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Serum trace metals and ceruloplasmin variability in individuals treated for pulmonary tuberculosis

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SUMMARY

SETTING: Investigation of trace metals behaviour during the treatment of active pulmonary tuberculosis patients of Romanian residence.
OBJECTIVE: To assess, follow and identify serum iron, copper and zinc levels in patients diagnosed and treated for active pulmonary tuberculosis.
DESIGN: Chemical and statistical analysis of various biochemical parameters in 47 subjects diagnosed with active pulmonary tuberculosis and 170 healthy individuals of Romanian residence.
RESULTS: Copper and ceruloplasmin levels were found to be increased in patients with active pulmonary tuberculosis compared to the control group ($p<0.01$), while serum zinc level was significantly lower than in healthy subjects ($p<0.01$) or within the normal range. The present study shows that there is a significant correlation between serum copper concentrations and ceruloplasmin.
CONCLUSIONS: This study provides preliminary evidence that zinc and iron redistribution is operating as primary host defence mechanism to reduce metals availability for microbial metabolism during infection. The study also calls attention to the fact that anti-tuberculosis treatment is sufficient to enhance the concentration of the antioxidant species (copper and ceruloplasmin). The obtained data suggest that serum Cu, Zn and Cu/Zn levels may serve as indirect pointers in the diagnosis of a disease, including also active TB, but they can't be considered as specific markers for tuberculosis. However, the monitoring of their evolution following anti-tuberculosis therapy administration may represent a very good tool to asses the favourable response of the host to a specific drug.

KEYWORDS: active pulmonary tuberculosis, iron, copper, zinc, ceruloplasmin

INTRODUCTION

Elements such as copper (Cu), iron (Fe) and zinc (Zn) are essential trace metals involved in several key biological processes.[1-3] While iron is a component of oxygen-binding molecules of cytochromes and of many enzyme cofactors with an important role in the transport and storage of molecular oxygen,[4] copper is needed for the proper utilization of Fe and it is involved in the natural defences of the organism against the damage induced by reactive oxygen species (ROS).[5] Iron and copper are interlinked through ceruloplasmin, which presents considerable ferroxidase activity and is of significance in assessing free radical activity.[6,7] On the other hand, zinc plays a central role in the intracellular immunologic system.[8]

Because of their immunomodulatory functions, Fe, Cu and Zn are elements which might influence the susceptibility of the human body to the course and the outcome of various infections.[9,10] In active pulmonary tuberculosis (TB) or in the presence of inflammation, the status of Fe, Cu and Zn might be strongly perturbed. The reduction in the circulating Fe or Zn levels is mainly a protective effect to reduce micronutrients availability for microbial metabolism during infection.[9,11] There is also suggestion that metabolic alterations may occur before the development of the immunity, as a necessary part of the integrated host response to inflammatory stress.[12] Advances in microbiology, immunology and clinical studies are presently strengthening the relationship between Fe and TB.[13] The role of Zn and Cu on health and immune function has been largely reviewed,[8,14] and deficiencies of Zn and vitamin A are associated with active pulmonary TB.[15] Zinc depletion and supplementation may influence the activity of normal human bronchial epithelial cells;[16] its deficiency is known to cause impaired cell-mediated immunity and to compromise neutrophil functions, which may result into increased susceptibility to TB.[8]

Monitoring the outcome of a treatment is essential to estimate the effectiveness of the administered therapy.[17] An assessment can be made by measuring the level of various biochemical parameters in body fluids, such as serum or plasma.[9] Cu and/or Zn levels in serum/plasma are quite often used to investigate the effectiveness of an ongoing anti-tubercular or thoracic emphysema therapy[18]. Furthermore, their levels in tissues[19,20] or in serum[19,21] are used in the diagnosis process of lung affections including TB, or in the assessment of risk factors for the development of carcinoma.

There is suggestion that the severity of the TB problem in Iasi County, Romania, is presently underreported.[22] Moreover, microelements status in individuals of Romanian residence is not yet reported. Because of the important role of Fe, Cu and Zn in the human body, the present study was designed to evaluate changes in their serum concentrations upon anti-tuberculosis therapy administration in Romanian residents diagnosed with active pulmonary TB. The results regarding the behaviour of serum Fe, Cu, Zn, erythrocyte sedimentation rate and fibrinogen levels, upon

chemotherapy administration, are also discussed in terms of possible co-assisting factors in the setting of the inflammation associated with active pulmonary TB.

