VARIATIONS IN PLASMA VOLUME AND MICRONUTRIENT BIOMARKERS ACROSS THE MENSTRUAL CYCLE AMONG WOMEN OF REPRODUCTIVE AGE

A Dissertation in Nutritional Sciences

by Sixtus Aguree

© 2019 Sixtus Aguree

Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

August 2019
The dissertation of Sixtus Aguree was reviewed and approved* by the following:

Alison D. Gernand  
Assistant Professor of Nutritional Sciences  
Dissertation Advisor  
Chair of Committee

Laura E. Murray-Kolb  
Associate Professor of Nutritional Sciences  
Professor-in-Charge of the Graduate Program

Francisco J. Diaz  
Associate Professor of Reproductive Biology

Lacy Alexander  
Associate Professor of Kinesiology

Matthew L. Reimherr  
Associate Professor of Statistics

*Signatures are on file in the Graduate School
ABSTRACT

Background: Micronutrient deficiencies are a global health problem most commonly occurring in women of reproductive age and children. Plasma volume is an important marker of health status and large changes have been associated with variation in plasma-based micronutrient biomarkers. Further, some evidence suggests that plasma volume and micronutrient concentrations may vary across the menstrual cycle. Accurate knowledge of micronutrient variability in the prepregnancy state would help improve decisions on appropriate times in the menstrual cycle to assess a woman’s nutritional status and to evaluate micronutrient deficiencies in a population. Accurate determination of micronutrient concentrations and plasma volume in the nonpregnant state could also improve how changes during pregnancy are estimated, and ultimately could be useful in deciding when to intervene in pregnancy.

Objectives: The objective of this study was to prospectively evaluate the variations in plasma volume and selected micronutrient biomarker concentrations across the menstrual cycle, under free-living conditions, in a cohort of healthy women of reproductive age. The primary outcomes were 1) plasma volume in the early and late follicular and midluteal phases; and changes in plasma volume between timepoints; 2) serum zinc, copper, magnesium, manganese, iron, ferritin, and retinol concentrations and total circulating mass at the three timepoints and prevalence of deficiencies, as well as changes in concentrations between timepoints; and 3) the relationship between plasma volume and micronutrient biomarkers.

Research Design and Methods: This was a longitudinal prospective study wherein subjects were examined at three time points within a single menstrual cycle: the early follicular phase (EFP; ~day 2), the late follicular phase (LFP; ~day 12), and the midluteal phase (MLP; ~day 21) — based on a 28-day cycle length. The actual days of study visits were adjusted for the length of the woman's menstrual cycle and urinary hormone results from a fertility monitor. Visits were timed to key ovarian states and the hormone concentrations helped us determine those states. Blood samples were collected followed by the injection of a bolus dose of 0.25 mg/kg body weight of indocyanine green dye (ICG). Starting at 2
minutes post-ICG injection, a series of 5 blood samples timed to occur every 45 seconds were collected and used to estimate plasma volume. Pre-injection blood samples were used to measure ovarian hormones (serum estradiol and progesterone) and micronutrient biomarkers (serum zinc, copper, magnesium, manganese, iron, ferritin, retinol), and to conduct a complete blood count. All biomarkers were measured at three timepoints in the cycle. For practical purposes, we assumed that serum and plasma concentrations of micronutrients are comparable, and therefore, total mass for each biomarker was calculated by multiplying plasma volume and serum concentrations. Linear mixed-effects models were used to investigate the bivariate association between menstrual phase and plasma volume, and menstrual phase and micronutrient biomarkers. Fractional polynomial regression (prediction plots) were used to examine bivariate relationships between plasma volume and biomarkers including micronutrient biomarker concentrations and mass.

**Results:** A total of 47 women were enrolled in the study and 35 completed all study visits. Blood data was available for 45 subjects at visit 1 (EFP), 39 at visit 2 (LFP), and 35 at visit 3 (MLP). The mean ± SD plasma volume was highest at 2276 ± 478 mL in the EFP; it declined slightly to 2232 ± 509 mL in the LFP and 2228 ± 502 mL in the MLP. On average, plasma volume fell by 55 mL (2.4%) between EFP and MLP ($P = 0.536$).

The mean ± SD concentrations for micronutrients in EFP were 81.8 ± 16.2 µg/dL (zinc), 80.1 ± 12.8 µg/dL (copper), 17.9 ± 1.4 mg/L (magnesium), 1.51 ± 1.97 µg/L (manganese), 106.7 ± 1.7 µg/dL (iron), 26.4 ± 2.3 µg/L (ferritin), and 39.4 ± 9.3 µg/dL (retinol). All micronutrient biomarker concentrations decreased between EFP and LFP. Reductions in zinc and magnesium concentrations were relatively large, 6.6% ($P = 0.009$) and 4.6% ($P < 0.001$), respectively; changes for other micronutrients ranged from <1 to 4.3%, and were not statistically significant (all $P > 0.05$). Correspondingly, the prevalence of micronutrient deficiencies changed across the cycle. Overall, the highest prevalence of deficiencies was in the LFP and MLP. For instance, zinc deficiencies rose from 22% to 37% from EFP to MLP and magnesium went up from 17% to 49%.
Plasma volume and biomarker concentrations showed a weak correlation or no correlation. Values were -0.05 to 0.10 (all \( P > 0.05 \)) across all biomarkers. The correlations between plasma volume and biomarker mass showed a strong positive correlation: 0.26 to 0.95 (all \( P < 0.01 \)). The correlation coefficient was greater than 0.70 (\( P < 0.001 \)) for the mass of five out of the eight biomarkers.

**Conclusions:** The results from this study suggest that the menstrual cycle state or phase has a relatively small influence on plasma volume in healthy nonpregnant women of reproductive age. Changes in serum zinc and magnesium concentrations across the menstrual cycle were quite large. In healthy nonpregnant women, plasma volume and micronutrient concentrations were not associated. Therefore, in normal healthy women of reproductive age, it is not expected that plasma volume is needed to aid in the interpretation of micronutrient status.

Though more research is needed to determine whether menstrual phase should be considered in the design of studies examining micronutrient biochemical markers in healthy nonpregnant women, the results from this study suggest that the menstrual cycle phase may influence how concentrations of zinc and magnesium are interpreted. Further work is needed to build a body of evidence on the variability of micronutrient biomarker concentrations across the menstrual cycle, and to determine whether the menstrual phase should be considered in evaluating micronutrient status in women of reproductive age, in research and clinical practice. Such studies will also provide the basis on which to determine the appropriate time to measure micronutrient status that reflects the true status of an individual and at the population level.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xi</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xii</td>
</tr>
<tr>
<td>Chapter 1 Background</td>
<td>1</td>
</tr>
<tr>
<td>Micronutrients</td>
<td>2</td>
</tr>
<tr>
<td>Zinc</td>
<td>3</td>
</tr>
<tr>
<td>Copper</td>
<td>4</td>
</tr>
<tr>
<td>Magnesium</td>
<td>5</td>
</tr>
<tr>
<td>Manganese</td>
<td>6</td>
</tr>
<tr>
<td>Iron and hemoglobin</td>
<td>7</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>8</td>
</tr>
<tr>
<td>Serum versus plasma</td>
<td>9</td>
</tr>
<tr>
<td>Effect of inflammation on micronutrient biomarkers</td>
<td>10</td>
</tr>
<tr>
<td>Plasma volume</td>
<td>11</td>
</tr>
<tr>
<td>The menstrual cycle</td>
<td>13</td>
</tr>
<tr>
<td>The menstrual cycle and plasma volume</td>
<td>15</td>
</tr>
<tr>
<td>The menstrual cycle and micronutrient biomarkers</td>
<td>15</td>
</tr>
<tr>
<td>Plasma volume and micronutrient biomarkers</td>
<td>17</td>
</tr>
<tr>
<td>Methods for measuring plasma volume</td>
<td>19</td>
</tr>
<tr>
<td>Following the menstrual cycle in research</td>
<td>21</td>
</tr>
<tr>
<td>Hydration</td>
<td>24</td>
</tr>
<tr>
<td>Summary of research gaps</td>
<td>25</td>
</tr>
<tr>
<td>Aims and objectives</td>
<td>26</td>
</tr>
<tr>
<td>Aims</td>
<td>26</td>
</tr>
<tr>
<td>Specific objectives</td>
<td>26</td>
</tr>
<tr>
<td>Conceptual framework</td>
<td>28</td>
</tr>
<tr>
<td>Chapter 2 Research Design and Methods</td>
<td>29</td>
</tr>
<tr>
<td>Research Design</td>
<td>29</td>
</tr>
<tr>
<td>Statistical power and sample size estimation</td>
<td>33</td>
</tr>
<tr>
<td>Inclusion and exclusion criteria</td>
<td>34</td>
</tr>
<tr>
<td>Inclusion criteria</td>
<td>34</td>
</tr>
<tr>
<td>Exclusion criteria</td>
<td>35</td>
</tr>
<tr>
<td>Early withdrawal</td>
<td>35</td>
</tr>
<tr>
<td>Pregnancy test</td>
<td>36</td>
</tr>
<tr>
<td>Blood pressure</td>
<td>36</td>
</tr>
<tr>
<td>Anthropometry</td>
<td>37</td>
</tr>
<tr>
<td>Weight measurement</td>
<td>37</td>
</tr>
<tr>
<td>Height measurement</td>
<td>37</td>
</tr>
<tr>
<td>Questionnaire</td>
<td>38</td>
</tr>
<tr>
<td>Measurement of urine specific gravity</td>
<td>38</td>
</tr>
<tr>
<td>Biomarkers</td>
<td>39</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Discussion</td>
<td>107</td>
</tr>
<tr>
<td>Clinical and research implications</td>
<td>109</td>
</tr>
<tr>
<td>Chapter 6 The relationship between plasma volume and micronutrient</td>
<td>118</td>
</tr>
<tr>
<td>biomarker concentrations and mass in women of reproductive age</td>
<td></td>
</tr>
<tr>
<td>Abstract</td>
<td>118</td>
</tr>
<tr>
<td>Introduction</td>
<td>120</td>
</tr>
<tr>
<td>Subjects and Methods</td>
<td>122</td>
</tr>
<tr>
<td>Study design and participants</td>
<td></td>
</tr>
<tr>
<td>Blood collection and plasma volume determination</td>
<td></td>
</tr>
<tr>
<td>Measurement of micronutrient biomarkers</td>
<td></td>
</tr>
<tr>
<td>Statistical analysis</td>
<td></td>
</tr>
<tr>
<td>Results</td>
<td>126</td>
</tr>
<tr>
<td>Characteristics of the study samples</td>
<td>126</td>
</tr>
<tr>
<td>Micronutrient biomarker concentration and mass</td>
<td>126</td>
</tr>
<tr>
<td>Correlation of plasma volume with micronutrient biomarker concentrations and mass</td>
<td>127</td>
</tr>
<tr>
<td>Discussion</td>
<td>128</td>
</tr>
<tr>
<td>Strengths and limitations</td>
<td>130</td>
</tr>
<tr>
<td>Conclusions and recommendations for future research</td>
<td>130</td>
</tr>
<tr>
<td>Chapter 7 Research Summary, Discussion and Future Perspectives</td>
<td>136</td>
</tr>
<tr>
<td>Appendix A Recruitment and screening materials</td>
<td>140</td>
</tr>
<tr>
<td>Recruitment material for ResearchMatch</td>
<td>140</td>
</tr>
<tr>
<td>Screening form 1a</td>
<td>141</td>
</tr>
<tr>
<td>Screening form 1b</td>
<td>143</td>
</tr>
<tr>
<td>Appendix B Study consent form</td>
<td>145</td>
</tr>
<tr>
<td>Appendix C Questionnaire</td>
<td>154</td>
</tr>
<tr>
<td>Background, and health &amp; pregnancy histories</td>
<td>154</td>
</tr>
<tr>
<td>Blood collection form</td>
<td>157</td>
</tr>
<tr>
<td>Fertility monitor daily testing sheet</td>
<td>158</td>
</tr>
<tr>
<td>References</td>
<td>159</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1-1: Clearblue fertility monitor with indicators of fertility status typical record from daily urine testing..........................................................23

Figure 1-2: The interplay between the menstrual cycle, plasma volume and micronutrient concentrations in healthy women of reproductive age.................................28

Figure 2-1: Summary of the flow process of screening and data collection process at each visit.32

Figure 2-2: ATAGO™ Digital hand-held urine specific gravity “pen” clinical refractometer. 39

Figure 3-1: ICG preparation and injection: A, ICG powder and sterile water; B, syringes and alcohol pad for disinfecting; C, transferring the sterile water into the ICG powder vial to prepare the ICG solution; D, mixing the ICG powder and sterile water to ensure that all the power is dissolved; E, pipetting the required volume of ICG solution for injection; F, ICG solution ready for injection; G, injecting ICG solution into the IV line established; H, 3-way stopcock replacement system for blood collection; I, Remaining ICG solution in vial and the aliquoted ICG solution (in cryovial) for lab work........................................................................51

Figure 3-2: Steps for 3-way stopcock connections during the ICG injection and blood collection process................................................................................................................52

Figure 3-3: Standard curve for estimating the concentration of ICG in unknown plasma samples (example from one participant). ...............................................................................58

Figure 3-4: The decay curve for plasma ICG concentrations back-extrapolated to time, t = 0 (left) and t = 1 minute (right) using data collected from t = 2 to 5 minutes (example from one participant). ..........................................................59

Figure 3-5: Local polynomial regression plot (regression line) of height, weight, BMI and BSA against plasma volume (at time, t = 0). The Spearman’s correlation coefficient and regression estimates were: BMI on plasma volume (r = 0.23, P = 0.547; β = 0.032, 95% CI: -0.088, 0.152); BSA on plasma volume (r = 0.74, P = 0.022; β = 3.023, 95% CI: 0.597, 5.457); weight on plasma volume (r = 0.57, P = 0.111; β = 0.030, 95% CI: -0.009, 0.069), and height on plasma volume (r = 0.68, P = 0.043; β = 0.055, 95% CI: 0.002, 0.107). Body mass index (kg/m²); BSA, body surface area (m²)..................................................................59

Figure 4-1: Flow chart of participants. IV; intravenous..........................................................83

Figure 4-2: Fractional polynomial with regression (line) of plasma volume on day of cycle when measurement occurred (left), cycle day relative to presumed date of ovulation (right). Number visits = 118 (95 graph on right). Black dots represent data from study participants; solid black line represents prediction based on all data; short dash gray line represents the 95% CI around the prediction. ...............................................................................87

Figure 4-3: Fractional polynomial with regression (line) of plasma volume on plasma osmolality (A), systolic blood pressure (B), diastolic blood pressure (C), age (D), weight (E), BMI (F), lean body weight (G), body fat (%) (H), body surface area. Number visits = 118 (114 for
plasma osmolality). Black dots represent data from study participants; solid gray line represents prediction based on all data; short dash gray line represents the 95% CI around the prediction.

