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CCS mRNA transcripts and serum CCS protein as copper marker in adults suffering inflammatory processes

Magdalena Araya · Ricardo Gutiérrez · Miguel Arredondo

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Abstract The chaperone to Zn–Cu superoxide dismutase (CCS) has been postulated as a candidate copper indicator, changing in a consistent manner in induced and recovered copper deficiency, in experimental cell and animal models. In real life people have various conditions that may modify molecules acting as acute phase proteins, such as serum ceruloplasmin and copper concentration and could alter CCS responses. With the hypothesis that CCS mRNA transcripts and protein would be different in individuals suffering inflammatory processes in comparison to healthy individuals, we assessed adult individuals who, although not ill had conditions known to induce variable degrees of inflammation. Screening of 600 adults resulted in two study groups, formed on the basis of their clinical history and levels of serum C reactive protein (CRP): Group 1 ($n = 61$, mean (range) CRP = 0.9 (0.3–2.0 mg/dL) and Group 2 ($n = 150$, mean (range) CRP = 6.1 (4.3–8.7 mg/dL). Results showed that mRNA transcripts relative abundance was not different for CCS, MTIIA, TNF-alpha and Cu–Zn-SOD by group ($p > 0.05$, one way Anova), nor between sexes ($p > 0.05$, one way Anova). Distribution of CCS mRNA transcripts and CCS protein in serum did not show any differences or trends. Results disproved our hypothesis that CCS abundance of transcripts and CCS protein would be different in individuals suffering inflammatory processes, adding further support to the idea that CCS may be a copper marker.

Keywords Copper · Marker · CCS transcripts · CCS protein · Humans

M. Araya (✉) · R. Gutiérrez · M. Arredondo
Institute of Nutrition and Food Technology (INTA), University of Chile, Av. El Líbano, 5524 Macul, Santiago, Chile
e-mail: maraya@inta.uchile.cl

Introduction

Modern nutritional studies on micronutrients require detecting changes of nutritional status at early stages, such that detrimental effects can be prevented or reverted. In the case of copper, this is difficult to achieve. The National Institutes of Health defines biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers and surrogate endpoints: preferred definitions and conceptual framework 2001).

As for any micronutrient, assessing a copper biomarker requires evaluating the proposed test against a gold standard. Since there is no gold standard for copper status, the only way to validate a newly proposed marker is demonstrating that it measures what it is supposed to measure, which adds again another difficulty because Cu status in the population is not easily measured (Danzeisen et al. 2007; Harvey

and McArdle 2008; Hooper et al. 2009). In recent years the chaperone to Zn–Cu superoxide dismutase (CCS) has been reported to change in a consistent manner after copper deficiency, in experimentally induced and recovered copper deficiency (Bertinato et al. 2003; Iskandar et al. 2005; Prohaska et al. 2003; West and Prohaska 2004). So far, a few studies in humans have approached the idea that CCS may be a copper biomarker (Araya et al. 2012; Suazo et al. 2008), but there are relevant pieces of information still lacking. Most studies in animals and cell lines have measured CCS protein (Bertinato et al. 2003; Iskandar et al. 2005; Prohaska et al. 2003; West and Prohaska 2004, while studies in humans assessed mRNA CCS transcripts (Araya et al. 2012; Suazo et al. 2008).

A recent study successfully evaluated this chaperone in healthy adults, who were extensively screened before accepting them as "healthy" volunteers (Araya et al. 2012). Although interesting, this approach had the limitation that the assessment did not represent a "real life" situation, condition necessary to advance in the line of a marker applicable to human population, where persons have variable degrees of overweight/obesity, high blood pressure, altered lipid profile, inflammation, among others. All these conditions modify molecules acting as acute phase proteins, as is the case of historical copper markers (serum copper concentration and ceruloplasmin). For this reason, in this study we set as objective to assess both CCS protein and CCS mRNA abundance of transcripts in adult individuals who, although not ill had conditions known to induce variable degrees of inflammation. Our hypothesis was that in individuals suffering inflammatory processes their CCS mRNA transcripts and protein would be different when compared to healthy individuals.

