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Isotopic analysis of Cu in blood serum by multi-collector ICP-mass spectrometry: a new approach for diagnosis and prognosis of liver cirrhosis?

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Abstract

The isotopic composition of blood serum Cu has been investigated as a potential parameter for diagnosis and prognosis of liver cirrhosis. Serum samples from supposedly healthy women (reference population) and from a group of female patients suffering from liver cirrhosis of different etiologies were analysed. The procedure for isolation of serum Cu and the measurement protocol for its isotopic analysis by multi-collector inductively coupled plasma-mass spectrometry (MC-ICP-MS) were evaluated. Significant differences in the isotopic composition of Cu were observed between the reference population and the patients. A wide spread in $\delta^{65}$Cu was observed within the cirrhosis population and $\delta^{65}$Cu seems to be linked to the severity of the disease. Patients with end-stage liver disease showed a significantly lighter serum Cu isotopic composition. Many clinical parameters used for the diagnosis and monitoring of liver diseases, *i.e.* the levels of aspartate aminotransferase, de ritis ratio, prothrombin and international normalized ratio, albumin, bilirubin, Na and C-reactive protein, correlate well with the $\delta^{65}$Cu values, as did the ceruloplasmin level and the ceruloplasmin / Cu concentration ratio. The isotopic composition of serum Cu appears to reveal the synthetic and hepatocellular function of the liver synergistically with inflammation and fluid retention in the cohort studied. A relevant relationship was also observed between $\delta^{65}$Cu and scores of mortality risk, such as the Model for End-stage Liver Disease (MELD) and MELD-Na. Thus, the isotopic composition of serum Cu shows potential as a new approach for prognosis of liver disease, and although further investigation is required, for evaluation of the mortality risk in end-stage liver disease and prioritization of liver transplants.
Keywords: Copper isotopic composition, blood serum, liver cirrhosis, MC-ICP-MS, diagnosis, prognosis
1 Introduction

Liver cirrhosis is the end-stage of chronic liver disease that can arise from many etiologies, such as metabolic disorders, obesity, cholestasis, viral hepatitis, excessive alcohol consumption, the occurrence of autoimmune events, the intake of toxic substances, infections or congenital diseases. It is characterized by irreversible scarring (fibrosis) of the liver and the lack of its function.\textsuperscript{1} The pathogenesis of liver cirrhosis has been gradually uncovered; hepatic stellate cells seem to play a central role in the initiation and progression of the disease. Next to the hepatic stellate cells, numerous other cells (hepatocytes, macrophages inflammatory cells, …) collaborate with these stellate cells. Many complications, such as ascites (fluid retention), encephalopathy or hepatocellular carcinoma (HCC), can be involved and no curative treatment is available at present. Advanced stage cirrhosis is life-threatening; about 15\% of the cirrhotic patients with ascites succumb within a period of 1 year.\textsuperscript{2}

The liver plays a key role in the homeostatic regulation of essential mineral elements, such as Cu. The homeostasis of Cu needs to be strongly regulated as the high oxidative potential of free Cu ions induces reactive free radicals (via the Haber-Weiss reaction) that can give rise to cellular damage.\textsuperscript{3,4} Cu seems to be involved in the stimulation of the Kupfer cells (liver macrophages), with the subsequent release of reactive oxygen and nitrogen species and cytokines.\textsuperscript{5} Liver diseases are associated with serious oxidative stress.\textsuperscript{6} Given the key role of the liver in Cu homeostasis, an impaired Cu metabolism occurs in patients with chronic liver disease.\textsuperscript{7}

Under normal conditions, the liver takes up the Cu present in the circulation, Cu enters the hepatocytes and is subsequently distributed by Cu chaperones for its incorporation
into cytochrome C oxidase, Cu-transporting P-type ATPase (ATP7B) and Cu/Zn superoxide dismutase (SOD). The ATP7B protein facilitates incorporation of Cu into ceruloplasmin, its release into the bloodstream and its biliary excretion.\(^8\),\(^9\) Ceruloplasmin, representing \(~90\%\) of plasma Cu, prevents free Cu ions from inducing oxidative damage.