Figure 4-4: Locally weighted regression (line) of estrogen (left) and progesterone (right) concentrations versus cycle day from ovulation. Number visits = 95. Black dots represent data from study participants; solid black line represents prediction based on all data. ...............88

Figure 4-5: Locally weighted regression (line) of plasma volume versus log concentration of estrogen (left) and progesterone (right). Number visits = 118. Black dots represent data from study participants; solid gray line represents prediction based on all data; short dash gray line represents the 95% CI around the prediction. ..............................89

Figure 5-1: Prevalence of micronutrients deficiencies and anemia across the menstrual cycle. EFP, early follicular phase; IDA, iron deficiency anemia (hemoglobin<12g/dL and ferritin<15µg/L); LFP, late follicular phase prospective; MLP, Midluteal phase. There was no vitamin A deficiency (i.e., retinol (< 20µg/dL)) at any visit. .................................................90

Figure 5-2: Fractional polynomial regression (prediction line) of estradiol concentration (log) on biomarker concentration; log of progesterone concentration on zinc (A), copper (B), magnesium (C), manganese (D), log of iron (E), log of ferritin (F), retinol (G), and hemoglobin (H). Solid black lines represent the prediction line. Short-dash gray lines represent the 95% prediction interval for the prediction line. (118 data points, n=45). .................................113

Figure 5-3: Fractional polynomial regression (prediction line) of progesterone concentration (log) on biomarker concentration: log of progesterone concentration on zinc (A), copper (B), magnesium (C), log of manganese (D), log iron (E), log ferritin (F), retinol (G), and hemoglobin (H). Solid black lines represent the prediction line. Short-dash gray lines represent the 95% prediction interval for the prediction line (118 data points, n=45). ..................115

Figure 5-4: Fractional polynomial regression (prediction line) of micronutrient biomarker concentration on day of cycle when measurement occurred; zinc (A), copper (B), magnesium (C), manganese (D), log of iron (E), log of ferritin (F), retinol (G), and hemoglobin (H). Solid black lines represent the prediction line. Short-dash gray lines represent the 95% prediction interval for the prediction line. (118 data points, n=45). .................................116

Figure 5-5: Fractional polynomial regression (prediction line) of plasma volume on biomarker concentration: plasma volume on zinc (A), copper (B), magnesium (C), manganese (D), iron (E), ferritin (F), retinol (G), and hemoglobin (H). Solid black lines represent the prediction line. Short-dash gray lines represent the 95% prediction interval for the prediction line. (118 data points for 44 women). ..........................................................133

Figure 6-1: Fractional polynomial regression (prediction line) of plasma volume on biomarker concentrations: plasma volume on zinc (A), copper (B), magnesium (C), manganese (D), iron (E), ferritin (F), retinol (G), and hemoglobin on plasma volume (H). Solid black lines represent the prediction line. Short-dash gray lines represent the 95% prediction interval for the prediction line. (118 data points). ...............................................................134
LIST OF TABLES

Table 2-1: Study schedules and activities across visits ......................................................... 43

Table 3-1: Preparation of standard concentrations for ICG (using six 25 mL volumetric flasks, total volume for each is 25 mL) ................................................................. 56

Table 3-2: Final concentration for standard curve (using six 1.5 mL microcentrifuge tubes, total volume for each is 400 µL) ................................................................. 56

Table 3-3: Participants and plasma volume (n = 9) ............................................................. 58

Table 4-1: Baseline descriptive characteristics and menstrual cycle information of study participants (n = 45) ................................................................. 84

Table 4-2: Mean within-person differences in plasma volume, blood pressure and body weight across a single menstrual cycle (n = 45) ......................................................... 85

Table 4-3: Mean adjusted within-person differences in plasma volume across a single menstrual cycle (n = 45) (adjusted) ................................................................. 86

Appendix 4-1: Associations between within-person changes in plasma volume and changes in plasma osmolality, blood pressure, and body weight across the menstrual cycle (n = 34) 91

Table 5-1: Mean differences in micronutrient biomarker concentrations across a single menstrual cycle in women of reproductive age (n= 45) ................................................................. 111

Table 5-2: Adjusted mean differences in nutritional biomarkers concentrations across a single menstrual cycle in women of reproductive age (n = 45) ......................................................... 112

Table 5-3: Correlations between micronutrient biomarker concentrations and hemoglobin (n = 45) ................................................................. 114

Table 5-4: Mean serum circulating mass of micronutrient biomarkers and inflammatory marker at early follicular phase, late follicular phase, and midluteal phase in menstrual cycle of reproductive age women (n=45) ......................................................... 114

Table 6-1: Characteristics and health measurements of women of reproductive age (n = 45) 131

Table 6-2: Micronutrient biomarker and hemoglobin concentrations and mass of women of reproductive age (n = 45) ................................................................. 132

Table 6-3: Spearman’s correlations of plasma volume with micronutrient biomarker and hemoglobin concentrations and mass of women of reproductive age (n = 45) ................................................................. 132

Appendix 6-1: Spearman’s correlations of plasma volume and micronutrient biomarkers and hemoglobin concentrations and mass of women of reproductive age across the menstrual cycle (n=45) ................................................................. 135
Appendix 6-2: Spearman’s correlation between change (%) in plasma volume with change (%) in biomarker concentration and mass (n=45)..................................................................................135
ACKNOWLEDGEMENTS

There are many people I would like to express my sincere gratitude to for assisting me in diverse ways throughout my graduate program. Foremost among them is my advisor, Dr. Alison Gernand. Her invitation to join her laboratory in the study of ‘micronutrients and pregnancy’ started my journey as a graduate student. Throughout that journey, Dr. Gernand has supported me in different ways by providing guidance and mentorship at every stage. She challenged me to think critically and to think outside of the box. Thanks to her, my approach to research and writing has seen a dramatic improvement over time. She also encouraged me to take classes in statistics, and critically question statistical data when reading scientific publications. I also developed an interest in data science through her encouragement, and I’m very grateful for that.

I also thank my dissertation committee members, Dr. Laura Murray-Kolb, Dr. Francisco Diaz, Dr. Lacy Alexander, and Dr. Matthew Reimherr for their expertise, guidance and support throughout the research process. I am grateful for all that they have done for me, both as a collective group and as individuals.

Special thanks also go to the Gernand laboratory team members - Leigh Taylor, Anita Subramanian and Celeste Beck -, including the many talented undergraduate research assistants in the lab and on this project especially Alysha, Grace, Lauren, Nicole, Casey, and Carissa. You have created a very supportive environment, and it has been wonderful working with all of you.

A very special gratitude to the Faculty, administrative staff, especially Tammy, Mary, Scott, Barb, Deb, and Julie, and fellow graduate students of the Department of Nutritional Sciences at The Pennsylvania State University who have supported me along the way. I am thankful for all their sacrifices.

I also extend a hand of appreciation to the following individuals and groups at The Pennsylvania State University: Dr. A. Catharine Ross’s laboratory; Suzanne Strickland Simons of Dr. Murray-Kolb’s laboratory; Drs. Hilary Bethancourt and Asher Y. Rosinger of Dr. Rosinger’s laboratory; Ellen M.
Bingham, Kristen J. Koltun and Dr. Mary Jane De Souza of the Women's Health and Exercise laboratory; Matthew S. Gonzales and David R. Welsch of the Laboratory for Isotopes and Metals in the Environment (LIME).

Special thanks also go to the medical staff, especially Tracey Allen and Cyndi Flanagan, at the Clinical Research Center of The Pennsylvania State University’s Clinical and Translational Science Institute. I extend my gratitude to Dr. James A. Pawelczyk, Associate Professor of Physiology and Kinesiology at The Pennsylvania State University, for his support, particularly on the use of indocyanine green. I also thank the volunteers for their participation in the study.

Finally, I will like to thank my family and friends for their continuous support and encouragements.
Chapter 1

Background

Micronutrient deficiencies are a global problem most commonly occurring in women of reproductive age and children (1-4). Concerted public health effort by the World Health Organization and the Centers for Disease Control and Prevention among other governmental groups have advocated for improving micronutrient status among reproductive-age women before pregnancy and during pregnancy to improve health outcomes (5, 6). In the US, vitamin D and iron deficiency are particularly common among women of child bearing age (7, 8) but other deficiencies such as vitamin C are found at a low prevalence (9). Dietary assessment provides valuable information about the nutritional status of a population (e.g., macronutrients or micronutrients that are likely to be deficient) (10-14). Biochemical assessment of nutritional status provides insight about the totality of macro- and micronutrients status—which depends on the dietary intake, absorption, and endogenous production from non-dietary sources (e.g., vitamin D) (15, 16). Therefore, blood-based micronutrient biomarkers which are often assessed in serum or plasma samples are considered a more accurate (compared to dietary assessment) reflection of the nutritional status of a population (17-20). Serum and/or plasma-based micronutrient biomarkers are often used to estimate nutritional deficiencies in a population and public health interventions (e.g., iron and folic acid supplementation or multivitamin supplements) are often made based on the level of severity of deficiencies (9, 21-24).

Significant changes are observed in micronutrient concentrations during physiological adaptations that affect plasma volume, such as those seen during pregnancy. Some of the physiological changes of pregnancy, including increases in estrogen and progesterone as well as activity of renin-angiotensin-aldosterone system (RAAS), are observed to a lesser extent during the normal menstrual cycle (25). Higher concentrations of some RAAS components, including renin, plasma renin activity, and
aldosterone, have been observed during the luteal phase compared with the follicular phase (25, 26). Estrogen regulates the expression of angiotensinogen genes (27) and correlate positively with plasma renin activity (28-30). Both exogenous estrogen administration and endogenous estrogen production increase the activity of RAAS, leading to vasodilation (31). Administration of estrogen and progesterone has been associated with increased plasma volume (32, 33). Concentrations of estrogen and progesterone increase across pregnancy, similar to that observed for plasma volume, reaching peak in the third trimester. If plasma volume increases in pregnancy is driven by the rise these hormones, it is reasonable to expect plasma volume to fluctuate across the menstrual cycle in a similar manner to the concentration of estrogen and progesterone. Overall, we expect that plasma volume, ovarian hormones, and micronutrient concentrations should follow a defined pattern across the menstrual cycle.

**Micronutrients**

The term micronutrient is a generic term for vitamins and minerals that are needed in small quantities but play a central role in metabolism, reproduction and growth, and other functions. Nutritional status is important across the whole spectrum of the female reproductive life. Good nutritional status in the preconception period and during pregnancy are associated with optimal birth outcomes (34, 35). Micronutrient deficiencies, however, often co-exist in low-resource settings during pregnancy (36-38). Globally, micronutrient deficiencies are more common in low- and middle-income countries. Iron, zinc, iodine, and vitamin A are the most common micronutrient deficiencies worldwide, with the highest prevalence in pregnant women from developing countries (39). Accordingly, most research in this area has often focused on micronutrient supplementation (40-50), which can improve birth outcomes such as birthweight and length (23, 24). Multiple micronutrient supplementation has been shown to increase concentrations of iron, folate, and vitamin D biomarkers (51). Understanding micronutrient status in nonpregnant women can provide valuable information for public health planning at the population level.
At the individual level, it can help a woman improve her nutrition status, for instance, through a combination of dietary sources and micronutrient supplementation, as appropriate. Our broad interest was to examine different micronutrient biomarkers across the menstrual cycle in nonpregnant women. The current work focuses on biomarkers that resources allowed us to measure, at the present time: zinc, copper, magnesium, manganese, iron, and vitamin A.

**Zinc**

Zinc deficiency is an important problem worldwide particularly affecting populations whose main dietary source is high in cereal and low in animal source food (52). Several biological processes in the human body require zinc to function, including immune function, fertility, embryogenesis, implantation, milk secretion, and fetal growth and development (52-56). Common sources of zinc include beef, lamb, fish, poultry, and dairy products. Animal sources such as meat, eggs and seafood have higher bioavailability than whole grains and legumes. Plasma (serum) zinc is the recommended marker for population zinc status (57-59). Plasma zinc concentration is not a good marker of individual zinc status, and no biomarker has been identified for routine measurement individual zinc status (52). Zinc homeostasis is primarily maintained by adjustments in total zinc absorption and endogenous intestinal excretion, and zinc is not stored in the body, except in infants (52). Serum zinc concentration decline across the day, with the highest estimates in morning samples (58).

**Copper**

Copper deficiency is uncommon in the general population but can result from several causes such as anemia and genetic copper deficiency (Menkes syndrome) (60). Copper is an essential cofactor for oxidation-reduction reactions involving copper-containing oxidases and cuproenzymes (60, 61). Some
of the copper dependent enzymatic processes are energy production, iron metabolism, neurotransmitter synthesis, formation and maintenance of myelin, antioxidant functions, and regulation of gene expression (60-63). Major sources of copper are organ meats, shellfish, nuts, and seeds (including nuts and grains) (60). Copper absorption is relatively high from copper-rich sources but is dependent on dietary intake – a higher percentage is absorbed at lower intakes (60). Uptake of copper is competitively inhibited by divalent metals ions of manganese and zinc (60). The relative amount of copper in the diet seems to be the major predictor of absorption levels. Serum copper is a commonly measured biomarker of copper status in population studies (64-66). Copper is generally not stored and homeostasis is regulated by excretion (67). Concentrations of serum copper and ceruloplasmin show small fluctuation across the day, with highest estimates in the morning (68), though other studies have reported no diurnal variation in serum copper concentration (69).

**Magnesium**

In the United States, close to half of the population (45-48%) does not meet the estimated average requirements (EAR) for magnesium (70, 71). Among women 19 years, 56% had dietary magnesium intakes below the EAR (71). Some studies have reported nearly half (47%) of study participants had low serum magnesium concentrations (72). Severe magnesium deficiency may impede vitamin D and calcium homeostasis (73). Magnesium is involved in many physiologic pathways including energy production, protein synthesis, ion transport, signaling, cellular and tissue integrity as well as structural functions (74). Major dietary sources include green leafy vegetables, unrefined grains, legumes, beans, and nuts (e.g., Brazil nuts, cashews, almonds). Inadequate dietary intakes and/or low serum concentrations of magnesium are linked to increased risk of cardiovascular disease, osteoporosis, elevated C-reactive protein, and metabolic disorders; hypertension, and type 2 diabetes mellitus (71, 73). There is currently no recommended biomarker of magnesium status at the population or individual level. However, serum
magnesium concentration has been established for the US population and is often cited (73, 75, 76). Homeostasis is regulated primarily by the kidney but to some extent by dietary intake. Urinary magnesium excretion is increased with high levels and decreased with low intakes (73). Day-to-day variations are negligible but within day fluctuations have been reported with highest concentration in the morning (69, 77).

