Zn ratio in male and female at different age. *Box* plots of plasma Cu to Zn ratio in 20- to 40-year-olds ($n = 23$ M and $n = 33$ F), 41- to 60-year-olds ($n = 36$ M and $n = 62$ F) and the elderly population subdivided in into three age levels: 61–70 years ($n = 144$, 74 F, 70 M), 71–80 years ($n = 199$, 110 F, 89 M) and 80–100 years ($n = 155$, 92 F, 63 M). The *box* plots display the median, interquartiles range, the 5th and 95th percentile. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Cigarette smoking and anti-inflammatory medication were not associated with plasma Cu/Zn levels in the elderly population (Table 1).

A BMI index ≥ 25 was associated with decreased Cu/Zn levels in females aged 71–80 years and octa-nonagenarians (males and females), whereas the presence of stable CVD was invariably associated with increased Cu/Zn (Table 1).

Influence of inflammatory parameters and albumin on Cu/Zn

All elderly men and women were grouped within each age level into three tertiles of Cu/Zn. Ranges of the intermediate tertile (Med Cu/Zn) were: 1.41–1.65 for women and 1.29–1.49 for men (age range 61–70 years); 1.53–1.76 for women and 1.41–1.62 for men (age range 71–80 years); 1.63–2.06 for women and 1.47–1.78 for men (age range 81–100 years). Subjects included in the low Cu/Zn and high Cu/Zn tertiles had plasma Cu/Zn values, respectively, below and above these ranges.

High plasma levels of Cu/Zn were associated with high CRP and ESR as well as with low serum albumin independently by gender within each age group (Table 2). A significant association between high Cu/Zn and high IL-6 was also found in men within each age group, as well as in women aged 61–80 years (Table 2). Significant differences were found between low Cu/Zn and median Cu/Zn for albumin in men aged 61–70 years and women aged 81–100 years, as well as for ESR in men aged 71–80 years and CRP in men aged 81–100 years (Table 2).

Plasma Cu/Zn ratio correlated directly with CRP, ESR and inversely with albumin (Table 3). The correlations remained significant even after correcting for the age (data not shown). Plasma Cu/Zn ratio correlated with plasma IL-6 in elderly men but not in women (Table 3).

Mortality risk

Over the 3.5 years follow-up there were 32 deaths (four females and six males aged 71–80 years; 13 females and 9 males aged 81–100 years). The cause of death was due to heart failure/attack ($n = 18$), stroke ($n = 4$), airway or other infection ($n = 4$) and cancer ($n = 6$). Univariate analysis using age as a covariate showed that plasma Cu/Zn was significantly higher in old subjects who died when compared to those still living after 3.5 years of follow-up [mean ± SEM log(Cu/Zn) 0.53±0.06 vs. 0.43± 0.02, $P < 0.001$], where gender played no significant role. Figure 2 illustrates the Kaplan–Meier survival plots of the population grouped into tertiles of plasma Cu/Zn. Cox analysis stratified by gender showed significant association of Cu/Zn tertiles with all-cause mortality in multivariate models adjusted for the effects of age, gender, smoking, BMI, presence of stable CVD, hyperlipidemia, hypertension and anti-

Table 1 Plasma levels of Cu/Zn in elderly according to demographic characteristics and presence of stable cardiovascular disease or anti-inflammatory treatment

