Trace element deficiency is highly prevalent and associated with infection and mortality in patients with alcoholic hepatitis

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Authorship statement
AD, SA and MT conceived and designed the study; AD, SA and NT collected samples, DE performed statistical analysis; AF and RC performed experimental work; AD drafted manuscript; MC and MT finalised manuscript. AD is guarantor of the article. All authors approved the final version of the manuscript.

Abbreviations
AH: alcoholic hepatitis; ALD: alcohol-related liver disease; DF: discriminant function; HRA: Health Research Authority; HV: healthy volunteer; ICP-MS: inductively coupled plasma mass spectrometry; MELD: model of end-stage liver disease; NAC: N-acetylcysteine; NHS: National Health Service

Abstract

Background and Aim

Malnutrition is common in patients with alcohol-related liver disease and is associated with outcome in patients with alcoholic hepatitis. Trace elements (cobalt, copper, iron, selenium and zinc) are micronutrients essential for many cellular processes including antioxidant pathways. The prevalence and relevance of trace element deficiency is unknown in alcoholic hepatitis. We aimed to determine prevalence of trace element deficiency and their association with clinical outcomes in patients with alcoholic hepatitis.

Methods

Serum was obtained from patients with alcoholic hepatitis, alcohol-related cirrhosis and healthy volunteers as part of clinical trials, cohort studies and a biobank. Trace element concentration was measured by inductively-coupled plasma mass spectrometry. Association of trace element levels with development of infection within 90 days and mortality within 28 and 90 days was evaluated by multivariate logistic regression.

Results

Serum from 302 patients with alcoholic hepatitis, 46 with alcohol-related cirrhosis and 15 healthy controls was analysed for trace element concentration. The prevalence of zinc and selenium deficiency in alcoholic hepatitis was significantly higher than in alcohol-related cirrhosis at 85% and 67%, respectively. Zinc, selenium, copper and chromium were significantly different between groups. Iron was a predictor of development of infection within 90 days. Zinc was a predictor of mortality within 28 and 90 days.

Conclusion

Trace element deficiency in patients with alcoholic hepatitis is highly prevalent and associated with infection and mortality. Supplementation with selected trace elements may improve clinical outcomes in this patient group but further insight is required of their biological and clinical effects.

Keywords

Alcoholic hepatitis; alcohol-related liver disease; trace elements; zinc; selenium; iron; infection

Background

Trace elements are mineral elements present in small quantities in the human body. These include cobalt (Co), copper (Cu), iron (Fe), selenium (Se) and zinc (Zn) which are essential for a number of physiological, cellular and immune functions. Healthy dietary requirement has been studied and daily intake recommended by the World Health Organisation. [1]

Copper, selenium and zinc are particularly important as they are required in the synthesis of antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase, which are impaired in chronic liver disease and may contribute to its progression.[2] Additionally, these elements undergo biliary excretion, which is altered in liver disease. Copper also plays a role in haemoglobin formation, thyroid hormone production and myelin production.[3] However, a pro-oxidant role has also been described.[4] Chromium (Cr), although no longer considered an essential element, is involved in redox reactions in cells and in carbohydrate and lipid metabolism.[5]

Malnutrition is common in patients with chronic liver disease[6,7] and has a negative impact on their health related quality of life.[7] Malnutrition has been best studied in patients with alcoholic hepatitis (AH), a serious complication of alcohol-related liver disease (ALD). Malnutrition in AH is almost universal and severity of malnutrition correlates with severity of AH.[8] Intervention to increase calorie intake is effective in improving survival in this group of patients.[9,10] However, trace element deficiency is a component of malnutrition that has not yet been investigated in patients with AH.