Materials and methods

Initial screening and study groups

Candidates that responded to posters inviting to participate in the study were interviewed and received detailed explanation about the protocol (Fig. 1). Those willing to participate ($n = 600$) signed an informed consent and underwent a first assessment for inclusion criteria. These included being 25–50 years of age, of both sexes, with no known diagnosis of current acute infections (previous week and coming fortnight), chronic conditions (mainly alcoholism, morbid obesity, cancer, diabetes, autoimmune conditions), or chronic consumption of multiple drugs, vitamins or minerals. Exclusion criteria included detection of previously unidentified diseases, such as liver damage (secondary to alcoholism or other conditions), pregnancy, any other defined diagnosis considered relevant to this study or that the participant declared his/her wish to leave the protocol. Individuals with pre diabetes, overweight and cardiovascular risk were accepted. Groups were formed following individuals' C reactive protein (CRP) values, above and below the cut off (≥ 3). Clinical information was obtained during a semi structured interview, registering symptoms, medical controls, laboratory studies performed, diets prescribed and ingestion of drugs (including vitamins and minerals), height, weight, waist circumference and blood pressure.

Procedures

After overnight fasting, at 8–9 AM a first 10 mL blood sample was obtained from the antecubital vein in the forearm. Hematological indicators were performed using an electronic counter (CELL-DYN 3200, ABBOTT Diagnostics, Abbott Park, IL). Serum copper, iron and zinc concentrations were measured by atomic absorption spectrometry (Perkin Elmer Model 2280, Norwalk, Conn.). Commercial kits were used to measure serum liver aminotransferases activities: GOT (aspartate aminotransferase or AST), GTP (alanine aminotransferase or ALT) and γ-glutamyltransferase (GGT) (Química Clínica Aplicada SA, Amposta, Spain). Cut offs for AST, ALT and GGT were obtained from http://www.nlm.nih.gov/medlineplus/ency/article/003458.htm. Altered liver function was defined by the presence of at least two of the three enzymatic activities measured ≥ 50 % above the cut off used for medical purposes (upper limits set at 35, 35 and 38 U/L, respectively). Total cholesterol, LDL, HDL, triglycerides and glucose were measure by commercial kits Química Clínica Aplicada SA, Amposta, Spain); GSH (in erythrocytes) by ELISA kit, Cayman Chemical Com, Michigan, USA), glycosylated hemoglobin was measured in whole blood (Pointe Scientific, Inc. Lincoln Park, MI) and Zn/Cu-SOD (from now on referred to as SOD) activity (total activity in erythrocytes) by Bioxytech SOD-525 Assay, OXIS International Inc,