Manganese

Data on manganese deficiencies in the population are scarce (78). Manganese is a constituent of several enzymes (metalloproteins) and activates several enzymatic actions. It is involved in antioxidant function, and metabolism bone development and wound healing (79-82). Metabolism and regulation of manganese is not well defined but it appears that it shares a common pathway with iron. Higher intakes of iron may interfere with manganese absorption and lead to lower concentration of manganese (83). Rich sources of manganese include whole grains (wheat germ, oats, and bran) and rice; nuts (hazelnuts, almonds, and pecans) contain the highest amounts of manganese (84). Recent reviews showed that manganese is an essential metal that is required for proper immune function, regulation of blood sugar and cellular energy, reproduction, digestion, bone growth, blood coagulation, and hemostasis and defense against reactive oxygen species (78, 84). Serum manganese concentration is commonly reported in population studies (85, 86). Under normal condition, manganese level is tightly regulated by dietary absorption and biliary excretion (87). Total body content of manganese in adult man range from 12-20 mg (88). Data on diurnal variations in serum manganese concentration are currently lacking.
Iron and hemoglobin

Globally, iron deficiency is the leading cause of anemia, with approximately 33% (i.e., more than 2.2 billion people) of the population affected in 2010 (89). About 29% of nonpregnant women and 38% of pregnant women are anemic (defined as low hemoglobin) (90). Iron deficiency anemia (IDA) in the US ranges from 4.5% to 18% across age and sex (91). IDA is associated with gastrointestinal disturbances and impaired cognitive function, immune function, and physical performance (91-94). In infants and children, IDA can result in psychomotor and cognitive abnormalities that, without treatment, can lead to learning difficulties (93). Rather than eliminate excess iron that is not immediately needed, iron is stored in ferritin for times of need (67). Iron regulatory proteins 1 and 2 (IRP1 and IRP2) control cellular iron homeostasis by binding to iron-responsive elements (IREs) which are involved in iron uptake, storage, utilization and export (95, 96). In times of demand, iron is liberated from ferritin to fulfill essential functions in oxygen transport and energy metabolism. Heme iron is the essential constituent for oxygen transport in hemoglobin, oxygen storage in myoglobin, and electron transport for cytochrome function in aerobic respiration. Hepcidin (a hepatic hormone) regulates intestinal dietary iron absorption, plasma iron concentrations, and tissue iron distribution (93, 97). When iron is depleted from the body, dietary iron absorption increases to meet the demand for iron, although no known regulated pathway exists for excretion of excess iron. Humans typically lose only small amounts of iron in urine, feces, the gastrointestinal tract, and skin. Losses are greater in menstruating women because of blood loss.

The main dietary sources are lean meat, seafood, and poultry; other dietary sources include nuts, beans, vegetables, and fortified grain products. Enhancers of iron absorption include ascorbic acid, dietary protein, lysine, cysteine, and methionine, while oxalic acid, tannins, phytate, and fiber inhibit iron absorption. The ferritin index—a combination of serum ferritin, transferrin saturation, and the serum soluble transferrin receptors—is recommended in classification of iron status (93, 98-101). However, ferritin and transferrin saturation (TSAT) can also be used alone (102-105). Iron homeostasis is maintained at the level of dietary iron absorption to prevent toxic accumulation while adequate amounts
are provided to offset losses (106). Diurnal variations in and hemoglobin and hematocrit values are small (107).

Among healthy adults, day-to-day variation in iron markers is considerably low for hemoglobin and hematocrit, and but very high for serum iron and transferrin saturation. Day-to-day variability for hemoglobin and hematocrit range from 3.1% to 4.5% (108-111) and 2.3% to 3.1% (108-110), respectively. Day-to-day variation of serum iron and transferrin saturation range from 17.1% to 27.8% (108-110) and 16.7% to 28.5% (108-110), respectively. Day-to-day variation ranges for serum ferritin, serum transferrin receptor, and TIBC are 8.2% to 18.8% (108-112), 8.1% to 12.7% (108, 109, 111, 112), and 2.3% to 3.9% (108-110), respectively.

**Vitamin A**

Vitamin A is a generic term that refers to a groups of related compounds with the biological activity of retinol (113). Vitamin A is a fat-soluble vitamin. Its absorption is enhanced by the presence of fat in the small intestines where the bile acid facilitates the formation of micelles. Absorption of carotenoids (provitamin) varies greatly and depends on the presence of fat, the nature of the food matrix and human variability. The carotenoids are transported in blood exclusively by lipoproteins (114). They are converted to retinol as varying efficiencies depending on the type and source of the carotenoid (115, 116). Retinol in circulation is bound to and is carried by retinol binding protein for delivery to different body tissues. Each molecule of retinol recirculates several times in the plasma compartment before it leaves the body largely through fecal matter (117). There are two forms of vitamin A: preformed vitamin A and pro-vitamin A. Preformed vitamin A is found in animal products such as meat, liver, fish, poultry and dairy foods; pro-vitamin A is found in plant-based foods such as fruits and vegetables, e.g., broccoli, spinach, carrots, tomatoes, sweet potatoes and most green leafy vegetables.
Some of the functions of vitamin A in the human body include vision, growth, and anti-infection activity (113, 117, 118). Vitamin A deficiency is a common micronutrient deficiency in many countries resulting in night blindness (119). Serum retinol concentration is the recommended marker of vitamin A status at the population level (120, 121). Retinoid are transported in the bloodstream bound largely to albumin (122). The liver is the primary storage site for vitamin A and it also controls vitamin A metabolism (123). Day-to-day variation in retinol are negligible and blood samples can be collected to analyze for vitamin A status at any time of the day (124). Other studies have reported that serum retinol concentration remains stable across the day and does not appear to be influenced by menstrual cycle (125).

**Serum versus plasma**

Serum and plasma micronutrient concentrations are the most widely used biochemical indicators of nutritional status (52, 59, 76, 121, 126-128). For practical purposes, plasma and serum are often used interchangeably, though there are some concerns that biomarker concentration could vary between plasma and serum values. Serum zinc and copper concentrations has been reported to be comparatively higher than those measured from plasma by 12% to 16% (129, 130) and 9%, respectively (130). These differences are attributed to the prolonged time allowed for samples to clot to prepare serum samples, whereas plasma is centrifuged immediately (131). No change was observed for copper, magnesium, sodium, potassium, chloride, calcium or albumin when samples were allowed to clot up to two hours, plasma and serum estimates were not different (131). This would suggest that the estimated differences in these micronutrient biomarkers will be negligible between plasma and serum (131). Other studies have reported no differences in serum and plasma zinc concentrations, a little of 1% higher in serum (not significant) (132). One study have reported ~3% higher plasma zinc than serum concentration but it was not significant (133). Overall, there are limited studies that evaluated the differences in micronutrient
biomarker concentrations comparing plasma and serum and which one is appropriate for assessing nutritional status. Though this may need to be evaluated, at present, both serum and plasma estimates are used in defining biomarker deficiencies.

**Effect of inflammation on micronutrient biomarkers**

Inflammation has the tendency to influence micronutrient biomarker concentrations. For instance, inflammation may lead to an overestimation (e.g. vitamin A deficiency as measured by low retinol concentration; and zinc deficiency as measured by low serum zinc concentration) (134-136), or underestimation (e.g. iron deficiency as measured by low serum ferritin concentration) (137, 138), of the population prevalence of micronutrient deficiencies. These changes in biomarker concentrations are thought to result from hemodilution, sequestration, and increase or decrease rates of synthesis (e.g. breakdown of muscle) (139).

The influence of inflammation of micronutrient deficiencies prevalence estimates have reported by several studies. For instance, prevalence of vitamin A deficiency (low serum retinol-binding protein (RBP), defined as RBP<0.7 μmol/L)) among Liberian children was estimated to be 24.7% but after adjusting for inflammation, the prevalence dropped to between 7.3% to 11.6% (depending on the methods of correction used) (134). Conversely, another study reported an increase in serum ferritin by as much as 30%, resulting in 14% underestimation of iron deficiency (137) as a result of inflammation. Furthermore, one study among Indonesian infants reported underestimating iron deficiency anemia by more than 15%, and overestimating vitamin A deficiency by more than 16% in infants with elevated acute phase proteins (APPs) compared with those infants without inflammation (140).

To address the effect of inflammation on micronutrient biomarkers, there has been ongoing research, particularly by the BRINDA (Biomarkers Reflecting Inflammation and Nutrition Determinants
of Anemia) project—a collaborative research project between The Centers for Disease Control and Prevention (CDC), National Institute for Child Health and Human Development, and Global Alliance for Improved Nutrition. The BRINDA project uses population-based micronutrient biomarker and acute-phase proteins (AGP, a1-acid glycoprotein; CRP, C-reactive protein) data of preschool children and women of reproductive age from 16 surveys and 14 countries data (141, 142). The collaboration was formed to address issues related to inflammation, micronutrient biomarkers, and anemia etiology (142).

The BRINDA project uses five approaches to adjust for micronutrient biomarker concentration across different studies (134, 141, 143-146). Approach 1; no adjustment for inflammation as defined by elevated CRP (>5mg/L) and/or AGP (>1 g/L). Approach 2; data from participants with elevated two APPs (CRP>5mg/L and/or AGP>1 g/L) are eliminated. Approach 3; generate internal correction factors based on a four-level model of inflammation (both CRP and AGP data available): the incubation period (CRP>5mg/L and AGP≤ 1 g/L), early convalescence (CRP>5mg/L and AGP>1 g/L), late convalescence (CRP ≤5mg/L and AGP>1 g/L) and apparently healthy (CRP ≤5mg/L and AGP≤1 g/L (reference group)). The level-specific adjustment factors (correction factors) are calculated as the ratios of mean concentration of the biomarker for apparently healthy individuals (reference group) to mean concentration the biomarker for those in the incubation period, the early convalescence period and the late convalescence period. Each individual’s biomarker concentration value (raw data as measured) is then multiplied by their corresponding correction factor (based on their stage of inflammation) to estimate the adjusted biomarker concentration. A two-group model of inflammation is used if only CRP or AGP data are available. Individuals are classified into one of two groups, inflammation or no inflammation (reference); For CRP: 1) no inflammation (CRP ≤5 mg/L) (reference); 2) inflammation (CRP >5 mg/L). Or AGP: 1) no inflammation (AGP ≤1 g/L) (reference); 2) inflammation (AGP>1 g/L).

Approach 4; use external correction factors established by Thurnham et al. (147). Thurnham et al. meta-analysis used data from several countries that also had AGP and CRP values to generate a four-group model of inflammation: 1) non-elevated state or reference (CRP ≤5 mg/L and AGP ≤1 g/L); 2)
incubation (CRP >5 mg/L and AGP ≤1 g/L); 3) early convalescence (CRP >5 mg/L and AGP >1 g/L); 4) late convalescence (ACP ≤5 mg/L and AGP >1 g/L). Approach 5; use a multiple linear regression model to obtain adjusted biomarker concentrations, where CRP and or AGP are added as independent variables and the biomarker of interest is the dependent variable. The adjusted slopes from the regression equations are multiplied by the CRP and AGP values, and then subtracted from the corresponding unadjusted values to obtained the adjusted biomarker concentration (134, 141-143).

Overall, CRP and AGP values are used to adjust for the effect of inflammation on ferritin, retinol and zinc concentrations, and deficiencies at the population level. External correction factors are widely used to adjust for micronutrient concentration when AGP and CRP are measured to correct for infection.

**Plasma volume**

Plasma volume is a component of the extracellular fluid volume – fluid outside of body cells. It represents the total volume of plasma in circulating blood. Under normal physiological conditions, plasma volume is tightly controlled in healthy individuals. Plasma volume changes in response to temperature (148-150), exercise (151, 152), disease states (153, 154), and hydration status (155). For instance, during and immediately following intense exercise, plasma volume declines by 11% to 19% (150, 156). During prolonged exercise or ~24 hours following an intense exercise, plasma volume increases by 5% to 12% (151, 152, 156, 157). The combination of exercise and heat can lead to an expansion of plasma volume up to 18% (157). Other studies have reported contraction in plasma volume by 4% and 11% during heat-induced dehydration and exercise-induced dehydrations, respectively (155).

A large proportion of the body weight is made up of water (60% or 42 L in a 70 kg man of normal BMI). This is distributed between the intracellular (fluid found within cells) and extracellular (water in blood and in between cells) compartments (158). About 65% (37 L) of the water is found in intracellular fluid and 35% (15 L) extracellular fluid (interstitial fluid and plasma). Intracellular fluids are
found within cells and interstitial fluids are fluids found between cells. Plasma is the fluid in the blood that transports blood cells around the body (158). The extracellular fluid surrounds all cells in the body, and the intracellular fluid surrounds all cells not in the blood.

Several factors including blood pressure, hormones and the kidney are thought to be involved in plasma volume regulation (153, 154). Plasma volume may also change at different phases of the menstrual cycle (159, 160). Plasma volume expansion is a known biological process in pregnancy, though the amount of increase across gestation is not well established. Plasma volume expansion starts at about six to eight weeks in pregnancy (161-163) and continues throughout the second trimester and early third trimester (162, 164) but whether this continues up till term is inconclusive (165). The expansion of plasma volume serves to increase uteroplacental blood flow and improve nutrient transport to the fetus.

Marked plasma volume reduction occurs following parturition (165-167) with half of the postpartum fall within the first three to five days after delivery (168). The level of reduction can vary considerably during the first 3 or 4 days (164). Plasma volume is thought to return to nonpregnant volume by week 6-8 postpartum, which is often used as proxy for nonpregnant plasma volume to retrospectively estimate volume expansion during pregnancy (169-174). Notably, others have reported that plasma volume does not return to pre-pregnancy status until 12 weeks postpartum or later (175, 176). It is unknown if plasma volume returns to normal values at different time between women who breastfeed and those not breastfeeding.

While opinions vary as to what drives plasma volume expansion, some propose that activation of the RAAS in early pregnancy drives plasma volume expansion (177), while others speculate that plasma volume increases in response to changes in hemodynamics (178, 179). Some argue that changes in the RAAS are responsive to the preceding changes in blood volume (179). Estrogen and other hormones initiate systemic vasodilation (178). Along with the reduced tone of the vascular walls, this leads to a cascade of events including a fall in afterload (resistance against which the heart must work to eject blood during contraction, into systemic circulation), increase in stroke volume, release of vasopressin,
hemodilution of blood, reduced vascular resistance, and finally, plasma volume expansion (178, 180). As well, angiotensinogen slowly rises across pregnancy up to 36 weeks (181), corresponding approximately to the peak in plasma volume expansion.

**The menstrual cycle**

The menstrual cycle is a series of monthly physiological and physical changes a woman’s body undergoes in preparation for a pregnancy. It is physiologically regulated by a set of hormones, and physically manifested in monthly bleeding. The menstrual cycle is the time interval from the first day of one menstrual bleeding to the day before the next menstruation (182). The cycle is usually divided into the follicular and the luteal phases, each of which could further be divided into sub-phases; early, mid, and late follicular or luteal phases. Ovulation divides the cycle into the two phases. In women aged 19-42 years, the average follicular phase and luteal phase lengths are 14.6 days and 13.6 days, respectively (183). Activities of the menstrual cycle are hormonally regulated by the hypothalamus–pituitary–ovarian axis. Six major reproductive hormones regulate the cycle activities, namely, Gonadotropin-releasing hormone (GnRH), follicular stimulating hormone (FSH), inhibin, estrogen (estradiol and estrone are the major forms of estrogen in non-pregnant women; the two are metabolically interconvertible), luteinizing hormone (LH) and progesterone.

FSH is secreted by the pituitary gland and stimulates the growth of ovarian follicles in the early follicular phase of the cycle. As the follicles grow, they secrete estrogen, estrogen (and inhibin) acts on the hypothalamus and the pituitary gland to inhibit FSH (184, 185). Rising concentration of FSH leads to an emergence of a wave (cohort) of follicles, and the transition to low FSH concentration culminates in dominant follicle selection; the surrounding follicles undergo atresia in the mid-follicular phase of each cycle (186-188). The selected dominant follicle continues to secrete estrogen. When estradiol (the predominant circulating estrogen in humans) concentration is sustained beyond a specific threshold for a
period of approximately 48 hours (189), it triggers a GnRH surge which then causes LH surge (190) leading to an LH surge. The rise in LH triggers ovulation (around the midcycle), occurring within 24-48 hours after the LH surge ovulation (191-194) or ~0.5 to 1 day after LH peak, or 3 days after urinary estrone-3-glucuronide (E3G, a major metabolite of estradiol) surge (195).

