

Present situation of biomarkers for copper status^{1–3}

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ABSTRACT

Serum or plasma copper and ceruloplasmin concentrations are the most widely used laboratory indicators to evaluate copper status. Both indicators are decreased in moderate or severe copper deficiency. The activity of several cuproenzymes is decreased in mild copper deficiency. However, their use is limited by the nonexistence of standardized assays and high interindividual variability and because some of these indicators are affected by other conditions. Recently, it was shown that the protein expression of the copper chaperone for superoxide dismutase (CCS) is increased in erythrocytes of rodents with mild copper deficiency. However, no traditional laboratory indicators have been identified as potential early markers of copper excess. It is possible that the biomarkers studied so far are not sensitive enough to detect an increase in body copper before the appearance of functional or clinical effects or that the homeostatic mechanisms are so strong that no significant changes in body copper occur with mild-to-moderate copper exposure. The identification of appropriate biomarkers for early detection of an increase in body copper represents a major challenge for further research, and the development of new approaches, such as network biology, allows us to search and propose new candidates to be studied. Recently, we found that CCS mRNA abundance in mononuclear blood cells significantly decreased after copper supplementation. The usefulness of this indicator to detect an increase in body copper should be assessed in clinical trials.

INTRODUCTION

Significant health risks from both deficient and excessive copper intakes are described. The challenge in hand is to find appropriate biomarkers that allow the early detection of copper deficiency and copper overload in at-risk groups at the population level as well as in the individual patient.

ASSESSMENT OF COPPER STATUS

Serum or plasma copper and ceruloplasmin concentrations are the most widely laboratory indicators used to evaluate copper status (1). Because these indicators are tightly controlled, they are only decreased in moderate or severe copper deficiency (1). Both indicators experience changes related to age, sex, and pregnancy and are increased by other conditions not related with copper status (inflammatory or infectious processes, neoplasm, and estrogen therapy) (1). A decrease in serum or plasma copper and ceruloplasmin concentrations is described in Wilson disease and nephrosis. It was shown that the ratio of enzymatic ceruloplasmin activity to immunoreactive protein concentration may

be a better indicator of copper deficiency, with the additional advantage that age, sex, or hormonal therapy does not influence this ratio (1).

Intracellular copper in blood cells and hair copper content have not provided additional useful information. Furthermore, urinary copper excretion is only decreased in severe copper deficiency.

The measurement of the activity of several cuproenzymes was used to evaluate copper status in both animals and humans. A decrease in the activity of erythrocyte copper-zinc superoxide dismutase (SOD1) was found in patients deficient for copper and in subjects with low copper intakes (2, 3). However, it was also observed that SOD1 activity does not change quickly in subjects receiving an experimental diet with low copper, because of the rate of erythrocyte turnover ($\approx 1\%$ per day) and because erythrocytes are not able to synthesize proteins. A higher SOD1 activity can be found in conditions that produce oxidative stress (1).

Copper deprivation studies in humans have shown that cytochrome-c activity of mononuclear leukocytes and platelets is reduced in copper deficiency. This decrease occurs before the appearance of a change of SOD1 activity, suggesting this may be a more sensitive marker of copper status (1, 3).

Animal and human studies have shown that plasma diamine oxidase (DAO) activity is reduced in mild copper deficiency (4, 5). However, tissue injury may also increase plasma DAO activity, thus confounding its interpretation. Moreover, women have higher plasma DAO activity than men. Furthermore, because plasmatic activity of this enzyme in humans is low, it is hard to detect a decrease in DAO activity as a result of copper deficiency. Kehoe et al (6) observed, in humans, an increase in serum DAO after copper supplementation with 3 or 6 mg/d for a period of 6 wk. The investigators suggest that this laboratory measurement is sensitive to changes in copper intake. However, it is not possible to discard that the increase in serum DAO could be the consequence of an alteration of intestinal integrity secondary to an oxidative stress induced by copper supplementation.

A decreased peptidyl glycine α -amidating monooxygenase activity in plasma and tissues has been described in both rodents with mild copper deficiency and patients with Menkes disease (7). However, the usefulness of this test should be analyzed in copper depletion studies in humans.

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Activity of lysyl oxidase is decreased in patients with Menkes disease. However, this laboratory indicator is not suitable to use in the evaluation of copper status because a tissue sample obtained by biopsy is required for the measurement of its activity.

The main limitations in the use of cuproenzymes for the evaluation of copper status are the following. 1) Standardized assays are nonexistent; for this reason each laboratory should define its own normal values. 2) There is high interindividual variability of enzymatic activities. 3) Some of these measurements are affected by other conditions. 4) Some enzymes are labile, which limits their use in field studies. 5) The performance (sensitivity, specificity, and likelihood ratio) of these tests to detect mild copper deficiency in humans has not been appropriately studied.