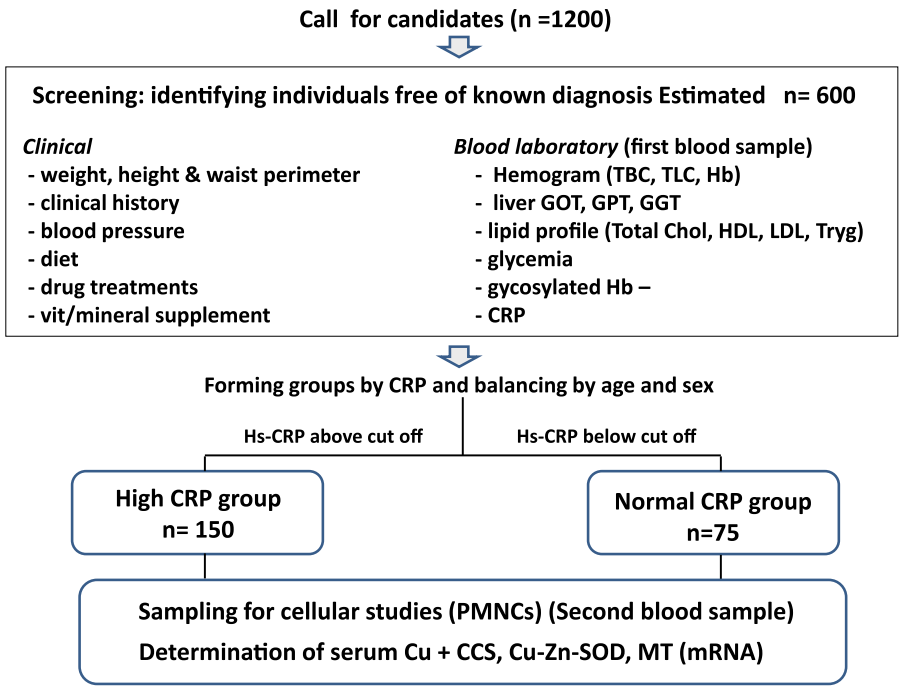

Fig. 1 Algorithm

Portland OR. CRP was the last analysis performed such that the time between the first and second blood sampling was minimized (Química Clínica Aplicada SA, Amposta, Spain).

Cell studies

After forming the study groups a second blood sample (20 mL) was obtained following the same protocol. Peripheral mononuclear cells (PMNCs) were isolated as previously described (Araya et al. 2012) using Histopaque gradients (Histopaque 1077, density: 1.119, Sigma Diagnostic, St. Louis, MO). For total metal content quantification PMNCs were processed as described by Tapia (Tapia et al. 2003) and Suazo (Suazo et al. 2008). Cu and Fe were measured by means of a graphite furnace AAS (Perkin Elmer, SIMMA 6100, Shelton, CT). Zn content was determined by flame AAS (Perkin Elmer, Model 2280, Norwalk, Conn). Calibration was against a standard curve made using dilutions of a Cu, Fe or Zn standard (J T Baker, Phillipsburg, NJ) and the PMNCs Cu, Fe and Zn content was normalized to cell total protein.

Quantitative polymerase chain reaction (qPCR)

CCS, MTII A, Cu–Zn-SOD and TNF-alpha mRNA were studied. Total RNA extraction, synthesis of single strand cDNA and qPCR reactions were performed as previously described (Suazo et al. 2008). Briefly, reactions are carried out on the LightCycler System (Roche Applied Science, Mannheim, Germany) using the LightCycler- FastStart DNA Master SYBR Green kit (Roche Applied Science). Reaction mixes are at a final volume of 10 μL and contains 2 mM $MgCl_2$, 10 pmol each primer, and 100 ng cDNA. A standard curve is plotted for each primer set with isolated PCR-products obtained from amplification of pooled PMNCs cDNAs. MTIIA, Cu/Zn-SOD and TNF-alpha were measured as markers of cell copper status, oxidative stress and inflammation, respectively. Human RPLP0 (Large Ribosomal Protein) was used as reference to normalize the mRNA abundance levels between the samples. Abundance data for each gene is expressed as arbitrary unit (AU). The following primers were used (5′ and 3′ respectively): ***MT2A:*** ATGGATCCCAACTGCTCCTGCG and GGGGCTGTCC CAGCATCAGGC; ***CCS:*** AC AGCTGACCCCTGAGCG and ACAGAGCCAAGG TGAGGTC; ***SOD1***: CTGAAGGCCTGCATGGATT C and CCAAGTCTCCAACATGCCTCTC; and TNF-alpha CCTCACACTCAGATCATCTTCTC and GGT CTTTGAGATCCATGCC; ***RPLP0*** GGCGACCTGG AAGTCCAACT and CCATCAGCACCACAGCCTT C. Primers were designed using the Primer Premier 5.0

software (Premier Biosoft International, Palo Alto, CA, USA) except for SOD1 and RPLP0.