Estrogen drops before or immediately after ovulation and starts to rise again around day 5 post-ovulation, with a surge (lower than during the follicular phase) at midluteal phase. Progesterone begins to surge around 3-5 days post ovulation, peaks around 9 days after LH surge or 7-8 days following LH peak (196-198). Pregnanediol-3-glucuronide (P3G, a progesterone metabolite) peaks around day 7.5 post-ovulation (195). Following ovulation, the dominant follicle transforms into a corpus luteum in preparation for a pregnancy. The corpus luteum takes over the production of estrogen and progesterone, and if no pregnancy occurs, it regresses gradually, leading to menstruation (199, 200). In 80% of ovulatory women, the average blood loss during menstruation is about 33.2 mL (10-80 mL), with the heaviest bleeding occurring on cycle day 2 (199).

Although the average menstrual cycle length is often quoted as 28 days, there is a considerable variability in length across women and within an individual woman (201-207). Intra-individual cycle length can vary as much as 7 days between cycles in about 36% to 70% of women (204, 205, 207). For instance, in one study among 14 healthy women studied across 3-13 cycles, an intra-cycle variability of 7 days was reported in 42.5% of the women (202). The variability in cycle length is mostly driven by variation in follicular phase length, while the luteal phase length is relatively constant within and between women (201, 202, 206, 208). Mean follicular phase length declines with age but the luteal phase length remains constant until menopause (209). Regular normal menstrual cycle length can vary from 21-42 days (210, 211). Intra-cycle variations of 8-20 days between cycles are considered irregular cycles (205).
The menstrual cycle and plasma volume

Estrogen and progesterone play important roles in extracellular fluid volume and plasma volume regulation across the menstrual cycle (33, 212-217). Stephenson and Kolka have reported that plasma volume varies across the menstrual cycle, with the highest estimates in the preovulatory phase, where the estrogen surge is expected (218). Similarly, exogenous administration of estrogen increases plasma volume in women (216, 217). However, some studies have reported higher plasma volume in the early follicular phase compared to the late follicular phase and/or the luteal phase (159, 160, 219).

The few studies that measured plasma volume across the menstrual cycle showed an inconsistent pattern across time. While Spaanderman et al. found a higher volume in the luteal phase vs. the follicular phase in a small group of healthy controls (an increase of 13%) (n = 10) (220), Bernstein et al. reported higher plasma volume during the early follicular phase vs. late follicular or luteal phases among healthy women planning a pregnancy (n = 21), a decrease of 1-5% (159). Other studies have reported a decrease of 4% between the onset of menstruation to cycle day 10 (n = 18) (160, 219).

The menstrual cycle and micronutrient biomarkers

Cyclic variations in estrogen and progesterone concentrations across the menstrual in women of reproductive age influence several biological processes and can impact research findings (221, 222). Several lipoproteins including HDL and apolipoproteins concentrations decreases during the follicular phase and increases during the luteal phase, whiles TAG, cholesterol and free-fatty acid as well as de novo synthesis increases during the follicular phases and decline in the luteal phase in women of reproductive age (223). Furthermore, women reproductive frequent craving for foods as well as increased consumption of food higher in fat, carbohydrate and proteins during the luteal phase compared to follicular phase (224-231). It is speculated estrogen suppresses appetite during the periovulatory phase while progesterone stimulates appetite in the luteal phase (232, 233).
A limited number of studies have examined changes in micronutrients homeostasis during the menstrual cycle. Among women of reproductive age, Deuster et al. found that plasma zinc concentrations were higher during the menses and follicular phase than at ovulation (n = 5) (234). However, Michos et al., observed the highest plasma zinc concentrations at the time of ovulation and the lowest estimates at menstruation (n = 14) (235). Changes in plasma zinc and estradiol were positively correlated (235). In the same study plasma copper concentration decline across the menstrual cycle with highest concentration at during menses. Changes in plasma concentrations of copper and estradiol were negatively correlated (n = 14) (235).

Serum magnesium concentration has been reported to decline between menses and the follicular phase, and rose between the follicular phase and luteal phase (n = 25-50) (236, 237). Similarly, Deuster et al. found a decline in plasma magnesium concentration across the menstrual cycle with highest concentration observed during menses (222). Furthermore, Deuster et al., reported that plasma magnesium concentration was highest during menses and lowest during the late follicular phase and ovulation (n = 5) (234). However, Rosenstein et al. observed no changes in three blood measures (serum, red blood cell, and mononuclear blood cell) of total magnesium across the menstrual cycle in healthy women with regular cycles (n = 16) (238).

Inconsistent results have been reported for biomarkers of iron across the menstrual cycle (111, 239, 240). The lowest serum ferritin concentration was reported during menses in some studies (239, 240), but in iron-depleted women no change were observed across the menstrual cycle in (111). Using data from the National Health and Nutrition Examination Survey (NHANES II), Kim et al. reported a significantly lower serum ferritin during menses compared with the luteal phase (n = 1712) (239). Laine et al. observed lowest concentration of transferrin saturation, serum iron and hepcidin during menses, increasing at mid-cycle before stabilizing during the luteal phase, while hemoglobin remain relatively stable across the menstrual cycle in these group of healthy women (n= 90)( 54 of them used oral contraception) (241).
Belza et al. further reported no significant change in hemoglobin, serum transferrin receptors, and body Fe stores across the menstrual cycle (n = 20) (111). Laurence and Sobel have shown that serum retinol concentration changes across the menstrual cycle in healthy women, with lowest estimates reported during menstruation, and rose to peak values at around cycle day 14 (n = 6) (242). Mumford et al. reported the lowest serum retinol concentration during menses compared to the mid-, late follicular phases, early- and midluteal phases, but not the late luteal phase. Serum retinol concentration was positively associated estradiol concentration (n = 259) (243).

Overall, it appears that serum(plasma) micronutrient concentrations fluctuate during the menstrual cycle but the exact pattern is largely unclear.

**Plasma volume and micronutrient biomarkers**

Plasma-based micronutrient biomarkers are commonly measured to evaluate a person’s nutrition status and to estimate the prevalence of micronutrient deficiencies in a population. A biomarker (biological marker) is defined as “an indicator that provides evidence on the magnitude and distribution of individual nutrient deficiencies/excesses, which has been subjected to scientific review and for which there is international consensus” (244). Examples includes serum ferritin, retinol, and zinc. Because blood samples are easy to collect, biochemical assessment of micronutrient concentrations are often measured in plasma or serum. For instance, a decline in plasma zinc concentration reflects change in total body zinc status and is a recommended marker of population zinc status (52). These elements are usually transported in circulation bound to transport proteins e.g., retinol is transported in circulation bound to serum albumin (122). Nutritional requirements and status of several micronutrients were established based on micronutrient concentrations, and cutoff values are often used to define deficiencies (57, 245-249). Commonly used micronutrient cutoffs include: serum zinc below 70 µg/dL (57, 246) defines zinc deficiency, and retinol below 20 µg/dL (0.70 µmol/L) (120) defines vitamin A deficiency, at
the population level. Similarly, serum ferritin concentration of less than 15 µg/dL in the presence of anemia (hemoglobin < 12.0 g/dL) is used to define iron deficiency anemia in women of reproductive age (250).

These micronutrient concentrations can be influenced by hemodilution arising from plasma volume expansion, a common physiological event during pregnancy. Plasma or serum zinc concentration declines by 15%–35% (251-253) during pregnancy while plasma volume increases by about 42% to 50% (168, 254, 255). The decline in zinc concentration is attributed to physiological responses to pregnancy, hemodilution, hormonal changes, increased zinc uptake by maternal tissues, and active maternal-fetal transfer of zinc (256). Maternal plasma zinc concentration returns to prepregnancy or early pregnancy values within 7-8 weeks postpartum (252).

The increase in plasma volume parallels the fall in plasma zinc and rise in copper concentrations (257). Similarly, concentrations of folate (258) and hemoglobin (259, 260) fall as the plasma volume increases. However, the circulatory mass of both zinc and copper rise as plasma volume increases during pregnancy (257). This suggests that plasma volume may be related directly to the micronutrient biomarker mass but exhibits negative or no apparent relationship with the micronutrient concentration. The impact of plasma volume on biomarker concentration were evaluated across the menstrual cycle in two studies. In both studies, plasma volume was not measured but calculated based on hemoglobin and hematocrit values (160, 219). Across these studies a reduction of ~4% was reported in plasma volume, while lipoproteins concentrations increased by about 10%.

Though plasma volume is often discussed as a potential factor influencing micronutrient concentration or mass, it is not often measured to examine its influence on the concentration of micronutrients. Understanding how plasma volume influences micronutrient concentrations could lead to improved evaluation of micronutrient status and interpretation of research findings.
Methods for measuring plasma volume

There is no true direct method for measuring the volume of plasma in the body, yet some existing techniques are thought to provide a close approximation of circulating plasma volume (261). Overall, these techniques for measuring plasma volume involve injecting a known amount of an indicator (radiolabeled or dye) into a blood vessel, allowing time for the substance to mix throughout the vascular space, and measuring the amount of the indicator in a post-injection plasma sample (153, 261). Human serum albumin, radiolabeled with iodine isotopes (\(^{125}\text{I}-\text{HSA}\) or \(^{131}\text{I}-\text{HSA}\)) is considered the best method for measuring plasma volume (262). This method provides accurate results but cannot be used in pregnancy because it labelled with radioactive iodine. It also requires specialized skills and training as well as a facility for storage. The method provides accurate results but is cumbersome and costly (263).

Colored dyes that can be measured by spectrophotometry have been commonly used in pregnancy, but also in nonpregnant patients, to avoid the safety issues and difficulty of use for the radiolabeled method. In pregnancy, Evans blue dye (T-1824) has been the most widely used for measuring plasma volume. This dye rapidly binds with albumin and other plasma proteins for distribution throughout the vascular space. Although Evans blue is considered safe and reliable for measuring plasma volume in pregnancy (264, 265) there are some concerns that the dye could be toxic or carcinogenic, as reported in one animal study (261, 266), and it is no longer sold in the US.

A promising method using indocyanine green (ICG) has been widely used for other purposes – cardiac output, liver function, and fluorescent imaging in surgery (267) – but deserves more attention as an indicator for plasma volume(268-278). Indocyanine green (ICG) is a cyanine dye with a peak spectral absorption of about 805 nm. This green dye binds to plasma proteins (279), and has a very short half-life of about 3 minutes (280). It is quickly cleared from circulation by the liver (281, 282), does not accumulate in the blood or the body if the liver is functioning well (279, 283), and appears to be non-toxic (261). Several papers, including our own work (Chapter 3) report tested and validated protocols so that ICG is now faster, less-invasive, and similarly accurate compared to using radiolabeled HRA or Evan’s
blue dye (268, 284, 285). ICG has been used safely in pregnancy (281, 282, 286-288) and does not appear to cross the placenta (281, 288, 289), therefore it should be explored for plasma volume measurement in pregnancy.

Other methods of plasma volume measurement have been used less often and have limitations. Dextran-70 has been used to measure plasma volume and if the process is properly done, results are comparable to that of the standard method (290). However, this method requires several hours to produce reliable results unlike the ICG method, which takes less than 2 hours to complete. A major drawback is that dextran-70 can actually result in plasma volume expansion (291, 292) and has been used as a plasma volume expander (293). Some case reports have been made of anaphylactic reaction even in doses as low as 0.8 ml of dextran injection (294). Complications resulting from dextran-induced anaphylactoid reactions have also been reported in pregnancy, and the use of this in pregnancy is not recommended (292).

The BVA-100 blood volume analyzer, is a semi-automatic method that utilizes the tracer dilution technique to measure plasma volume, red cell volume, and total blood volume using low-level $^{131}$I-labeled HSA tracer (radioactive Volumex®, Daxor Corp) (263). The BVA-100 has an automated well counter interfaced with a computer. The method is reported to be reliable and accurate and can provide blood volume results within an hour. The method also provides the individual participant’s normal blood volume and compares the measured volume to what is ideal blood volume for that patient based on deviation from ideal weight. This method has been approved by the FDA (263, 295). But because of the use of radioactive iodine, it is not used in pregnancy and children. Furthermore, a noninvasive method (pulse dye densitometry) using finger photosensor detection of ICG exists, making this method even easier on participants and investigators by not requiring sampling and processing of blood (261). Estimates from the noninvasive method are comparable with both $^{131}$I-HSA and the invasive ICG method (268, 284, 285), but the method has not been approved by the FDA.
Finally, there is an indirect method using an equation to calculate the percent change in plasma volume, between any two timepoints using hemoglobin and hematocrit values (296). Several other equations for calculating plasma volume have been reviewed but are yet to receive wide recognition in practice (297). Further attention should be given to developing less invasive methods that could be field-friendly to large populations including pregnancy studies.

**Following the menstrual cycle in research**

Understanding the inter- and intra-person variability in ovarian hormones across the menstrual cycle is extremely important in appropriately designing studies to prospectively predict when key hormones are dominant. The most accurate method for determining menstrual cycle phase is by the use of ultrasound (sonography) but this method is expensive, and is not feasible for use field studies (298). It involves a high degree of participant’s burden for the scan itself and the need to visit a special facility for assessments. Laboratory measurements of serum and urine samples are relatively inexpensive, reliable, and accurate for tracking ovarian hormone concentrations across the menstrual cycle.

Menstrual cycle hormones are released in a pulsatile manner (299-301) at different times in the cycle. A common practice in research is to collect urine samples timed across the cycle as often as possible to measure the hormone concentration and chart them across the cycle. This practice allows for detecting surges in estrogen, LH and progesterone concentrations. Elevation in the concentrations of estradiol and LH in serum and urine can indicate impending ovulation (191, 302-305), and progesterone concentration measured in the luteal phase can help establish whether ovulation has occurred (306, 307). Other approaches to predicting or determining ovulation include tracking changes in body temperature and cervical secretions. A less invasive and simple method for tracking changes in menstrual cycle hormone is the use of personal fertility monitors designed to track E3G and LH concentrations.
The Clearblue® Advanced Fertility Monitor (Swiss Precision Diagnostics (SPS) GmbH, Procter and Gamble, Cincinnati, OH) is an easy to use, battery-operated, hand-held, portable electronic device developed for tracking changes in urine hormone concentrations (308). The device is comprised of test sticks and the hand-held monitor (308). The device was designed to help predict more fertile days in the woman’s cycle when sexual intercourse would enhance the chance of conception (see Figure 1-1). The disposable test sticks (contains monoclonal antibodies, conjugated to blue latex particles, which detect and identify E3G and LH in urine) collect hormones from the first urine of the day and convert them into information which can be read by the monitor. Using an internal algorithm, intensity of the blue lines formed on the sticks are measured and transformed into concentrations. The monitor reads the test stick, interprets the hormone levels, and displays the fertility status of the woman as low, high or peak, for the next 24 hours (211, 308-311). The monitor tracks two key fertility hormones – LH and estrone (E3G, primary metabolite of estradiol) – to identify up to 6 fertile days in each cycle (308, 309), 1- 5 additional high fertility days prior to ovulation. Levels of E3G and LH results from fertility monitors parallel the increase in the diameter of the dominant follicle as measured with transvaginal ultrasonography (312).