Reduced serum levels of trace elements are present in patients with a range of chronic liver diseases[11,12] and correlate with severity.[12-14] Causes of deficiency are multifactorial but mainly due to reduced dietary intake and altered gut absorption. Zinc and selenium are the trace elements most studied in the context of liver disease. Zinc deficiency is associated with severity and prognosis in chronic liver disease.[15] Selenium deficiency in chronic liver disease has also been reported in a number of studies but has not been associated with clinical outcomes.[14,16,17]

The aim of this study was to determine whether there were trace element deficiencies in patients with AH compared to those with ALD cirrhosis and healthy controls and whether these were related to clinical outcome.

Methods

Biological samples

Written informed consent was obtained from all participants. Serum samples were obtained from five sources as described in detail below. (1) A prospective cohort study in Plymouth; (2) the Steroids or Pentoxifylline for Alcoholic Hepatitis (STOPAH) clinical trial; (3) the Imperial College Healthcare NHS Trust Tissue Bank; (4) the mechanism of action of N-Acetylcysteine for reducing risk of infection in Alcoholic Hepatitis (NACAH) clinical trial; (5) healthy volunteers in Plymouth.

Patients with severe AH or ALD cirrhosis were prospectively recruited to an observational cohort study conducted in University Hospitals Plymouth NHS Trust under NHS Health Research Authority (HRA) approval (15/LO/1501). Severe AH was defined according to the criteria applied to the STOPAH RCT: new onset jaundice within 3 months, bilirubin > 80 μmol/L, coagulopathy, heavy alcohol use (> 80 g ethanol/day in males and > 60 g ethanol/day in females within the preceding 4 weeks), discriminant function (DF) ≥ 32.[18] ALD was defined as similar alcohol consumption as the AH group but without jaundice and with established cirrhosis based on imaging, biopsy or transient elastography (stiffness > 20 kPa). Blood was taken by standard venepuncture into a serum separating tube within 72 hours of presentation prior to commencement of corticosteroids. Serum was obtained by centrifugation after standing for 20-30 minutes and stored in aliquots at -80°C. Patients with severe AH were treated with prednisolone 40 mg daily in the absence of active infection, hepatorenal syndrome or active gastrointestinal bleeding. Baseline biochemistry including DF and Model of End-stage Liver Disease (MELD) scores were recorded. Development of infection within 90 days of recruitment was prospectively recorded. Outcome at day 28 and day 90 was determined.

Serum was obtained from patients recruited to the STOPAH randomised controlled trial (NHS HRA reference: 09/MRE09/59). Participant selection criteria and trial methodology has previously been described in detail.[19] Serum from 192 participants were selected based on sample availability for trace element analysis.

Serum samples were obtained from patients with ALD cirrhosis from Imperial College Healthcare NHS Trust Tissue Bank under HRA approval. Samples were provided fully

anonymised with alcohol consumption data but no further demographic or clinical information.

Serum samples collected from participants recruited to the NACAH study (HRA reference: 15/LO/0153) were also analysed. Peripheral blood samples were taken directly into serum saving tubes, which were kept at room temperature up to 20-30 minutes prior to centrifugation. Serum was stored at -80°C until thawing for trace element analysis. NACAH participant selection criteria were the same as the STOPAH trial. Participants were treated with prednisolone 40 mg daily for 28 days with or without NAC for 5 days.

Healthy volunteer (HV) samples were obtained from laboratory and healthcare workers in University Hospitals Plymouth NHS Trust under HRA approval (South West – Cornwall and Plymouth reference: 1703). Written informed consent was taken and volunteers provided a self-declaration that they did not have any known acute or chronic health condition and consumed alcohol within UK government recommended guidelines (less than 112 g ethanol per week). Serum was taken and stored in aliquots as described above.