CCS protein quantification

For protein quantification, PMNCs were isolated as above and homogenized by mechanical breakage in lysis buffer (150 mM NaCl, 3 mM MgCl2, 0.5 % Nonidet P40, 10 mM Tris/HCl, pH 7.0) and complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN). After centrifugation (15 min at 4 °C at 12000 g) the supernatant was stored at −80 °C. Protein content was determined using Protein Assay reagent Bio-Rad. CCS quantification was performed by 1) ELISA kit (100 µL) (CSB-EL004854HU, Cusabio Biotech., Wuhan, Hubei, China), following manufacturer instructions and 2) proteins separations by microfluidics system (Agilent Protein 80 kit, Agilent Technologies, Palo Alto, CA) prior immunoprecipitation using Dynabeads (Invitrogen, Paisley, United Kingdom) with anti-CCS (10 µg) (sc-20141, Santa Cruz Biotechnology, Inc, Santa Cruz, CA), following manufacturers recommendations. Chip-based analysis was performed with the Agilent 2100 Bioanalyzer system (Agilent, Waldbronn, Germany). All chips were prepared according to the protocols provided with the individual LabChip kits. Briefly, samples (1–4 µL) were diluted in sample buffer with or without 1 M dithio-threitol solution (DTT). The samples were denatured by placing the vials in boiling water for 5 min, cooled down, centrifuged for 15 s, and then 84 µL deionized water were added to the ladder and samples. A 6 µL aliquot of this solution was loaded onto the chip, which was first filled with a gel/dye mix and destaining solution. Separated proteins were detected by laser-induced fluorescence. The sample buffer used included an upper and a lower marker at a known molecular weight.

Sample size and data analysis

This was calculated following Suazo's (Suazo et al. 2008) report of figures of mRNA in PMNCS obtained from humans. Calculation was first performed by difference of the mean (mRNA), which in this paper was 59.6 %. Estimating a difference of 26 %, with alpha error of 0.01 and power of 0.9, the sample size was 75 per group. Using proportions, the highest reported mean ± 2 SD, alpha error of 0.01 and power of 90 %, sample size is 49 per group. We chose a final size of 75, 150 in the study group (two experimental cases for each control) and 75 in the control group. Statistical analysis included parametric and non-parametric tests and multiple regression analyses to detect associations of the measured variables and CCS. We first used the customary cut offs used in biomedicine and then varied them, analyzing by the indicator and then by group or sex, as needed. Additional analyses included Pearson correlations and path analyses (SISTAT package).

Ethical considerations

This protocol was approved by the INTA IRB, which complies with the Helsinski Declaration. INTA's Committee on Ethics is certified by the Office for Human Research Protections (OHRP, USA) (IRB00001493).

Results

A total of 211 women and men without firm clinical diagnoses as evaluated by clinical history and CRP provided all the data necessary and were analyzed as Group 1 ($n = 61$, mean (range) CRP = 0.9 (0.3–2.0 mg/dL) and Group 2 ($n = 150$, mean (range) CRP = 6.1 (4.3–8.7 mg/dL). As expected, anthropometric and blood indicators tended to be higher in G2, reaching statistical significance in waist circumference, total and LDL cholesterol and triglycerides in blood, when comparing individuals of same sex, between groups (T test, $p < 0.05$, Table 1). Table 2 shows serum concentrations of Cu and Zn and SOD and GSH. Comparison of individuals of the same sex in Group 1 and Group 2 showed significant differences for SOD and GSH (T test, $p < 0.05$).

mRNA transcripts relative abundance was not different for CCS, MTIIA, TNF-alpha and Cu–Zn-SOD by group ($p > 0.05$, one way Anova), nor between sexes ($p > 0.05$, one way Anova) (Fig. 2, panels A, B, C and D). Also, determination of CCS protein concentration in serum by ELISA (Fig. 3a) and immunoprecipitation (Fig. 3b) did not show differences by group or by sex ($p > 0.05$, one way Anova). Figure 4 shows that the distribution of CCS mRNA transcripts and CCS protein in serum did not show any differences or trends.