The user conducts daily urine test starting on cycle day 6 up until the monitor stops requesting tests, usually, a few days after ovulation has occurred. At the early few days of testing (mid-follicular phase), the monitor establishes a baseline for the woman as “Low” fertility (indicated by one ‘bar’ on the LCD screen). A rise in E3G is detected by the device as “High” fertility, and a surge in LH is displayed as “Peak” fertility.

This presents a unique opportunity for researchers interested in examining the influence of the reproductive hormones on plasma volume and micronutrient biomarker concentrations to be adapted for data collection. Because of the high accuracy with which this device compares with sonography in predicting ovulation, it can be adapted for data collection timed to key hormone levels. The researcher can also use the information recorded by the fertility monitor to predict women who are unlikely to experience ovulatory cycle. In so doing, if non-ovulatory cycles are of no interest, the participants can be
dropped to save time and resources, and the researcher has an opportunity to enroll more participants for replacement. Because urine is more concentrated in the morning, the use of early morning urine collection is preferred. These fertility monitors can be useful in research because of the simplicity of use, and the ability to predict when the concentration of key hormones is high in the menstrual cycle (211, 313).

Low fertility = very small chance of conceiving
High fertility = increased chance of conceiving
Peak fertility = highest chance of conceiving

Figure 1-1: Clearblue fertility monitor with indicators of fertility status typical record from daily urine testing
Hydration

Hydration is defined as the balance between water input and output from the human body. Dehydration results from water loss without replacement, e.g., water loss via urine, sweat, feces, and respiratory vapor (314, 315). Body water regulation is a highly controlled process and the loss of 1% of body water is replaced within 24 hours; a variation of more than 1% can influence cognitive and physical performance (316). Intra-individual day-to-day variations range from 0.8% to 1.4% (2–4 mmol/kg) (317). Increase in solute (negative water balance) triggers a physiological response by decreasing urine output (water reabsorption by the kidney) and stimulating thirst to increase body water (158), including plasma volume and blood volume. Increased plasma osmolality would suggest a decrease in plasma volume (155, 318). Methods for measuring hydration status include body weight changes, plasma, urine, and saliva osmolality, bioelectrical impedance, urine specific gravity (USG), and urine color. Urinary markers, USG, urine osmolality, and color charts, are non-invasive, cheap, and do not require high technical expertise to perform measurements.

Osmolality reflects the number of solute particles in urine or plasma. Specifically, it is the concentration of a solution expressed in milliosmoles of solute particles per kilogram of water (315). Urine osmolality is the concentration of osmotic solutes in urine and plasma osmolality reflects plasma solute concentration (314, 315). There is no known standard reference method for assessing hydration status (317, 319-321) but for field studies, the urine specific gravity (USG) is recommended (315).

The USG method uses a refractometer to measure moderate changes in fluid volume. Concentration is measured in terms of the specific density of water. This density is compared to that of the density (rho) of pure water (water, rho = 1.000 g/cm³). The more dehydrated a participant, the higher the density, or specific gravity of the urine. A urine specific gravity of 1.000-1.019 is considered a hydrated state, 1.020-1.027 is considered a minimal to moderate dehydrated state, and 1.028-1.035 is considered severely dehydrated (322). USG can easily be used for determining hydration status in large-sample studies with a high degree of reliability (314). USG has been used even in places where there is
low availability of resources, like the Amazon (319). Falsely elevated USG and urine osmolality values can result from the presence of large molecules such as glucose and protein in the urine (314).

One issue to consider is whether to use an early morning urine sample or a 24-hour urine sample to measure USG or urine osmolality. Early morning urine sample is convenient and less demanding of the participant’s time but is also more concentrated than the 24-hour urine sample (323). For this reason, some authors prefer to use urine samples collected after the first morning urine has been voided – a good balance between convenience and urine that is not too concentrated (324). Urine osmolality and USG are strongly correlated in both early morning urine and 24-hour samples (323). Furthermore, urine samples collected from athletes prior to training showed a strong positive correlation between osmolality values and USG (325).

Plasma osmolality fluctuates day-to-day and by phase of the menstrual cycle (326), but fluctuations are minimal and are tightly regulated within a range of 1 to 2% (314, 316). A suggested normal range for euhydration is serum osmolality of 293 to 294 mOsm/kg and urine osmolality of 549 to 705 mOsm/kg. The American College and Sport Medicine cutoff values for dehydration as measured by urine osmolality are ≥700 mOsmol/kg H₂O and USG ≥1.020 (327). Plasma osmolality is often suggested as the best method for examining dehydration but this is not universally agreed upon (321). It indicates intracellular fluid change and is therefore a good maker of acute change in hydration status (314).

**Summary of research gaps**

Although much is known about plasma volume expansion during pregnancy, little is known about how much plasma volume changes across the menstrual cycle. Further, there is no clear understanding as to whether plasma volume is associated with the menstrual phase or ovarian hormone concentrations across the menstrual cycle. Previous studies evaluating plasma volume fluctuation across the menstrual cycle were based on a comparatively small sample size. In addition, evidence on micronutrient
biomarkers and menstrual cycle phase is scarce. Though limited studies suggest that increases in plasma volume are associated with hemodilution, studies on how micronutrient biomarkers may change across the menstrual cycle are needed, especially for public health efforts to quantify the burden of malnutrition. Finally, the association between plasma volume and micronutrient biomarker concentrations has not been evaluated in nonpregnant women. The overall conceptual framework is represented in Figure 1-2.

Aims and objectives

Aims

The overall goal of this study was to examine the influence of the menstrual cycle on micronutrient concentrations and plasma volume in healthy women of reproductive age. Secondly, we were interested in examining the relationship between plasma volume and micronutrient concentration, an area that has received little attention. By studying the variation of micronutrient biomarker concentrations across the menstrual cycle within the context of plasma volume, we have the potential to expand our understanding of how cyclic variation affects micronutrient concentrations, in addition to informing public health efforts and research aimed at improving preconception nutritional status in women of reproductive age.

Specific objectives

AIM 1: To quantify changes in plasma volume across the menstrual cycle.

Hypothesis:

1. We hypothesized that plasma volume would decrease between the early and late follicular phase and have a slight rise at the midluteal phase.
AIM 2: To quantify changes in micronutrient biomarker concentrations (serum zinc, copper, magnesium, manganese, iron, ferritin and retinol) across the menstrual cycle.

Hypotheses:

1. We hypothesized that zinc concentration would increase across the menstrual cycle
2. We hypothesized that copper concentration would decrease across the menstrual cycle
3. We hypothesized that magnesium concentration would decrease across the menstrual cycle
4. We hypothesized that manganese concentration would decrease across the menstrual cycle.
5. We hypothesized that serum iron and ferritin concentrations would increase across the menstrual cycle
6. We hypothesized that retinol concentration would increase across the menstrual cycle.

AIM 3: To evaluate the relationship between plasma volume and micronutrient biomarker concentrations in healthy women of reproductive age.

Hypothesis:

1. We hypothesized a negative association between plasma volume and micronutrient concentrations.
Conceptual framework

Cyclic phase
- Follicular phase: day 2-5
- Ovulatory: day 10-14
- Luteal phase: day 20-24

Micronutrient concentrations
- Zinc
- Copper
- Magnesium
- Manganese
- Iron/Ferritin
- Retinol (vitamin A)

Figure 1-2: The interplay between the menstrual cycle, plasma volume and micronutrient concentrations in healthy women of reproductive age.
Chapter 2

Research Design and Methods

Research Design

This was a longitudinal prospective study wherein subjects were examined at three time points within a single menstrual cycle: the early follicular phase (~day 2), the late follicular phase (~day 12), and the luteal phase (~day 21) — based on an approximate 28-day cycle length. The actual days of visits were adjusted for the length of the woman’s menstrual cycle and based on the results from the fertility monitor. Nonpregnant women aged 18 to 44 years were recruited as a convenience sample of eligible women in the State College area and surrounding towns within Centre County. Women responded to the recruitment material posted on several platforms and were pre-screened (they also completed a screening consent form) to determine eligibility. Those eligible (Appendix A) were assigned a screening ID and invited for a full screening and enrollment at the Clinical Research Center (CRC), a center run by Penn State University's Clinical and Translational Science Institute. The CRC is a research facility with on-site nurses and doctors and a wide-range of equipment and resources for clinical research.

Women provided the expected date of the beginning of their next menstrual period, and were tentatively scheduled for the study visit (for in-person screening and enrollment) at the CRC approximately 1 day after this date (avoiding weekend days). Several days before the scheduled visit, a study staff contacted the woman to remind her of the scheduled visit and asked her to notify the research office when her menses began. Visit days were adjusted based on the actual start of menses. All enrollment visits occurred within cycle day 1-5, and when this was not possible, participants were rescheduled for their next cycle.

Before each study visit, the woman was asked to fast for 12 hours (nothing but water to drink) the night before her visit. She was also asked to avoid alcohol intake for 12 hours preceding the scheduled visits to the CRC, and to drink plenty of water. On the morning of the visit, the woman arrived at the
CRC and went through complete screening (including height, weight, blood pressure, and pregnancy test) (Table 2-1). Pulse was measured (together with blood pressure) but was not used for assessing eligibility. If eligible for the main study, the woman then went through the consent process for the study and completed study measurements (including a questionnaire and body composition measurement) (Figure 2-1).

The blood draws were done by a CRC nurse. An IV was inserted into the vein of one arm and an initial baseline blood draw taken (~14 mL) on the same arm into trace element-free vacutainer tubes (BD, Becton Dickinson, Franklin Lakes, NJ). Next, 25 mg of indocyanine green lyophilized powder was dissolved in 10 mL of sterile water to make ICG solution. The volume of the ICG drawn for injection was based on the subject’s weight (0.25 mg/kg body weight). The syringe was weighed to measure the exact amount of dye injected using a high precision balance. Injection was followed by a saline flush (10 mL) to clear all the dye into the IV system into the circulating system. A timer was started at injection, and starting at ~ 2 minutes, five 3 mL blood samples were drawn at intervals of 45 seconds over the next 3 minutes (5 EDTA-treated tubes in total).

Blood samples for whole complete blood count assays were not centrifuged but sent immediately for analysis. EDTA-treated tubes were centrifuged at 3200 rpm (1500 x g) for 15 minutes and the plasma (supernatant) aliquoted into cryovials. Plasma samples were analyzed immediately for plasma volume and the remaining plasma was frozen at -80°C until analysis. EDTA-free tubes were allowed to clot and centrifuged as above, and the supernatant (serum) was aliquoted into microcentrifuge tubes. Serum samples were frozen in -80°C, until analysis.

Participants were debriefed at the end of the first visit about the use of the fertility monitor and the terms related to fertility; high and peak fertility were explained to allay potential fears of a woman before she was instructed on how to use this fertility monitor at home. They were instructed on how to conduct and record the daily urine testing with the fertility monitor.
A research staff maintained contact with each woman on a regular basis (1-5 times via email and/or phone) to ensure that she was using the monitor correctly. Women conducted a daily urine test with the monitor, which uses urine hormone concentrations to track the stage of the menstrual cycle for the participants by displaying on the monitor LCD, low, high, or peak fertility. “Low fertility” corresponds to low estrogen and LH; “High fertility” corresponds to high estrogen; and “peak fertility” corresponds to LH surge. Women were provided a daily check sheet to record fertility status. The participant immediately notified the study staff via email or phone when the monitor read “high fertility” and she was immediately scheduled to make her second study visit at the CRC as soon as possible within the next few days, the first day when it was feasible. The woman continued to use the fertility monitor at home until “peak fertility” was reached and passed. The third visit was scheduled approximately 9 days from the peak fertility (LH surge). This last visit scheduled to coincide with the predicted progesterone peak and the second estrogen peak for the menstrual cycle. Concentrations of serum estradiol and progesterone were measured at the end of the study from serum samples (collected prior to ICG injection).

Women were dropped if they did not reach high fertility because it was determined that they would not ovulate for that cycle. If a woman reached high fertility but not peak fertility, we used the high fertility information together with her cycle length to schedule the third visit. At the third visit, the participant returned the monitor to the CRC with the completed Daily Home Testing sheet. A trained staff retrieved the fertility results stored in the monitor and compared them to those recorded on the checking sheet to confirm the records (when there was any discrepancy, we relied on the results from the fertility monitor).

Each woman received $90 cash for completing the study; $30 per completed visit ($5 if she only completed part of the study). At the end of the third visit her study participation was completed but we kept in touch with her to record the first date of her next cycle to enable us calculate the length of her current cycle.
Figure 2-1: Summary of the flow process of screening and data collection process at each visit.
Statistical power and sample size estimation

The sample size for this study was estimated based on the results of our pilot study, and the findings from a previous study among 14 healthy reproductive age women with a regular menstrual cycle by Michos et al. (235). In that study, which reported variations in plasma copper and zinc concentrations at different time points in the menstrual cycle, there was an 11-13% change in zinc concentration between the follicular and luteal phases and an 8-10% change in copper concentration between phases. In the proposed study, we aim to be able to detect a 10% change in both copper and zinc concentrations between any two points in the menstrual cycle. The mean (SD) serum concentrations for copper and zinc in the follicular phase were 107.1 (16.2) µg/dL and 75.6 (12.4) µg/dL, respectively, from our pilot study (n = 8). Using the copper and zinc concentration from our pilot study, a 10% change in copper and zinc concentration would result in an effect size of 0.66 and 0.61 respectively. We wanted to have statistical power of 80% and a type I error of 5% in a two-sided test, to be able to detect a difference between any two of three time points. Because our initial plan was to test simultaneously for four micronutrient concentrations as our response variable, we applied a Bonferroni correction for a family-wide error rate to give a type I error rate of 1% (i.e., 0.05/5 = 0.01). From this data, we estimated a sample size of 31 for copper and 35 for zinc (using the Power Analysis package in R) to determine the minimum sample needed was 35 women with complete data. This sample size was determined to be adequate for detecting differences in concentrations between any two phases of the menstrual cycle for other micronutrients biomarkers under concentration.

Based on a study by Bernstein et al. (n = 21) (159), we estimated that with a minimum sample of size of 28 subjects, we could detect a significant difference in plasma volume between any two timepoints of the menstrual cycle with a statistical power of 80% and type I error of 5% in a two-sided t test. Thus, a sample size of 35 was estimated to be sufficient to detect differences in zinc and copper concentrations and also plasma volume. Applying a 29% expected dropout rate (loss to follow) yielded a sample size of 45.2, which rounds up to 46 subjects needed for enrollment. We also anticipated that some women may
not ovulate with the cycle under investigation. In the BioCycle Study among women with a mean age of 27.5 years and BMI of 24.1 kg/m², anovulatory cycle incidence varied widely from 3.4% to 18.6% (328, 329). If anovulation occurred, participants did not have complete data and were not scheduled for visits 2 or 3. Thus, we accounted for an additional 10% of women that may not ovulate, and added 5 additional enrolled women for a total of 51. Thus, we aimed to enroll 51 women to reach our desired sample size.