The functions of ATP7B seem to be impaired in the case of liver cirrhosis. For patients with primary biliary cirrhosis (PBC) without decreased biliary excretion, the hepatic accumulation of Cu is a result of the reduced incorporation of Cu into ceruloplasmin and/or an increased hepatic Cu uptake.\(^10\) However, when Cu transportation is disturbed due to collapse of the bile ducts or cholestasis, ceruloplasmin activity increases to metabolize the excess of Cu in the liver.\(^11\) The liver also induces the synthesis of metallothioneins (MTs) as scavengers to remove the excess of Cu. Increased plasma MT concentrations were observed during the progression of diseases associated with high liver Cu concentrations, such as PBC and Primary Sclerosing Cholangitis (PSC). However, normal MT levels were observed in liver diseases not accompanied with increased liver Cu concentrations, as can be the case in alcoholic or cryptogenic cirrhosis and acute viral or chronic active hepatitis.\(^12\) Normal ceruloplasmin and CuZn-SOD and reduced catalase and glutathione peroxidase activities were observed in patients with alcoholic liver cirrhosis as a result of the antioxidant imbalance.\(^13\)

As many features can occur during cirrhosis, serological tests, liver histology and imaging are generally combined for diagnosis and management of liver cirrhosis. In this context, many practical clinical guidelines, issued by international associations, are available.\(^14\),\(^15\) However, effective strategies for the identification of patients with rapidly
progressing disease, for making decisions on adequate therapeutic management and for
prioritization of liver transplants are still lacking. Recent papers reported a possible
occurrence of a significant effect on the isotopic composition of Cu when the uptake or
excretion is jeopardized. Patients with Wilson’s disease, caused by the alteration of
ATP7B gene expression, showed a lighter Cu isotopic composition in serum compared
with the reference population.\textsuperscript{16} High-precision Cu isotopic analysis after administration
of a stable isotopic tracer also pointed towards poor control in Cu metabolism in
patients with Parkinsonism.\textsuperscript{17} Thus, the possibility of using the isotopic composition of
Cu in serum as a diagnostic parameter for liver cirrhosis deemed promising.

The aim of this work was to investigate the potential use of the isotopic composition of
Cu for diagnosis and prognosis of liver cirrhosis. The procedure for isolation of serum
Cu and the measurement protocol for its isotopic analysis by multi-collector inductively
coupled plasma-mass spectrometry (MC-ICP-MS) have been evaluated prior to analysis
of actual samples. Serum samples from healthy women and female liver cirrhosis
patients have been analyzed. Within the group of patients, different liver diseases were
included. Possible relationships between the isotopic composition of Cu and i) relevant
clinical parameters used for diagnosis and management of the liver cirrhosis, Cu and
ceruloplasmin levels and ii) mortality risk scores have been evaluated.

\section{Experimental}

\subsection{Reagents and standards}
Ultrapure water (resistivity > 18.2 MΩ cm) obtained from a Milli-Q Element water purification system (Millipore, France) was used throughout. Pro analysis purity grade 14 M HNO₃ and 12 M HCl (both from ProLabo, Belgium) were further purified by sub-boiling distillation in PFA and quartz equipment, respectively. Ultrapure 9.8 M H₂O₂ was acquired from Sigma-Aldrich (Belgium) and used as such.

Polypropylene chromatographic columns filled with AG MP-1 strong anion exchange resin (100-200 mesh, chloride form) purchased from Bio-Rad (Belgium) were used for chromatographic Cu isolation.

The Cu isotopic standard reference material NIST SRM 976 was purchased from the National Institute of Standards and Technology (NIST, USA). A standard solution of 1,000 mg L⁻¹ of Cu (Inorganic Ventures, the Netherlands; lot D2-ZN02061) was used as in-house isotopic standard (further referred to as A&MS-Cu) for checking the quality of the isotope ratio measurements.

Single-element standard stock solutions (1,000 mg L⁻¹) used for mass bias correction (Ni) and for quantification purposes (Cu and some major elements) were acquired from Inorganic Ventures. Standard working solutions were prepared by adequate dilution in 0.42 M sub-boiled HNO₃.

All manipulations were carried out in a class-10 clean lab. Teflon Savillex® beakers used for sample handling and storage were thoroughly pre-cleaned in several steps with HNO₃ and HCl (pro analysis) and subsequently rinsed repeated times with Milli-Q water before use.
2.2 Samples

A total of 55 serum samples, obtained from the Ghent University Hospital (UZGent, Belgium), were analysed in this study. 30 samples were from supposedly healthy donors and 25 from patients with liver cirrhosis. The reference population was formed by women ranging from 28 to 91 years old and the cirrhosis population by women between 39 and 67 years old. Within the set of patients, different liver features and etiologies were present: alcoholic cirrhosis (AC), toxic cirrhosis (TC), cryptogenic cirrhosis (CC), PBC, PBC + autoimmune hepatitis (PBC+AIH, overlap syndrome), PSC and non-alcoholic steatohepatitis (NASH) + alcoholic steatohepatitis (ASH). Ethical approval was obtained for this research by an independent commission connected to the Ghent University Hospital. Patients and individuals forming the reference population signed an informed consent.

The serum samples were originally collected in a BD Vacutainer blood tube suitable for trace element analysis. After centrifugation, serum samples were subjected to different analyses at the Ghent University hospital to determine various clinical parameters. An aliquot of about 500 µL of sample was transferred to a pre-cleaned Eppendorf tube and was kept at -20 °C until sample preparation in the clean lab and subsequent Cu isotopic analysis.

