the subjects with NOx ≥80 μmol/L. In particular, body fat and skinfold thickness were 1.6-fold higher in the subjects with NOx ≥80 μmol/L than in the subjects with NOx <20 μmol/L. These results indicate that moderately increased NOx concentrations are relevant to BMI and body fat. In this study, we investigated whether serum lipid profiles show significant changes as NOx concentrations increase; however, unlike body fat mass, there were no significant differences in serum lipid profiles between the subjects with NOx <20 μmol/L and with NOx ≥80 μmol/L.

In conclusion, our results suggest that obesity leads to increased NO production in humans. Increased serum NOx correlate strongly with body fat but poorly with serum lipid concentrations.

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References


10. Verdon CP, Burton BA, Prior RL. Sample pretreatment with nitrate reductase subjects with NOx


Effects of Anticoagulants and Contemporary Blood Collection Containers on Aluminum, Copper, and Zinc Results, Elizabeth L. Frank, 1 Martin Patrick Hughes, 2 Daniel D. Bankson, 3 and William L. Roberts 1 (1) Department of Pathology, University of Utah Health Sciences Center, Salt Lake City, UT 84132; 2 Department of Laboratory Medicine, University of Washington, Seattle, WA 98195; 3 Veterans Affairs Puget Sound Health Care System, Seattle, WA 98108; * address correspondence to this author at: c/o ARUP Laboratories, 500 Chipeta Way, Salt Lake City, UT 84108; fax 801-584-5207, e-mail william.roberts@arup-lab.com

Laboratory analysis is used to evaluate both deficiency and excess of trace elements (1). The intravascular concentrations of many of these elements are maintained within narrow limits. For this reason, preanalytical loss of analyte and spuriously high values attributable to contamination are concerns. Blood collection, processing, and storage before analysis are critical for accurate trace element analysis. Decreased concentrations of analytes may result from adsorption onto collection container surfaces or from the use of anticoagulants that complex metals (2, 3). Sources of contamination include patient clothing and skin; blood collection materials, including needles, anticoagulants, stoppers, serum separators, and glass containers; and particulate matter in laboratory air.

Aluminum, copper, and zinc are three metallic elements commonly monitored by the clinical laboratory. Although aluminum may have a physiological role in the action of a few enzymes, such as succinic dehydrogenase and porphobilinogen synthase, it is typically monitored to evaluate toxicity in patients subjected to hemodialysis for renal failure (3, 4). These patients may be exposed to high aluminum concentrations in their treatment regimen but lack an efficient physiological means to remove this element. Aluminum accumulation may lead to dialysis encephalopathy and osteomalacia. Toxicity is known to occur at concentrations >100 μg/L, although symptoms may occur at 60 μg/L or lower in dialysis patients (3, 5). Because aluminum is ubiquitous and pervasive, contamination is a serious concern for the analytical laboratory. Falsely high concentrations measured in contaminated specimens may affect clinical decisions.

Copper is an essential trace element necessary for the function of several enzymes involved in electron transport, free radical defense, and other biological oxidation-reduction reactions (1). Copper has a role in iron metabolism and is an important indicator of Wilson disease and Menkes kinky hair syndrome. Copper concentrations vary with age, gender, and ethnic group.