**Inclusion and exclusion criteria**

**Inclusion criteria**

- Female
- 18 to 44 years of age
- General good health (does not have a known, ongoing health condition/medical issue that requires regular monitoring by a doctor or regular visits to the hospital)
- BMI 18.5-24.9 kg/m²
- Regular menstrual cycle (26-35 days)
- Non-smoker
- Nonpregnant
- If pregnant before, ≥12 months since last pregnancy
Exclusion criteria

- Know allergy to shellfish or iodine
- Blood pressure on the day of measurements is low or high (SBP < 90 or ≥ 130 mmHg and/or DBP < 60 or ≥ 80 mmHg)
- Currently has low or high blood pressure (SBP < 90 or ≥ 130 mmHg and/or DBP < 60 or ≥ 80 mmHg), self-reported
- Current hypertension or previous hypertensive disorder in pregnancy (gestational hypertension or preeclampsia)
- Taking regular medication(s) (physician’s prescribed medications for a health condition)
- Currently trying to conceive
- Currently breastfeeding
- Currently using hormonal birth control or used within last 3 months
- Used depot medroxyprogesterone acetate (DMPA) in the past 12 months
- Diagnosis of polycystic ovary syndrome
- Women taking vitamins or mineral supplements will not be excluded. These criteria were chosen to minimize the risk of confounding variables that could influence plasma volume and/or to reduce potential risks to participants.

Early withdrawal

A subject was removed from the study if she experienced any form of following;

- did not reach “high fertility” (visits 2 and 3 canceled)
- tested positive for pregnancy
- had high or low blood pressure (at the beginning of a visit; see exclusion criteria)
• gained or lost 10% of body weight since the last visit

**Pregnancy test**

The Participants provided fresh urine samples for early pregnancy test (The QuickVue One-Step hCG Combo test, Quidel corporation, San Diego, CA). This test kit is used for qualitative determination of human chorionic gonadotropin (hCG) in urine, to detect early pregnancy. Pregnancy tests were conducted at each visit.

**Blood pressure**

The blood pressure measurement was adapted from Whelton *et al.* (330). Blood pressure was measured after the woman had emptied her bladder and rested for at least 5 minutes. The woman sat upright with her feet flat on the floor (a stool was used where necessary) in a comfortable chair before and during measurements. During the rest and measurement no activity was allowed (including no cell phone, no talking etc.). Measurements were always taken on the left arm. The measurements were repeated at least 1 minute after the cuff has completely deflated. Both the first and second systolic blood pressure, diastolic blood pressure, and pulse measurements were recorded on the form. At the end of the visit, the blood pressure measurements were repeated as before. Blood pressure was measured with Omron Blood Pressure Monitor (OMRON HEM-712C Blood Pressure Measuring Machine, Omron Healthcare, Inc., Bannockburn, Il, USA) with cuff size adjusted for each participant based on the size of their mid-upper arm circumference.
Anthropometry

Weight measurement

Trained research assistants weighed the subject using a digital scale (Scale–Tronix, Inc., White Plains, NY). The weighing scale was placed on a flat surface, in a fixed location at the CRC for measuring weight for study participants. Participants wore minimal, light clothing during measurements. They removed their shoes and any heavy clothing or objects, such as jewelry, large ornaments, or any bags or filled pockets. The participant always stood on the scale in the center of the platform, with her hands at her sides while looking straight ahead towards the research staff taking the measurements. The participant was instructed not hold anything or anyone else to support herself. The subject was weighed to the nearest 0.1 kg (100 g) at each visit and recorded on the form.

Height measurement

We used a self-standing stadiometer (Seca 213 Portable Stadiometer Height-Rod, seca Deutschland, Medical Measuring Systems and Scales, seca gmbh & co. kg, Hamburg, Germany) for height measurements. Participants were instructed to remove any hair ornaments, jewelry, buns, or braid from the top of the head before each measurement started.

The woman was instructed to stand on the base with her back straight against the backboard with the body weight evenly distributed and both feet flat on the base, with her heels together and toes apart. The research staff checked to ensure that the back of the head, shoulder blades, buttocks, and heels made contact with the backboard, and the head was aligned so that the Frankfort plane was horizontal. She was instructed to look straight ahead, then the research staff lowered the stadiometer headpiece so that it rested firmly on top of the woman’s head, with sufficient pressure to compress the hair. The participant
was then instructed to stand as tall as possible and take a deep breath, and this position was held by the headpiece and recorded to the nearest 0.1 cm.

**Questionnaire**

The questionnaire, form 2, was administered verbally by trained study staff. The questions included basic socio-demographic information (e.g. age, marital status, education, income, and employment) and were self-reported by the participants. Information on health status (e.g. diabetes, hypertension) and previous pregnancies were also asked (Appendix B).

**Measurement of urine specific gravity**

Hydration status was assessed with a digital hand-held urine specific gravity "Pen" refractometer (Atago PEN Urine S.G. Refractometer; ATAGO U.S.A., Inc.) to the nearest 0.0001 unit. The USG pen was standardized each morning before the start of the study using distilled water. The standardized value read “1.0000” each testing day before it was used for conducting tests (Figure 2-2). The woman provided fresh urine samples for pregnancy test and part of this was used for the hydration test as well. Urine samples were covered and allowed to cool (for at least 20 minutes) before testing. The refractometer was held at the top in a vertical position with the “pen” pointing downward, and lowered into the urine specimen. The tip of the pen is tipped into the into the sample, and the “START” key is pressed, and the results are displayed within 2 seconds. The refractometer is cleaned with distilled water, wiped with tissue paper and a second reading is taken. The results are recorded on the form (appendix 4). This is followed by preparation for blood collection.
Plasma volume was measured within 2 hours of blood collection in Dr. Alison D. Gernand’s laboratory. Dye dilution principle was used to measure plasma after measuring concentration of Plasma ICG collected over 5 minutes post-dye injection. We used a previously developed method from our laboratory (331). In brief, a standard curve was constructed for each participant using her plasma samples collected before (1 sample) and after and part of the remaining ICG solution that was injected. Absorbance of plasma samples (pre-injection and post-injection) were read on Epoch plate reader (BioTek Instruments, Inc., Winooski, VT, USA) powered by Gen5™ Software, set to a wavelength of 805 nm. Concentration of the ICG in plasma collected after ICG injection were calculated from the standard curved. The plasma ICG concentrations were natural log transformed and plotted against the time they were collected, and extrapolated to time t = 0 to obtain the ICG concentration at time of complete mixing. The mean within-person coefficient of variation for a single study was 2.2%. Plasma volume measurements were completed within two hours of blood collection.

Serum samples were frozen at -80°C immediately after they were aliquoted into microcentrifuge tubes. At the end of the study, all samples were transferred (on dry ice) to the Women's Health and
Exercise Laboratory (Penn State University, University Park, PA, USA) directed by Dr. Mary Jane De Souza. Serum estradiol and progesterone were measured for each sample. Samples from each subject were assayed together within a single run to limit analytic variability. Serum samples were run for estradiol and progesterone using ELISA kits produced by Siemens for the Immulite, SAP #10702832 and SAP #10381128, respectively (Siemens Medical Solutions Diagnostics, Norwood, MA, USA) on the Diagnostics Product Corporation Immulite 2000 Analyzer (Siemens Medical Solutions Diagnostics, Deerfield, IL, USA). The Inter- and intra-assay coefficients of variation (CV) for estradiol were 16.0% and 15.0%, and for progesterone, 13.2% and 12.5%.

Three markers of hydration were measured. USG was measured from fresh urine samples at each study visit using the USG pen. Remaining urine samples were frozen at -20°C until the participant has completed the study or drop from continuing. Similarly, plasma samples were stored at -80°C and until data from all visit for the participants were completed. Both urine and plasma samples were transferred to Dr. Asher Rosinger’s Laboratory (Department of Biobehavioral Health). Samples were assayed by freezing point-depression osmometry with the use of the K-7400S Semi-Micro Osmometer (KNAUER, Wissenschaftliche Geräte GmbH, Berlin, Germany). The coefficient of variation across assays was < 1%.

Whole blood samples were assayed within one hour of blood collection in Dr. Laura Murray-Kolb’s laboratory. Samples were run together with 3-level quality-control material (Beckman Coulter, Inc. Brea, CA) on Beckman Coulter Ac-T Diff 2 hematology analyzer (Beckman Coulter Inc, Brea, CA). Serum ferritin was also assayed in the Murray-Kolb laboratory using enzyme-linked immunosorbent assay (ELISA) methods (Ramco Laboratories, Inc., Strafford, Texas). ELISA methods were used to assay acute phase proteins: C-reactive protein (CRP; Kent Laboratories Inc., Bellingham, Washington) and serum α1-acid glycoprotein (AGP; Kent Laboratories Inc., Bellingham, Washington) in the Gernand laboratory.

Serum concentration of zinc (Zn), copper (Cu), magnesium (Mg), manganese (Mn) and iron (Fe) were measured at the Laboratory for Isotopes and Metals in the Environment (LIME) at The Pennsylvania
State University. Analysis were performed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) with Collision Cell Technology (CCT) (ICP-MS, Thermo Fisher Scientific X Series 2). The method used was adapted from previous studies (332-334). Serum samples for each sample were aliquoted into 15 trace element-free tubes in duplicates and diluted to 25 folds, 200 µL of serum to 4.8 mL of 0.1N double distilled NHO₃ (HNO₃, trace metal grade, EMD Millipore Corporation, Darmstadt, Germany). Samples were vortexed at high speed for 15 seconds stored at 24 hours and analyzed. Samples were weighed using high precision scales to calculate actual dilution factors. Quality control check were performed using seronorm reference materials (Seronorm™ trace element serum L-1 and L-2 RUO, SERO AS, Billingstad Norway). NIST traceable standard were used to prepare calibration standards for each element.

To monitor stability of and repeatability of analysis, external standards traceable to NIST (National Institutes of Standards and Technology, NIST 1640a) were run for every 10 samples. The intra- and inter-assay coefficients of variations were 2.0% and 2.6%, 3.7% and 4.0%, 3.4% and 7.0%, 5.1 and 4.9% and 2.3 and 3.1% for Zn, Cu, Mg, Mn, and Fe, respectively. Serum retinol was measured in Dr. Catharine Ross’ laboratory using ultra-performance liquid chromatography (ACQUITY UPLC System, Waters Corporation, Milford, MA). Quality checks for retinol were performed by measuring retinol in NIST standard (SRM® 1950, Gaithersburg, Maryland) along with samples.

As part of general quality control, all glassware was acid washed (3% nitric acid and 0.3% HCL) overnight, rinsed with double distilled water, and air dried before use. High purity deionized water (18 MΩ cm⁻¹) was obtained from a milli-Q water purification system (Millipore, Bedford, MA) and used in all analytic processes.
Ethics

The protocol was approved by The Office for Research Protections (ORP) at The Pennsylvania State University (STUDY00008383) and conducted in line with the Declaration of Helsinki. All participants provided written informed consent before enrolling into the study.

We conducted a single visit pilot study at Penn State to develop and test an ICG method for measuring PV in nonpregnant women of reproductive age (Chapter 3). Currently, PV measurement is not one of the Food and Drug Administration (FDA) approved uses of ICG but the method uses ICG in the same manner as the approved uses, measuring cardiac output, liver function and hepatic blood flow. As a result, our work was granted an investigational new drug (IND) exemption from the FDA (the pilot study and the longitudinal study). ICG has been used safely in pregnancy, and does not appear to cross the placenta (281, 286) but is not currently approved for use in pregnant women in the US. Thus, our protocol in women of reproductive age includes a pregnancy test the same day of the measurement (we additionally screened out women who were trying to get pregnant). The trial is registered at www.clinicaltrials.gov (NCT03422809).
Table 2-1: Study schedules and activities across visits.

<table>
<thead>
<tr>
<th>Study phase</th>
<th>Screening (on phone and in-person)</th>
<th>Enrollment (early follicular phase)</th>
<th>Follow-up visit (late follicular phase)</th>
<th>Follow-up visit (Midluteal phase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visit number</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Menstrual cycle days</td>
<td>Before enrollment</td>
<td>Day 2-5</td>
<td>Day ~12</td>
<td>Day ~21</td>
</tr>
<tr>
<td>Screening consent</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Main study consent</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Review inclusion/exclusion criteria</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Demographics</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medical history</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnancy history</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Weight*</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Height*</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnancy test</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Blood pressure*</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Hydration</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Body fat assessment</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Blood collection</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>ICG injection</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Fertility monitor use</td>
<td></td>
<td>from cycle day 6 until a few days after visit 2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*self-reported in phone screening
Chapter 3

An efficient method for measuring plasma volume using indocyanine green dye

*Authors: Sixtus Agray, MS, Alison D. Gernand, PhD, MPH

*Affiliations: Department of Nutritional Sciences, The Pennsylvania State University, 110 Chandlee Laboratory, University Park, PA, 16802

*Contact email: adg14@psu.edu

Abstract

Plasma volume (PV) can be an important marker of health status and may affect the interpretation of plasma biomarkers, but is rarely measured due to the complexity and time required. Indocyanine green (ICG) is a water-soluble tricarbocyanine dye with a circulatory half-life of 2-3 minutes, allowing for quick clearance and repeated used. It is used extensively in medical diagnostics tests including ophthalmologic imaging, liver function, and cardiac output, particularly in critical care. ICG has been validated for measuring PV in humans, however previous work has provided minimal published details or has focused on a single aspect of the method. We aimed to develop a detailed, optimal protocol for the use of ICG to measure PV in women of reproductive age. We combined best practices from other studies and optimized the protocol for efficiency.

- This method reduces the time from blood collection to PV determination to ~2 hours and the amount of plasma required to estimate PV to 2.5 mL (1.5 mL before ICG injection and 1.0 mL post-injection).
- Participant inconvenience is reduced by inserting an intravenous (IV) catheter in only one arm, not both arms.
Five post-injection plasma samples (2-5 minutes after ICG bolus) are enough to accurately develop the decay curve for plasma ICG concentration and estimate PV by extrapolation.

Introduction

Plasma is the water component of blood in which nutrients, hormones, and other biomarkers circulate. Plasma volume (PV) is the total amount of plasma in blood. It is an important biomarker in pregnancy, in chronic heart failure patients, as well as in situations where blood transfusion is critical. PV changes to a small extent from temperature changes and exercise (149, 150, 152), and some evidence has shown changes in PV at different points in the menstrual cycle (159, 160). In pregnancy, PV increases on average by 50% from nonpregnant values, causing hemodilution (168). Abnormal PV expansion has been associated with adverse pregnancy outcomes (335-338). Because proteins and biomarkers of health are transported in the plasma, the amount of PV could impact how biomarker concentrations are interpreted.

Despite the substantial value of PV assessment, it is not routinely measured in clinical settings or during pregnancy, partly because the method is cumbersome and time consuming. PV could be a useful diagnostic tool if it could be safely and easily measured. The method recommended by the International Committee for Standardization in Hematology (ICSH) for measuring PV uses radioiodine-labeled human
serum albumin ($^{125}\text{I–HSA}$) (262). $^{131}\text{I–HSA}$ has also been used for PV measurement (295). It is not ethical to use radioactive tracers in some populations including children and pregnant women. As well, it is challenging and expensive to perform measurements with such tracers. Other methods using dyes that bind to albumin, and therefore distribute throughout the vascular space with albumin, have been developed. The two main dyes used are Evans blue (339) and indocyanine green (ICG) (284), each of which have been validated against $^{125}\text{I–HSA}$ in humans. Evans blue dye is no longer available for purchase in the US (340). ICG is currently produced and sold and is advantageous because it is rapidly cleared from circulation. ICG also has a short circulatory half-life of 2.5-3 minutes (280, 341). These properties allow for quick assessment and repeated measurements of plasma ICG concentration and PV within a day (342), even as early as 30 minutes following the first injection (280, 343). In clinical settings, ICG has been used extensively in ophthalmologic imaging to examine the eye structure (344-346).