Charactristics	Age group																	
	61–70 Years						71–80 Years						81–100 Years					
	Women			Men			Women			Men			Women			Men		
	n	Cu/Zn	*P*	*n*	Cu/Zn	*P*	*n*	Cu/Zn	*P*	*n*	Cu/Zn	*P*	*n*	Cu/Zn	*P*	*n*	Cu/Zn	*P*
Smoking Status																		
Current or ex-smoker	16	1.64 (1.36–1.78)	NS	43	1.40 (1.28–1.55)	NS	13	1.69 (1.59–1.87)	NS	60	1.54 (1.33–1.68)	NS	7	2.11 (1.50–3.00)	NS	47	1.62 (1.40–1.85)	NS
Non-smoker	58	1.48 (1.34–1.72)		27	1.46 (1.21–1.62)		97	1.67 (1.43–1.88)		29	1.53 (1.29–1.70)		85	1.86 (1.58–2.16)		16	1.63 (1.32–1.96)	
BMI																		
<25	30	1.55 (1.38–1.78)	NS	25	1.38 (1.28–1.48)	NS	53	1.73 (1.53–2.00)	0.009	36	1.49 (1.33–1.66)	NS	69	1.99 (1.61–2.25)	0.05	35	1.75 (1.43–1.94)	0.03
≥25	44	1.45 (1.32–1.68)		45	1.41 (1.22–1.56)		57	1.57 (1.42–1.80)		53	1.56 (1.31–1.71)		23	1.67 (1.46–2.00)		28	1.54 (1.34–1.78)	
Hyperlipidemia																		
No	28	1.51 (1.39–1.76)	NS	32	1.46 (1.34–1.69)	0.036	62	1.67 (1.48–1.90)	NS	70	1.57 (1.33–1.68)	NS	54	2.00 (1.63–2.29)	0.003	55	1.66 (1.39–1.91)	NS
Yes	46	1.49 (1.31–1.73)		38	1.35 (1.22–1.48)		48	1.59 (1.48–1.83)		19	1.49 (1.25–1.62)		38	1.65 (1.44–2.02)		8	1.57 (1.51–1.82)	
CVD																		
Yes	19	1.72 (1.36–1.80)	0.04	9	1.56 (1.37–1.89)	0.039	29	1.73 (1.53–2.21)	0.008	29	1.54 (1.34–1.69)	0.035	47	1.89 (1.68–2.27)	0.04	29	1.75 (1.40–2.08)	0.030
No	55	1.43 (1.29–1.54)		61	1.38 (1.18–1.50)		81	1.65 (1.42–1.86)		60	1.42 (1.23–1.60)		45	1.63 (1.46–2.05)		34	1.53 (1.39–1.77)	
Hypertension																		
Yes	24	1.46 (1.37–1.72)	NS	29	1.39 (1.25–1.49)	NS	45	1.57 (1.40–1.87)	NS	38	1.52 (1.30–1.66)	NS	46	1.88 (1.54–2.25)	NS	24	1.63 (1.35–1.87)	NS
No	50	1.54 (1.32–1.78)		41	1.44 (1.28–1.59)		65	1.66 (1.52–1.86)		51	1.57 (1.33–1.70)		46	1.76 (1.53–2.14)		39	1.67 (1.47–1.81)	
Anti-inflammatory treatments																		
No	57	1.50 (1.34–1.73)	NS	53	1.43 (1.23–1.59)	NS	75	1.70 (1.47–1.81)	NS	62	1.70 (1.50–2.01)	NS	45	1.80 (1.56–2.06)	NS	35	1.89 (1.57–2.35)	NS
Yes	17	1.48 (1.36–1.74)		17	1.39 (1.29–1.47)		10	1.72 (1.50–2.02)		12	1.44 (1.25–1.56)		13	2.01 (1.57–2.30)		9	1.66 (1.38–2.00)	

Values are median (interquartile range) and two-tailed *t*-test. Plasma Cu/Zn is log transformed in the *t*-test. Presence of stable cardiovascular disease (angina, arrhythmia, heart failure) not affecting independent life represent CVD. Medicines with anti-inflammatory effects includes acetylsalicylic acid, corticosteroids and non-steroidal anti-inflammatory drugs, statins, ACE-inhibitors and fibrates