Inductively coupled plasma mass spectrometry

Serum samples were analysed for their Co, Cr, Cu, Fe, Se and Zn content immediately on receipt at the Analytical Research Facility, University of Plymouth using inductively coupled plasma mass spectrometry (ICP-MS). All laboratory ware was cleaned by soaking in 10% nitric acid for at least 24 hours before use followed by rinsing in copious amounts of high purity water (18 MΩ cm, Elga, High Wycombe, UK). Prior to analysis all serum samples were diluted fifty-fold into 2% nitric acid. Multi-element calibration standards were prepared

from elemental stock solutions (10,000, mg L^{-1}, SCP Science, QMX Laboratories, Thaxted, UK) to cover the expected range of each analyte in the serum samples. The ICP-MS instrument, an iCAP RQ (Thermo Fisher Scientific, Hemel Hempstead, UK) was tuned and checked for conformance to the manufacturer's specifications daily before use. The instrument was operated in collision cell mode, with helium as the cell gas, to negate the effect of any polyatomic interferences formed in the plasma or interface regions of the instrument. Indium and iridium were added, to give a final concentration of 10 µg/L, to all samples and calibration standards for use as internal standards to monitor for instrumental drift and sample matrix effects. All analyses were conducted under the ISO 9001:2015 certification held by the Analytical Research Facility and all balances and pipettes used were checked for accuracy of operation daily before use. Quality assurance was provided for by the analysis of Seronorm (Billingstad, Norway) L1 and L2 trace elements in serum reference materials, which were prepared daily before use according to the manufacturer's instructions. The found concentrations for all analytes were within the certified ranges for each Seronorm reference material.

Statistical analysis

Trace element concentration was described by mean and standard deviation. Mean trace element concentrations between sample categories (AH, ALD and HV) were compared by one-way ANOVA. Multiple comparisons between groups for each trace element were performed using t-tests with Bonferroni correction.

For AH cases only, a stepwise logistic regression was performed including in the model trace elements and MELD where variables with univariate logistic regression of $p < 0.25$ were

included in the multivariate model. Multicollinearity was checked using variance inflation factor. Model goodness-of-fit was checked using Hosmer and Lemeshow test. Models were fitted for the dependent variable of presence or absence of infection within 90 days, death within 28 days and death within 90 days. All analysis was performed using SPSS version 24 (IBM, Armonk, NY, USA).

Results

Participant characteristics

Serum was analysed from 302 patients with severe AH (60% male, mean age 49, mean MELD 22.8), 43 patients with ALD cirrhosis (55% male, mean age 54, mean MELD 13.3) and 15 HVs (61% male, mean age 31; table 1). Of those with ALD cirrhosis, 18 were actively consuming alcohol at potentially harmful levels (> 112 g [14 units] ethanol per week), 3 at low levels (< 112 g/week) and 22 were abstinent.

Serum trace element deficiency is highly prevalent in patients with AH and ALD

85% AH patients compared to 72% ALD patients had zinc deficiency (p=0.046) and 67% AH compared to 37% ALD patients were selenium deficient (p<0.001; table 2). Prevalence of copper and iron deficiency was similar in patients with AH and ALD (table 2). There is no established normal range for chromium or cobalt.

We note that zinc is bound to albumin in the circulation and hypoalbuminaeamia may result in a falsely low serum zinc level.[20] However, in patients with AH and ALD, serum zinc did not correlate with serum albumin (rho = 0.06; p=0.36). Additionally, diuretics and intravenous

human albumin solution (HAS) can alter serum levels of zinc and other trace elements.[21] At the time of the baseline sample, 29% and 16% of the 192 AH patients from the STOPAH cohort received diuretics or HAS, respectively, but neither was associated with differences in any serum trace element level. The presence of hepatic encephalopathy (26% of the included STOPAH cohort of patients) was not associated with differences in trace element levels.

Serum trace element concentration is lower in patients with AH or ALD than healthy volunteers

Serum concentration of, chromium, copper, selenium and zinc were all significantly different (all $p<0.001$ except copper $p=0.04$) between patients with AH, ALD and healthy volunteers (Figure 1). There were no significant differences between groups in serum concentration of iron or cobalt.