2.3 Sample preparation
About 500 µL of serum were digested in a Savillex® PFA vessel using 2 mL of 14 M HNO$_3$ and 0.5 mL of 9.8 M H$_2$O$_2$ at 110 °C overnight. The digests thus obtained were subsequently evaporated to dryness at 95 °C and re-dissolved in 5 mL of 8 M HCl + 0.001% H$_2$O$_2$. A blank, the A&MS-Cu standard at a concentration typically found in serum (1.5 mg L$^{-1}$) and the Seronorm™ Trace Elements Serum L-1 reference material were also included in each set of digestions for validation purposes.

In a next step, the samples were subjected to chemical purification by means of anion exchange chromatography.$^{18,19}$ For this, Bio-Rad Poly-Prep® columns were filled with 1 mL of AG MP-1 resin. The resin was gently cleaned with 10 mL of 7 M HNO$_3$ and 10 mL of Milli-Q H$_2$O. Afterwards, it was conditioned with 5 mL of 8 M HCl + 0.001% H$_2$O$_2$. The sample was loaded onto the column and 3 mL of 8 M HCl + 0.001% H$_2$O$_2$ were passed through for matrix elution. Subsequently, Cu was eluted using 9 mL of 5 M HCl + 0.001% H$_2$O$_2$. The purified Cu fraction was collected and evaporated to dryness at 95 °C to remove residual chloride. This procedure was performed twice. The final residue was re-dissolved in 0.42 M HNO$_3$.

2.4 Instrumentation and measurements

Cu isotope ratio measurements were carried out using a Thermo Scientific Neptune MC-ICP-MS instrument. A PFA nebulizer mounted onto a double spray chamber, consisting of a cyclonic and a Scott-type sub-unit, was used as sample introduction system. The Ni sampler and skimmer cones had an aperture diameter of 1.1 mm and 0.8 mm, respectively. The measurements were performed by static collection,
using five Faraday collectors connected to $10^{11}$ Ω amplifiers. The instrument settings and data acquisition parameters used are shown in Table 1. Gain calibration and baseline correction were performed before each measurement session. The mass position for the isotope ratio measurements was selected away (to a lower mass) from the peak centre in the plateau visualized via a peak scan.

The concentrations of Cu were adjusted to 200 µg L$^{-1}$ in all measurement solutions to avoid variations in analyte concentration from affecting the extent of mass bias, and all samples were measured in a standard-sample-standard bracketing sequence with NIST SRM 976 as the standard. The in-house standard A&MS-Cu previously characterized isotopically$^{19}$ was included every 4 to 5 samples to check the validity of the measurements.

The isotope ratios obtained were treated off-line after 2s-rejection of outliers. Correction for mass discrimination was performed through the combination of internal correction (with Ni) by means of a regression line and Russell’s exponential law and external correction in a sample-standard bracketing approach.$^{20}$ The isotopic composition of Cu is expressed in delta notation ($\delta^{65}\text{Cu}$, ‰), calculated as follows (equation 1):

$$\delta^{65}\text{Cu}_{\text{sample}} = \left( \frac{^{65}\text{Cu}^{/}_{\text{63}\text{Cu}}}_{^{65}\text{Cu}^{/}_{\text{63}\text{Cu}}_{\text{NIST SRM 976}}} - 1 \right) \times 1000 \quad (1)$$

A Thermo Scientific Element XR sector field ICP-MS instrument (Germany) was used for element quantification purposes. The instrument was equipped with a quartz nebulizer, a cyclonic spray chamber and Ni cones (1.1 and 0.8 mm aperture diameter for the sampler and skimmer, respectively). Table 1 provides the instrument settings and
data acquisition parameters used for the elemental assays. Concentrations of Cu and some major elements that can give rise to spectral interference were determined in the samples after acid digestion and after Cu isolation. Ga was used as an internal standard in this context.

2.6 Statistical methods

The unpaired t-test was used to establish significant differences between the reference and cirrhosis population. Bivariate analysis was used for determining pairwise associations between the isotopic composition of Cu and clinical parameters. Principal Component Analysis (PCA) was performed to visualize these associations. IBM® SPSS Statistics 22 package for Windows (SPSS Inc. Chicago Illinois, USA) was used for the statistical analysis of the data.