Table 1 Characteristic of the studied population

	Group 1		Group 2	
	Men	Women	Men	Women
n	24	37	72	78
Age (years)	33.0 ± 8.2	32.9 ± 6.4	32.5 ± 8.3	31.4 ± 7.6
Weight (kg)	67.3 ± 7.9	57.4 ± 6.3	67.2 ± 10.1	64.8 ± 7.4
Height (m)	170.1 ± 7.6	158.4 ± 6.0	169.6 ± 7.3	170.0 ± 6.0
BMI $(kg/m)^2$	23.3 ± 2.4	22.9 ± 2.0	23.3 ± 2.8	22.4 ± 2.2
n (%) BMI > 25	4 (16.5)	4 (10.8)	19 (26.4)	16 (20.5)
Waist Circ. (cm)	84.5 ± 5.6	80.0 ± 5.5	85.1 ± 8.4	81.7 ± 7.6*
Systolic BP	120.3 ± 10.1	109.5 ± 11.7	122.8 ± 8.4	116.3 ± 12.8
Diastolic BP	71.3 ± 9.0	68.1 ± 9.7	74.5 ± 9.7	71.1 ± 11.8
n (%) HBP*	6 (25.0)	2 (5.4)	17 (23.6)	3 (3.8)
Glycaemia (mg/dL)	94.6 ± 10.1	90.4 ± 9.0	94.8 ± 11.6	96.7 ± 9.8
Hb (g/dL)	16.5 ± 1.1	14.2 ± 1.6	16.2 ± 1.3	14.0 ± 0.9
n (%) glycaemia > 100	10 (41.7)	3 (8.1)	16 (22.2)	17 (21.8)
Tot Chol (mg/dL)	170.0 ± 35.3	152.4 ± 22.4	182.7 ± 33.4	177.1 ± 33.3*
HDL Chol (mg/dL)	49.6 ± 9.9	51.4 ± 11.4	48.9 ± 13.3	41.3 ± 13.9
LDL Chol (mg/dL)	100.2 ± 31.0	85.6 ± 23.7	110.2 ± 32.9	113.7 ± 27.3*
TGC (mg/dL)	100.9 ± 44.0	77.4 ± 32.0	117.4 ± 54.4	110.0 ± 44.0*
n (%) TGC > 150	4 (16.7)	2 (5.4)	18 (25.0)	11 (14.1)
CRP (mg/dL)	0.9 (0.4–2.0)	0.6 (0.3–1.4)	6.2 (4.4–8.7)*	6.1 (4.3–8.6)*
GOT (UI/L)	34.7 ± 11.7	34.2 ± 12.8	33.4 ± 12.6	31.4 ± 12.9
GPT (UI/L)	33.6 ± 17.0	31.5 ± 16.4	34.3 ± 15.6	28.2 ± 14.2
GGT (UI/L)	25.1 ± 9.1	12.4 ± 8.5	26.2 ± 10.2	14.0 ± 6.3

BMI Body Mass Index, *Hb* Hemoglobin, *HBP* High blood pressure, *TGC* Triglycerides

* *t* test, individuals of same sex ($p < 0.05$)

Table 2 Fe, Cu, Zn and SOD and GSH levels in serum

	Group 1		Group 2	
	Men $n = 2$	Women $n = 37$	Men $n = 72$	Women $n = 78$
Fe (μg/dl)	116.4 ± 45.2	110.0 ± 39.0	111.7 ± 42.1	111.9 ± 46.9
Cu (μg/dl)	77.9 ± 20.2	87.3 ± 30.8	75.0 ± 17.0	92.4 ± 38.0
Zn (μg/dl)	98.8 ± 5.2	92.5 ± 9.9	98.6 ± 8.7	89.6 ± 10.6
SOD (U/mg Hb)	2.59 ± 0.30	2.69 ± 0.20	3.00 ± 0.55	2.82 ± 0.18*
GSH (μmol/L)	14.3 (5.2–39.0)	20.6 (9.4–45.4)	9.2 (3.3–25.1)*	12.6 (5.7–27.6)

* *t* test, individuals of same sex ($p < 0.05$)

Discussion

Results disproved the hypothesis that CCS abundance of transcripts and CCS protein would be different in individuals suffering inflammatory processes, adding further support to the idea that CCS may be a copper marker. As CCS responded to copper changes in a variety of experimental controlled conditions, to mimic a controlled experimental conditions in humans, we conducted a first study assessing adults that underwent a strict and wide screening before declaring them "healthy" (Araya et al. 2012). This yielded results