Serum trace elements are lower in patients with AH than ALD cirrhosis

Patients with severe AH had significantly lower selenium (0.63 v 0.79 µmol/L; $p<0.001$), chromium (36.7 v 61.7 nmol/L; $p<0.001$) and copper (16.2 v 18.6 µmol/L; $p=0.03$) compared to patients with ALD cirrhosis.

Active alcohol consumption does not explain differences in serum trace elements in patients with ALD cirrhosis

To determine whether active alcohol consumption was related to serum trace element levels, a subgroup analysis of patients with ALD cirrhosis obtained from the Imperial biobank

was performed. There were no differences between trace element levels in patients who were actively consuming alcohol > 112 g/week (n=7) compared to those that consumed less than this (n=25) (**supplementary table 1**). There were similar findings if the three patients with low level alcohol consumption were changed to the "actively consuming alcohol" group.

Subgroup analysis of all ALD patients showed that samples from patients hospitalised with a complication of their cirrhosis had lower levels of cobalt, copper and selenium than those obtained from the biobank, who were outpatients with clinically stable cirrhosis (**supplementary table 2**).

Serum trace elements are associated with clinical outcomes

In patients with AH, trace element concentrations were not significantly associated with disease severity measured by MELD and DF. Univariate analysis demonstrated that MELD, bilirubin, albumin and iron were significantly different between patients who developed infection within 90 days and those that didn't (**table 3**). Multivariate analysis found that MELD and iron were independently associated with infection within 90 days. For every 1 µmol/L increase in iron, the odds of infection reduced by 4.2% (95% confidence interval 0.9-7.3%; p=0.02). Serum iron was not correlated with C-reactive protein (rho = -0.14; p=0.63) or total white blood cell count (rho = -0.24; p=0.16).

MELD and bilirubin were significantly different between survivors and non-survivors at day 28 and day 90. Multivariate analysis demonstrated that MELD and zinc were independent

predictors of 28-and 90-day mortality (**table 4 and 5**). At day 90, for every 1 µmol/L increase in zinc, the odds of survival increase by 14% (95% CI 3.3-23.5%; p=0.01; table 5).

Discussion

Trace element deficiencies are highly prevalent in patients with ALD cirrhosis and even more so in patients with AH with the most striking deficiencies seen with zinc and selenium; 85% of AH patients were zinc deficient and 67% were selenium deficient. Furthermore, trace element deficiencies were associated with clinical outcomes. Deficiency in zinc was an independent predictor of 28- and 90-day mortality and iron was an independent predictor of infection within 90 days. Heavy alcohol consumption itself does not account for trace element deficiencies, which are likely to be multifactorial due to disease severity and nutritional status.

Protein-calorie malnutrition is almost universal among AH patients and is already recognised as an important contributor to severity of AH.[8] Indeed, calorie supplementation improves survival.[9,10] In addition to protein-calorie malnutrition, our data demonstrate the importance of micronutrient deficiencies. The cause of such deficiencies is due not only to reduced dietary intake but also altered absorption, metabolism and excretion.[21,22] Therefore, although these data cannot determine whether trace element deficiencies are independent of overall nutritional status, they are likely to have additional clinical impact above protein-calorie malnutrition alone.

Trace elements are essential for a wide range of cellular and immune functions. Approximately 10% of proteins encoded by the entire human genome have the potential to bind zinc,[23] thus influencing many cellular processes including proliferation, differentiation and apoptosis.[24] Zinc is an essential cofactor for many enzyme systems including antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase.[25] Superoxide dismutase is a particularly important antioxidant in ALD as alcohol metabolised to aldehyde can produce superoxide radicals. Additionally, zinc is involved in maintaining gut epithelial integrity and in wound healing.[26]

Zinc deficiency has been documented in patients with chronic liver disease including those with ALD cirrhosis[12,13,27] and correlates with stage of cirrhosis.[12] Additionally, lower zinc levels are associated with heavier alcohol consumption and raised markers of liver inflammation in patients without known liver disease.[28] However, zinc deficiency in AH has not been fully investigated. There has been a single report in a cohort of 6 patients with mild to moderate AH in which hepatic zinc content was reduced.[29] Serum zinc levels in AH patients have not been previously reported.