Table 2 Tertiles of plasma Cu to Zn ratio, inflammatory markers and albumin

Age	Factor	Women						Men					
		Low Cu/Zn Median	Low Cu/Zn IQR	Med Cu/Zn Median	Med Cu/Zn IQR	High Cu/Zn Median	High Cu/Zn IQR	Low Cu/Zn Median	Low Cu/Zn IQR	Med Cu/Zn Median	Med Cu/Zn IQR	High Cu/Zn Median	High Cu/Zn IQR
61–70 Years	CRP (mg/dl)	*0.21*[b]	0.08–0.31	0.25	0.08–0.36	0.27	0.10–0.47	*0.18*[b]	0.08–0.34	0.24	0.12–0.54	0.26	0.15–0.80
	ESR (mm/h)	*9.24*[b]	6.22–12.50	11.00	8.00–16.00	13.00	9.00–20.00	*7.00*[b]	3.00–9.32	6.00	4.50–10.17	7.55	5.00–15.00
	IL-6 (pg/ml)	*15.10*[b]	11.35–24.74	17.15	14.42–23.18	20.13	12.21–27.56	*19.81*[b]	14.94–27.83	27.77	16.63–33.14	22.56	16.92–38.30
	Albumin (g/dl)	*4.00*[b]	3.80–4.08	3.90	3.80–4.00	3.80	3.60–4.00	*4.00*[b,c]	3.90–4.11	3.90	3.74–3.97	3.80	3.75–4.01
71–80 Years	CRP (mg/dl)	*0.19*[a]	0.08–0.38	0.25	0.16–0.39	0.42	0.21–0.66	*0.26*[b]	0.11–0.41	0.38	0.14–0.75	0.51	0.11–1.00
	ESR (mm/h)	*10.87*[b]	8.45–14.50	11.00	7.00–17.00	13.00	9.00–16.01	*8.00*[a,d]	4.00–10.00	11.30	8.27–16.00	11.85	10.00–17.00
	IL-6 (pg/ml)	*19.45*[b]	15.92–21.44	19.39	15.73–23.33	26.33	16.27–37.81	*18.03*[a]	13.60–23.56	20.46	15.10–25.76	31.81	18.20–41.36
	Albumin (g/dl)	*3.89*[b]	3.70–4.01	*3.89*[b]	3.76–3.98	3.73	3.61–3.85	*3.96*[b]	3.90–4.10	3.84	3.78–3.90	3.76	3.66–3.84
81–100 Years	CRP (mg/dl)	*0.20*[b]	0.11–0.45	0.24	0.14–0.49	0.27	0.15–0.73	*0.12*[b,c]	0.08–0.25	0.33	0.20–0.77	0.23	0.10–0.49
	ESR (mm/h)	*9.00*[a]	7.00–16.00	*12.00*[a]	9.00–16.74	19.00	11.00–31.00	*10.49*[b]	6.00–16.00	13.54	8.00–16.00	13.00	9.75–18.00
	IL-6 (pg/ml)	19.01	13.52–29.37	17.69	13.60–32.68	17.78	14.71–40.00	*18.78*[b]	14.41–23.06	19.78	10.13–33.38	22.82	15.82–34.22
	Albumin (g/dl)	*3.69*[b,c]	3.60–4.99	3.56	3.40–3.72	3.40	3.20–3.60	*3.80*[b]	3.70–4.00	3.79	3.60–3.90	3.60	3.40–3.90

Statistical analysis was performed separately for men and women. For each gender group,

[a] $P < 0.01$ with respect to High Cu/Zn

[b] $P < 0.05$ with respect to High Cu/Zn

[c] $P < 0.05$ with respect to Med Cu/Zn

[d] $P < 0.01$ with respect to Med Cu/Zn

Table 3 Correlations among plasma Cu to Zn ratio, inflammatory markers (CRP, ESR and IL-6) and serum albumin

	Variable Cu/Zn	IL-6	CRP	ESR	Albumin
All					
Women	Pearson	0.058	**0.208**	**0.305**	**−0.420**
	Sig.	*0.434*	*0.001*	*<0.001*	*<0.001*
Men	Pearson	**0.172**	**0.272**	**0.386**	**−0.410**
	Sig.	*0.031*	*<0.001*	*<0.001*	*<0.001*

