



High-precision isotopic analysis of serum and whole blood Cu, Fe and Zn to assess possible homeostasis alterations due to bariatric surgery

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Abstract

Bariatric surgery is an effective procedure to achieve weight loss in obese patients. However, homeostasis of essential metals may be disrupted as the main absorption site is bypassed. In this study, we determined Cu, Fe and Zn isotopic compositions in paired serum and whole blood samples of patients who underwent Roux-en-Y gastric bypass (RYGB) surgery for evaluation of longitudinal changes and their potential relation to mineral element concentrations and relevant clinical parameters used for monitoring the patient's condition. Samples from eight patients were collected pre-surgery and at 3, 6 and 12 months post-surgery. Multi-collector inductively coupled plasma-mass spectrometry (MC-ICP-MS) was used for high-precision isotope ratio measurements. Alterations in metal homeostasis related to bariatric surgery were reflected in the serum and whole blood Cu, Fe and Zn isotopic compositions. The serum and whole blood Cu became isotopically lighter (lower $\delta^{65}\text{Cu}$ values) after bariatric surgery, reaching statistical significance at 6 months post-surgery ($p < 0.05$). The difference between the serum and the whole blood Zn isotopic composition increased after surgery, reaching significance from 6 months post-surgery onwards ($p < 0.05$). Those changes in Cu, Fe and Zn isotopic compositions were not accompanied by similar changes in their respective concentrations, making isotopic analysis more sensitive to physiological changes than elemental content. Furthermore, the Zn isotopic composition correlates with blood glycaemic and lipid parameters, while the Fe isotopic composition correlates with glycaemic parameters.

Keywords Cu · Fe · Zn · Isotopic analysis · MC-ICP-MS · Bariatric surgery

Introduction

Obesity, defined as a body mass index (BMI) of 30 kg/m² or more [1], is a risk factor contributing to numerous medical pathologies, including diabetes mellitus, cardiovascular disease, and cancer [2], and is associated with elevated mortality

[1, 2]. Obesity influences metabolic homeostasis as a result of an enhanced occurrence of inflammation, insulin resistance, and oxidative stress [3]. While the prevalence of obesity is about 12% worldwide [4] and less than 30% in Europe [5], more than one third of the US population is obese [6]. A change of the individual's lifestyle is usually the first option to reduce the BMI, as this can be effective with minimal health risk when done in a proper manner. However, this option is frequently ineffective at long term, as the desired BMI reduction is often not achieved and/or there is a lack of patient commitment.

Bariatric surgery is a common clinical practice for the weight management of obese patients, resulting in an effective and durable weight loss in patients with severe obesity. It is offered for adults with a BMI ≥ 40 kg/m² without co-morbidities or a BMI ≥ 35 kg/m² with co-morbidities, such as type 2 diabetes mellitus, hypertension, etc, and without excessive risk of post-operative morbidity [7]. Depending on

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the surgical procedure, bariatric surgery reduces food absorption by restricting the food intake, by inducing malabsorption or by a combination of both. A restrictive procedure reduces the stomach volume, e.g. by placing a band around the upper portion of the stomach, thus creating a small pouch, or by surgically removing part of the stomach. The procedure aiming at malabsorption limits digestion and absorption, e.g. by delivering chyme directly to the ileum in the case of biliopancreatic diversion (BPD) [8]. The most common surgical procedure is the Roux-en-Y gastric bypass (RYGB) [9], in which a small pouch in the stomach is connected to the last part of the jejunum, thereby bypassing most of the stomach, duodenum and upper jejunum [8]. The weight loss results from the reduced food intake accompanied by changes in metabolism and gut microbiota. Increased secretion of glucagon-like peptide-1 (GLP-1) is thought to be a major factor involved in the improvement of diabetes mellitus after bariatric surgery. This hormone induces insulin biosynthesis and secretion, along with β cell proliferation and differentiation [10]. Together with other so-called gut satiety hormones, which also include peptide YY (PYY), it may reduce the appetite via the gut–brain axis [3]. Furthermore, changes in gut microbiota which commonly occur after bariatric surgery may contribute to a reduced inflammation in the intestine [11].

As a consequence of a smaller absorption site and malabsorption, deficiency of essential nutrients is a common feature in bariatric surgery patients. Fe deficiency is reported in up to 50% of the patients, with menstruating women showing the highest risk [12]. Other micronutrients that are commonly depleted include water-soluble vitamins (thiamine, folic acid, vitamin B12), fat-soluble vitamins (vitamin A, vitamin D) and essential mineral elements (Ca, Zn, Cu) [8]. A nutritional deficiency can be developed not only in a relatively short post-surgery time, but also at long term. Vitamin B12 deficiency, for example, increases up to 62% 5 years after the bariatric surgery, while the prevalence at 12 months post-surgery is 4% only [8]. Therefore, long-term post-operative monitoring and patient follow-up are encouraged [7].

High-precision isotopic analysis of essential mineral elements is a powerful clinical tool complementary to their quantitative determination, as small changes in the isotopic composition due to homeostatic alterations can be detected. Additionally, changes in isotope ratios accompanying diseases or variation in isotope ratios across body compartments indicate isotope fractionation accompanying biochemical processes and can provide an enhanced insight into the role of these elements in health and disease. The serum Fe isotopic composition for example has been shown to be heavier in chronic kidney disease (CKD) patients with iron-deficiency anaemia, but not in CKD patients with erythropoietin-related anaemia, compared to the control group [13]. Patients with end-stage liver disease show a lighter serum Cu isotopic composition than that of a healthy population [14, 15]. Normalised values

after liver transplantation signal a restored liver function [16]. Recently, it has been shown that reduced Cu excretion through the bile, as in cholestatic liver disease, induced in mice via common bile duct ligation resulted in a light whole body Cu isotopic composition [17]. A lighter serum Cu isotopic composition was also observed in Wilson's disease [18] and cancer patients [19, 20]. The whole blood Zn isotopic composition was shown to be affected by dietary habits [21]. Although tumour tissue was shown to be isotopically light, the serum/whole blood Zn isotopic composition was not significantly altered in the case of breast cancer [22].

The goal of the present study was to examine potential changes in the serum and whole blood Cu, Fe and Zn isotopic compositions as a result of bariatric surgery in a case series of eight patients. Paired serum and whole blood samples were collected from obese female patients before the gastric bypass and at 3, 6 and 12 months post-surgery. We investigated the association between the isotopic compositions, element concentrations and clinical parameters used for evaluation of the patient's condition.

Materials and methods

Sampling

Whole blood and serum samples were withdrawn from eight patients who underwent laparoscopic gastric bypass at the Mayo Clinic (AZ, USA). All patients were female and their age ranged between 18 and 50 years at the time of surgery. The sampling was performed before the surgery (on the day of or 1 day before the surgery) and at 3, 6 and 12 months post-surgery. After the surgery, all patients received multivitamin supplements (also containing mineral elements) for preventing nutritional deficiencies. A wide set of clinical parameters were determined at the Mayo Clinic for monitoring the patient's condition using standardised clinical protocols [23].