Zinc replacement in patients with chronic liver disease may restore antioxidant enzyme pathways to protect the liver from oxidative stress related damage.[30] In endothelial cells, deficiency activates pro-inflammatory transcription factors Nuclear Factor kappa B (NF-κB) and activator protein-1 (AP-1) and downregulates the anti-inflammatory peroxisome proliferator-activated receptor (PPAR) pathway.[31,32] These alterations are partially ameliorated by zinc supplementation.[32] A clinical trial of zinc sulphate supplementation in patients with chronic ALD cirrhosis is ongoing (NCT02072746). Preliminary results show an

improvement in serological markers of liver inflammation.[33] A trial of zinc sulphate with anakinra (interleukin-1 antagonist) and pentoxifylline versus corticosteroids alone for AH will soon report findings (NCT01809132). However, this study will not be able to determine the role of zinc alone on clinical outcome. Further studies are required to fully evaluate the role of zinc supplementation in altering immune function in patients with ALD and AH.

Selenium is an essential cofactor in several antioxidant pathways and forms a number of redox enzymes such as glutathione peroxidase (a selenopeptide), which are important to protect against oxidative injury.[14] Selenium deficiency in chronic liver disease has been reported in a number of studies[13,16,17] and is associated with severity of cirrhosis.[13] Our data confirm these previous findings of selenium deficiency in ALD cirrhosis and also add that deficiency is even more prevalent and severe in patients with AH.

Reduced serum transferrin but not serum iron was identified as an independent predictor of mortality in over 800 patients from the STOPAH cohort.[34] Negative correlations with inflammatory parameters suggested that reduced transferrin was a marker of hepatocellular stress rather than altered iron metabolism. In the present study, we are unable to determine how low serum iron influences infection, but it was not correlated with markers of inflammation. Iron is involved in a number of biochemical pathways including oxygen transport. The majority of iron in the body is stored in erythrocytes in the form of heme but is present in hepatocytes and macrophages within ferritin, a storage protein.[35] The toxic effects of iron overload to human tissues are well established due to its ability to generate oxygen free radicals. However, iron has bactericidal properties through the action of two metalloproteins, lactoferrin and neutrophil gelatinase-associated lipocalin (NGAL).[36,37]

Lactoferrin interacts directly with bacteria causing cell death while NGAL inhibits bacterial iron uptake, a critical factor for bacterial proliferation. This bactericidal role may explain why iron is an independent predictor of development of infection in patients with AH.

Given the deficiencies in trace elements and their potential role in contributing to the damaging inflammatory response and perturbed immune function found in AH, there is a rationale to replace these to test whether there is a clinical benefit. Two clinical trials of trace element supplementation for AH have been performed. In one, 56 patients with severe AH were randomised to treatment with placebo or supplementation with an antioxidant cocktail including selenium and zinc.[38] Those with active treatment had an improvement in bilirubin and reduced mortality at 28 days compared to the placebo group. The second trial randomised 70 patients with severe AH to an antioxidant cocktail and N-acetylcysteine or corticosteroids and failed to demonstrate a survival difference at 6 months.[39] Both trials included other intervention in addition to trace elements making it different to determine the specific effects of the trace elements themselves. Further studies to investigate the biological and clinical effects of zinc and selenium supplementation in AH are warranted.

In conclusion, trace element deficiency is highly prevalent among patients with ALD and AH. In those with severe AH, zinc and iron levels are independent predictors of survival and infection, respectively. These observations need further exploration to determine the biological role of trace elements in ALD and AH disease pathogenesis.