3. Results and Discussion

3.1 Validation of the methodology

The sample preparation procedure of the serum samples entailed an acid digestion and the isolation of Cu from the matrix elements by means of anion exchange chromatography to minimize spectral and non-spectral interferences. In each set of samples, a blank, the A&MS-Cu in-house standard and the Seronorm™ Trace Elements Serum L-1 reference material were included. Element determinations were accomplished using SF-ICP-MS in all samples and standards before and after the isolation procedure. The recoveries of Cu were quantitative in all cases (95±5%), ensuring absence of any effect from on-column isotope fractionation. The efficiency of the anion exchange chromatographic procedure to remove the matrix elements was also
tested. The presence of Na and Mg as elements potentially forming interfering ions was monitored in the Cu pure fractions. In all cases, the concentrations were lower than 2 mg L$^{-1}$ and 0.5 mg L$^{-1}$, respectively. At these levels of concentrations, no effect on the $\delta^{65}$Cu values was observed. The use of medium resolution and a measurement position approximately 0.038 amu away from the peak centre (to a lower mass) avoid any effect of the Na- and Mg-related spectral interferences.

In addition, the Seronorm$^{\text{TM}}$ Trace Elements Serum L-1 reference material was doped gravimetrically with the A&MS-Cu in-house standard prior to acid digestion for validation purposes. Approximately 1.5 mg L$^{-1}$ of A&MS Cu, which corresponds to the level of Cu already present in the reference material, was added. The A&MS-Cu standard, the Seronorm$^{\text{TM}}$ material and the mixture of both were digested, after which the respective Cu fractions were isolated and subjected to isotopic analysis. This test was carried out in duplicate. The Cu recovery was quantitative (97±5 %). The $\delta^{65}$Cu value obtained for the A&MS-Cu standard was 0.20±0.03 ‰, that for the Seronorm$^{\text{TM}}$ Trace Elements Serum L-1 reference material :0.09±0.05 ‰ and that for the mix A&MS-Cu + Seronorm$^{\text{TM}}$ material 0.08±0.04 ‰. By using the equation reported in a previous work, the $\delta^{65}$Cu value for the reference material was also deduced from the result for the mixture and the isotopic composition of the A&MS Cu standard. The $\delta^{65}$Cu value thus obtained was -0.04 ‰ and thus, well in agreement with the value for the Seronorm$^{\text{TM}}$ Trace Elements Serum L-1 reference material obtained directly. The average $\delta^{65}$Cu value for five replicates of the in-house standard obtained in one measurement session was 0.22 ± 0.08 (2s) ‰.
The contribution of the procedural blanks, treated in the same way as the samples, was <0.1%. As a result, the maximum bias observed between the results with and without blank correction was <0.04‰. This maximum difference was within two times the standard deviation and thus, no blank correction was done before mass bias correction.

A set of samples was measured using both Ni and Zn as internal standard. No significant differences were obtained between the results obtained using the respective internal standards. However, a bias of 0.1‰ with and without blank correction was obtained when using the Zn as internal standard. As a result, Ni was preferred as internal standard.

3.2 Cu isotopic composition in serum

The average isotopic composition of serum Cu obtained for the reference population and the individual data for the liver cirrhosis population, expressed in delta values, is presented in Table 2. The δ notation provides the deviation in parts per mil of the ${^{65}}\text{Cu}/^{63}\text{Cu}$ isotope ratio relative to that of NIST SRM 976 reference material. For each individual, also the medical diagnosis, the clinical parameters with abnormal values and some remarks are indicated in this table. The reference population included 10 young (21 - 39 years), 19 middle-aged (40 - 60 years) and 1 old (91 years) women and the liver cirrhosis population was formed by middle-aged women. As no statistical difference was observed between age groups of the controls, all samples (N=29, after removal of 1 outlier) were considered for the statistical comparison with the liver cirrhosis population. The average δ$^{65}$Cu value obtained for the reference population was -0.29 ± 0.27‰ (also in Table 2), which is in good agreement with previously reported data. An average δ$^{65}$Cu value of -0.26 ± 0.40 (2s)‰ (N=49) was obtained by Albarède et al. for
serum samples from women and men between 19 and 38 years old. The isotopic composition Cu in serum does not seem to be affected by gender.

δ^{65}Cu values for the controls and liver cirrhosis patients are shown in Figure 1. Significant differences were observed between both populations (t-test, p=0.000). In general, cirrhosis patients show a lighter isotopic composition of serum Cu. The deviation between the individual patient δ^{65}Cu data and the average value for the reference population ranged between 0.04 and 1.15 ‰. The wide spread was also observed within sub-groups of different diagnosis, i.e. AC, PBC, PSC, PBC+AIH. No statistical evaluation was carried out within sub-groups due to the small number of patients in each group. The observed spread rather seems to be linked with the severity of the disease. Lower δ^{65}Cu values were observed in end-stage liver disease and in the case of ascites, encephalopathy or hepatocellular carcinoma (HCC) (Table 1). In general, cirrhotic patients with ascites and associated complications show low probability of long-term survival without liver transplantation.