Ten serum samples from assumed healthy (non-obese) subjects collected at the Ghent University Hospital (UZ Gent, Belgium) and literature-reported data on serum Cu, Fe and Zn isotopic compositions were used for comparison purposes. These healthy subjects were all female with ages from 23 to 50 (average 42.5) years old (Electronic Supplementary Material (ESM), Table S1).

This study was approved by the Mayo Clinic Institutional Review Board (expedited review procedure 45 CFR 46.110, item 2). All patients provided written informed consent. Healthy volunteers provided informed consent at UZ Gent.

Sample preparation for isotopic analysis

Whole blood and serum samples were prepared for subsequent elemental and isotopic analysis in a class-10 clean lab

(PicoTrace™, Germany), as described elsewhere [24]. The samples were digested with 14 M HNO₃ (pro analysis, Chem-Lab, Belgium) and 9.8 M H₂O₂ (TraceSELECT®, Sigma-Aldrich, Belgium) at 110 °C and then evaporated to dryness. HNO₃ was subjected to additional purification via sub-boiling distillation in a Savillex DST-4000 system (Savillex Corporation, MN, USA) prior to use. The samples were re-dissolved in 8 M Optima grade HCl (Fisher Chemical, UK) also containing ~0.001% H₂O₂. Cu, Fe and Zn were sequentially isolated from the sample digests by anion exchange chromatography using AG-MP1 resin (Bio-Rad Laboratories, CA, USA). A second chromatographic separation following the same protocol was applied to the serum Cu fractions to achieve negligible levels of Na [24], as the ratio Na/Cu in serum is about 2-fold higher than that in whole blood. Each purified metal fraction was re-dissolved in concentrated HNO₃ and dried twice, and finally re-dissolved in 0.28 M HNO₃ for further elemental and isotope ratio measurements. Ultrapure water used throughout was obtained from a Milli-Q Element water purification system (Merck Millipore, NE, USA).

Multi-collector and single-collector ICP-MS

The isotopic compositions of Cu, Fe and Zn were determined using a Thermo Scientific Neptune (Germany) multi-collector ICP-MS (MC-ICP-MS) unit, equipped with a high-transmission jet interface. The samples were measured in a sample-standard bracketing (SSB) approach using the isotopic reference materials Cu NIST SRM 976 (National Institute of Standards and Technology, NIST, MD, USA), Fe IRMM-014 (Institute for Reference Materials and Measurements, IRMM, Belgium) and Zn IRMM-3702 (IRMM, Belgium). The concentrations of the target element in the samples were matched within ±5% to that in the isotopic standard. Ga, Ni or Cu (Inorganic Ventures, VA, USA) was always added as an internal standard for the Cu, Fe and Zn isotope ratio measurements, respectively. The isotope ratios were corrected for instrumental mass discrimination using the combination of internal correction by means of the revised Russell's law and external correction (SSB) [25].

For QA/QC, single-element standard solutions of Cu, Fe and Zn (obtained by dilution of 1000 mg/L stock solutions from Inorganic Ventures, VA, USA) were measured every five samples in each measurement session. These standards were previously characterised isotopically (measurements spread over ~7 years [21]). The δ -values obtained (mean ± 2 times the standard deviation (SD)) along this work are $0.22 \pm 0.04\%$ ($N = 30$) for $\delta^{65}\text{Cu}$, $0.45 \pm 0.06\%$ ($N = 29$) for $\delta^{56}\text{Fe}$ and $-7.04 \pm 0.06\%$ ($N = 29$) for $\delta^{66}\text{Zn}$; these values are in good agreement with earlier reported data [21, 24]. For method (including sample digestion and target element isolation) evaluation, the Seronorm™ Trace Elements serum L-1 (lot number: 1801802, Sero, Norway) and whole blood L-1 (lot number: 1406263, Sero, Norway) reference

materials were used. The results obtained for the Seronorm™ serum L-1 (mean ± SD, $N = 3$) and for the Seronorm™ whole blood L-1 (mean ± SD, $N = 3$) reference materials are -0.22 ± 0.07 and $0.09 \pm 0.06\%$ for $\delta^{65}\text{Cu}$, -1.52 ± 0.03 and $-2.35 \pm 0.01\%$ for $\delta^{56}\text{Fe}$ and 0.01 ± 0.04 and $-0.02 \pm 0.05\%$ for $\delta^{66}\text{Zn}$, respectively; these values are in agreement with earlier reported data [21].

The elemental concentrations were determined using an Element XR (Thermo Scientific, Germany) single-collector sector-field ICP-MS instrument. External calibration, with Ga as internal standard (10 µg/L) to correct for matrix effects, signal drift and instrument instability, was applied for quantification.

Statistical analysis

Results are presented as mean ± SD, unless otherwise specified. Shapiro–Wilk's test was used to determine the normality of the data set. Parametric tests were used for data with normal distribution; otherwise, equivalent non-parametric tests were used. One-way ANOVA (parametric) or Kruskal–Wallis (non-parametric) tests were used to compare the data between groups. Paired *t* test (parametric) or Wilcoxon signed rank test (non-parametric) was used to compare paired data from the same patient. The correlation between two variables was determined using Spearman's rank-order correlation. A *p*-value ≤ 0.05 indicated significance. Statistical analysis was performed using SPSS Statistics 25 (IBM Analytics, Brussels, Belgium). SigmaPlot 13 (Systat Software Inc., CA, USA) was used for constructing graphs.

Results and discussion

Clinical parameters and mineral element concentrations

Table 1 compiles relevant clinical parameters used for the clinical follow-up of the bariatric surgery patients. In this cohort, the BMI was reduced by 27% at 12 months post-surgery on average. The levels of total protein, glycated haemoglobin HbA1c, cholesterol and low-density lipoproteins (LDL) had also decreased significantly at 3 and 6 months post-surgery ($p < 0.05$). The total protein, cholesterol and LDL values were within the normal range in all patients at all sampling points. One patient (#002) showed an abnormal HbA1c and an elevated glucose level pre- and post-surgery and two patients (#006 and #007) reached normal HbA1c and glucose levels 6 months post-surgery. The improvement of the glycaemic and lipid metabolism after bariatric surgery is consistent with other studies [26]. Protein deficiency (serum albumin < 3.5 mg/dL) is a severe complication associated with malabsorptive surgical procedures, e.g. RYGB and BPD, occurring during the first post-operative months, and is attributed