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Tables

Table 1. Characteristics of AH, ALD and HV cohorts. *ALD patient characteristics were only available for the Plymouth cohort as the remaining samples were provided fully anonymised from a biobank. [+]BMI data only available for STOPAH cohort of AH patients. DF: Discriminant Function; MELD: Model of End-stage Liver Disease; BMI: body mass index

	AH (n=302)	**ALD (n=11)***	**HV (n=15)**
Age	49.3 (10.4)	53.7 (11.6)	31.4 (8.6)
Gender (% male)	60%	55%	61%
DF	67.7 (28.5)	38.6 (12.5)	n/a
MELD	22.8 (6.8)	13.3 (3.5)	n/a
Albumin (g/L)	25.8 (6.6)	29.0 (5.4)	n/a
Bilirubin (µmol/L)	320.2 (157.8)	71.0 (49.8)	n/a
BMI (kg/m^2)[+]	28.3 (6.0)	n/a	n/a
Presence of hepatic encephalopathy	26%	18%	n/a

Table 2. Published normal ranges for trace elements and mean (SD) serum concentration in AH, ALD and HVs. Prevalence of deficiency is defined as the percentage of individuals with trace element concentration below the published normal ranges. There is no agreed serum reference value for chromium and cobalt. *Based on values from the American Medical Association manual of style.[40]

	Normal range (µmol/L)	**AH**		**ALD**		**HV**	
		Mean (SD)	Prevalence of deficiency (%)	Mean (SD)	Prevalence of deficiency (%)	Mean (SD)	Prevalence of deficiency (%)
Co	n/a	17.3 (8.0)	n/a	17.4 (7.7)	n/a	17.0 (5.5)	n/a
Cr	n/a	36.7 (21.9)	n/a	61.7 (26.5)	n/a	57.6 (16.2)	n/a
Cu*	11.0-22.0	16.2 (5.6)	16	8.6 (7.7)	12	16.5 (4.2)	0

Fe*	10.7-26.9	21.7 (10.4)	11	22.4 (12.8)	12	27.3 (11.2)	0
Se*	0.7-3.0	0.6 (0.3)	67	0.8 (0.4)	37	1.3 (0.2)	0
Zn*	11.5-18.5	8.3 (3.4)	85	9.0 (3.1)	72	17.6 (4.5)	0

Table 3. Multivariate logistic regression model for development of infection at within 90 days. SE: standard error.

Variable	Coefficient (β)	SE	Wald	p value	Odds ratio	95% confidence interval
MELD	0.74	0.02	11.7	0.001	7.7	3.2-12.3
Fe	-0.43	0.02	6.2	0.013	4.2	0.9-7.3
Constant	-1.71	0.62	7.7	0.005		

Table 4. Multivariate logistic regression model for mortality within 28 days.

Variable	Coefficient (β)	SE	Wald	p value	Odds ratio	95% confidence interval
MELD	0.12	0.03	22.4	<0.001	12.4	7.1-17.9
Zn	-0.11	0.06	3.9	0.047	10.6	0-20.0
Constant	-3.13	0.71	19.5	<0.001		

Table 5. Multivariate logistic regression model for mortality within 90 days.

Variable	Coefficient (β)	SE	Wald	p value	Odds ratio	95% confidence interval
MELD	0.08	0.03	8.8	0.003	8.4	2.8-14.3
Zn	-0.15	0.06	6.3	0.012	14.0	3.3-23.5
Constant	-1.55	0.73	4.4	0.035		

Figure legends

Figure 1. Serum trace element concentrations in patients with AH (n=302), ALD (n=43) and healthy volunteers (HV; n=15). ANOVA between all groups and comparisons between individual groups are shown. *p<0.05; **p<0.01; ***p<0.001. Co: cobalt; Cr: chromium; Cu: copper; Fe: iron; Se: selenium; Zn: zinc.

Statement of interests

Authors' declaration of personal interests

AD has received grant funding from the Medical Research Council, ERAB and the Association of Physicians of Great Britain and Ireland.

MT has served as an advisory board member for Novartis and Durect.

MC has served as a speaker and an advisory board member for AbbVie and Gilead.

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