Substantially lower serum δ^{65}Cu were previously observed in patients with Wilson’s disease. In Wilson’s disease, the fractionation towards the lighter Cu isotope appears to be related with the non-efficient incorporation of Cu into ceruloplasmin. In the case of liver cirrhosis, the possibly reduced incorporation of Cu into ceruloplasmin, impaired biliary excretion of Cu and redox changes could fractionate the isotopic composition of Cu towards lower δ^{65}Cu values.

3.3 Correlation with clinical parameters
A large selection of clinical parameters are routinely determined in biological fluids for diagnosis and monitoring of liver disease. Unfortunately, most of these parameters are not specific of liver disease and can be also influenced by physiological and lifestyle factors (age, sex, diet, consumption of alcohol and/or tobacco, etc) in absence of the disease. The typical clinical parameters used for these purposes are compiled in Table 3. The relevance of these parameters can differ depending on the type of liver disease. Clinical data are included in the supplementary material (Table S1) and the abnormal values of the parameters used to manage the liver cirrhosis are indicated in Table 2 for each patient.

Significant bivariate correlations were observed between the $\delta^{65}$Cu values and many clinical liver cirrhosis-related parameters (Table 3). The $\delta^{65}$Cu values decrease when INR, AST, bilirubin and CRP levels increase and when PT, albumin and Na levels decrease. The most significant relationship established was between the $\delta^{65}$Cu value and the serum bilirubin concentration. Bilirubin, produced from the breakdown of heme proteins, is an index of hepatocellular liver dysfunction and cholestasis in liver cirrhosis. An enhanced level of bilirubin can result from an increased production, decreased liver uptake or conjugation and/or decreased biliary excretion. It was observed that the $\delta^{65}$Cu values of cirrhosis patients approximate the reference value when the clinical parameters studied are within the reference values. Patients with $\delta^{65}$Cu values within ±2s of the average value for the reference population showed normal values for most of the liver disease parameters (Figure 1 and Table 2). We have currently no explanation for the light Cu isotopic composition for the two samples 16-PBC and 17-PBC+AIH, originating from patients at an early stage of the disease. These samples are indicated in all figures. Thus, when these two samples were excluded from
the bivariate analysis, all the correlations improved. The Spearman's rho values were higher than 0.589 (absolute value) and p<0.004 for all of the correlations obtained (Table 3).

Principal Component Analysis was performed to visualize these relationships in terms of the information contained in the different parameters. The loading plot is shown in Figure 2. Three principal components (PCs) described 76% of the variance. The first PC was loaded by δ^{65}Cu, PT, albumin, bilirubin, CRP and Na concentration, the second PC by AST and ALT and the third PC by ALP and GGT. Thus, δ^{65}Cu seems to provide similar information as do PT, albumin, bilirubin, CRP and Na concentration in the liver cirrhosis population studied. It points out that the isotopic composition of Cu is revealing the hepatocellular and synthetic dysfunction of the liver, synergistically with the inflammation and water retention (Table 3). The synthesis of proteins, including ceruloplasmin, can also be upregulated with inflammation. The excretion of Na depends on the functional state of the liver and on the content of salts in the body.

Also Cu and ceruloplasmin concentrations (Table S1) were checked. While the isotopic composition of Cu did not correlate with the Cu concentration, a significant relationship was observed between the δ^{65}Cu value and both the ceruloplasmin (ρ=-0.493, p=0.012) and the ceruloplasmin / Cu concentration ratio (ρ=-0.476, p=0.016). It was noted that both ceruloplasmin levels and the ceruloplasmin / Cu ratios (in some cases ~100% saturation) were high in the patient population. The increased serum ceruloplasmin levels observed in the patient population could result from the estrogens effect, inflammation and/or hepatocellular hypoxia. In contrast to the isotopic composition, Cu concentrations and ceruloplasmin levels do not seem to be distinctive of the disease.
as they did not correlate with any liver cirrhosis-related parameter studied. However, as both parameters can be altered by the disease, further investigation is required to reveal the entire message embedded in the isotopic composition of Cu.

3.4 δ^{65}Cu for prognosis of liver cirrhosis

Many scores are being used by the medical community for prognosis of liver cirrhosis, to predict the mortality risk in end-stage liver disease and to prioritize liver transplants. The traditional Child-Pugh score, used for about three decades, and the Model for End-stage Liver Disease score (MELD), proposed by the Mayo Clinic in 2001, are the most frequently used.

The Child-Pugh score includes the following clinical parameters: bilirubin, albumin, INR, presence of ascites and encephalopathy (medically and poorly controlled). To estimate the severity of the disease, three classes are established. To evaluate the capability of the δ^{65}Cu for prognosis in end-stage liver disease, the isotopic composition of Cu was plotted versus the Child-Pugh score in Figure 3A. Class A indicates a good medium term survival (85 % of 2 years survival), class C corresponds to 35 % of survival in 2 years, but class B is a heterogeneous group, i.e. the clinical condition may remain stable for more than a year or deteriorate rapidly. As can be seen in Figure 3A, lighter δ^{65}Cu values were established for classes B and C than for Class A, but no difference was observed between classes B and C.