Table 1 Selected clinical parameters for the RYGB patients pre- and post-surgery

| Clinical parameter | Pre-surgery | Post-surgery | | |
|--------------------------------|--|---|---|---|
| | | + 3 months | + 6 months | + 12 months |
| Age at surgery (years) | 42.5 (23–50) | | | |
| Weight (kg) | 124 ± 14 <i>n</i> = 8 | 107 ± 11* <i>n</i> = 7 | 97 ± 12* <i>n</i> = 8 | 92 ± 13* <i>n</i> = 7 |
| BMI (kg/m ²) | 46.1 ± 4.3 <i>n</i> = 8 | 38.3 ± 4.0* <i>n</i> = 7 | 36.3 ± 4.4* <i>n</i> = 8 | 33.7 ± 5.3* <i>n</i> = 7 |
| WBC (billion cells/L) | 7.0 ± 1.6 <i>n</i> = 8 | 6.1 ± 1.6 <i>n</i> = 8 | 6.7 ± 1.8 <i>n</i> = 7 | 6.2 ± 2.4 <i>n</i> = 5 |
| Platelet (billion/L) | 299 ± 79 <i>n</i> = 8 | 278 ± 72 <i>n</i> = 8 | 276 ± 81* <i>n</i> = 7 | 287 ± 93 <i>n</i> = 5 |
| Haemoglobin (g/dL) | 13.1 ± 0.6 <i>n</i> = 8 | 12.7 ± 0.8 <i>n</i> = 8 | 13.1 ± 0.5 <i>n</i> = 7 | 12.6 ± 0.9 <i>n</i> = 5 |
| Total protein (g/dL) | 6.99 ± 0.43 <i>n</i> = 8 | 6.74 ± 0.24* <i>n</i> = 8 | 6.59 ± 0.30* <i>n</i> = 7 | 6.98 ± 0.86 <i>n</i> = 5 |
| Albumin (g/dL) | 4.10 ± 0.29 <i>n</i> = 8 | 4.13 ± 0.18 <i>n</i> = 8 | 4.09 ± 0.27 <i>n</i> = 7 | 4.12 ± 0.33 <i>n</i> = 5 |
| Ferritin (ng/mL) | 57 ± 47 <i>n</i> = 4 Median = 41 | 70 ± 53 <i>n</i> = 8 Median = 67.5 | 85 ± 40 <i>n</i> = 6 Median = 78 | 45 ± 51 <i>n</i> = 5 Median = 21 |
| HbA1C (%) | 5.55 ± 0.67 <i>n</i> = 8 | 5.19 ± 0.49* <i>n</i> = 8 | 5.07 ± 0.43* <i>n</i> = 6 | 5.28 ± 0.53 <i>n</i> = 5 |
| Glucose (mg/dL) | 109 ± 24 <i>n</i> = 8 | 96.5 ± 9.7 <i>n</i> = 8 | 91 ± 28* <i>n</i> = 5 | 98 ± 18 <i>n</i> = 4 |
| Vitamin D (ng/mL) | 28 ± 14 <i>n</i> = 7 | 33.1 ± 7.1 <i>n</i> = 8 | 31 ± 10 <i>n</i> = 5 | 34 ± 10 <i>n</i> = 4 |
| Vitamin B ₁₂ (ng/L) | 410 ± 210 <i>n</i> = 7 Median = 372 | 650 ± 630 <i>n</i> = 8 Median = 446 | 730 ± 440 <i>n</i> = 6 Median = 545 | 530 ± 190 <i>n</i> = 4 Median = 456 |
| Cholesterol (mg/dL) | 179 ± 22 <i>n</i> = 7 | 150 ± 20* <i>n</i> = 8 | 162 ± 16* <i>n</i> = 6 | 164 ± 18 <i>n</i> = 5 |
| HDL (mg/dL) | 43.4 ± 7.5 <i>n</i> = 7 | 44 ± 13 <i>n</i> = 8 | 47 ± 12 <i>n</i> = 6 | 50 ± 14 <i>n</i> = 5 |
| LDL (mg/dL) | 109 ± 22 <i>n</i> = 7 | 81 ± 20* <i>n</i> = 8 | 89 ± 13* <i>n</i> = 6 | 87 ± 22 <i>n</i> = 5 |
| Triglyceride (mg/dL) | 129 ± 30 <i>n</i> = 7 | 123 ± 43 <i>n</i> = 8 | 131 ± 68 <i>n</i> = 6 | 112 ± 46 <i>n</i> = 5 |
| Creatinine (mg/dL) | 0.74 ± 0.18 <i>n</i> = 8 | 0.70 ± 0.16 <i>n</i> = 8 | 0.73 ± 0.16 <i>n</i> = 7 | 0.76 ± 0.18 <i>n</i> = 5 |
| Total bilirubin (mg/dL) | 0.43 ± 0.16 <i>n</i> = 7 | 0.59 ± 0.23* <i>n</i> = 8 | 0.50 ± 0.17 <i>n</i> = 7 | 0.54 ± 0.11 <i>n</i> = 5 |
| ALT (U/L) | 170 ± 380 <i>n</i> = 8 Median = 26.5 | 35 ± 29 <i>n</i> = 8 | 16.3 ± 4.8 <i>n</i> = 7 | 600 ± 1400 <i>n</i> = 5 Median = 20 |
| AST (U/L) | 34 ± 21 <i>n</i> = 8 | 29 ± 18 <i>n</i> = 8 | 19.3 ± 2.6 <i>n</i> = 7 | 20.4 ± 3.8 <i>n</i> = 5 |
| ALP (U/L) | 66 ± 12 <i>n</i> = 8 | 74 ± 15 <i>n</i> = 8 | 77 ± 17 <i>n</i> = 7 | 84 ± 18 <i>n</i> = 5 |
| Bicarbonate (mmol/L) | 24.1 ± 1.7 <i>n</i> = 8 | 24.4 ± 2.1 <i>n</i> = 7 | 25.7 ± 2.0 <i>n</i> = 7 | 27.2 ± 2.7 <i>n</i> = 5 |
| Phosphate (mg/dL) | 3.48 ± 0.52 <i>n</i> = 4 | 4.10 ± 0.73 <i>n</i> = 8 | 4.33 ± 0.49 <i>n</i> = 6 | 3.88 ± 0.73 <i>n</i> = 4 |
| Chloride (mmol/L) | 102.1 ± 1.2 <i>n</i> = 8 | 102.9 ± 2.7 <i>n</i> = 8 | 102.0 ± 2.2 <i>n</i> = 7 | 101.8 ± 2.6 <i>n</i> = 5 |

