Emily Watson is a senior majoring in nutrition science at Purdue University with a minor in biology. She spent two years in Dr. Gletsu-Miller’s lab where she completed an honors thesis and was part of the Transdisciplinary Obesity Prevention Research Science (TOPRS) program. Upon graduation she will continue doing research under a pediatric endocrinologist at the Indiana School of Medicine. She plans to attend medical school in the future.

Nana Gletsu-Miller is currently an assistant professor of nutrition science at Purdue University. She obtained a PhD in nutrition and metabolism from the University of Alberta in Edmonton, Canada, in 1998. Her dissertation studies involved the effect of obesity on insulin signal transduction in the cell nucleus. Thereafter she was a postdoctoral fellow in the Department of Pathology at Emory University, Atlanta, with Kenneth Bernstein, studying the transcriptional regulation of angiotensin converting enzyme. In 2002 she transitioned to focus on human studies in the field of bariatric surgery, in the Department of Surgery, Emory University, as a research instructor. In 2006 she became an assistant professor of research in surgery in the Hubert Department of Global Health. Her studies focused on the mechanisms responsible for resolution of type 2 diabetes following bariatric surgery Gletsu-Miller joined the Department of Nutrition Science at Purdue in 2011. Her studies investigate the nutritional status of bariatric surgery patients and focus on deficiencies in iron, copper, vitamin D, and essential fatty acids.
**LITERATURE REVIEW AND RATIONALE**

Obesity is a rising epidemic in the United States, affecting 35% of adults (body mass index, BMI ≥ 30 kg/m²), with 4.5% of men and 8% of women being severely obese (BMI ≥ 40 kg/m²) (Flegal, Carroll, Kit, & Ogden, 2012). It is forecasted that incidence of moderate (BMI ≥ 35 kg/m²) and severe obesity will continue to increase in the United States (Finkelstein et al., 2012). Obesity is a major risk factor for several chronic diseases, including diabetes, cardiovascular disease, and cancer (WHO, 2016). For those patients who wish to lose weight, there are several methods that can be used to induce weight loss, including diet, exercise, medication, and surgery.

Bariatric surgery is considered to be one of the most effective treatments for obesity and is recommended for individuals who are moderately or severely obese, suffer from an obesity-related health condition, and have not responded to other methods of weight loss (Jensen et al., 2014; Sjöström et al., 2004). On average, a patient’s BMI drops between 8–10 units (kg/m²), with maximum weight loss 12 months post-surgery (Schauer et al., 2014). Bariatric surgery is also very popular; in 2013, 154,276 people with severe obesity in the U.S. underwent the procedure (Angrisani et al., 2015).

There are multiple types of bariatric surgery procedures. Of those, Roux-en-Y gastric bypass (RYGB), seen in Figure 1A, used to be the most commonly performed bariatric surgery procedure, but is currently declining in popularity (Angrisani et al., 2015). In the RYGB surgery, the duodenum of the small intestine and most of the stomach is bypassed, leaving a 30 mL stomach pouch (MacLean, Rhode, & Nohr, 2000). This procedure induces weight loss through food restriction and nutrient malabsorption (Gletsu-Miller & Wright, 2013). Another type of bariatric surgery procedure is the sleeve gastrectomy (SG) surgery, seen in Figure 1B, which is currently the most popular type of bariatric surgery procedure. In this procedure, the surgeon removes a portion of the stomach but leaves the intestines intact. This induces weight loss through food restriction (Gletsu-Miller & Wright, 2013).

There are many documented benefits of receiving bariatric surgery. These benefits include resolution or improvement of diseases, such as type 2 diabetes, cardiometabolic disease, hypertension, depression, and reduction in mortality rates (Adams et al., 2012; Christou et al., 2004; Dixon et al., 2008; Schowalter et al., 2007; Sjöström et al., 2007; Sjöström et al., 2012). However, there are also some negative
consequences associated with undergoing bariatric surgery. Bariatric surgery alters dietary intake and nutrient absorption, which may result in nutritional deficiencies following surgery (Gletsu-Miller & Wright, 2013). Some of the nutritional complications are well-known and screened for by health care providers, including malnutrition of protein, vitamin B12, vitamin D, calcium, and iron (Fe). Others that are not typically screened for by physicians include B vitamins, fat-soluble vitamins, and minerals such as copper (Cu) and zinc (Clements et al., 2006; Gasteyer, Suter, Gaillard, & Giusti, 2008). Without screening, patients who have undergone bariatric surgery are at risk for postsurgical nutrient deficiencies that are not being monitored or treated appropriately.