As the interpretation of Child-Pugh score can be subjective in terms of the degree of clinical abnormalities, the MELD score is often preferred. This score is calculated according to the equation 2:
\[ \text{MELD} = 3.8 \times \ln(\text{bilirubin concentration}) + 11.2 \times \ln(\text{INR}) + 9.6 \times \ln(\text{creatinine concentration}) + 6.4 \times (\text{etiology}; 0 \text{ if cholestatic or alcoholic, 1 otherwise}) \quad (2) \]

The Cu isotopic composition showed a significant correlation at a \( p < 0.05 \) level with the MELD score (Table 3), suggesting that \( \delta^{65}\text{Cu} \) could be useful to estimate the mortality risk also. In some cases, the MELD score can also fail, e.g., patients with persistent ascites and a low serum Na level can show a relatively low MELD score, but a high risk of early death.\(^{32}\) Hyponatremia is a common complication during cirrhosis due to the solute-free water retention and thus, the serum Na concentration can also be included to complement the MELD score (MELD-Na, equation 3).

\[ \text{MELD-Na} = \text{MELD} - \text{Na concentration} - [0.025 \times \text{MELD} \times (140 - \text{Na concentration})] + 140 \quad (3) \]

In severe cirrhosis patients awaiting liver transplantation, MELD-Na can be more predictive of mortality than MELD.\(^{33}\) However, the influence of many factors (e.g., administration of diuretics) on the Na concentration makes a correct interpretation difficult. The risk of death within a 6 months period is 6, 16 and 37 % for MELD-Na scores of 20, 30 and 40, respectively.\(^{34}\) As expected, the correlation between the isotopic composition of Cu and the MELD score improved when the serum Na concentration was included (\( p < 0.01 \), Table 3). This relationship between \( \delta^{65}\text{Cu} \) and MELD-Na is shown in Figure 3B. These results point out that \( \delta^{65}\text{Cu} \) shows promise as a parameter for the prognosis of cirrhosis, for assessing the mortality risk in end-stage liver disease and for prioritization of liver transplants. Although further investigation is required and a study comprising a larger number of patients, for instance including additional samples of different etiologies and severities of disease, needs to be carried.
out, it seems that the Cu isotopic composition can be a potential indicator for liver cirrhosis.

4. Conclusions

The analytical methodology used was shown adequate for the precise isotopic analysis of Cu in blood serum samples by MC-ICP-MS. Anion exchange chromatography provided quantitative recovery of Cu and an efficient removal of the matrix. The isotopic composition of Cu in blood serum of women with liver cirrhosis was significantly different from that of a reference population, consisting of supposedly healthy female individuals. The isotopic composition of Cu seems to be correlated with the severity of the disease, since a more pronounced fractionation towards the lighter isotope was observed in end-stage liver disease patients. The $\delta^{65}$Cu values were significantly positively correlated with the liver cirrhosis-related parameters AST, INR, bilirubin and CRP and inversely correlated with PT, albumin and Na. Especially between serum bilirubin concentrations and the isotopic composition of serum Cu, a strong correlation was established within the liver cirrhosis population. The isotopic composition of Cu provided the same information than did PT, albumin, bilirubin, Na concentration and CRP. It suggests that $\delta^{65}$Cu values reveal the synthetic and hepatocellular function of the liver, synergistically with the inflammation and the fluid retention. A good relationship between the $\delta^{65}$Cu values and mortality risk scores was also observed. $\delta^{65}$Cu values also showed a correlation with the ceruloplasmin levels and the ceruloplasmin / Cu concentration ratios, but the latter parameters did not correlate with the liver cirrhosis-related parameters. Although further investigation is necessary, the results from this exploratory study suggest that the $\delta^{65}$Cu value for serum could be
used for the diagnosis and prognosis of cirrhosis, for assessing the mortality risk in end-stage liver disease and for prioritization of liver transplants.

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**Figure captions**

**Figure 1.** Delta Cu values for reference and liver cirrhosis populations. The continuous line indicates the average $\delta^{65}\text{Cu}$ of the healthy population and the discontinuous lines $\pm 2$ times the standard deviation.

**Figure 2.** Loading plot obtained via PCA for the isotopic composition of Cu in serum samples and clinical parameters used for the diagnosis and management of liver cirrhosis.

**Figure 3.** Relationship between the isotopic composition of serum Cu and the mortality risk, as provided by the Child-Pugh (A) and MELD-Na (B) scores.
Table 1. Instrument settings and data acquisition parameters for (A) the Neptune multi-collector and (B) Element XR single-collector ICP-mass spectrometers.