All correlations were calculated on the Log transformed variables

Sig. Significance (*P* values)

Table 4 Multivariate cox regression analysis of plasma Cu/Zn and all-cause mortality in elderly subjects aged 70+ years

Plasma Cu/Zn	HR	Multivariate analysis	
		CI (95%)	*P*
Low Cu/Zn	1		
Med Cu/Zn	4.75	1.30–17.32	0.018
High Cu/Zn	7.31	2.05–26.13	0.002

Multivariate model is adjusted for the effect of age, gender, smoking, body mass index, presence of stable CVD, hyperlipidemia, hypertension and intake of anti-inflammatory medicine. $n = 218$; 32 deaths within the 3.5 years of follow-up

HR hazard ratio

inflammatory medication (Table 4). Univariate analysis showed that in the present study population serum albumin ($P = 0.001$; HR = 0.09; 95%CI = 0.04–0.21), CRP ($P = 0.01$, HR = 1.46, 95%CI = 1.18–1.82) and ESR ($P = 0.048$; HR = 1.04; 95% CI = 1.02–1.08) were significantly associated with mortality. However, when all these variables were included in the multivariate analysis, only Cu/Zn, albumin and age remained significantly associated with mortality (Table 5). Similar results were also obtained after grouping all the variables into tertiles or using cut-off values derived from ROC curves (data not shown).

Age related changes of Cu, Zn and Cu/Zn in the elderly with or without stable CVD

In order to clarify whether the age-related changes of plasma Cu/Zn observed in the elderly subjects with or without stable CVD were due to changes in Zn or Cu, the age related patterns of both these trace elements were studied by linear regression. Figure 3 shows the scatter plots of Cu, Zn and Cu/Zn versus age in elderly men and women with or without CVD. Plasma Cu showed no significant linear changes with age in both females and males irrespective of the presence of stable CVD. However, univariate

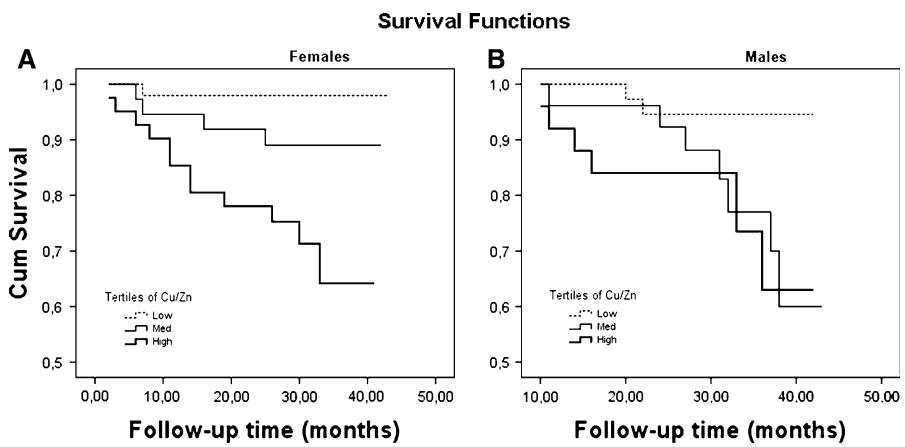

Fig. 2 Survival curves of elderly man and women in dependence of Cu/Zn ratio. Kaplan–Meier survival estimates of death from plasma Cu/Zn ratio in $n = 218$ elderly subjects (121 F; 97 M) aged 70–100 years. Subjects were assigned to tertiles of plasma Cu/Zn defined in the whole population separately for men and women. The ranges of the intermediate tertiles (Med Cu/Zn) were: 1.53–1.76 for females and 1.41–1.62 for males aged 71–80 years; 1.63–2.06 for females and 1.47–1.78 for males aged 81–100 years. Subjects assigned to the lowest tertiles (Low Cu/Zn) had Cu/Zn values lower than 1.53 for females and 1.41 for males. Subjects assigned to the highest tertiles (High Cu/Zn) had Cu/Zn values higher than 2.06 for females and 1.78 for males. The equality of the survival distributions for different tertiles of Cu/Zn was performed by Log Rank (Mantel-Cox) statistic. For females **a**: Chi-square = 14.464, $P < 0.001$; For males **b**: Chi-square = 4.854, $P = 0.028$