As one of the essential minerals, Cu plays many important roles in the body by acting as a cofactor for the enzyme function of ceruloplasmin (Cp) and superoxide dismutase 1 (SOD1) (Homberg & Laurell, 1947). It also assists in hemoglobin formation, cell signaling, and cellular respiration (Fox, 2003; Haremaki Fraser, Kuo, Baron, & Weinstein, 2007). Dietary Cu requirements are met by most healthy adults, because it is found in commonly consumed foods such as mushrooms, leafy green vegetables, and even cocoa (Trumbo, Yates, Schlicker, & Poos, 2001). However, cases of Cu deficiency have been found as a result of bariatric surgery. Cu is absorbed mostly in the duodenum of the small intestine, which is bypassed in patients who have had the RYGB procedure (Mason, 1979). A previous study reported from our group determined that incidence of copper deficiency following RYGB surgery was 18.8% (Gletsu-Miller et al., 2012).

One way Cu status can be altered is through interactions with Fe. A notable site of interaction between Fe and Cu is the divalent metal-ion transporter 1 (DMT1), a transporter on the basolateral membrane of the enterocyte, where they compete for absorption (Gulec & Collins, 2014). In addition, both minerals need to be in their reduced forms to be absorbed into the enterocyte, thus they compete for reduction through cytochrome B reductase 1 (CYBRD1). Previous studies have shown that high dietary Fe results in decreased Cu status in rats (Jung-Heun Ha, Zhao, Wang, Flores, & Collins, 2016). Copper-transporting ATPase 1 (ATP7A) is a Cu transporter that is upregulated during Fe deficiency (Collins, Franck, Kowdley, & Ghishan, 2005). Duodenal epithelial interactions are shown in Figure 2 (Gulec & Collins, 2014). One additional interaction that should be noted is Cu’s function in Fe homeostasis. Cp, a Cu binding molecule, mediates Fe release from sites of Fe storage and into the blood for Fe to perform essential functions (Hellman & Gitlin, 2002).

Currently, there are several methods of assessing Cu status. These methods include measuring concentrations of erythrocyte SOD1, plasma Cu activity, serum Cu, or liver Cu (Danzeisen et al., 2007). SOD1 has not been found to be a reliable or
sensitive indicator for Cu status because erythrocyte concentrations of SOD1 are affected by a variety of health and stress conditions (Danzeisen et al., 2007). Cp decreases during severe Cu deficiency but is also an acute-phase response protein that increases during inflammation and infection (Danzeisen et al., 2007). This may result in falsely elevated concentrations of Cp that could misrepresent Cu status. Serum Cu is tightly regulated but can also be elevated during inflammation; thus, it does not accurately correspond to Cu status (Danzeisen et al., 2007). Liver Cu is the only reliable Cu status indicator; however, it is difficult to measure in humans without an invasive procedure (Araya et al., 2003). Thus, there is a critical need for a more sensitive and specific biomarker to assess Cu deficiency and changes in humans. Studies have shown that copper chaperone for superoxide dismutase (CCS) could be a promising biomarker for assessing Cu status. CCS plays an important role in delivering Cu to the antioxidant enzyme SOD1 (Culotta et al., 1997). It was found in rats that CCS expression is more sensitive to mild reductions in Cu status compared to serum Cu, SOD1, and Cp activity (Iskandar et al., 2005). CCS is elevated in the erythrocytes of Cu-deficient rats and mice compared to rats and mice that are normal in Cu status, reflecting a negative correlation between CCS and serum Cu (West & Prohaska, 2004). Since previous animal models show that CCS is a promising biomarker for Cu status, we would like to look at this in human subjects. In this study, we tested CCS as a biomarker of Cu status in bariatric surgery patients. We hypothesized that CCS would respond to changes in Cu status in Cu-deficient patients supplemented with copper, where CCS was hypothesized to decrease, and when altering Cu status in response to high doses of Fe supplementation, where CCS was hypothesized to increase.

OBJECTIVE

The purpose of my research is to determine whether concentrations of CCS in erythrocytes are associated with serum concentrations of Cu in patients who have had bariatric surgery. We will additionally determine whether we can observe changes in CCS concentrations in erythrocytes in response to changes in Cu status by supplementing patients with high doses of Cu and Fe. We expect CCS to increase for patients supplemented with Fe and decrease for patients supplemented with Cu. From this data, we can evaluate whether CCS would be a viable biomarker to test Cu status.