Table 1 (continued)

| Clinical parameter | Pre-surgery | Post-surgery | | |
|--------------------|-----------------------------|------------------------------|-----------------------------|-----------------------------|
| | | + 3 months | + 6 months | + 12 months |
| Na (mEq/L) | 140.3 ± 1.4 <i>n</i> = 8 | 140.9 ± 2.7 <i>n</i> = 8 | 140.7 ± 3.3 <i>n</i> = 7 | 141.2 ± 2.3 <i>n</i> = 5 |
| K (mmol/L) | 4.20 ± 0.28 <i>n</i> = 8 | 3.99 ± 0.22* <i>n</i> = 8 | 4.26 ± 0.54 <i>n</i> = 7 | 4.20 ± 0.68 <i>n</i> = 5 |
| Ca (mg/dL) | 9.08 ± 0.49 <i>n</i> = 8 | 9.01 ± 0.57 <i>n</i> = 8 | 9.16 ± 0.51 <i>n</i> = 7 | 9.02 ± 0.56 <i>n</i> = 5 |

*Significantly different from the pre-surgery value (paired *t* test or Wilcoxon signed rank test)

to an acquired food intolerance towards protein-rich foods [27]. In spite of this, the individuals enrolled in this study showed normal serum albumin levels post-surgery. One patient (#007) developed iron-deficiency anaemia (reduced haemoglobin, ferritin and serum Fe levels; elevated total iron-binding capacity (TIBC)) after the bariatric surgery and remained so during the whole post-operative period of the study.

The intra-individual metal concentrations were compared pre- and post-surgery (one-tailed paired *t* test or Wilcoxon signed rank test). All individual data of Cu, Fe and Zn concentrations are provided in ESM Tables S1–S2. The intra-individual serum and whole blood Cu concentrations were significantly reduced post-surgery ($p < 0.05$) (Fig. 1). However, a significant decrease of the serum and whole blood Cu concentrations was not observed between groups (ANOVA or Kruskal–Wallis test) due to the large inter-individual variability. The serum and whole blood Fe and Zn concentrations did not reach a significant difference during the post-operative period compared to the pre-surgery values (Table 2).

Mineral absorption mainly takes place in the duodenum and proximal jejunum, i.e. the portion of the intestine that is bypassed, resulting in a reduced gastric capacity and intestinal

mineral absorption. Cu, Fe and/or Zn deficiency is frequently developed in RYGB patients, a condition that can remain for years [29–32] due to reduced intake, reduced absorption and/or increased intestinal losses, despite supplementation [29, 33, 34]. A prevalence of ~19% for Cu deficiency at 24 months post-surgery [29] and of ~24% for

Fe deficiency anaemia after 18 months have been reported for RYGB patients [33]. Adequate multivitamin and mineral supplements may restore the Cu, Fe and Zn status at long term after bariatric surgery, but regular monitoring of the clinical parameters is still needed.

Serum and whole blood Cu, Fe and Zn isotopic compositions

Metal isotopic compositions were examined in the serum and whole blood pre- and post-surgery (Fig. 2). Individual data are provided in ESM Tables S3–S4. As can be seen in Fig. 2, the whole blood was enriched in the heavy ^{65}Cu isotope (+0.3‰) and enriched in the light ^{54}Fe isotope (−0.2‰) compared to serum, whereas the Zn isotopic compositions of serum and whole blood were similar. This difference in isotopic

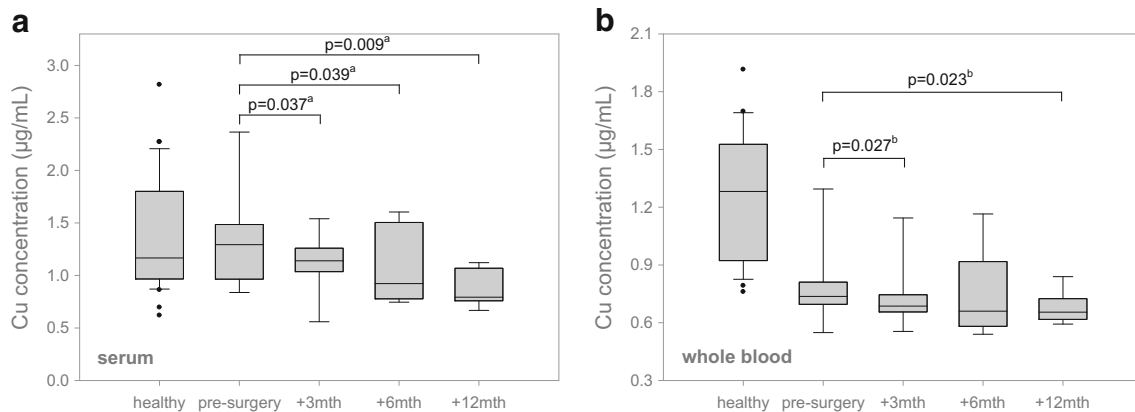


Fig. 1 Box plots for the Cu concentration in serum (a) and whole blood (b) for the bariatric surgery patients and assumed healthy subjects. Cu concentrations did not show a significant difference between groups (ANOVA or Kruskal–Wallis test). Paired comparisons reveal significant difference compared to pre-surgery values (one-tailed *t* test or Wilcoxon

signed rank test). Data of healthy subjects (European non-obese females) were compiled from this study and literature [28]. Healthy subjects were not included in the statistical tests for data comparison. ^a*p* value from one-tailed *t* test; ^b*p* value from one-tailed Wilcoxon signed rank test

Table 2 Fe and Zn concentrations in serum and whole blood sampled pre-surgery and 3, 6 and 12 months post-surgery

| | Serum | | | | Whole blood | | | |
|-------------------------|-----------------|-----------------|-----------------|-----------------|------------------------------|------------------------------|------------------------------|------------------------------|
| | Pre-surgery | Post-surgery | | | Pre-surgery | Post-surgery | | |
| | | + 3 months | + 6 months | + 12 months | | + 3 months | + 6 months | + 12 months |
| Fe ($\mu\text{g/mL}$) | 0.82 \pm 0.24 | 0.75 \pm 0.29 | 0.71 \pm 0.20 | 0.77 \pm 0.29 | 0.44 \pm 0.02 ^a | 0.42 \pm 0.03 ^a | 0.42 \pm 0.04 ^a | 0.41 \pm 0.04 ^a |
| Zn ($\mu\text{g/mL}$) | 0.73 \pm 0.13 | 0.70 \pm 0.09 | 0.64 \pm 0.16 | 0.62 \pm 0.14 | 5.11 \pm 0.85 | 5.08 \pm 0.75 | 4.88 \pm 0.71 | 4.94 \pm 0.73 |

^aData $\times 10^3$

compositions between serum and whole blood can be hypothetically attributed to the effect of redox processes and erythropoiesis and is in agreement with previous observations [28, 37].