Table 5 Multivariate cox regression analysis of plasma Cu/Zn, CRP, IL-6, ESR and albumin considered as continuous variables and all-cause mortality in elderly subjects aged 70+ years

Plasma Cu/Zn	HR	Multivariate analysis	
		CI (95%)	*P*
Age	1.101	1.04–1.17	0.001
Cu/Zn	4.58	1.70–12.33	0.018
CRP	1.24	0.91–1.70	0.175
IL-6	0.98	0.95–1.02	0.390
ESR	0.97	0.97–1.02	0.203
Albumin	0.25	0.81–0.80	0.019

Multivariate model is adjusted for the effect of age, gender, smoking, body mass index, presence of stable CVD, hyperlipidemia, hypertension and intake of anti-inflammatory medicine. $n = 218$; 32 deaths within the 3.5 years of follow-up

HR hazard ratio

analysis using age as a covariate showed that Cu levels were significantly higher in elderly subjects with stable CVD than in those in the same age group without CVD: both in females ($P = 0.003$) and males ($P = 0.014$). Plasma Zn levels declined significantly with age both in females and males, independently of the presence of stable CVD ($P < 0.019$ at least, with linear regression). No significant differences were found between plasma Zn in subjects with stable CVD versus subjects without CVD. Plasma Cu/Zn significantly increased with advancing age both in male and female subjects without CVD ($P < 0.001$). Cu/Zn increased with age in females with CVD ($P = 0.004$) and showed a tendency to also increase in men with CVD ($P = 0.08$).

Discussion

In this study, we found that plasma Cu/Zn is associated with different inflammatory markers, as well as with the nutritional marker serum albumin and may predict mortality in the elderly above 70 women had a higher Cu/Zn ratio, irrespective of age, though the Cu/Zn ratio increased with advancing age both in men and women. Cu/Zn plasma levels were not related to smoking, hypertension or anti-inflammatory medication, but were, to stable CVD and BMI. Taking into account the reported association of Zn or Cu with BMI in adults (Song et al. 2007), it might come as unexpected to find that women over 70 and men over 80 with a BMI $\leq$ 25 display higher Cu/Zn levels than their respective counterparts with a BMI $\geq$ 25 (Table 1). However, these data are consistent with previous findings which suggest that weight loss rather than weight gain appears to be more important factor in predicting mortality in very old subjects (Diehr et al. 2008). Indeed, being lean in very old age may represent a risk factor as a result of nutrient deficiency, frailty, and a reduced functional status (Stevens et al. 1998). Cu/Zn may be a potential biomarker of these conditions, as supported by its correlation with serum albumin CRP, ESR and IL-6 .

As in the case of albumin, all these inflammatory parameters have a predictive value for mortality in the elderly (Alley et al. 2007; Campbell et al. 1985; Natali et al. 2003; Harris et al. 1999; Sullivan et al. 2007), thus the finding that plasma Cu/Zn itself is a sensitive predictor of mortality (Table 4) draws attention to its clinical relevance in the geriatric population. In the univariate analysis, CRP and ESR were also found to be predictive of mortality in the present population. However, in the multivariate analysis, only plasma Cu/Zn and serum albumin remained significant predictors of mortality after the 3.5 years follow-up. Conversely, low plasma Cu/Zn seems to confer a survival advantage in elderly subjects older than 70.