METHODS AND MATERIALS

Subjects and definition of cases

Definite cases of male and female gender were patients with untreated active pulmonary TB admitted to the Clinic of Pulmonary Diseases, "Gr.T. Popa" Medical School of Iasi, Romania, from July 2008 to August 2009. Patients with positive cultured sputum had a definitive diagnosis of active pulmonary TB. Chest radiological examination was also undertaken for each patient.

From the initial cohort of identified definite cases (65 subjects), a subpopulation of 47 subjects (36 males, 11 females) was drawn for the convenience of the follow-up. Relapsed cases and patients with partial attendance to the program were excluded from the present study. Also excluded from the study were patients with extra-pulmonary tuberculosis, hypertension, diabetes mellitus, chronic liver disease, rheumatoid arthritis, malignancy, human immunodeficiency virus (HIV), pregnancy, women on oral contraceptive, which are physiological and pathological states and drugs likely to affect the serum concentration of essential micronutrients (copper and zinc). Investigations to establish the glycemic status and tests for liver and renal functions have been undertaken in all patients, to identify diabetes mellitus, chronic renal failure and hepatic dysfunctions. Information regarding age, gender, height, mass and other individual status (rural or urban residence, studies and employment) was recorded in the *pro forma* of every subject of the study.

Zinc concentration was selected as a key criterion. Its status was classified as serum Zn lower than the lower limit of the normal range - Zn low, and serum Zn level within the normal range - Zn normal. Patients with definite cases of active pulmonary TB were classified in subgroups of similar behaviour during anti-tuberculosis therapy administration. The identified male subgroups are quoted in the present work as M_I (Zn low) and M_{II} (Zn normal), the female subgroups as F_I (Zn low) and F_{II} (Zn normal).

For the patients diagnosed with active pulmonary TB, various laboratory parameters were investigated soon after the disease diagnosis (time 0) and after 7, 14, 30, 60 and 180 days of anti-tuberculosis therapy administration. Cohorts of healthy subjects (80 males, mean age 30±11, and 90 females, mean age 33±12), were investigated in order to establish reference values (RV) for the parameters of interest. Data are accompanied by their standard deviation (SD).

Treatment and nutrition during hospitalisation

All the patients diagnosed with active pulmonary TB were treated with a regimen made of isoniazid, rifampicin, pyrazinamide and ethambutol in usual doses. Most patients had a relatively fast response to the therapy, with the bacillary count decreasing progressively to negative within one month of treatment initiation. As the cultures didn't remain positive for more than two months,

there were not suspected cases of non-adherence, mal-absorption of drugs, drug resistance or a combination of these factors.

An equilibrated dietary habit, prescribed also for pulmonary TB patients with common TB forms, suggests a contribution of 3000 calories per day and 36 g of animal proteins as recommended by the internal protocol of the Diet Department in the Clinic of Pulmonary Diseases. Therefore, during the hospitalisation (80% of the enrolled subjects were hospitalised for one month and 20% for two months) the nutritional regime was of 2857±129 calories (mean±SD) and 127±18 g (mean±SD) of proteins per day. Moreover, the administered diet included dairy and meat products. We should mention that no records exist in the *pro forma* of the enrolled patients after hospitalisation regarding their dietary habits, although their evolution was followed over a total period of 6 months after chemotherapy initiation.

Ethics

Approval No. 4950/04.09.2008 was obtained from the local regional ethics committee, Bioethics Committee for the Medical Directorate, Iasi, Romania. All the study subjects signed an informed consent during the interview.

Data collection for selected laboratory parameters

Fe, Cu and Zn measurements were performed on a MicroLab 200 spectrophotometer. Determination of Fe was performed at 546 nm with Ferrozine as chromogen,[23] which supplies the advantage of accurate iron determination in patients with high concentrations of ferritin.[24] The measurement of serum Cu was performed at 580 nm with the colour reagent 4-(3,5-dibromo-2-pyridylazo)-N-ethyl-N-sulfopropylaniline, also called 3,5-diBr-PAESA.[25] For Zn, 2-(5-bromo-2-pyridylazo)-5-(N-n-propyl-N-3-sulfopropylamino)phenol (5-Br-PAPS) was used as chromogen because it may yield comparable results with FAAS.[26] Serum ceruloplasmin was measured by its p-phenylenediamine oxidase activity at pH 5.4.[27] The Westergren method was used to determine erythrocyte sedimentation rate (ESR) values and fibrinogen was measured on a Spekol 11 spectophotometer. Bacilli in the sputum and serial determination of sputum colony-forming unit (cfu) counts were measured in all patients suspected of pulmonary TB.