An age- and gender-matched European (French and Belgian) population consisting of assumed healthy (non-obese) individuals was included (ESM Table S5) to provide a general overview of variability in the isotopic compositions of these metals. For comparative purposes, age- and gender-matching is crucial, especially for $\delta^{56}\text{Fe}$, as the isotopic compositions differ significantly between male and female subjects and between pre-menopausal and post-menopausal female subjects [38]. To the best of our knowledge, there are no data available on metal isotopic compositions for either non-obese US individuals or obese Europeans to perform a strict comparison. Previous literature shows that for different European (French, Swedish and Swiss) cohorts of healthy individuals investigated, no significant differences in $\delta^{65}\text{Cu}$, $\delta^{56}\text{Fe}$ and $\delta^{66}\text{Zn}$ values were established [39]. The overall serum and whole blood Cu isotopic composition and serum Fe isotopic compositions of the obese patients were lighter than those of healthy (European) subjects, while whole blood $\delta^{56}\text{Fe}$ and serum $\delta^{66}\text{Zn}$ were heavier in obese patients. No difference was observed in the whole blood $\delta^{66}\text{Zn}$ value between the healthy subjects and obese patients. A light Fe isotopic composition could be expected due to a low-grade inflammation typically accompanying obesity, a high Fe status and/or reduced intestinal Fe absorption [35, 40]. Furthermore, a high BMI is associated with reduced Fe absorption [41] and elevated serum hepcidin concentration [42]. Increased levels of the hormone hepcidin, as a result of increased cytokine production in obese patients [43], downregulate Fe release from enterocytes and thus intestinal uptake [42]. A low dietary intake of bioavailable heme-Fe can also lead to an iron-restricted erythropoiesis. However, as the healthy subjects are from different geographical origins, firm conclusions as to whether the difference is due to obesity and/or other factors cannot be derived. Populations from different geographical areas may have different metal isotopic compositions because of diet, metabolic activity, genetics, etc [44]. For example, healthy subjects from the circumpolar area of Russia showed significantly different $\delta^{65}\text{Cu}$ and $\delta^{66}\text{Zn}$ values compared to

healthy subjects from Europe and Japan, while the latter also showed a $\delta^{56}\text{Fe}$ value that was significantly different from that observed in healthy French subjects [39].

The use of healthy subjects from the same geographical origin receiving the same essential metal-containing multivitamin supplementation as the controls could be meaningful for comparison with the metal isotopic compositions of bariatric surgery patients. However, the absorption of micronutrients in healthy subjects and bariatric surgery patients is at different levels as the latter tend to absorb more nutrients from the multivitamin to address the deficiencies [45]. Moreover, as a common challenge in a nutritional study, the level of baseline nutrients (i.e. before intervention) also varies between healthy subjects, while a fraction of the healthy volunteers constituting a reference population often also show a sub-optimal level of micronutrients without any clinical symptom [46]. In this study, we preferred to use the pre-surgery value as control to evaluate whether Cu, Fe and Zn isotopic compositions are altered after bariatric surgery. By using the same individual as control, stronger conclusions can be achieved with a smaller number of participants [47]. All pre-surgery samples were acquired on the day of or 1 day before the surgery to reduce the variability due to different disease severity.

A significant difference was observed for serum and whole blood Cu isotopic compositions between the pre-surgery and 6 months ($p = 0.011$ and $p = 0.037$, respectively) and 12 months ($p = 0.013$ and $p = 0.031$, respectively) post-surgery samples (two-tailed paired t test or Wilcoxon signed rank test) (Fig. 2). The group-averaged Cu isotopic composition became gradually lighter over time, reaching significance at 12 months ($p = 0.029$ for serum and $p = 0.016$ for whole blood). This difference was not observed for the group-averaged Cu concentration (vide supra) in either serum or whole blood (Fig. 1), and thus, the Cu concentration was not conclusive. For this purpose, the small inter-individual variability in the Cu isotopic composition provides benefit compared to the Cu concentration.

The enrichment in the light ^{63}Cu isotope in the serum/whole blood over time could be hypothetically associated with the reduced duodenal Cu absorption and mineral bioavailability as the amount of digestive juice in the stomach is limited after the gastric bypass. When the Cu level in the body is