Zinc is an essential trace metal that is second in abundance among the essential metals only to iron (1). Zinc is a necessary component of the active sites of many enzymes and contributes to the structural stability of numerous metalloenzymes and other proteins. More than 75% of whole blood zinc is present in erythrocytes. Hemolysis can falsely increase zinc concentration and should be avoided in specimens used to measure zinc. Copper and zinc
usually are measured to evaluate deficiency. Higher concentrations, however, may be associated with toxicity, and laboratory methods to determine increased concentrations are necessary. We evaluated the effects of common blood collection tubes on the measured concentrations of these three trace elements in serum and plasma. The sampling protocol was devised to minimize contamination during specimen collection and sample processing. This study was approved by the University of Washington Human Subjects Review Committee. Specimens (n = 164) were collected from 23 fasting subjects by venipuncture using sterile butterfly needles. The first milliliter of drawn blood was discarded. Seven different commercial evacuated glass tubes were cooled to 4 °C before filling. The order of filling and tube characteristics are shown in Table 1. Filling order was based on NCCLS guidelines devised to minimize cross-contamination from tube additives (6). Royal-blue top tubes are acid-washed and have been tested for trace element background concentration. Plasma tubes were centrifuged cold within 10 min of collection. Serum tubes were placed in a 22 °C water bath for 30 min, and then centrifuged at room temperature. Specimens were transferred immediately into two acid-washed plastic tubes, capped, and stored at −70 °C before analysis. One aliquot was used for the determination of the aluminum concentration, and the second aliquot was used for analysis of copper and zinc by inductively coupled plasma mass spectrometry. This sensitive technique allows simultaneous multielement measurement and the use of an internal standard to monitor background signal, instrument drift, and matrix effects (7). Aluminum was analyzed separately to minimize contamination of the specimen. This is consistent with our usual laboratory protocol, which was adopted to decrease potential aluminum contamination from particulate matter and other sources in the laboratory that cannot be entirely controlled. Ions generated by the argon plasma were separated by their mass-to-charge (m/z) ratios and measured by mass spectrometry. We monitored masses 27, 65, and 64 for aluminum, copper, and zinc, respectively. Quantification was accomplished by comparison with an internal standard. Beryllium (isotope mass, 9) was used as an internal standard in the measurement of aluminum. Yttrium (isotope mass, 89) was used as an internal standard in the measurement of copper and zinc.

### Table 1. Characteristics of specimen collection containers by tube top color.

<table>
<thead>
<tr>
<th>Tube top color</th>
<th>Order of filling</th>
<th>Additive</th>
<th>Separator</th>
<th>Interior coating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Royal-blue</td>
<td>1</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Red</td>
<td>2</td>
<td>None</td>
<td>None</td>
<td>Silicone</td>
</tr>
<tr>
<td>Tiger stripe</td>
<td>3</td>
<td>Clot activator</td>
<td>Yes</td>
<td>Silicone</td>
</tr>
<tr>
<td>Royal-blue</td>
<td>4</td>
<td>Sodium heparin</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Green tiger stripe</td>
<td>5</td>
<td>Lithium heparin</td>
<td>Yes</td>
<td>None</td>
</tr>
<tr>
<td>Lavender</td>
<td>6</td>
<td>Potassium EDTA</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Green</td>
<td>7</td>
<td>Lithium heparin</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

The mean and standard deviation for each analyte are plotted by collection container type in Fig. 1. Two specimens collected in tiger top serum separator tubes produced spuriously high aluminum values and were excluded from further analysis. For all other tube types and analytes, results from 23 specimens are plotted. In general, plasma values were lower than serum concentrations, but these differences were not statistically significant.

![Fig. 1. Concentrations of aluminum (A), copper (B), and zinc (C) plotted by blood collection container type.](image-url)

Mean concentrations of each analyte are displayed as columns; error bars, 1 SD. Mean concentrations of aluminum and zinc in EDTA plasma are 28.0 and 9401.7 µg/L, respectively. *, statistically significant difference. LiHep, lithium heparin.
cant. We recommend a royal-blue top collection tube with no additive or with heparin for trace element testing. Mean values for specimens collected in these tubes were compared with mean values for the other anticoagulant collection tube systems using a paired *t*-test.

Comparison of aluminum measured in serum collected in a royal-blue top trace metal collection tube with aluminum measured in other collection containers showed a statistically significant difference only for EDTA plasma collected in the lavender top tube. Comparison of aluminum in plasma collected in a royal-blue heparinized tube with aluminum in all other plasma collection containers gave similar results, i.e., only the aluminum concentration found in the EDTA plasma was significantly different. We found no statistically significant differences between serum copper concentrations measured in any collection container and the copper concentration measured in serum collected in a royal-blue top trace metal collection tube. Likewise, no statistically significant differences were seen between plasma copper concentrations compared with copper measured in plasma collected in a royal-blue top tube, although the *P* value for the EDTA result was 0.09. Copper concentrations measured in EDTA plasma were lower than copper measured in any other collection container. Chelation of copper in the specimen by EDTA should not substantially affect the copper concentration measured by inductively coupled plasma mass spectrometry; it is possible that less copper contamination was present in these tubes. Comparison of zinc measured in serum collected in a royal-blue top trace metal collection tube showed statistically significant differences for serum collected in red top tubes and for plasma collected using lithium heparin and a polymer gel separator as well as for specimens collected in EDTA. Zinc concentrations in EDTA plasma were much higher than zinc measured in other collection systems. This could reflect contamination of the collection container or the EDTA anticoagulant with zinc. Plasma zinc concentrations in all tubes other than EDTA showed no statistically significant differences when compared with the royal-blue heparinized tube.