Kinetic treatment in the serum levels of the investigated parameters

A simple conceptual kinetic model in the form

$c = c_0 \times e^{-k \times \Delta t}$ (eq. 1)

where c_0 is the concentration of the parameter at the initial time, c its concentration at the time t and k the enhancement rate (increase or decrease) of the interest parameter in a period of time Δt, allowed us to determine the value for k. Natural logarithm applied to (eq. 1) leads to the equation

$$\ln \frac{c_0}{c} = k \times \Delta t \qquad \text{(eq. 2)}$$

From (eq. 2), the value of k can be derived as the linear slope of $\ln(c_0/c)$ vs. Δt.

Statistical analysis

Mean and standard deviation (SD) are used for reporting the data. Independent sample t test was used to assess the difference between patients and controls. The association between various laboratory parameters has been investigated by the Spearman rank analysis. The p level considered as significant was of <0.01.

RESULTS

Table 1 presents subjects characteristics and data related to the body mass index (BMI) and tuberculosis severity as determined at the initial time of disease diagnosis, for the subjects included in M_I, M_{II} (male gender) and F_I, F_{II} (female gender) groups.

Serum Fe, Cu, Zn and ceruloplasmin levels

Table 2 presents serum Fe, Cu and Zn concentrations, accompanied by their standard deviation (±SD), in patients diagnosed with active pulmonary TB and in healthy cohorts of individuals (reference values, RV), for both male and female genders. Ceruloplamin concentrations and the Cu/Zn ratio, defined as the ratio between serum Cu and serum Zn concentrations, are also given in Table 2.

During the anti-tuberculosis therapy, serum micronutrients status returned toward normal values. To better observe the time variability of Fe, Cu and Zn, examples are presented in Figure 1 for both males (1a,b) and females (1c,d) subgroups. After therapy initiation, an increase in serum Fe level was observed in all patients diagnosed with active pulmonary TB. In contrast, Zn showed different tendency (increase or decrease) depending on its initial status (Zn low or Zn normal, respectively). As presented in Figure 2, also the Cu/Zn ratio showed a tendency toward normalisation after anti-TB chemotherapy initiation.

Data in Table 3 indicate the % change in the serum concentration of Fe, Cu and Zn at the therapy completion, relative to the initial values of each interest parameter. The values obtained for the enhancement rate k are also reported in Table 3.

Baseline characteristics for the tracers of the inflammation

In the patients diagnosed with active pulmonary TB, at the initial time of the disease diagnosis, fibrinogen and ESR levels were significantly higher than in healthy individuals. Fibrinogen serum levels as high as 4.96±0.68 mg mL^{-1} for males and 4.95±0.89 mg mL^{-1} for females were initially determined in the patients with active pulmonary TB. These values should be compared with the reference ones determined in healthy males and females, which were 2.65±0.62 mg mL^{-1} and 2.52±0.50 mg mL^{-1}, respectively. At the initial time of disease diagnosis, the measured ESR levels in the patients with active pulmonary TB were 90.75±43.74 mm h^{-1} for males and 88.38±52.88 mm h^{-1} for females. The ESR values are significantly higher than those of <15 mm h^{-1} or <20 mm h^{-1} suggested for males and of <20 mm h^{-1} or <30 mm h^{-1} suggested for females.

Correlation of the measured serum biochemical parameters

Microelement concentrations (Fe, Cu, Zn) in the investigated subjects were highly variable, most probably depending on the individual metabolism. However, in a stepwise multiple regression analysis, serum Cu, Fe and Zn concentrations showed significant association with the erythrocyte sedimentation rate (ESR) indices. The correlation between trace metal concentration (Cu, Fe, Zn) and ESR is presented in Figure 3 for both males and females. The correlations are significant at a probability level $p<0.01$ in each investigated subgroup. Positive slope was observed for the association between Cu and ESR and negative slope for the (Fe, ESR) pair, while for the pair (Zn, ESR) both negative (M_I, F_I, Zn low) and positive (M_{II}, F_{II}, Zn normal) slopes were observed. Although not shown, significant correlation ($p<0.01$) was observed between ESR and fibrinogen levels and between fibrinogen and Cu, Fe and Zn levels in the investigated subgroups. Significant correlation ($r=0.87$, $p<0.01$) was also observed between serum Cu and ceruloplasmin levels in the patients diagnosed with active pulmonary TB (Figure 4). The ceruloplasmin concentration increased with the serum Cu levels.