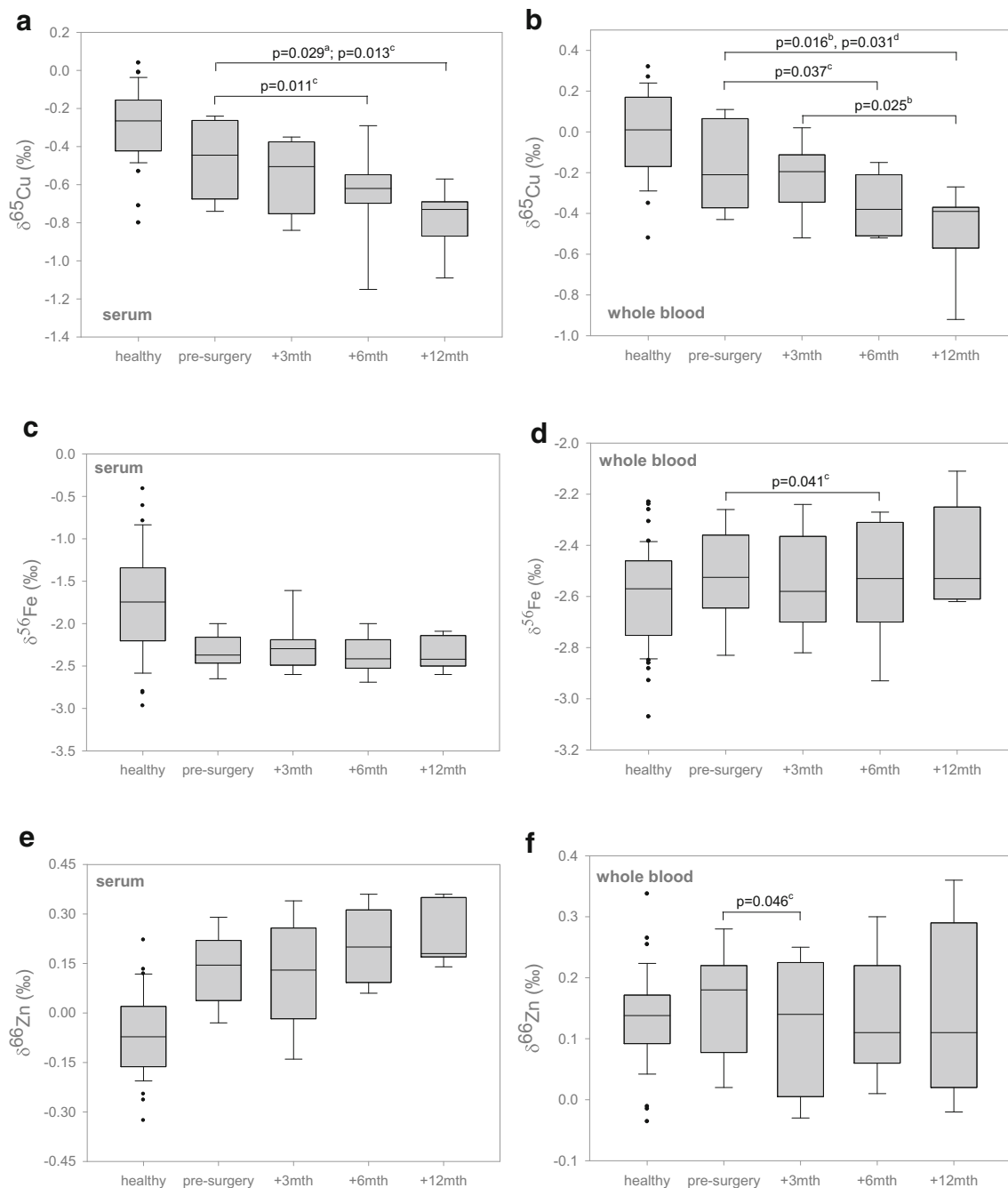


Fig. 2 a–f Box plots for the metal isotopic compositions ($\delta^{65}\text{Cu}$, $\delta^{56}\text{Fe}$ and $\delta^{66}\text{Zn}$) in serum and whole blood for the bariatric surgery patients over time and for assumed healthy subjects (non-obese European females). The $\delta^{65}\text{Cu}$ values among time points were significantly different in serum (ANOVA, $p = 0.037$) and whole blood (Kruskal–Wallis test, $p = 0.038$). Paired comparisons of $\delta^{65}\text{Cu}$ show significant differences compared to pre-surgery values (two-tailed t test or Wilcoxon signed rank test). Data for healthy subjects were compiled from

literature [28, 35, 36] and completed by additional analysis performed along this study, as summarised in ESM Table S5. The $\delta^{66}\text{Zn}$ data from reference [28] were recalculated relative to the IRMM-3702 isotopic reference material [36]. Healthy subjects were not included in the statistical tests for data comparison. ^a p value from Tukey's post hoc test of ANOVA; ^b p value from pairwise comparison of Kruskal–Wallis test, ^c p value from two-tailed t test; ^d p value from two-tailed Wilcoxon signed rank test

insufficient, the level of CTR1 (the major Cu importer) is up-regulated in the intestine [45, 48]. This process may affect the Cu isotope fractionation accompanying intestinal absorption. It has been shown in vitro that a high level of Ctr1 favours a lighter Cu isotopic composition in yeast [49]. Furthermore,

changes in the composition and distribution of gut microbiota after bariatric surgery [11] can also contribute to an altered serum/whole blood Cu isotopic composition [50]. It has been shown that region-specific Cu isotopic changes in the intestine of antibiotics-treated mice, particularly in the duodenum and

colon, are accompanied by an increased level of Cu–Zn superoxide dismutase (SOD1, an antioxidant enzyme) in the duodenum and a reduced level of the Cu transporters CTRL and ATP7A in the colon [50].

Fe and Zn isotopic compositions did not show any systematic difference after surgery compared to the pre-surgery values in the intra-individual comparisons (two-tailed paired *t* test or Wilcoxon signed rank test) (Fig. 2). Among patients, those suffering from severe diabetes (#002) and Fe deficiency anaemia (#007) showed the heaviest whole blood Fe isotopic compositions (Fig. 3). The heavier Fe isotopic composition at pre-surgery in patient #007 might indicate that Fe deficiency had started before surgery, although the Fe concentration had not been altered yet. On the other hand, the Fe concentrations of those patients were within the range of the others, highlighting the superiority of isotopic composition over concentration. Thus, unlike the Fe concentration, which has high intra- and inter-individual variability and is barely changed, alteration in the Fe isotopic composition reflects a change in the Fe homeostasis.

Longitudinal change of the difference between the serum and whole blood Zn isotopic compositions

The serum Zn isotopic composition 6 months post-surgery was slightly heavier than that during pre-surgery, but the

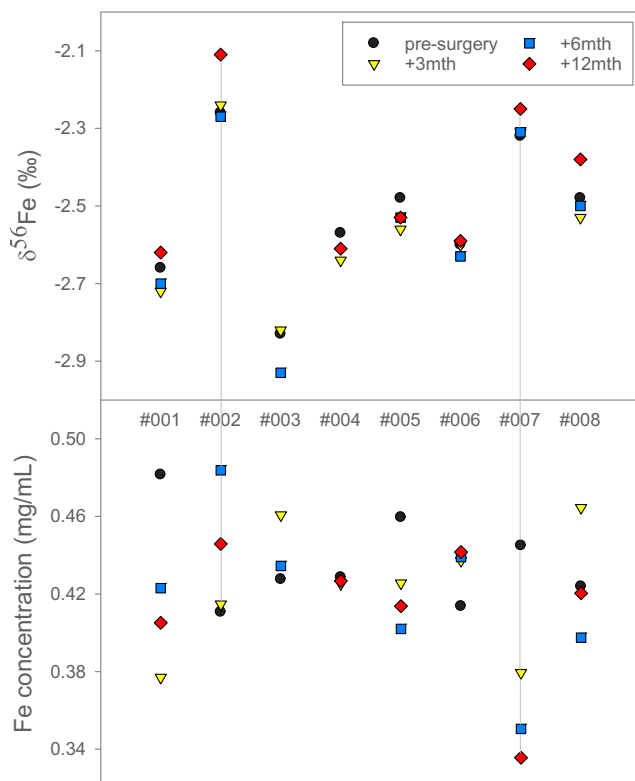


Fig. 3 Whole blood Fe isotopic compositions and concentrations for each patient before and after bariatric surgery. Patient #002 was diagnosed with severe diabetes and patient #007 with iron-deficiency anaemia

difference did not reach significance (Fig. 2e). This enrichment in the heavier Zn isotopes was not observed in the whole blood (Fig. 2f). However, the difference between the $\delta^{66}\text{Zn}$ value in serum and that in whole blood (expressed as $\Delta^{66}\text{Zn}$) became gradually larger over the post-operative time (Fig. 4). The $\Delta^{66}\text{Zn}$ values at 6 and 12 months post-surgery showed a significant difference compared to the pre-surgery value (Kruskal–Wallis test, $p = 0.023$ and 0.004 , respectively). This effect was not observed for the $\Delta^{65}\text{Cu}$ and $\Delta^{56}\text{Fe}$ values (Kruskal–Wallis test, $p = 0.197$ and $p = 0.343$, respectively). The $\Delta^{66}\text{Zn}$ seems to reflect the disrupted Zn homeostasis, impaired Zn status and/or reduced Zn absorption capacity in the bariatric surgery patients. Ruz et al. observed a reduced Zn absorption in bariatric surgery patients from 6 to 18 months post-surgery [34]. When the Zn supply is low, Zn is taken from the storage to fulfil Zn requirements. Intracellular Zn is bound to metallothionein via a Zn–S bond in cysteine clusters and can be released when the Zn level is low [51]. As the Zn–S bond favours the lighter Zn isotopes, the heavier isotopes would be preferentially released from intracellular metallothionein to serum in which it is transported to organs sensitive to Zn depletion. Additionally, changes in the Zn absorption involve many metal transport proteins, e.g. ZIP4 as a primary regulator of intestinal Zn uptake and ZnT1 for the Zn efflux from the enterocyte. The expression of Zn transporters is regulated by cytokines, hormones and the metal itself [52], and changes in their expressions can impact Zn homeostasis, as potentially reflected in the $\Delta^{66}\text{Zn}$ value.