To evaluate the contribution of an osmotic effect on differences seen between serum and plasma, we measured the albumin concentration in serum collected in royal-blue top serum collection containers and in plasma anticoagulated with heparin (royal-blue top tube) and EDTA (laverender top tube). The mean albumin concentration was decreased by 2.6% in heparinized plasma (44.6 g/L; SD, 2.4 g/L) and by 6.6% in EDTA plasma (42.8 g/L; SD, 2.1 g/L) compared with the mean serum albumin concentration (45.8 g/L; SD, 2.1 g/L). The difference was significant for EDTA specimens (*P* < 0.01), but not significant for heparinized specimens (*P* = 0.08). The decrease in the albumin concentration observed for the heparinized royal-blue top tube was equivalent to the decrease in the albumin measured in the same tube type and could be explained by the osmotic redistribution of water between cells and plasma. This observation is consistent with those of several other investigators and has been attributed to a fluid shift from erythrocytes into plasma in response to increased osmolality produced by the addition of anticoagulants such as citrate or EDTA (8). These authors found decreased concentrations of albumin, cholesterol, and total protein in plasma specimens compared with the same analytes measured in serum specimens. Heparinized plasma concentrations of copper and zinc were much lower, reduced by 7% and 16%, respectively.

Metal contamination of EDTA in the collection container was evaluated by drawing a volume of distilled, deionized water into each of three royal-blue top and lavender top tubes and analyzing each sample. The mean concentrations of aluminum, copper, and zinc measured in the royal-blue top serum containers were 0.67 µg/L (SD, 0.59 µg/L), 0.38 µg/L (SD, 0.18 µg/L), and 4.8 µg/L (SD, 0.34 µg/L), respectively. The mean concentrations of aluminum, copper, and zinc measured in the EDTA plasma containers were 13.1 µg/L (SD, 2.61 µg/L), 0.67 µg/L (SD, 0.15 µg/L), and 698.9 µg/L (SD, 442.7 µg/L), respectively. We conclude that some component of the lavender top collection container, most likely the EDTA, contributes to the aluminum and zinc concentrations of specimens collected in these tubes. The extent of zinc contamination varies considerably from tube to tube.

Although there were significant differences between serum and plasma values, with the exception of samples anticoagulated with EDTA, measured aluminum, copper, and zinc values fell within expected reference intervals for all tube types. Results of this study indicate that the best choice for trace metal testing is a royal-blue top tube designed for trace element analysis. Lavender top EDTA tubes should not be used, but other blood collection tubes may be adequate for routine testing. Specimens collected in glass collection containers must be processed and transferred to plastic containers within 1 h of collection to minimize alteration of aluminum concentration (2, 3). Plastic vacuum collection containers are available and provide a reasonable alternative if specimen processing will be delayed. Mean concentrations for serum aluminum, copper, and zinc can be expected to be slightly higher than mean plasma concentrations. Plasma specimens may offer an advantage for zinc determinations to minimize release of the high concentration of zinc in erythrocytes and platelets (1).

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References
New Missense Mutation in the Human Ferrochelatase Gene in a Family with Erythropoietic Protoporphyria: Functional Studies and Correlation of Genotype and Phenotype, Urszula B. Rüfenacht,1 Anita Gregor,2 Laurent Gouya,3 Sylwia Tarczynska-Nosal,2 Xiaoye Schneider-Yin,1* and Jean-Charles Deybach 1 (Zentrallabor, Stadtpital Triemli, Birmensdorferstrasse 497, CH-8063 Zurich, Switzerland; 2 Porphyria Center, Institute of Hematology and Blood Transfusion, Chocimska 5, 00-957 Warsaw, Poland; 3 Centre Francais des Porphyries, INSERM U 409, Hopital Louis Mourier, F-92701 Colombes, France; * author for correspondence: e-mail xiaoye.schneider@triemli.stzh.ch)