DISCUSSION

Trace metals variability

Data in Table 2 show that in the cohorts of healthy individuals, serum concentrations of the investigated trace metals were within their normal ranges for both male and female subjects. Variable serum concentrations of trace metals are mainly characteristic of the patients diagnosed with active pulmonary TB. In these patients, the concentration of Fe was significantly lower ($p<0.01$) than that of the control group, while the concentration of Cu and the Cu/Zn ratio were significantly higher ($p<0.01$). An exception was observed for the F_{II} subgroup, where the difference in the Cu/Zn ratio compared to the healthy cohort of individuals (90 females) was not statistically significant. The data in Table 2 show that a significant number of patients (83% for males and 64% for females) have low serum Zn and normal-to-high Cu concentrations at the initial stage of disease diagnosis, compared to the reference values.

The data in Table 3 indicate that, following therapy administration, the Fe levels showed the most rapid and pronounced increase toward the reference values for both males and females, when compared with other indices. The high serum Cu concentrations seem to be the most persistent in active pulmonary TB patients, probably because of ceruloplasmin abundance and its relatively long half time.[28] Compared to Cu, a more rapid return of Zn concentrations to the normal range is observed. The high levels of serum ceruloplasmin, observed at the initial time of disease diagnosis in subjects with active pulmonary TB, allow us to suggest that increased release of stimulus to the inflammatory process may occur during the development of the infection.

Variability of inflammation clinical targets

Inflammation plays an important role in active pulmonary TB, which is known to be associated with hemostatic changes (also monitored through the ESR) and can result into a hypercoagulable state. Plasma fibrinogen is a major determinant of platelet aggregation and blood viscosity, through mechanisms that play a central role in the formation of thrombi and thrombosis.[29] In the present work, a ~70% decrease in serum fibrinogen levels was observed at the end of the anti-tuberculosis therapy, for both males and females. However, the levels remained well above the corresponding reference values. Significant correlation between serum fibrinogen levels and ESR ($r=0.83$, $p<0.01$) was also observed, suggesting common mechanistic action. Famodu et al. reported serum fibrinogen levels as high as 8.52 ± 1.84 mg L^{-1} and 7.71 ± 2.02 mg L^{-1}, respectively, in male and female patients with pulmonary TB.[30] High levels of fibrinogen in active pulmonary TB were also observed by Robson et al., who suggested that complications due to deep venous thrombosis (DVT) are inherent to severe pulmonary TB.[31]

Although not shown, we observed significant correlation between ceruloplasmin and fibrinogen and between ceruloplasmin and ESR, with $r>0.9$ at a probability level of $p<0.01$. These observations suggest that ceruloplasmin also behaves as an acute-phase reactant. Fibrinogen is a known acute-phase reactant, the level of which increases in response to a high cytokines state. Under these circumstances, prothrombic and proinflammatory states may be metabolically interconnected.[32] In our study, under inflammatory state (ESR>20 mm h^{-1}) all patients presented increased ceruloplasmin concentrations, most probably because of increased ceruloplasmin release in serum in response to the inflammatory process through the release of cytokines. However, the increase in ceruloplasmin may also be associated with its role as antioxidant.

Trace metals role in the inflammation associated with active pulmonary TB

From Figure 3 it can be observed that in patients with active pulmonary TB, the inflammatory condition (ESR>20 mm h^{-1}) may persist under high Cu, low Fe and variable Zn serum levels. In a study performed by Rice et al., it has been shown that metals such as Fe, Cu and Zn may induce pulmonary inflammation by different pathways or combinations of signals.[33] They found that Cu is the most proinflammatory metal, which induces the synthesis of macrophage inflammatory protein-2 (MIP-2) mRNA, followed by Fe(II) and Zn. The latter induce similar levels of inflammation. In the present study, we observed that the patients with active pulmonary TB usually have low serum Fe and Zn and high serum Cu concentrations (Table 2).