Most PV measurements specify that the dye (tracer) be injected in one arm and blood collected on the contralateral arm to avoid possible contamination of the tracer with post-injection blood (280). This has been followed by others (347). The inconvenience and challenge of inserting an intravenous (IV) catheter line in both arms can be overcome by replacing all or parts of the blood collection system following the dye injection, before post dye-injection blood is collected (348). The objective of this study was to develop an efficient and less-invasive ICG method for measuring PV in women of reproductive age by combining best practices from the literature and further reducing 1) the number of blood samples needed and 2) the amount of plasma needed per subject.
Methods

Study design

We conducted a single visit study at Penn State to develop and test an ICG method for measuring PV in nonpregnant women of reproductive age. Currently, PV measurement is not one of the Food and Drug Administration (FDA) approved uses of ICG but the method uses ICG in the same manner as FDA approved uses such as cardiac output, liver function and hepatic blood flow. As a result, our work was granted an investigational new drug (IND) exemption from the FDA. ICG has been used safely in pregnancy, and does not appear to cross the placenta (281, 286) but is not currently approved for use in pregnant women in the US. Thus, our protocol in women of reproductive age includes a pregnancy test the same day of the measurement (we additionally screened out women who were trying to get pregnant).

Visits were conducted in the Clinical Research Center (CRC), a service unit in The Pennsylvania State University's Clinical and Translational Science Institute, University Park, PA. Participants were healthy women of reproductive age that were nonpregnant, non-breastfeeding, and not using hormonal birth control methods. Eligibility also included normal blood pressure (systolic blood pressure 90 to < 140 mmHg and/or diastolic blood pressure 60 to < 90 mmHg) because we wanted healthy subjects, and because there are previous reports of blood pressure dropping after ICG use (349, 350). The study visit was scheduled to occur within the early follicular phase of the menstrual cycle, aiming for cycle day 2 (because previous studies have found variation in PV across the menstrual cycle) (159, 160). Two days before the study visit, participants were asked to drink plenty of water and to abstain from any form of alcohol because we wanted each person to be well hydrated at the time of measurement. All participants fasted for 12 hours (overnight) before the study visit, which took place in the morning between 7 am and 10 am. This component of the protocol was needed to standardize methods for biomarker measurement (not described here), but it also served to standardize the timing of PV measurement.
At the visit, women were asked a series of questions about their health history and lifestyle. Blood pressure, height, and weight were measured using standard methods. Weight was needed to calculate the amount of ICG solution to inject. Participants provided a fresh urine sample for a human chorionic gonadotropin (hCG)-based pregnancy test. We measured body fat percentage using the Tanita InnerScan Body Composition Monitor.

After these measurements, the specific protocol for ICG measurement of PV began.

**Blood collection and processing**

Women rested for 15 minutes in a supine position in a hospital-style room with a small heating pad over the inside of the arm selected for IV insertion (either arm was used, based on participant preference). At the end of 15 minutes, a temporary tourniquet was applied to aid in identifying an antecubital vein and an IV (BD Insyte Autoguard 18G and 20G, size of gauge varied depending on size of the participant, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was inserted by a nurse. To standardize the point of entry, we only used the antecubital vein and did not consider other locations (e.g., hand or wrist) if the IV was unsuccessful in the arm. After IV insertion, blood was drawn into a 6 mL vacutainer blood collection tube coated with K\textsubscript{2} ethylenediaminetetraacetic acid (K\textsubscript{2} EDTA). Of note, a 4 mL tube would be sufficient for measuring PV, but we took extra plasma to aid in method development. We also collected blood into tubes for serum and whole blood at this time for measurement of other biomarkers. After ICG injection, 5 more EDTA tubes were collected as described below. We used 3 mL tubes for method development, but 2 mL would be more than enough for our final method. EDTA tubes were gently inverted 10 times immediately after the tube was filled. Tubes were centrifuged (within 10 minutes of collection) at 3,200 rpm for 15 minutes (Quest Diagnostics, Horizon model 642E centrifuge) to separate plasma from blood cells. The plasma samples were transported to the laboratory to determine the ICG concentrations. The PV was calculated from the laboratory-measured ICG values. PV determination was completed within 2 hours of blood collection.
Indocyanine green injection post-injection and blood collection

ICG doses up to 5.0 mg/kg body weight have been reported to be safe in humans (351). This amount has been used in pregnant women without any adverse effects (281). Haneda et al. used between 5.0 to 10.0 mg in children and 10.0 to 15.0 mg ICG in adults (339). Other researchers have used 25.0 mg to study ICG clearance by the liver (352). The most commonly used doses for injection in the determination of PV and ICG plasma disappearance rate studies are 0.25 mg/kg body weight (284, 347, 353, 354) or 0.50 mg/kg body weight (352, 355-359). Plasma disappearance rates of ICG using 0.25 mg/kg body weight are comparable to 0.50 mg/kg body weight (360). In this study, we chose the lower dose of 0.25 mg/kg because it is expected that lower doses will clear faster from the body than higher ones, and it will use less ICG overall.

For this study, we used an ICG kit that contained a 10 mL ampule of sterile water and 25 mg of ICG powder in a vial (IC-Green®, AKORN Inc, Lake Forest, IL, USA). The study nurse added the water to the ICG vial to create a concentration of 2.5 mg/mL, immediately before injection. The solution must be used within 6 hours of mixing, so we waited until the IV was successfully placed for each participant before mixing the ICG and water. A 10 mL syringe (Luer-LokTM) was rinsed with the ICG solution (~1 mL) and the calculated volume (0.25 mg/kg x body weight (kg) ÷ 2.5 mg/mL) was drawn for injection. The 10 mL syringe was weighed with the full content for injection, then reweighed after the injection to determine the exact weight of the ICG injected using a high-precision scale. The weight of the ICG injection was used later for PV determination (see below).

A bolus dose of ICG (0.25 mg/kg body weight) was injected evenly over 5 seconds into the antecubital vein through an IV line with a 3-way stopcock (Baxter Healthcare Corporation, Deerfield, IL, USA) attached, and flushed with 10 mL saline (McKesson Medical-Surgical, Inc., Richmond, VA, USA). The 3-way stopcock (with rotating male luer lock) was replaced after the dye injection to prevent contamination of ICG and post-injection blood to be collected (see Figure 3-1 and Figure 3-2). A timer was started at the beginning of ICG injection (and used to count out loud 5 seconds as the nurse
performed the injection). Starting at 2 minutes, blood samples were collected into 3 mL EDTA vacutainer blood collection tubes every 45 seconds, up to 5 minutes (total of 5 draws at exactly (min:sec) 2:00; 2:45; 3:30; 4:15; and 5:00). The time in seconds was recorded at each draw. The method is still successful if the draw is not evenly spaced at each interval (but it is very important to know the exact time of each draw). Blood was drawn into 2 mL syringes (connected to the 3-way stopcock) before each draw and pushed back immediately after the draw to keep the IV line clear (Suppl. 1 video). The blood collection tubes and syringes were purchased from BD (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Blood samples were processed the same way as the 6 mL EDTA tubes described above, and were used for PV determination described below.
Figure 3-1: ICG preparation and injection: A, ICG powder and sterile water; B, syringes and alcohol pad for disinfecting; C, transferring the sterile water into the ICG powder vial to prepare the ICG solution; D, mixing the ICG powder and sterile water to ensure that all the powder is dissolved; E, pipetting the required volume of ICG solution for injection; F, ICG solution ready for injection; G, injecting ICG solution into the IV line established; H, 3-way stopcock replacement system for blood collection; I, Remaining ICG solution in vial and the aliquoted ICG solution (in cryovial) for lab work.
Figure 3-2: Steps for 3-way stopcock connections during the ICG injection and blood collection process
This is an overview of the full process:

**Participant visit**

Collect urine sample for pregnancy test; weigh participant; take blood pressure.

Have participant rest in a supine position for 15 minutes, with a small heating pad over the antecubital vein (for temperature control and to aid IV insertion).

Trained phlebotomist inserts an intravenous (IV) catheter with a 3-way stopcock (Baxter Healthcare Corporation, Deerfield, IL, USA).

Participant rests supine for 5 minutes. [During this time, ICG powder and water are mixed, and appropriate volume per participant’s weight (at 0.25 mg/kg) is drawn into a syringe. Keep remaining ICG mixture for lab work.]

Collect pre-injection blood sample(s) – minimum 4 mL blood in EDTA vacutainer tube needed for plasma. Collect additional blood here if other biomarkers will be measured.

Start timer and inject ICG through IV in a bolus dose over 5 seconds.

Flush with 10 mL saline solution.

Remove and replace 3-way valve system (3-way stopcock).

Attach a 2 mL syringe to the 3-way stopcock. Before each blood draw blood into the syringe and replace this after each tube is draw.

At exactly 2 minutes from start of ICG-injection, draw 2 mL blood using EDTA vacutainer tube; continue with 4 more blood draws every 45 seconds (will end at exactly 5 minutes post injection). Use a larger blood tube here (e.g., 3 mL) if other biomarkers will be measured.

Remove IV and let participant get up when comfortable; take blood pressure. [Participant involvement is complete]

**Laboratory procedure**

Process blood tubes per standard centrifugation methods; aliquot plasma.

Set up standard curve and 96-well plate per details below.

Measure ICG wavelength (805 nm) in a standard plate reader.
Plot standard curve and the decay curve for plasma ICG concentration.

Extrapolate the decay curve for plasma ICG concentration to estimate plasma volume.

**PV determination**

PV was measured by applying the indicator-dilution principle. Plasma obtained from the participant before injection (“blank”) and the five plasma samples obtained after the injection of ICG were used to determine PV for each subject. Calibration curves (285) were prepared by diluting the initial concentration of ICG (2.5 mg/mL) with MilliQ water (EMD Millipore Corporation, Billerica, MA, USA) to concentration of 5 mg/L to 30 mg/L (Table 3-1). A solution with 200 µL of ICG and 200 µL of the participant’s blank plasma were mixed together to obtain final standard concentrations of 2.5 mg/L to 15 mg/L (Table 3-2). We chose this range to so that absorbance readings from different subjects and clearance could be captured. The linear relationship between absorbance and concentration of ICG solution in plasma follows the Beer-Lambert’s law up 15 mg/L (361). All samples including standard solutions, blank samples and post-injection plasma samples were vortexed at high speed for 10 seconds for even mixing of solutes, and 100 µL of each was pipetted into 96 well plates, in triplicate. Absorbance was read on an Epoch plate reader (BioTek Instruments, Inc., Winooski, VT, USA) powered by GenS™ Software, set to a wavelength of 805 nm. Triplicate readings were taken for each blank, standard, and sample. The mean of 3 readings was calculated and used as the result.

We constructed a standard curve of absorbance against standard concentrations (Figure 3-3.) and used it to estimate the concentrations of serially collected plasma samples, obtained from t = 2 to 5 minutes, for each participant. The concentrations of ICG in serial plasma samples were transformed into natural logs and plotted against the time they were collected, from t = 2 to 5 minutes post-injection (converted to seconds, exact time of collection used). Traditionally, plasma volume estimation with ICG is made by back-extrapolation to time t = 0 minute (280, 284, 285, 348, 362). However, this method has been shown in some studies to underestimate PV partly due to incomplete ICG mixing at this time (i.e.,
the time of injection) (339). To overcome this problem, other researchers resorted to using a tourniquet (prior to ICG injection) to create a state of reactive hyperemia to speed up intravascular mixing and distribution of ICG(280, 284). Another solution, shown by Polidori and Rowley, is to use backward-extrapolation to time $t = 1$ minute, which produces consistent and more accurate PV than back-extrapolating to $t = 0$ (347). In this paper, we back-extrapolated the ICG concentration to both $t = 0$ and $t = 1$ minute so that the data is comparable to either approach for future work. We also showed back-extrapolation graphs for both timepoints (Fig. 3). The PV (L) for the participant was thus calculated as:

$$PV = \frac{D}{C_0}$$

(where $D =$ Dose of ICG administered (mg) and $C_0 =$ Plasma concentration of ICG (mg/L) at $t_0 = 0$ minute, back-transformed from natural log). This procedure was repeated for each participant with a new calibration curve constructed from the participant’s plasma (Figure 3-4). PV was also calculated by weight and body surface area (363). We also examine the relationship between PV and BSA, because strong association between the two have been reported (364, 365).

Overall, ~1.5 mL of pre-injection plasma (blank) and 0.2 mL of each post-injection plasma sample was sufficient to measure PV. This included five standard concentrations (2.5 mg/L to 10 mg/L), which were sufficient for the estimation of PV for all subjects. The total of ~2.5 mL of plasma needed per participant was much lower than the amount used in other studies (280, 361). While this is a small amount of plasma, we recommend a total of 14 mL (4 mL pre-injection and 2 mL x 5 post-injection) of blood collection to allow for repeat testing if needed. As well, although we used 5 post-injection samples, we found that 3 samples would be sufficient (i.e., if we missed a blood draw during the procedure). The laboratory work for each participant measurement of PV took approximately two hours to complete, after blood collection.
Table 3-1: Preparation of standard concentrations for ICG (using six 25 mL volumetric flasks, total volume for each is 25 mL)

<table>
<thead>
<tr>
<th>Standard concentrations (mg/L)</th>
<th>Volume of 2.5 mg/mL ICG solution to add (µL)</th>
<th>Volume of milliQ water (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>50</td>
<td>24950</td>
</tr>
<tr>
<td>7.5</td>
<td>75</td>
<td>24925</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>24900</td>
</tr>
<tr>
<td>15</td>
<td>150</td>
<td>24850</td>
</tr>
<tr>
<td>20</td>
<td>200</td>
<td>24800</td>
</tr>
<tr>
<td>30</td>
<td>300</td>
<td>24700</td>
</tr>
</tbody>
</table>

Mix thoroughly

Table 3-2: Final concentration for standard curve (using six 1.5 mL microcentrifuge tubes, total volume for each is 400 µL)

<table>
<thead>
<tr>
<th>Standard concentrations (mg/L) (from Table 1)</th>
<th>Amount of standard (µL) (from Table 1)</th>
<th>Amount of pre-injection plasma to add (µL)</th>
<th>Vortex at high speed for 10 seconds</th>
<th>Final concentration of standards (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>200</td>
<td>200</td>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>7.5</td>
<td>200</td>
<td>200</td>
<td></td>
<td>3.75</td>
</tr>
<tr>
<td>10</td>
<td>200</td>
<td>200</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>200</td>
<td>200</td>
<td></td>
<td>7.5</td>
</tr>
<tr>
<td>20</td>
<td>200</td>
<td>200</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>30</td>
<td>200</td>
<td>200</td>
<td></td>
<td>15</td>
</tr>
</tbody>
</table>
Results

Characteristics of study samples

A total of nine women enrolled and were included in the analyses. Eight women self-identified as white and one as African-American. Participants were college educated (n = 5) or were undergraduate students (n = 4). Two women were married (others were never married); all women were nulliparous except one. The mean ± SD age of participants was 25.0 ± 4.5 years, BMI was 23.5 ± 2.9 kg/m², and total body fat was 28.6 ± 5.0%. At the beginning of the visit, mean systolic blood pressure was 106 ± 8 mmHg and mean diastolic blood pressure was 71 ± 4 mmHg. Table 3-3 presents the PV of each participant sorted from low to high.