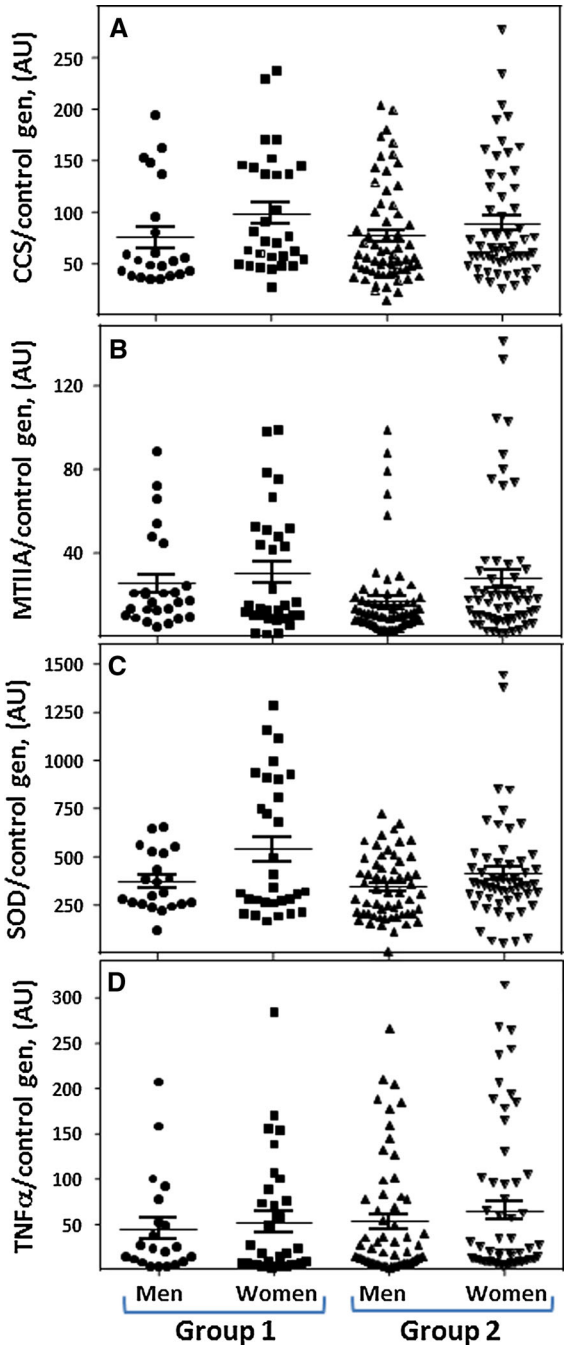

Fig. 2 CCS (**a**); MTIIA (**b**); TNFα (**c**) and SOD (**d**) mRNA relative abundance by sex