Correlations between isotopic compositions and clinical parameters

A schematic overview of the correlations between the Cu, Fe and Zn isotopic compositions and the clinical parameters used for the monitoring the patient's condition is

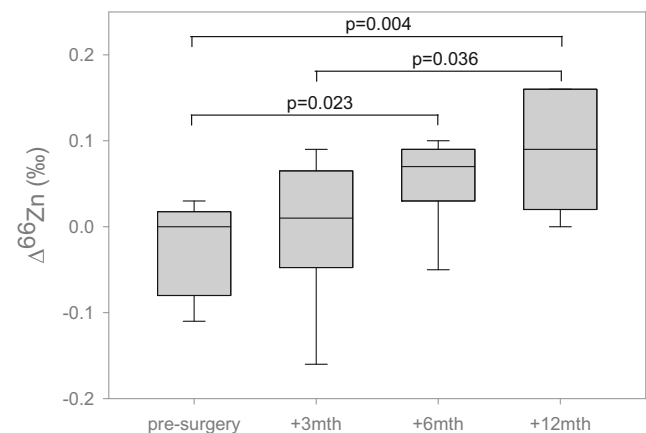
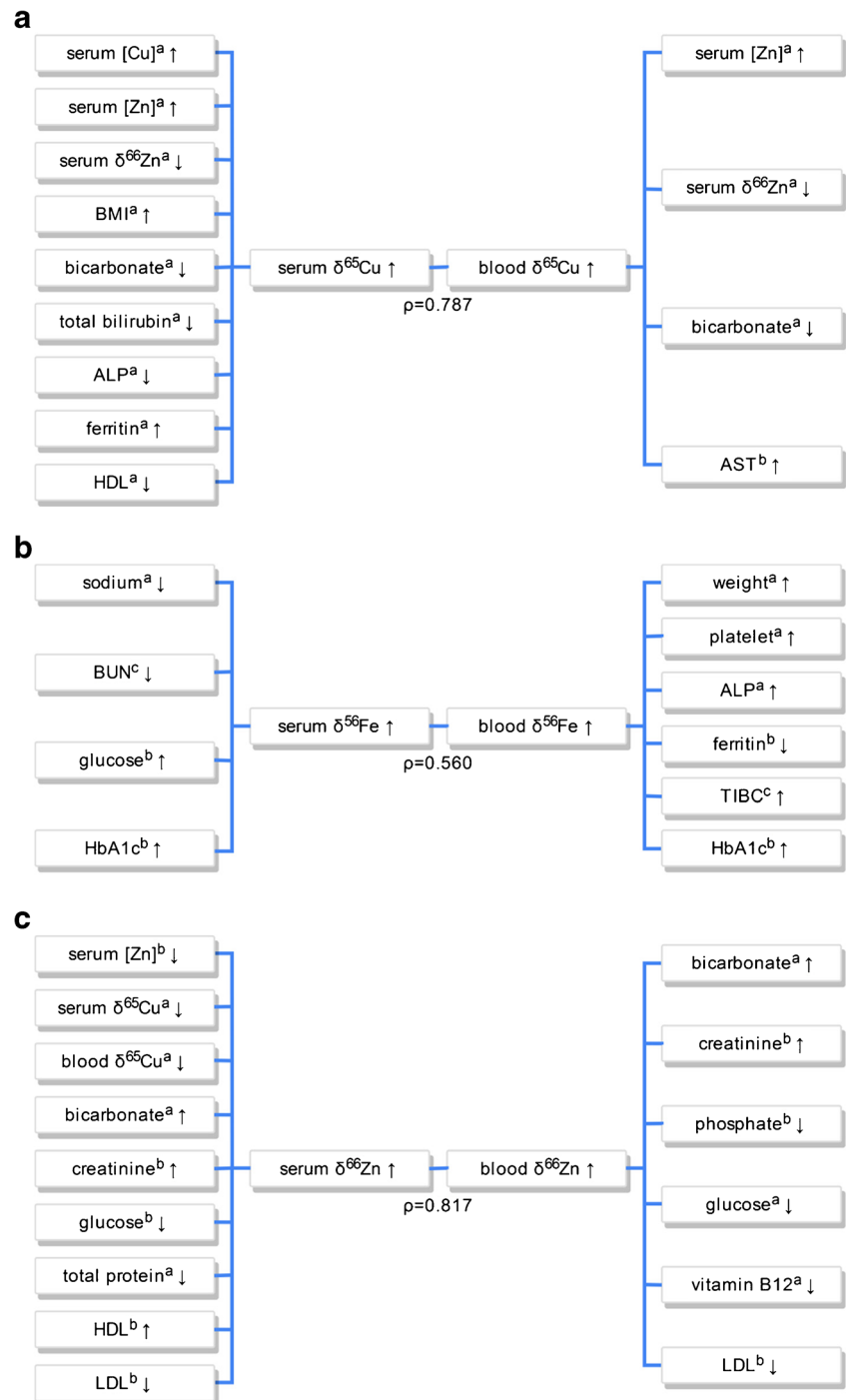


Fig. 4 Box plot for the difference between serum and whole blood Zn isotopic compositions (expressed as $\Delta^{66}\text{Zn}$). $\Delta^{66}\text{Zn}$ shows significant differences among time points (Kruskal–Wallis test, $p = 0.015$). The p value shown is derived from pairwise comparison using the Kruskal–Wallis test

Fig. 5 Schematic overview of the correlation between Cu (a), Fe (b) and Zn (c) isotopic compositions and clinical parameters. Only significant ($p < 0.05$) relationships are shown. Arrows with the same directions indicate positive correlation, while arrows with opposite directions indicate negative correlation. ^aCorrelation coefficient between 0.3 and 0.5. ^bCorrelation coefficient between 0.5 and 0.7. ^cCorrelation coefficient > 0.7 . The individual Spearman's correlation coefficients and the level of significance are provided in the Electronic supplementary information (ESM Table S6)



presented in Fig. 5. Serum and whole blood $\delta^{66}\text{Zn}$ values showed significant correlations with glycaemic and lipid parameters, i.e. glucose, high-density lipoproteins (HDL) and LDL. The link between Zn and diabetes has been firmly established. Zn^{2+} and insulin are secreted from pancreatic β cells and both synergistically induce glucagon secretion from nearby α cells. In the target cells, Zn maintains an active state of the insulin receptor, thus prolonging the insulin activity [31]. Zn is also involved

in lipogenesis and its deficiency disrupts lipid homeostasis [53, 54].