Presently it is known that in infection with microorganisms, phagocytosis represents the initial active mechanism of host defence against pathogens action.[12] In infections associated with leukocyte endogenous mediator (LEM), which are substances produced during phagocytosis, many metabolic alterations may occur. Phagocytosis may lead to a 50-100 % increase in plasma Cu and to a 25-50 % decrease in plasma Zn concentrations.[12] LEM mediates the redistribution of circulating Zn via intrahepatic sequestration. Therefore, with a net flow of Zn to the liver for the synthesis of acute-phase reactants including metalloenzymes, Zn redistribution may appear as a host defence that allows for more extensive phagocytosis by circulating leukocytes.[34] The increase in plasma Cu

may be the result of the requirements induced when high amounts of catecholamines should be metabolized.[12] Increased amount of catecholamines was previously reported in patients with pulmonary TB, as a result of the effective influence of adrenaline and noradrenaline in the catecholamines synthesis.[35]

CONCLUSIONS

In the present study we examined the variability in the serum levels, upon anti-tuberculosis therapy administration, of essential elements such as Fe, Cu, Zn and of the copper-binding protein ceruloplasmin. In patients with active pulmonary TB, Cu and ceruloplasmin levels are increased compared to the control group ($p<0.01$), while serum Fe and Zn levels are significantly lower ($p<0.01$). The results of the present study allow us to suggest that in individuals with active pulmonary TB, the synthesis of ceruloplasmin could be favoured in order to enhance the antioxidant defence mechanism of the host.

Serum Cu, Zn and Cu/Zn levels may serve as indirect pointers in the diagnosis of a disease, including also active TB, but they can't be considered as specific markers for TB. However, the monitoring of their evolution following anti-tuberculosis therapy administration may represent a very good tool to asses the favourable response of the host to a specific drug.

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RIC performed the analysis of laboratory parameters. TM coordinated the study and provided comments. MV supplied the samples and interpreted the data. DV provided vital comments. RIO interpreted the data and provided comments. CA interpreted the data and has written the manuscript.

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Table 1. Subjects characteristics at the initial stage of disease diagnosis. For comparison purposes, reference values (RV) determined in the present work in healthy cohorts or normal ranges suggested in the laboratory are also presented.

	n	Age	Body mass index (BMI)	Severity of disease based on X-ray of chest	Sputum colony-forming unit (cfu) counts	Other characteristics
Male gender						
M_I (Zn low)	30	43 ± 14	19.9 ± 3.5	70% POTB; 20% MLCL; 10% BLCL	30% with 1+ cfu counts; 17% with 2+ cfu counts; 53% with 3+ cfu counts	Smoking and alcohol on occasion; 5% jobless and homeless; 40% jobless; rural (64%); urban (36%)
M_{II} (Zn normal)	6	42 ± 10	20.0 ± 0.9	70% POTB; 20% MLCL; 10% BLCL	17% with 1+ cfu counts; 65% with 2+ cfu counts; 18% with 3+ cfu counts	Non-smokers; alcohol on occasion; 50% jobless; rural (60%); urban (40%)
Control group (RV)	80	30 ± 11	23.6 ± 2.7	-		60% non-smokers; employed; rural (20%); urban (80%)
Suggested range	-	-	20 - 25	-		-
Female gender						
F_I (Zn low)	7	38 ± 16	20.2 ± 3.9	86% POTB; 14% MLCL	14% with 1+ cfu counts; 72% with 2+ cfu counts; 14% with 3+ cfu counts	Non-smokers; 35% jobless; rural (57%); urban (43%)
F_{II} (Zn normal)	4	31 ± 3	17.5 ± 1.3	50% POTB; 50% MLCL	75% with 2+ cfu counts; 25% with 3+ cfu counts	Smokers; 25% jobless; rural (75%); urban (25%).
Control group (RV)	90	33 ± 12	22.3 ± 3.02	-		75% non-smokers; employed; rural (25%); urban (75%).
Suggested range	-	-	18 - 24	-		

Note: reference values (RV) were determined in the present work. POTB: parenchimal opacity tuberculosis; MLCL: mono-lateral cavity lesion; BLCL: bi-lateral cavity lesion.

Table 2: Serum iron, copper and zinc concentrations, Cu/Zn ratio and ceruloplasmin levels, in patients with active pulmonary TB (M_I, M_{II}, F_I, F_{II}) and in healthy cohorts of individuals (reference values).