METHODS

Human Subjects

The Purdue Institutional Review Board approved this study (#1410015305). Postcards were used to recruit patients who might be eligible for the study. We also used a recruitment service called ResNet, which is a database of patients who are receiving services through the Indiana University Health System. To qualify for the study, subjects must have been female, have had RYGB or SG bariatric surgery at least 6 months prior to enrollment, and have been at least 18 years of age. All subjects gave informed consent to participate in the study. Subjects were excluded if they were pregnant, had received intravenous (IV) Fe in the last month, or had surgical revisions to bariatric surgery. Men were excluded from the study because it is less common for men to undergo bariatric surgery (Buchwald & Oien, 2013).

Screening

To enroll in the study, patients had to qualify at a screening visit. Patients filled out three-day food logs before coming in for their screening visits. At the visit, a blood draw was performed in the morning after the patient had undergone an 8-hour overnight fast. Status of Fe and Cu were measured by Mid America Clinical Laboratories (MACL, Indianapolis, IN), a commercial reference laboratory. Information was collected regarding demographics and relevant medical history during an interview. Height and weight were also measured at the patient screening visit.

To enroll in the Fe supplementation intervention, patients had to have Fe deficiency based on two or more of the following criteria: ferritin < 20mcg/L, total iron binding capacity (TIBC) > 370mcg/dL, soluble transferrin receptor (sTfR) > 2012mcg/L, and sTfR: ferritin ratio > 500 (Grant et al., 2012). In order to enroll in the Cu supplementation intervention, patients must have had Cu deficiency at two separate screening visits, diagnosed by having a serum Cu concentration < 70mg/dL and Cp activity in plasma < 62.5 units/L (Gletsu-Miller et al., 2012).

Intervention

We enrolled one patient into the Cu supplementation intervention and five patients into the Fe supplementation intervention. We provided patients who were enrolled in the Fe supplementation intervention with Nature Made ferrous sulfate Fe supplementation at a dose of 65 mg taken three times
a day as recommended by the bariatric surgery guidelines (Mechanick et al., 2013). They received supplementation for 8 weeks with visits at weeks one, two, four, and eight. We provided the patient who enrolled in Cu intervention Cu gluconate supplementation from GNC at a dose of 8 mg/day for 8 weeks as previously determined by Griffith and colleagues (2009).

At each study visit, enrolled patients went to the Purdue Clinical Research Center after having undergone an 8-hour overnight fast to have their blood drawn. Supplements were provided at each visit. Controls for this study were bariatric surgery patients who had undergone screening for Fe and Cu deficiency but were not deficient.

**Fractionation of Erythrocytes**

A blood draw was obtained at all visits for both screened and enrolled patients. The blood was fractionated to separate erythrocytes, monocytes, and plasma as previously described (Araya et al, 2012). Measurements of serum Cu were measured as described (Lassi & Prohaska, 2012).

**Western Blotting**

Western blotting assays were performed using patients’ blood erythrocytes to test for presence of CCS as described by Lassi and Prohaska (2012). We did not regulate protein loading due to difficulty quantifying erythrocyte proteins. Primary antibody, mouse anti-rabbit polyclonal antibody against CCS, was obtained from West and Prohaska (2004), and diluted 1:500. Secondary antibody was obtained from Li-Cor diluted 1:10,000. Positive controls were run on every Western performed. We assessed CCS in all patients who were screened regardless of whether they were Cu-deficient or not. By assessing all patients, we were able to determine normal reference levels of CCS.

**STATISTICS AND DATA ANALYSIS**

All data is presented as median values and interquartile (IQ) ranges, unless otherwise specified on the figure. Serum Cu and erythrocyte CCS correlations were performed using Spearman correlations for non-parametric data. Two outliers were removed from the data before running the statistical measures. A dependent sample T-test was performed to compare changes in the Fe supplemented patients’ concentrations of CCS at visit 1 to those of visit 4. Coefficients of variation (CV) for Western blots were calculated based on positive control samples run for both intraday variation and interday variation.

**RESULTS**

**Patient Characteristics**

Table 1 shows the patient demographics and characteristics of all screened subjects. We screened 32 subjects; all screened subjects were female. The median age of the study population was 46 years old. Most subjects self-identified as Caucasian (94%). Their BMIs ranged from 22.3 to 55.9 kg/m². Most subjects underwent the RYGB surgery (94%), but one subject had undergone the SG.