The serum $\delta^{56}\text{Fe}$ correlated with the HbA1c and glucose levels in this population. HbA1c, which is the percentage of haemoglobin that binds glucose, is thought to be a more robust diabetic parameter as an erythrocyte is recycled every 120 days, thus carrying information of the blood glucose concentration over a long term [55]. However, the HbA1c value tends to be elevated when Fe is depleted because the erythrocyte

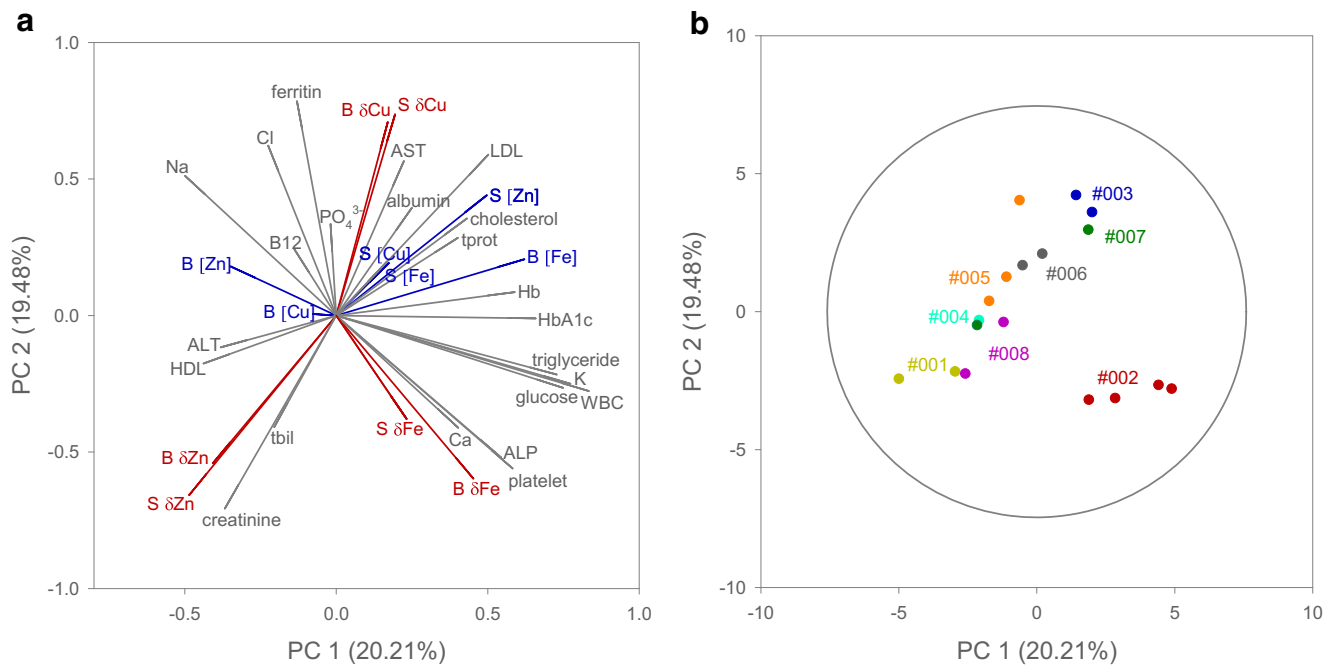


Fig. 6 Loading plot (a) and scores plot (b) of the principal component analysis (PCA). Observation scores from patient #002 are clustered separately. B denotes blood; S denotes serum; [...] corresponds to concentration; tbil is total bilirubin; tprot is total protein

cycle becomes longer [56]. Studies are needed to understand whether $\delta^{56}\text{Fe}$ reflects blood glucose concentration or merely Fe deficiency.

Principal component analysis (PCA) was conducted using 35 variables (Fig. 6). Three principal components (PCs) explained 54% of the total variance. PC1 was mostly loaded by the number of white blood cells (WBC) and the levels of potassium, glucose, triglyceride, HbA1C, haemoglobin, platelets and alkaline phosphatase (ALP). PC2 was mostly loaded by the ferritin concentration, serum $\delta^{65}\text{Cu}$, whole blood $\delta^{65}\text{Cu}$ and the levels of chloride, LDL and aspartate aminotransferase (AST). Meanwhile, PC3 was mostly loaded by the serum Cu concentration, whole blood Cu concentration, whole blood Zn concentration and whole blood Fe concentration. The PCA scores plot (PC1 vs. PC2) shows that data from patient #002 are distinct from those of the other patients (Fig. 6b). From the loading plot, it can be seen that the scores of these data are related to glycaemic parameters (HbA1c, glucose level, serum $\delta^{56}\text{Fe}$ and blood $\delta^{56}\text{Fe}$). This patient has a severe case of diabetes and receives a high dose of insulin and oral diabetes medication—higher than that for two other patients receiving this type of medication in this cohort dose (data not shown). Thus, from the PCA, the heavy serum and whole blood $\delta^{56}\text{Fe}$ of the patient can be hypothetically associated to severe diabetes.

Conclusions

The study of serum and whole blood Cu, Fe and Zn isotopic compositions showed to be useful for the follow-up of

bariatric surgery patients. The Cu isotopic composition became gradually lighter over time, while the difference in Zn composition between whole blood and serum ($\Delta^{66}\text{Zn}$) was significantly increased 6 months post-bariatric surgery. Gastric bypass patients showed an improvement in metabolic parameters, reaching normal values post-surgery, but the mineral isotopic compositions were not fully restored 12 months post-surgery. The Fe isotopic compositions are altered in patients with disrupted metabolism. Those changes in Cu, Fe and Zn isotopic compositions are not accompanied by similar changes in their respective concentrations, making isotopic compositions more sensitive to physiological changes than elemental content, thus providing added value and advocating for further research in this context. Additionally, correlations between metal isotopic compositions and glycaemic and lipid parameters were established. However, whether these metal isotopic shifts are caused by metabolic changes or due to metal imbalance remains to be elucidated.

Competing interests The authors declare that they have no conflict of interest.

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Compliance with ethical standards

This study was approved by the Mayo Clinic Institutional Review Board (IRB). All patients provided written informed consent at the Mayo Clinic. Healthy volunteers provided informed consent at UZ Gent.

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