<table>
<thead>
<tr>
<th><strong>(A) Neptune MC-ICP-MS</strong></th>
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<td>RF power (W)</td>
<td>1250</td>
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<tr>
<td>Guard electrode</td>
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<td>Ar flow rates (L min⁻¹)</td>
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<tr>
<td>Number of blocks</td>
<td>9</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>5</td>
</tr>
<tr>
<td>Integration time (s)</td>
<td>4.194</td>
</tr>
<tr>
<td>Cup configuration</td>
<td>L3: ⁶⁰Ni; L1: ⁶¹Ni; C: ⁶²Ni; H1: ⁶³Cu; H3: ⁶⁵Cu</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>(B) Element XR SF-ICP-MS</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RF power (W)</td>
<td>1250</td>
</tr>
<tr>
<td>Guard electrode</td>
<td>Connected</td>
</tr>
<tr>
<td>Ar flow rates (L min⁻¹)</td>
<td>Plasma 15; auxiliary 0.85; nebulizer 1.0-1.1</td>
</tr>
<tr>
<td>Sample uptake rate (µL min⁻¹)</td>
<td>200</td>
</tr>
<tr>
<td>Resolution mode</td>
<td>Medium</td>
</tr>
<tr>
<td>Acquisition mode</td>
<td>E-scan</td>
</tr>
<tr>
<td>Dwell time per point (ms)</td>
<td>10</td>
</tr>
<tr>
<td>Points per peak</td>
<td>20</td>
</tr>
<tr>
<td>Number of runs</td>
<td>5</td>
</tr>
<tr>
<td>Number of passes</td>
<td>5</td>
</tr>
</tbody>
</table>
Table 2. Isotopic composition of Cu in serum from the reference population (supposedly healthy female individuals) and from female liver cirrhosis patients. Abnormal values of the liver cirrhosis-related parameters are indicated for each patient (×).

<table>
<thead>
<tr>
<th>Sample/Diagnose</th>
<th>$\delta^{65}$Cu</th>
<th>Clinical parameters</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AST    ALT    ALP    GGT    Bili    Na    PT    Alb    CRP</td>
<td></td>
</tr>
<tr>
<td>Reference population</td>
<td>b -0.29 ± 0.27</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Liver cirrhosis population</td>
<td></td>
<td>**       **    **    **    **    **    **    **    **       **</td>
<td></td>
</tr>
<tr>
<td>1-AC</td>
<td>-0.65 ± 0.05</td>
<td>×       ×       ×       ×       ×       ×       ×       ×       ×       ×</td>
<td></td>
</tr>
<tr>
<td>2-AC</td>
<td>-0.82 ± 0.03</td>
<td>×       ×       ×       ×       ×       ×       ×       ×       ×       ×</td>
<td></td>
</tr>
<tr>
<td>3-PSC</td>
<td>-1.06 ± 0.06</td>
<td>×       ×       ×       ×       ×       ×       ×       ×       ×       ×</td>
<td>Ascites, HCC, died 5 months post-sampling</td>
</tr>
<tr>
<td>4-PBC</td>
<td>-0.69 ± 0.02</td>
<td>×       ×       ×       ×       ×       ×       ×       ×       ×       ×</td>
<td></td>
</tr>
<tr>
<td>5-PBC+AIH</td>
<td>-0.49 ± 0.08</td>
<td>×       ×       ×       ×       ×       ×       ×       ×       ×       ×</td>
<td></td>
</tr>
<tr>
<td>6-AC</td>
<td>-0.70 ± 0.12</td>
<td>×       ×       ×       ×       ×       ×       ×       ×       ×       ×</td>
<td></td>
</tr>
<tr>
<td>7-ASH+NASH</td>
<td>-1.38 ± 0.04</td>
<td>×       ×       ×       ×       ×       ×       ×       ×       ×       ×</td>
<td>Severe ascites, encephalopathy, died 2 months post-sampling</td>
</tr>
<tr>
<td>8-PBC</td>
<td>-0.82 ± 0.09</td>
<td>×       ×       ×       ×       ×       ×       ×       ×       ×       ×</td>
<td>Controlled ascites, HCC, liver transplant</td>
</tr>
<tr>
<td>9-PBC</td>
<td>-0.46 ± 0.03</td>
<td>×       ×       ×       ×       ×       ×       ×       ×       ×       ×</td>
<td></td>
</tr>
<tr>
<td>10-PSC</td>
<td>-0.23 ± 0.09</td>
<td>×       ×       ×       ×       ×       ×       ×       ×       ×       ×</td>
<td>Mirena</td>
</tr>
<tr>
<td>11-PBC</td>
<td>-0.25 ± 0.04</td>
<td>×       ×       ×       ×       ×       ×       ×       ×       ×       ×</td>
<td>gluten-free diet</td>
</tr>
<tr>
<td>12-PBC</td>
<td>-0.83 ± 0.08</td>
<td>×       ×       ×       ×       ×       ×       ×       ×       ×       ×</td>
<td>Ascites, died 22 months post-sampling (encephalopathy at this date)</td>
</tr>
<tr>
<td>13-PBC+AIH</td>
<td>-0.04 ± 0.01</td>
<td>×       ×       ×       ×       ×       ×       ×       ×       ×       ×</td>
<td>Oral contraceptive</td>
</tr>
<tr>
<td>14-TC</td>
<td>-1.28 ± 0.11</td>
<td>×       ×       ×       ×       ×       ×       ×       ×       ×       ×</td>
<td>Slight ascites</td>
</tr>
<tr>
<td>15-CC</td>
<td>-0.70 ± 0.05</td>
<td>×       ×       ×       ×       ×       ×       ×       ×       ×       ×</td>
<td>Slight ascites</td>
</tr>
<tr>
<td>16-PBC</td>
<td>-1.14 ± 0.07</td>
<td>×       ×       ×       ×       ×       ×       ×       ×       ×       ×</td>
<td></td>
</tr>
<tr>
<td>17-PBC+AIH</td>
<td>-0.99 ± 0.04</td>
<td>×       ×       ×       ×       ×       ×       ×       ×       ×       ×</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>18-PBC</td>
<td>-0.70 ± 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19-C</td>
<td>-0.80 ± 0.02</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>20-AC</td>
<td>-0.96 ± 0.08</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>21-PBC</td>
<td>-0.46 ± 0.12</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>22-PBC</td>
<td>-0.45 ± 0.05</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>23-AC</td>
<td>-1.44 ± 0.01</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>24-AC</td>
<td>-1.07 ± 0.01</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>25-PBC</td>
<td>-1.14 ± 0.05</td>
<td>×</td>
<td>×</td>
</tr>
</tbody>
</table>