	n	Fe	Zn	Cu	Cu/Zn	Ceruloplasmin
		mg L^{-1}			-	g L^{-1}
Males						
M_I (Zn low)	30	0.34±0.14	0.35±0.17	1.61±0.21	4.62±1.07	1.00±0.14
M_{II} (Zn normal)	6	0.41±0.18	1.06±0.28	2.27±0.31	2.14±0.82	1.23±0.30
Reference values	80	0.94±0.37	0.90±0.32	1.08±0.17	1.20±0.47	0.48±0.12
Normal ranges	-	0.40–1.75	0.50–1.50	0.70–1.40	-	0.40-0.60
Females						
F_I (Zn low)	7	0.40±0.15	0.41±0.14	1.72±0.17	4.21±0.32	1.08±0.19
F_{II} (Zn normal)	4	0.21±0.10	1.41±0.02	1.64±0.26	1.17±0.19	1.18±0.15
Reference values	90	0.80±0.30	0.93±0.30	1.08±0.20	1.16±0.52	0.49±0.13
Normal ranges	-	0.40–1.75	0.50–1.50	0.80–1.50	-	0.40-0.60

Table 3: Relative change (%) and enhancement rate (day^{-1}) in patients cured for active pulmonary TB. The change is estimated toward the initial values in the serum concentrations of Fe, Cu and Zn at the therapy completion.

Parameter	Unit	Gender	
		Male	Female
Fe	Relative change (%)	+ 150	+ 100
Zn		+ 100 (if Zn low)	+ 90 % (if Zn low)
		± 40 (if Zn normal)	± 50 % (if Zn normal)
Cu		- 30	- 25
Fe	Enhancement rate (day^{-1})	$+(3.58\pm0.69)\times10^{-2}$	$+(2.43\pm0.23)\times10^{-2}$
Zn		$+(2.38\pm0.25)\times10^{-2}$ (if Zn low)	$+(1.34\pm0.35)\times10^{-2}$ (if Zn low)
		$-(7.21\pm0.56)\times10^{-3}$ (if Zn normal)	$-(5.99\pm0.62)\times10^{-3}$ (if Zn normal)
Cu		$-(1.03\pm0.96)\times10^{-2}$	$-(8.72\pm0.96)\times10^{-3}$

Note: (+) indicates a rise while (-) suggests a decrease in the concentration of the parameter at the end of therapy.