Recent publications have shown that the copper chaperone for superoxide dismutase (CCS) protein expression, but not mRNA, is increased in liver and erythrocytes of rodents receiving a copper-deficient diet or in rats having a mild copper deficiency induced by moderately high dietary zinc (8–11). We have found that blood mononuclear CCS mRNA abundance is decreased after a copper supplementation with 10 mg/d for 2 mo, suggesting that this seems to be a promissory biomarker of copper exposure whose usefulness should be evaluated (12).

Liver copper concentration is the best indicator of copper status and is the standard to compare the performance of any test used to detect copper overload. However, this invasive procedure is only justified when there is evidence of liver damage as a result of copper overload. Because of the aforementioned ethical constraint, the usefulness of several tests in detecting an early increase in copper storage in humans was measured in different copper supplementation trials or in subjects receiving copper-enriched diets.

Serum copper, ceruloplasmin, and SOD1 do not change in infants from 3 to 12 mo of age receiving diets that provided $\approx 300 \mu\text{g Cu} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ (13) or in adults supplemented daily with 10 mg Cu for 2 mo or 8 mg Cu during 6 mo (14, 15). Similar results were observed in adult subjects receiving water with a copper concentration of $<0.1, 2, 4,$ or 6 mg/L (range of copper intake: $15\text{--}150 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) for 2 mo (16). In addition, we have shown that erythrocyte metallothionein, blood mononuclear copper concentration, and urinary copper excretion after a dose of a copper chelator did not change after the increase in copper exposure (13, 14).

The non-ceruloplasmin-bound copper pool was proposed by some as a marker of copper excess. We calculated this pool, subtracting the value obtained from total copper in the serum based on the assumption that each molecule of ceruloplasmin binds 6 copper atoms. We found an increase in non-ceruloplasmin-bound copper, which was attributable to copper supplementation, in only 1 of the 4 aforementioned studies (14). Because the calculation of non-ceruloplasmin-bound copper has some uncertainties, such as the number of copper atoms bound to ceruloplasmin and the proportion of apoceruloplasmin included in the ceruloplasmin assessment, the direct measurement of this serum copper fraction would be required to ascertain its usefulness as an indicator of an increased copper body load.

In summary, no laboratory indicators are universally accepted as an early marker of copper excess. It is possible that the biomarkers studied were not sensitive enough to detect an increase in body copper, before the appearance of functional or clinical effects, or that homeostatic mechanisms are so strong that no

significant changes in body copper occurred with mild-to-moderate copper exposure.

NEW INSIGHTS FROM THE SEARCH OF PROTEIN CANDIDATES AS BIOMARKERS OF COPPER STATUS

Recently, a new understanding of the mechanisms that determine the responses of the cell on nutritional or metabolic conditions was developed with the advance of network biology, which is a quantifiable description of the networks that characterize various biological systems and allows analyzing the interactions of several genes or proteins (17). The use of algorithms that enables one to construct networks made up of specific genes involved in specific processes is a valuable tool to study new relations; hence, network biology can be effective in determining and selecting new targets, eg, for drug discovery and for mutational analysis. A recent work featured the construction of a “human disease network,” which represents disorders and disease genes linked by known associations (18). However, significant further efforts are needed to identify new candidate genes that can help us understand the response of “borderline” populations to external variations and to increase our knowledge about disease-causing mutations.

We constructed a network representing the “copper interactome” with the aim to find new candidates as biomarkers of copper status and to analyze the value of current and proposed biomarkers. Within a network, genes or proteins harboring a high number of interactions with other partners are thought to be essential in the network, and alterations in these nodes (called “hubs” in network biology) can be relevant in the integrity of the network. Hence, we searched for the existence of hubs in the network constructed for copper homeostasis. For the construction of the network, we selected 7 proteins directly involved in copper metabolism, after reviewing the current literature. The proteins selected were ceruloplasmin, copper-transporting adenosine 5'-triphosphatase (ATPase) 7A (ATP7A), ATP7B, copper transporter CTR1 (CTR1), CCS, copper transport protein ATOX1 (ATOX1), and COMM domain containing protein 1 (COMM1). These proteins were used as templates, and we constructed, for each protein, a network with the STRING database (<http://string.embl.de/>), limiting the search only to information available in *Homo sapiens*. These networks were merged and analyzed with the CYTOSCAPE, which is a free software

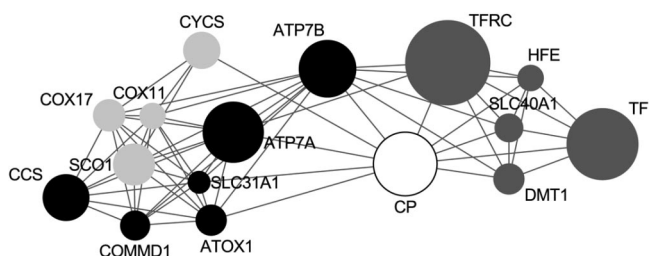


FIGURE 1. Subnetwork shows the main hubs identified in our analysis. The node color indicates the biological process in which the protein is involved: iron homeostasis (dark gray), cytochrome-*c* oxidase complex assembly or release from mitochondria (light gray), and copper homeostasis and transport (black). In the center of the network, ceruloplasmin (white node) plays a role in connecting both copper-related and iron-related proteins. However, ATP7B is also connecting both processes. Size of nodes indicates their degree or connectivity, *k*. Information about name and degree of the nodes is available in Table 1.