Although increased inflammatory markers and decreased serum albumin contribute to raising plasma Cu/Zn, this parameter may only be sensitive to increased Cu levels or decreased Zn levels.

In our elderly population, subjects with stable CVD display increased plasma Cu as compared to those without CVD, thus suggesting that Cu is the main factor affecting Cu/Zn in their plasma. In this case, altered compartmentalisation of Cu in the body caused by inflammatory or injurious processes could contribute to an increase in plasma Cu levels irrespective of diet (Reunanen et al. 1996).

Concomitantly, another factor that contributes to an increase in plasma Cu/Zn with advancing age is the progressive decline of plasma Zn. This phenomenon may occur due to decreased dietary intake, reduced absorption (Fairweather-Tait et al. 2008) and altered compartmentalisation caused by chronic low-level inflammation (Mocchegiani et al. 2000). Indeed, pro-inflammatory cytokines, such as IL-6, stimulates Zn uptake and metallothionein expression in different

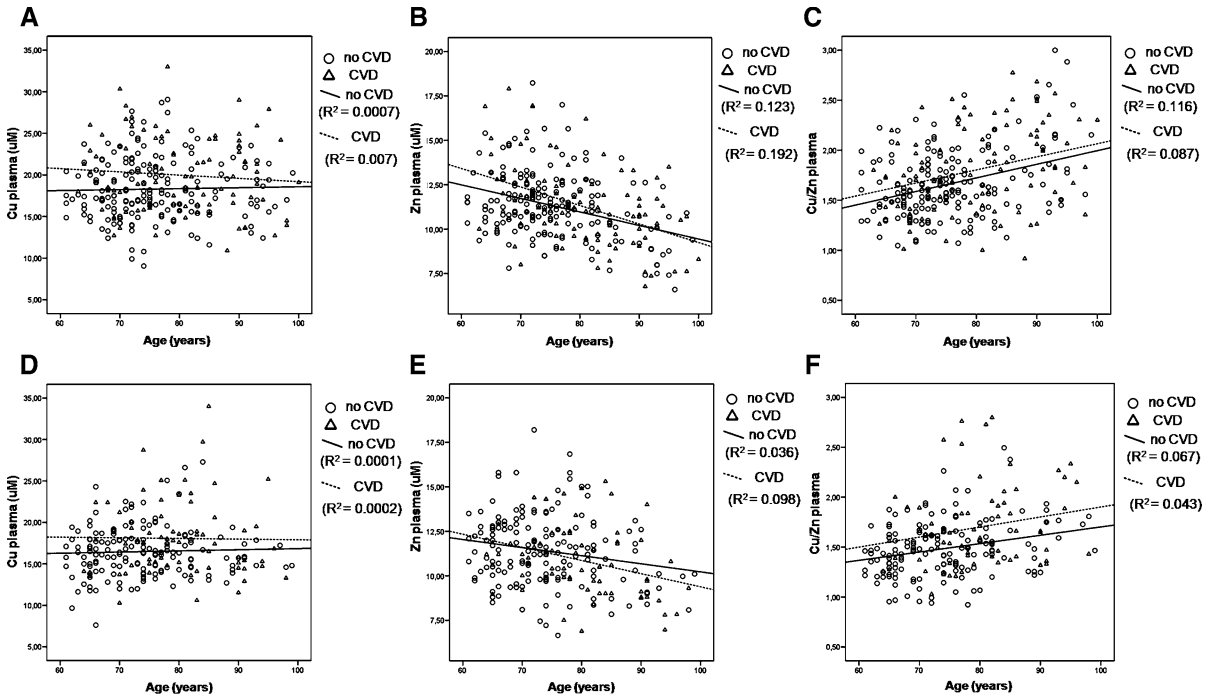

Fig. 3 Linear regression with age of plasma Cu, Zn and Cu/Zn ratio in Italian elderly population with or without CVD. Age-dependent changes of plasma Cu, Zn and Cu/Zn in an Italian elderly population aged 60–100 years. *Lines* are fitted with linear regression. *Circles* and *solid lines* represent subjects without stable CVD, whereas *triangles* and *dashed lines* represent subjects with stable CVD. Plasma Cu showed no significant linear changes with age in both females (**a**) and males (**d**) independently of presence of stable CVD. However, Cu levels where significantly higher in elderly subjects with stable CVD when compared to elderly without CVD, both in females and males (univariate analysis using age with covariate found $P = 0.003$ and 0.014 for females and males, respectively). Plasma zinc levels showed a significant linear decline with age both in females (**b**) and males (**e**), independently of presence of stable CVD ($P < 0.019$ at least with linear regression). However, the points in curves clearly show that the main decline of plasma Zn occurs in subjects aged 80 years and older. No significant difference where found between plasma Zn in subjects with CVD versus subjects without CVD. Cu/Zn ratio showed a significant increase with age both in man (**f**) and women (**c**) without CVD ($P < 0.001$ for both man and women). Cu/Zn ratio increased with age in women with CVD ($P = 0.004$) and showed a trend to increase also in man with CVD ($P = 0.08$)

tissues and cells (Kwon et al. 2007). Given the pivotal role played by metallothioneins in retaining intracellular Zn with the subsequent reduction of plasma Zn (Kwon et al. 2007), higher Cu/Zn plasma levels might in part reflect this phenomenon.