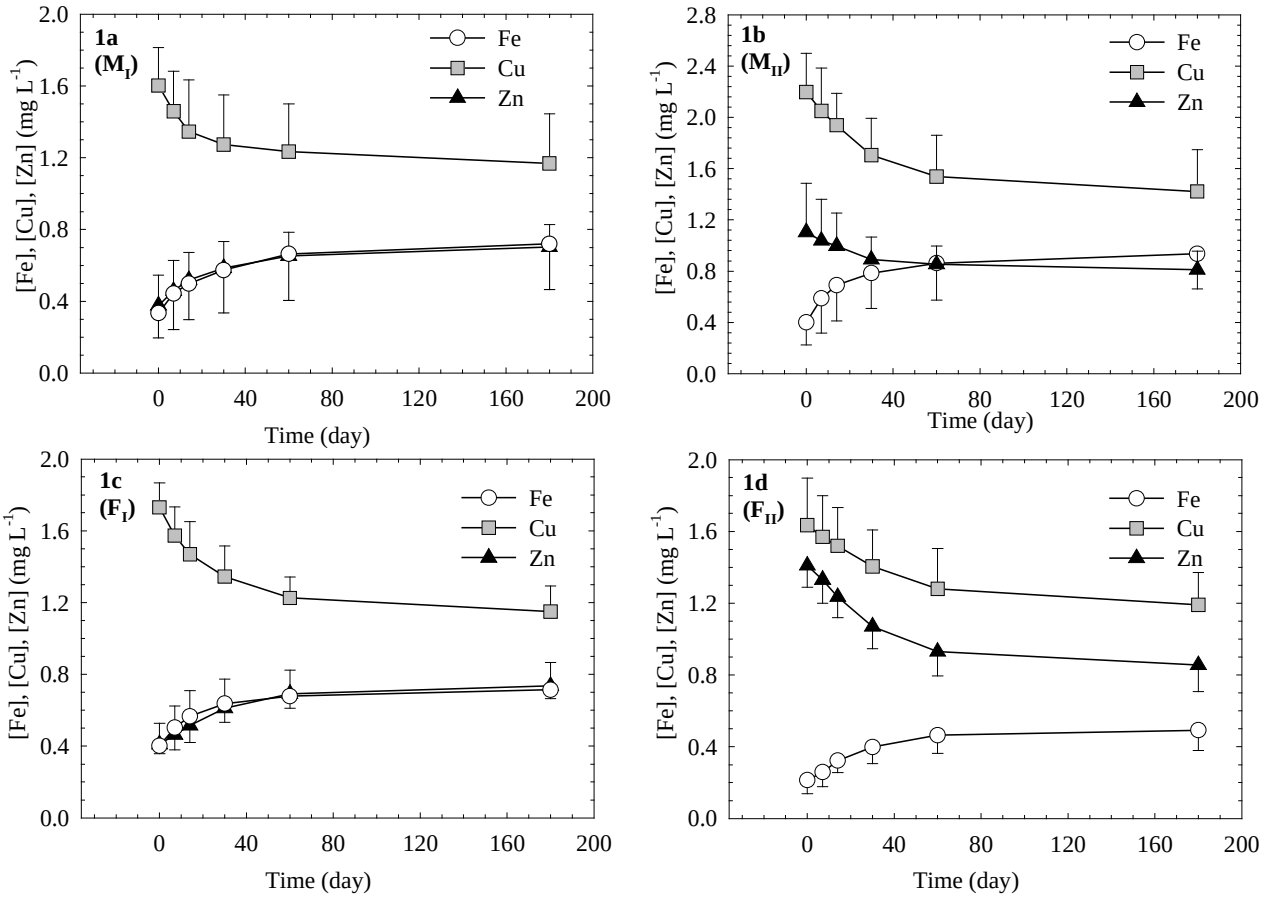

Figure 1 Time course of serum Fe, Cu and Zn concentrations both for male and female genders during anti-tuberculosis therapy.

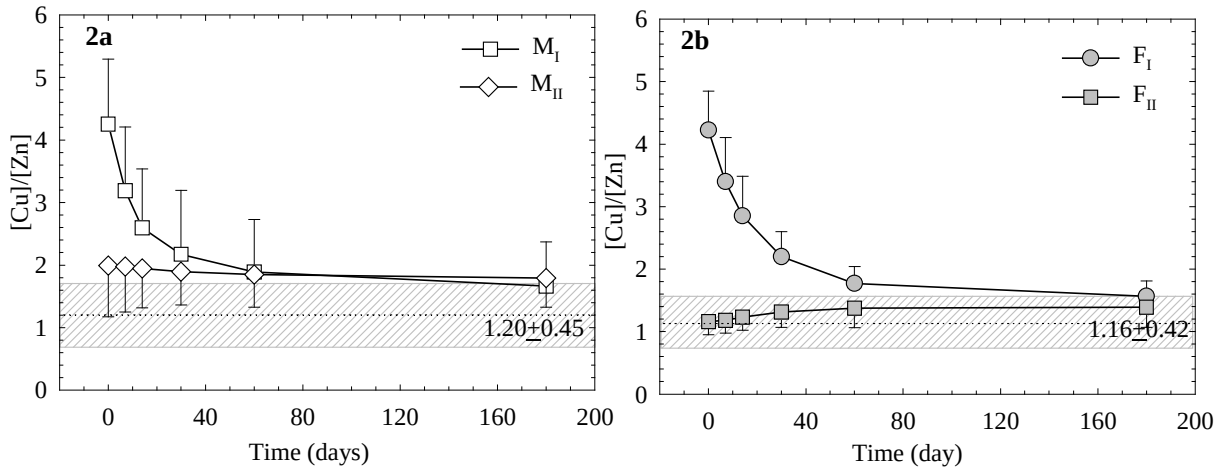

Figure 2 Time evolution of Cu/Zn ratio in patients under treatment for active tuberculosis. The shadowing represents the reference values.