<table>
<thead>
<tr>
<th>All Screened Patients (N = 32)</th>
<th>Median (Interquartile Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>46 (39–49)</td>
</tr>
<tr>
<td>Sex of subject, female N (%)</td>
<td>100 %</td>
</tr>
<tr>
<td>Race/ethnicity (%)</td>
<td>94, 3, 3</td>
</tr>
<tr>
<td>Type of surgery, RYGB N (%)</td>
<td>31 (94)</td>
</tr>
<tr>
<td>Years since surgery</td>
<td>6.82 (3.5–10.16)</td>
</tr>
<tr>
<td><strong>Anthropometrics</strong></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>30.5 (26.8–36.8)</td>
</tr>
<tr>
<td><strong>Nutritional Status</strong></td>
<td></td>
</tr>
<tr>
<td>Serum Cu (μg/dL)</td>
<td>109 (96–124.5)</td>
</tr>
<tr>
<td>Ferritin (μg/L)</td>
<td>13.75 (6.4–43.78)</td>
</tr>
</tbody>
</table>

CA: Caucasian; AA: African American; HI: Hispanic; BMI: body mass index.
Associations Between Erythrocyte CCS and Serum Cu Concentration

For the CCS assay, the intraday CV was found to be 16.1% and the interday CV was found to be 18.25%. All screened patients were used to assess the association between erythrocyte CCS and serum Cu. Figure 3 shows the association between the two measures. It was found that CCS and serum Cu were not significantly correlated ($p = 0.61$).

![Figure 3](image)

**Figure 3.** CCS and serum Cu were found not to be significantly correlated. $R = -0.092, p = 0.61$.

Impact of Cu and Fe Supplementation on Erythrocyte CCS Concentrations

The patient supplemented with Cu had a BMI of 22 kg/m². The patient had a serum Cu concentration of 79.1 μg/dL on visit 1 and 83.4 μg/dL on visit 4. Figure 4 shows the serum Cu concentrations of the patient supplemented with Cu gluconate (8 mg/day) for 8 weeks, where Cu status was normalized from baseline to visit 4. Figure 5 shows the CCS concentrations in erythrocytes before and after Cu supplementation. There was no change in CCS between the patient’s initial screening, baseline, and final visit.

![Figure 4](image)

**Figure 4.** After 8 weeks of 8mg/day Cu gluconate supplementation, the patient began with 64 μg/dL of serum Cu and ended above a normal level at 83.4 μg/dL. At the end of the intervention, serum Cu concentration was above the normal reference level.

Five patients were found to have Fe deficiency. Their serum Cu at screening averaged to be 145.8 μg/dL. Their ferritin concentrations increased from 5.1 μg/L to 18.76 μg/L, visit 1 to visit 4, respectively ($p = 0.001$). All patients had increased Fe status at the completion of the study. Figure 6 shows the changes in CCS concentrations in erythrocytes for patients who received Fe supplementation.

DISCUSSION

This study analyzed Cu status in normal and deficient patients following Cu and Fe supplementation. We evaluated whether a biomarker, called CCS, was associated with serum Cu concentrations, and how CCS responds to change in Cu status following Fe and Cu supplementation. Our study found no correlation between CCS concentrations in erythrocytes and serum Cu. However, a significant increase was seen in CCS from visit 1 to visit 4 in patients who underwent Fe supplementation. CCS concentrations in the patient who received Cu supplementation did not change at any point during the study.

This study did not find a significant correlation between CCS concentrations in erythrocytes and serum Cu, suggesting that CCS may not be a viable biomarker for Cu status in human subjects. This result was unexpected and contradicts previous research performed on rats where CCS was shown to be an effective biomarker in representing Cu status (West & Prohaska, 2004). Additionally, it contradicts studies done on humans that showed CCS as a biomarker in assessing Cu toxicity (Araya et al., 2012). As far as we know, this was the first study to test CCS concentrations, as it relates to Cu deficiency in human subjects. CCS may respond differently in humans than we had expected from previous studies conducted on rats. Because we had a high CV both between and within assays for our Western blots, it is possible that the assay itself was not reliably...
measuring CCS concentrations in the erythrocytes of human subjects. Further research should be conducted to assess the reliability of the methods used for this study.