*Delta values are expressed as average ± 2 times the standard deviation.*

*Number of samples: 29 (removal of 1 outlier)*

- Data not available
- Bili is bilirubin
- Alb is albumin
- Mirena is a levonorgestrel-releasing intra-uterine contraceptive device

Controlled ascites
Oral contraceptive
Died 16 months post-sampling
Encephalopathy, died 11 months post-sampling
Poorly controlled ascites
Table 3. Bivariate correlations between δ^{65}Cu, clinical parameters and scores used for the management of the liver cirrhosis population.

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Acronym</th>
<th>Information</th>
<th>ρ</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate aminotransferase</td>
<td>AST</td>
<td>Cells damage</td>
<td>-0.470</td>
<td>0.018</td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>ALT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>De ritis ratio</td>
<td>AST/ALT</td>
<td></td>
<td>-0.517</td>
<td>0.008</td>
</tr>
<tr>
<td>Gamma-glutamyltransferase</td>
<td>GGT</td>
<td>Liver excretory function (cholestasis)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>ALP</td>
<td>Liver excretory function (cholestasis), hepatocellular function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bilirubin</td>
<td></td>
<td>Liver excretory function (cholestasis), hepatocellular function</td>
<td>-0.576</td>
<td>0.003</td>
</tr>
<tr>
<td>Na concentration</td>
<td></td>
<td>Water retention and electrolytes balance (ascites)</td>
<td>0.454</td>
<td>0.023</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>PT</td>
<td>Water retention and electrolytes balance (ascites)</td>
<td>0.436</td>
<td>0.029</td>
</tr>
<tr>
<td>International Normalized Ratio</td>
<td>INR</td>
<td>Synthesis of proteins in the liver</td>
<td>-0.445</td>
<td>0.026</td>
</tr>
<tr>
<td>Albumin</td>
<td></td>
<td>Synthesis of proteins in the liver</td>
<td>0.479</td>
<td>0.018</td>
</tr>
<tr>
<td>C-Reactive Protein</td>
<td>CRP</td>
<td>Inflammation</td>
<td>-0.501</td>
<td>0.011</td>
</tr>
<tr>
<td>Model for End-Stage Liver Disease score</td>
<td>MELD</td>
<td>Severity of the disease, estimation of risk mortality</td>
<td>-0.496</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>MELD-Na</td>
<td></td>
<td>-0.523</td>
<td>0.007</td>
</tr>
</tbody>
</table>

ρ is Spearman’s rho coefficient
p is the level of significance (2-tailed)
Number of samples is 25.

* No correlation
Figure 1

Samples

Healthy

Liver Cirrhosis

Ascites

$\delta^{65}\text{Cu}$
Figure 3

(A) Child-Pugh

(B) MELD-Na

\[ \delta^{65}Cu \]