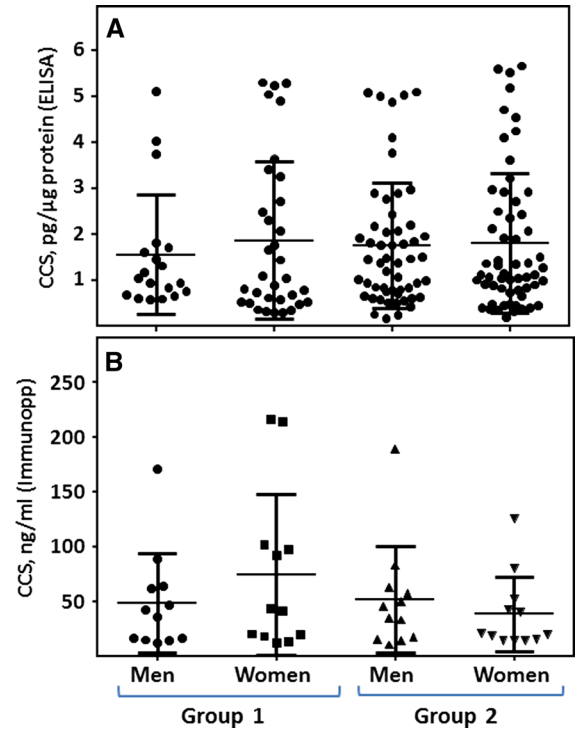

Fig. 3 Serum CCS concentration by ELISA and immunoprecipitation

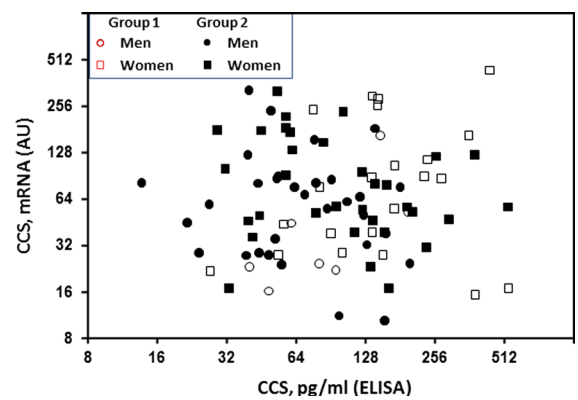

Fig. 4 Association between mRNA relative abundance of CCS and serum CCS concentration

concurrent to those obtained in cells and animals. Then, the next logical and necessary step to assess CCS as copper marker was to test it in individuals as they appear in real life, suffering the wide variety of conditions that induce inflammation and oxidative stress, such as depression (Chang et al. 2014), oxidative stress (Inoue et al. 2013; Ozturk et al. 2013), obesity (Azab et al. 2014), some forms of cardio vascular hypertrophy (Lind et al. 2012), some parasitic infections (Saad et al. 2013), diabetes mellitus (Lin et al. 2014), rheumatoid arthritis (Strecker et al. 2013), some forms of cancer (Khanna

et al. 2013), among many, all of which are frequently found in general population. Study groups were formed following clinical data and blood C reactive protein (CRP), the most used clinical indicator to assess inflammation; although ill individuals were excluded, groups thus formed were clearly separated by this indicator (CRP = 0.9 (0.3–2.0 mg/dL in Group 1 and CRP = 6.1 (4.3–8.7 mg/dL) in Group 2. In addition, blood biochemistry and nutritional anthropometry further supported that the groups assessed differed in their inflammatory condition. Thus, the protocol successful compared groups that suffered low and higher degrees of inflammation, providing a valuable piece of information in the task of assessing CCS.

Finding no differences between groups for Fe and Zn was the expected result, because in Chile deficiency of these minerals is low among adults (Ministerio de Salud and Gobierno de Chile, "Encuesta Nacional de Salud" (National Health Survey) 2003). Results of SOD and GSH, in individuals that represent preclinical cases, are interesting; although this protocol does not allow to draw conclusions in this respect, we think that the fact that SOD was higher and GSH was lower in the inflamed group (in both sexes) is consistent with the functions described for these molecules in the handling of inflammation and oxidative stress (McCord 1974; Salin and McCord 1975), i.e., SOD production is increased to manage the oxidative stress present and GSH decreases as it is consumed.

Obtaining concurrent results measuring CCS transcripts and protein represents another relevant finding of this study; considering that the studied individuals showed mild and moderate degrees of inflammatory processes we interpret this result as that neither of the measures was affected by inflammation. In other words, CCS transcripts and protein in serum did not follow the known response of several acute phase proteins, including copper indicators. Also of interest is that individuals were clearly different for CRP values and nevertheless serum copper were not different among groups, suggesting that serum copper would respond to more intense inflammation. If CCS finally proves being a copper marker, a practical kit applicable on clinical situations will be needed. Until now, the human studies available were performed measuring only CCS mRNA transcripts; CCS protein would have advantages when preparing the clinical test, not only because its development would have a lesser cost, but also it would be easier to set it up in less sophisticated clinical laboratories.

Although present results are the first to test CCS in human beings clearly closer to real life, there is still a long way to go before CCS can be declared a copper marker. Participants in this protocol represent preclinical cases and we do not know whether in more intense conditions results would be the same. On the other hand, the point made by Hooper et al. (Hooper et al. 2009) is still unanswered, if a biomarker of micronutrient status does have an effect on a health outcome, is this because the micronutrient has a specific direct effect on health or because the biomarker is a surrogate variable, namely, that it responds to another factor, e.g. obesity, which has a direct effect on health? Putting all arguments together and in summary, the fact that CCS abundance of transcripts and CCS protein measured were not different in the groups studied gives supports to the idea that CCS may be a copper marker useful for human studies and this justifies its further assessment.

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