The patient with Cu deficiency that we provided with high doses of Cu gluconate supplementation, 8 mg/day for 8 weeks, had a normalized serum Cu status (> 70 mg/dL) at the conclusion of the study. For this patient, 8 mg/day was a sufficient amount of Cu supplementation to replete her Cu status by measurement of her serum Cu. The CCS values for this patient did not appear to change from visit 1 to visit 4. It was surprising to only find one patient with Cu deficiency since our lab previously saw much higher incidence of Cu deficiency in the bariatric surgery population (Gletsu-Miller et al., 2012). Different geographical locations or a difference in diet may be reasons why we are seeing less incidence of Cu deficiency. Since Cu is affected by inflammation, high BMI in some of the patients may also be a factor that is affecting Cu status (Danzeisen et al., 2007). We suspect that dietary Cu is also associated with Cu status, but since we found only one patient with Cu deficiency, this remains to be determined. There is no evidence to suggest that blood loss through menstruation has an effect on Cu status. More patients with Cu deficiency would be needed to determine whether this supplementation treatment is sufficient to replete Cu status in patients who are Cu-deficient, and to evaluate whether erythrocyte CCS responds to changes in Cu status following high-dose Cu supplementation.

In the other intervention, we had five patients who were enrolled to receive Fe supplementation. As we had expected, all the patients had a normalized Fe status at the conclusion of the study. A previous study in our lab analyzed dietary recall data of the subjects and showed that dietary Fe was associated with Fe status independent of the level of obesity, meaning patients who consumed less dietary Fe were more likely to be deficient (Mischler et al., 2015). Blood losses through menstruation or gastrointestinal problems are other areas that may affect Fe status in patients. The Fe supplement successfully normalized deficient patients.

There was a consistent increase in CCS from visit 1 to visit 4 in patients who were supplemented with Fe. Previous studies have shown that Cu status is low when Fe is high in animal models that consume high dietary Fe, and this study saw a similar trend in terms of erythrocyte CCS indicator of Cu status (Gulec & Collins, 2014; Jung-Heun Ha et al., 2016). After receiving high doses of supplementary Fe for 8 weeks, patients had a significant increase in CCS. This implies that there is an interaction between Fe and CCS. Because no correlation was found between CCS and serum Cu, we cannot determine if a change in Cu status in these patients is a potential reason for the change in CCS.

The present study is limited by its low statistical power, having only one patient supplemented with Cu and five patients supplemented with Fe. However, as the study is ongoing, the sample size will continue to increase. The study also only recruited females and all of the study subjects were Caucasian, so the study may not be generalizable to men or other races. In addition, we do not know what the serum Cu concentrations are for those enrolled in the Fe supplementation study. We did not record where patients were in their menstrual cycle at the time blood draws were taken. This would have an effect on Fe status, but there is no evidence to suggest that the menstrual cycle affects Cu status. We are actively working to obtain those values to explore how

**Figure 6.** CCS between visits 1 and visits 4 significantly increase across all Fe supplemented patients ($p = 0.046$). (A). The image of the Western blot for Fe supplemented patients. GAPDH was used as the control across the patients. (B). All patients’ CCS concentrations in erythrocytes increased after undergoing Fe supplementation. Mean [SEM] CCS for visit 1 was 0.958 [0.164], which increased to 1.247 [0.154] at visit 4.
serum Cu may have changed compared to erythrocyte CCS in reaction to high doses of Fe supplementation.

While this study did not find a significant association between CCS in erythrocytes and serum Cu in human subjects, we did find a significant increase in CCS from visit 1 to visit 4 in patients who received high doses of Fe supplementation. We were also able to replete the Cu status of the Cu-deficient patient who took high doses of Cu supplementation, though the patient’s erythrocyte CCS concentrations were unchanged. We will continue to screen for patients who have Cu or Fe deficiency and treat them with high doses or oral supplementation. We will also continue to explore associations between CCS in erythrocytes and serum Cu in patients who have undergone bariatric surgery. Future studies should explore dietary records collected and how that relates to Cu status. Because obesity is an inflammatory disease, future studies should investigate whether BMI and body weight of patients affects Cu status, since serum Cu is elevated during inflammation (Danzeisen et al., 2006). Incidence of vitamin deficiency after laparoscopic Roux-en-Y gastric bypass in a university hospital setting. American Journal of Surgery. 72(12), 1196–1202; discussion 1203–1204.