This assumption may appear in contrast with our observation that Cu/Zn plasma levels do not correlate with IL-6 in women (Table 3). However, the absence of a correlation in women seems mainly due to the lack of association among these variables only in the oldest female subjects (Table 2). Two factors may account for this dichotomy: (A) the heterogeneity existing between the oldest Italian men and women (Franceschi et al. 2000), as is also demonstrated by the interquartile ranges of the inflammatory parameters reported in Table 2; (B) the activity of IL-6 could be limited or suppressed by certain compensating factors, such as reduced gp130 activity (Mocchegiani et al. 2004) or genetic polymorphisms exerting a gender specific effect (Passarino et al. 2006).

Conversely, the persistence of a close correlation between serum albumin and plasma Cu/Zn as well as the association between BMI and plasma Cu/Zn in the oldest elderly suggest that nutritional components play a major role in raising Cu/Zn in these subjects.

Therefore, multiple factors could contribute to raising plasma Cu/Zn with ageing, including chronic low-level inflammation (Mocchegiani et al. 2008), impaired nutritional status (Belbraouet et al. 2007) and specific underlying conditions which increase the risk of mortality, such as CVD (Reunanen et al.

1996). In longitudinal studies, also positivity for cytomegalovirus (CMV) infection, an inverted CD4/CD8 ratio and other markers of immunosenescence, collectively indicated as immune risk phenotype (IRP) (Wikby et al. 2006), resulted to be strong predictors of morbidity and mortality in octo-nonagenarians. Information about these parameters was not available in most subjects in the present study, but it could be hypothesized a possible relationship among plasma Cu/Zn and persistent CMV infection. Indeed, infection and inflammation produce systemic responses that induce hypozincemia (Liuzzi et al. 2005) which, in turn, has been also reported in pregnant women with CMV infection (Zhang et al. 2008). Therefore, it is reasonable to assume that conditions associated with increased inflammatory and/or impaired nutritional markers can be signalled by decreased Zn and/or increased Cu in the plasma. Moreover, the comparison of plasma Cu/Zn between elderly and young donors suggests that this parameter may be regarded as a potential biomarker of aging.

In conclusion, the Cu/Zn ratio seems to be of clinical importance as an inflammatory-nutritional biomarker and a sensitive predictor of mortality in elderly subjects aged 70 years and above. However, it is important to mention that the present study was performed using a limited study sample and further multicentre longitudinal investigations including subjects with various chronic diseases are needed to confirm if these results could be translated to the general population.

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