Erythropoietic protoporphyria (EPP) is an inherited disorder caused by a partial deficiency of the enzyme ferrochelatase. Ferrochelatase catalyzes the insertion of ferrous ions into protoporphyrin IX, the last step in heme biosynthesis. As a result of ferrochelatase deficiency, protoporphyrin accumulates in various tissues; this accumulation is responsible for the clinical symptoms of light sensitivity and, in rare cases, liver damage (1). Several mutations have been identified in the human ferrochelatase (FECH) gene among EPP patients (2). The results indicate a possible link between the “null-allele” mutations (i.e., mutations that lead to the formation of a truncated enzyme) in the FECH gene and liver complications in EPP (3). Although EPP is considered an autosomal dominant disorder, only ~10% of individuals with a defective enzyme develop clinical symptoms. A recent study suggested that symptomatic patients differ from asymptomatic gene carriers by the amount of mRNA output from the nonmutated allele. This so-called “low expressed” wild-type FECH allele, identified in EPP patients, is highly associated with a specific haplotype at two single nucleotide polymorphism (SNP) sites in the FECH gene namely, a G at the −251 position in the promoter region and a T at IVS1-23 (4). These latest developments in the pathogenesis of EPP allow prediction of the clinical course of the disease. We report here the first study conducted in an EPP family from Poland.

The proband was a 15-year-old female patient from Masuria in the northern part of Poland who had been suffering from photosensitivity since early childhood. The diagnosis of EPP was established based on the clinical symptom and laboratory analyses (Table 1). Ferrochelatase activity in the proband was reduced to 25% of the mean value, as is typical for EPP patients (5). A massive increase of protoporphyrin concentration was measured in both the red cells and feces. The patient had normal liver function. Although asymptomatic, the proband’s father exhibited a >50% reduction in ferrochelatase activity accompanied by a moderate increase of protoporphyrin concentration in the red cells. Neither clinical nor laboratory abnormalities were observed in the other two members of the family.

Peripheral blood samples were collected from all members of the family for isolation of genomic DNA. All 11 coding exons as well as the flanking intronic regions of the FECH gene were analyzed in the genomic DNA from the proband by denaturing gradient gel electrophoresis (DGGE) according to a method described previously (2). An abnormal DGGE pattern was observed in exon 5 of the FECH gene (Fig. 1A). Direct sequencing of the PCR-amplified fragment containing exon 5 revealed a missense mutation, T545G, which led to the conversion of Leu-182 to Arg in ferrochelatase.

Because T545G (L182R) was, to our knowledge, a novel missense mutation in the FECH gene, its effect on enzyme activity was examined in vitro. The L182R mutation introduced into a wild-type FECH cDNA cloned into prokaryotic expression vector pGEX-2 (2). Ferrochelatase activity of the mutant in the crude bacterial cell extracts was 0.17 ± 0.02 U (an average of four independent clones), which corresponds to <6% of the activity of 2.85 ± 0.5 U observed with the wild-type FECH cDNA. This result demonstrated that L182R was indeed a causative missense mutation. Because no other sequence abnormalities were found in the FECH gene, L182R was

Table 1. Clinical and biochemical findings among members of a Polish EPP family.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age, years</th>
<th>Clinical symptom</th>
<th>Ferrochelatase activity, U (1.84–3.15 U)*</th>
<th>Red cells, nmol/L (&lt;88.7 nmol/L RBC)b</th>
<th>Feces, nmol/g dw (&lt;100 nmol/g dw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proband</td>
<td>15</td>
<td>PS</td>
<td>0.6</td>
<td>9392.7</td>
<td>1730</td>
</tr>
<tr>
<td>Father</td>
<td>40</td>
<td>None</td>
<td>0.88</td>
<td>302.5</td>
<td>65</td>
</tr>
<tr>
<td>Mother</td>
<td>38</td>
<td>None</td>
<td>1.75</td>
<td>47.1</td>
<td>ND</td>
</tr>
<tr>
<td>Brother</td>
<td>17</td>
<td>None</td>
<td>3.15</td>
<td>51.7</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Values in parentheses are reference intervals.

b RBC, red blood cells; dw, dry weight; PS, photosensitivity; ND, not determined.