TABLE 1

Main proteins identified as hubs in the network for copper homeostasis, including biological process associations from Gene Ontology, classified from 292 proteins that are included in the network

| Protein | Degree ¹ | Biological process |
|--|---------------------|---|
| Cytochrome- <i>c</i> oxidase assembly protein (COX11) | 22 | Cytochrome- <i>c</i> oxidase complex assembly |
| Hereditary hemochromatosis protein (HFE) | 22 | Iron homeostasis |
| T cell surface glycoprotein CD4 precursor (CD4) | 22 | Syncytium formation |
| Solute carrier family 40 member 1; ferroportin-1 (SLC40A1) | 24 | Iron homeostasis |
| COMM domain containing protein 1; Murr1 protein (COMMD1) | 25 | Copper homeostasis |
| Copper transport protein ATOX1 (ATOX1) | 26 | Copper homeostasis |
| Divalent metal transporter 1 (DMT1; SLC11A2) | 26 | Iron homeostasis |
| Cytochrome- <i>c</i> oxidase copper chaperone (COX17) | 27 | Cytochrome- <i>c</i> oxidase complex assembly |
| α -2-Macroglobulin precursor (A2 M) | 28 | Regulation of receptor mediated endocytosis |
| Cytochrome- <i>c</i> , somatic (CYCS) | 31 | Release of cytochrome <i>c</i> from mitochondria |
| SCO1 protein homolog, mitochondrial precursor (SCO1) | 35 | Cytochrome- <i>c</i> oxidase complex assembly |
| Copper chaperone for superoxide dismutase (CCS) | 39 | Copper homeostasis |
| Copper-transporting ATPase 2 (ATP7B) | 48 | Copper homeostasis |
| Copper-transporting ATPase 1 (ATP7A) | 51 | Copper homeostasis |
| Ceruloplasmin (CP) | 54 | Iron homeostasis ($1.75e^{-30}$) ² ; copper homeostasis ($5.04e^{-25}$) ² |
| Transferrin (TF) | 60 | Iron transport |
| Transferrin receptor (TFRC) | 71 | Iron homeostasis |

¹ Degree or connectivity, ie, the number of proteins interacting with the protein indicated in the table.

² In the case of ceruloplasmin, ≥ 2 biological processes indicated have a similar *P* value; hence, we included, in this case, the respective *P* values for each Gene Ontology association.

package for visualizing, modeling, and analyzing molecular and genetic interaction networks (19). This network comprises 292 proteins and 1151 interactions, and 37 proteins are classified as hubs, according to the algorithms implemented (**Figure 1**). The main hubs observed are listed in **Table 1**. The principal observation is the high number of proteins related with iron metabolism classified as hubs. In addition, several proteins directly related with copper metabolism are present as hubs in the network, and ceruloplasmin plays a role as a bridge between copper and iron metabolism. In addition, in the network, ATPases 7A and 7B are connected with ceruloplasmin and with several proteins involved in iron metabolism. Because it is possible to speculate that proteins that are interacting with ceruloplasmin can respond, concomitantly, to copper overload or deficiency, ATPases 7A and 7B deserve to be further assessed as potential biomarkers of copper status.

It is interesting to note that CCS has a high number of interactions in the network. Considering the observations about the behavior of CCS under copper exposure, CCS could be an interesting candidate, because small changes in the amount of this protein within the cell, as well as alterations in its activity, can produce changes in others proteins directly associated with this chaperone. However, CCS is not directly associated with ceruloplasmin, the most traditional marker of copper status. Other candidate markers such as CTR1 and SOD1 are poorly associated with other copper-related proteins, suggesting that changes in these proteins are likely to be less appropriate to study as markers of copper homeostasis. A recent work screened for genetic variations in the proteins ATOX1, CTR1, CCS, and COX17 in persons with a copper-deficiency phenotype, and only a heterozygous polymorphism was found in CCS (20). Therefore, future directions of study should consider the screening of genetic variants (such as single nucleotide polymorphisms) in candidates such as ATPases 7A and 7B, ceruloplasmin, CCS, and copper-binding protein of the mitochondrial inner membrane (SCO1), because of their high connectivity with other proteins, and also in

candidates related with iron metabolism, as transferring and transferring receptor, because the relation between iron and copper metabolism was further supported by our analysis.

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