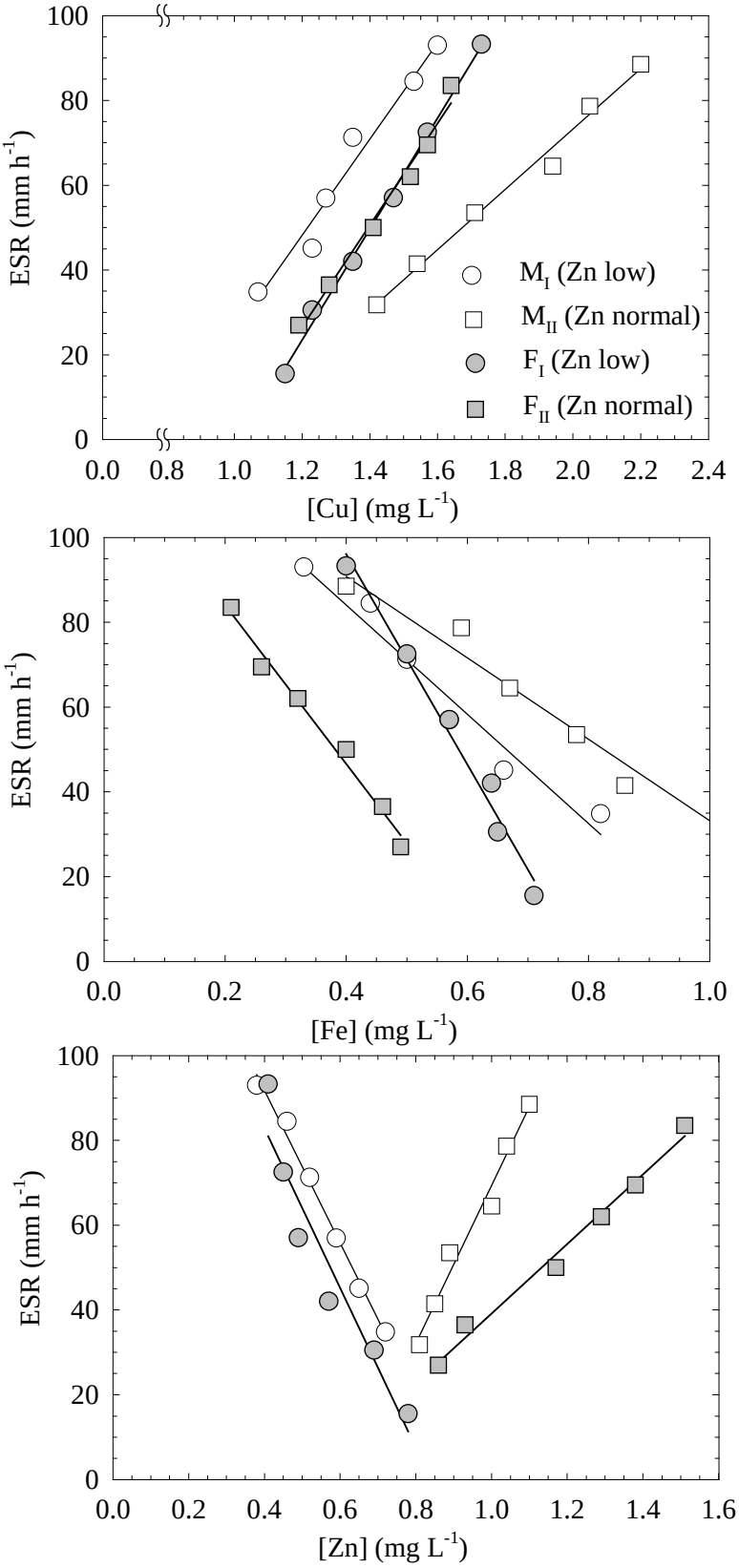

Figure 3 Association between serum Fe, Cu and Zn concentrations and ESR levels in patients treated for active pulmonary TB.

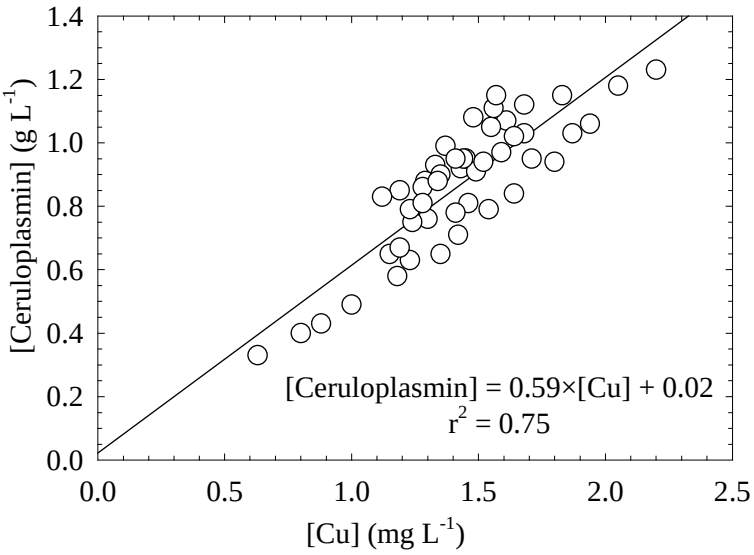

Figure 4 Association between serum Cu concentrations and ceruloplasmin levels in patients treated for active pulmonary tuberculosis.