The mean ± SD of the correlation coefficient for the standard curve was 0.989 ± 0.023; 6 out of 9 were 0.99. The correlation coefficient for the decay curve was 0.991 ± 0.013; 7 out of 9 were 0.99. The mean ICG elimination rate constant (k) was 0.25 ± 0.06 /min and the (hepatic) clearance of ICG was 402 ± 119 mL/min (t = 0). The mean coefficient of variation for PV across the nine participants was 1.7%. The mean PV was 1608 ± 394 mL (or 2067 ± 470 mL, when extrapolated to time, t = 1 minute). PV by body size was 25 ± 5 mL/kg body weight and 941 ± 193 mL/m² body surface area (t = 0). The relationships between PV and anthropometric measures are presented in Figure 3-5. The correlation was particularly strong for BSA and plasma volume (r = 0.74, P = 0.022). The correlation coefficients were high for both the calibration curves and ICG decay curves (back-extrapolation). A sample standard curve and ICG decay curve from the study are shown in Figure 3-3 and Figure 3-4, respectively. The dye had a circulatory half-life of 2.9 ± 0.9 minutes and the ICG-plasma disappearance rate was 25.4% per minute. No participant reported any adverse events for the duration of the study.
Table 3-3: Participants and plasma volume (n = 9)

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Weight (kg)</th>
<th>BMI (kg/m^2)</th>
<th>Body fat (%)</th>
<th>SBP (mmHg)</th>
<th>DBP (mmHg)</th>
<th>Plasma volume (mL)^2</th>
<th>Plasma volume (mL)^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>54.7</td>
<td>21.6</td>
<td>25.6</td>
<td>109</td>
<td>70</td>
<td>1250</td>
<td>1500</td>
</tr>
<tr>
<td>28</td>
<td>53.5</td>
<td>20.3</td>
<td>21.5</td>
<td>99</td>
<td>69</td>
<td>1220</td>
<td>1570</td>
</tr>
<tr>
<td>21</td>
<td>63.6</td>
<td>24.2</td>
<td>29.4</td>
<td>98</td>
<td>66</td>
<td>1200</td>
<td>1600</td>
</tr>
<tr>
<td>21</td>
<td>65.5</td>
<td>26.1</td>
<td>27.8</td>
<td>123</td>
<td>75</td>
<td>1510</td>
<td>1940</td>
</tr>
<tr>
<td>18</td>
<td>63.6</td>
<td>22.1</td>
<td>26.8</td>
<td>103</td>
<td>66</td>
<td>1600</td>
<td>2000</td>
</tr>
<tr>
<td>23</td>
<td>65.0</td>
<td>22.5</td>
<td>31.4</td>
<td>109</td>
<td>79</td>
<td>1700</td>
<td>2200</td>
</tr>
<tr>
<td>27</td>
<td>64.9</td>
<td>23.8</td>
<td>26.1</td>
<td>109</td>
<td>75</td>
<td>1640</td>
<td>2350</td>
</tr>
<tr>
<td>31</td>
<td>79.5</td>
<td>29.6</td>
<td>39.7</td>
<td>108</td>
<td>74</td>
<td>2000</td>
<td>2660</td>
</tr>
<tr>
<td>30</td>
<td>63.6</td>
<td>21.4</td>
<td>29.4</td>
<td>101</td>
<td>69</td>
<td>2410</td>
<td>2780</td>
</tr>
<tr>
<td>25.0 ± 4.5</td>
<td>63.8 ± 23.5</td>
<td>28.6 ± 5.0</td>
<td>106 ± 8</td>
<td>71 ± 4</td>
<td>1614 ± 397</td>
<td>2067 ± 470</td>
<td></td>
</tr>
</tbody>
</table>

1 Plasma volume values are sorted from lowest to highest; Mean ± SD (reported in the last row for each variable); BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.
2 Plasma volume extrapolated t = 0
3 Plasma volume extrapolated t = 1 minute (60 seconds)

Figure 3-3: Standard curve for estimating the concentration of ICG in unknown plasma samples (example from one participant).
Figure 3-4: The decay curve for plasma ICG concentrations back-extrapolated to time, \( t = 0 \) (left) and \( t = 1 \) minute (right) using data collected from \( t = 2 \) to 5 minutes (example from one participant).

Figure 3-5: Local polynomial regression plot (regression line) of height, weight, BMI and BSA against plasma volume (at time, \( t = 0 \)). The Spearman’s correlation coefficient and regression estimates were: BMI on plasma volume (\( r = 0.23, P = 0.547; \beta = 0.032, 95\% \text{ CI: -0.088, 0.152} \)); BSA on plasma volume (\( r = 0.74, P = 0.022; \beta = 3.023, 95\% \text{ CI: 0.597, 5.457} \)); weight on plasma volume (\( r = 0.57, P = 0.111; \beta = 0.030, 95\% \text{ CI: -0.009, 0.069} \)), and height on plasma volume (\( r = 0.68, P = 0.043; \beta = 0.055, 95\% \text{ CI: 0.002, 0.107} \)). Body mass index (kg/m\(^2\)); BSA, body surface area (m\(^2\)).
Discussion and Conclusion

This study developed and field tested an efficient method for measuring PV using ICG dye among healthy, nonpregnant women. We have shown that the measurement of PV can take less than 2 hours to accomplish compared to commonly used methods that can take several hours to obtain results (169, 366, 367). This makes the method practical for research, however challenges for use in clinical settings remain. Some have used the non-invasive pulse dye densitometry (353, 354, 360, 368-372) to avoid the need for blood draws when using ICG, which deserves further consideration for application to PV estimation. This densitometry method has not yet been approved the FDA for use in the US.

Similarly, BVA-100 (Blood Volume Analyzer), a semi-automated system for blood volume analysis reduces the time required to measure blood volume using $^{131}$I-labeled HSA as tracer. This method has been approved by the FDA (263, 295) but because it uses a radioactive iodine isotope, there are concerns using it in some populations like pregnant women and young children. We avoided using radioactive isotopes because our long-term goal is to have a method that works for maternal and child health research. Another concern in clinical settings is overestimation of PV due to the escape of albumin-bound ICG into the interstitial space. This has been documented in patients with capillary leaks (e.g., sepsis, burns, trauma, and inflammation) (369, 373). In healthy patients, losses are minimal and do not appear to affect PV estimates (348, 373, 374).

By adjusting methods and concentrations, we found that a small quantity of plasma (~2.5 mL) can be used to measure PV – needing only 14 mL of blood to be drawn from each person. Further, our method reduced the number of post ICG-injection samples needed to 5, which is fewer than other methods that require 7-10 post-injection samples (280, 348, 361, 375-377). PV can be estimated with as few as 3 post-injection plasma samples, but having 5 samples improves the accuracy of estimates. Though we collected blood every 45 seconds, other time intervals could be used. The most important factor is that timing of blood collection should be precisely recorded. Altogether, we have reduced the total amount of blood needed, which is helpful in all populations but can be important in certain clinical cases.
Currently, no universally accepted standard values for comparing PV across age and gender exist. However, our measured values (mean 2067 mL using the t = 1 extrapolation) are comparable to estimates from Pearson et al. who estimated PV among more than 400 males and females from different equations (for 67 females with PV data, the estimates range from 2029 mL to 2744 mL) (297). Although we did not compare our results with the method recommended by ICSH or other common methods for measuring PV, our estimates are comparable to what is commonly reported in literature for the age group examined. Furthermore, previous studies have shown that PV estimates from ICG were comparable to the ICSH recommended method – \(^{125}\)I-HSA(284) and/or that of Evans blue dye (339), and our goal was not to re-validate the ICG measurement.

Taken together, the short circulatory half-life and high plasma disappearance rate give further support of ICG rapid clearance from the plasma, and safety when used in humans. This also makes it an ideal tracer for repeated use in plasma volume determinations. The half-life reported in this study was consistent with previous estimates (280, 341). \(^{125}\)I–HSA, has a circulatory half-life of 60 days (262). This makes repeated use of the method unacceptable because of the possibility of accumulation in the body. Over the years, interest in and use of ICG has been increasing particularly in clinical settings. A review in 2012 showed that in 1970 there were 38 studies on the use of ICG in PubMed, compared to 397 studies in 2010. As more evidence becomes available in PV measurements, ICG use will become more common.

In conclusion, ICG is a safe, efficient method for the measurement of PV in adults. As reported here, we successfully piloted the method in nine participants. Since then, we have used the same method in a longitudinal study of 35 women with 3 visits across the menstrual cycle, resulting in over 100 PV measurements. In our method development, we have further improved the method by reducing the amount of time and the volume of plasma needed to measure PV. PV can be estimated within 2 hours using only 5 post-injection blood draws and only ~2.5 mL of plasma per participant. ICG should be a recommended method for PV measurement in future research.
Funding

The Pennsylvania State University, College of Health and Human Development

Conflict of interest

Authors declared no conflict of interest exist.

Ethical considerations

The protocol was approved by The Office for Research Protections (ORP) at the Pennsylvania State University (STUDY00004051) and conducted in line with the Declaration of Helsinki. All participants provided written informed consent before enrolling into the study.

Acknowledgements

We thank the medical staff, especially Cyndi Flanagan, at the Clinical Research Center of The Pennsylvania State University’s Clinical and Translational Science Institute. We also extend our gratitude to Dr. James A. Pawelczyk, Associate Professor of Physiology and Kinesiology at The Pennsylvania State University, for his support in the study design, particularly on the use of indocyanine green. The authors sincerely acknowledge the research support provided by several research assistants, particularly, Leigh A. Taylor. We also thank the volunteers for their participation in the study. The project described was supported by the National Center for Advancing Translational Sciences, National Institutes of Health, through Grant UL1 TR002014. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.
Chapter 4

Plasma volume variation across the menstrual cycle among healthy women of reproductive age: a prospective cohort study

Abstract

Background: Plasma volume increases substantially during pregnancy. Little is known about plasma volume and the menstrual cycle, but some evidence shows that plasma volume may decrease across the cycle.

Objective: Our aim was to evaluate within-person change in plasma volume across the menstrual cycle in healthy women of reproductive age.

Design: This was a longitudinal prospective study of healthy women aged 18–44 years with regular menstrual cycles. Subjects were examined at three timepoints within a single menstrual cycle: the early follicular phase (~day 2), periovulation (~day 12), and the mid-point of the luteal phase (~day 21) – based on a 28-day cycle length. The actual days of visits were adjusted for the length of the woman's menstrual cycle and results from a fertility monitor. Blood samples were collected to measure plasma volume, ovarian hormones, and plasma osmolality across the cycle. Initial blood samples were collected followed by the injection of a bolus dose of 0.25 mg/kg body weight of indocyanine green dye (ICG). A timer was set and starting at 2 minutes post-ICG injection, a series of blood samples were drawn every 45 seconds up to 5 minutes post-injection (5 tubes). Blood samples were spun to obtain plasma. Pre-ICG injection (blank) and post-ICG injection plasma samples were used to measure plasma volume. Each biomarker including plasma volume were measured at three points in the cycle. Out of 47 women enrolled, 45 has at least one complete study visit and 35 had complete data from all 3 study visits.

Results: Mean (± SD) plasma volume was highest during the early follicular phase at 2276 ± 478
mL; it declined to 2232 ± 509 mL at the late follicular phase, and 2228 ± 502 mL at the midluteal phase. On average, plasma volume went down by 55 mL (2.4%) between the early and the midluteal phase. Though no overall change was observed plasma volume change significantly for many women (18%) in one way or the other. Plasma volume showed a moderate correlation with anthropometry measures; ranged from a low of 0.22 ($P = 0.018$) to a high of 0.55 ($P < 0.001$), for BMI and body surface area, respectively. Plasma volume also correlated positively with age 0.32 ($P < 0.001$). After adjusting for hydration status, ovarian hormone concentrations, and participant characteristics, a very small reduction of 29 mL (1.3%) in plasma volume was observed between the early and late follicular phase, and an increase of 48 mL (2.1%) was found between the early follicular phase and midluteal phase. Adjusted plasma volume went up by 77 mL (3.4%) from the late follicular phase to the midluteal phase. All estimates of change in plasma volume had 95% confidence intervals that substantially overlapped positive and negative values.

Conclusions: This study found that overall variations in plasma volume are small across the menstrual cycle, but that some individuals have relatively large changes. Given that plasma volume is generally well controlled in healthy women, it is unclear if these small changes could have clinical implications or not.

TRIAL REGISTRATION NUMBER: The trial is registered at www.clinicaltrials.gov (NCT03422809).

Key words: plasma volume/plasma volume and menstrual cycle/plasma volume and micronutrient biomarker/indocyanine green
Introduction

Plasma volume is known to increase remarkably during pregnancy. An increase of up to 50% has been reported between the third trimester and the nonpregnant state (168, 254). Plasma volume expansion during pregnancy are estimated from the baseline values (nonpregnant state), but it is unclear how plasma volume varies across the menstrual cycle in women of reproductive age. Without an accurate understanding of plasma volume variability in the nonpregnant state, it could affect how expansion during pregnancy is estimated. If plasma volume fluctuates across the menstrual cycle, that could have a potential impact on to how ‘normal’ nonpregnant plasma volume is defined; and the appropriate time to measure plasma volume that represents the nonpregnant state, to estimate amount of increase of plasma volume across pregnancy. Although the exact mechanism is not clear, it is thought that ovarian hormones –estrogen and progesterone– regulate plasma volume expansion and extracellular fluid volume in women of reproductive age (33, 212-215). Furthermore, plasma volume has the potential to influence biomarker concentrations measured in blood, suggesting that the phase of the menstrual cycle when measurements are done could influence research findings and interpretations. Unfortunately, plasma volume is often not measured, particularly, in nonpregnant women across the menstrual cycle.

Spaanderman et al. measured plasma volume in a small group of healthy controls (n = 10), and found that plasma volume was highest during the luteal phase (day 22 ± 2) compared to the follicular phase (day 5 ± 2) (220). In another study, Bernstein et al. examined the influence of plasma volume on time to conception among women planning to conceive, and concluded that plasma volume was not associated with increased probability of conception. However, the authors reported that mean plasma volume was significantly higher during the early follicular phase (cycle day 3) than at late follicular phase (cycle day 10) and midluteal phase (luteinizing hormone [LH] surge, plus 9 days) (159). Cullinane et al. assessed the effect of estimated plasma volume (calculated from hemoglobin and hematocrit) on serum
lipids and lipoproteins at a 5-day intervals across the menstrual cycle (day 1 or 2, day 5, day 10, day 15, day 20, and day 25). They reported a non-significant decrease in plasma volume, 4.1% ± 8.9%, between the onset of menstruation and cycle day 10 (160).

These inconsistencies in previous studies maybe due to small sample sizes, inaccurate timing of plasma volume measurement to cycle phase, and calculating plasma volume based on prediction equations instead of actual measurements based on dilution principle or use of tracers. To address these challenges, we prospectively examined plasma volume across the menstrual cycle to evaluate within-person change between any two phases of the menstrual cycle. We hypothesized that plasma volume would decline between the early and late follicular phase and rise slightly at the midluteal phase.
Materials and Methods

Study design

We examined cyclic variation in plasma volume in a prospective, observational cohort of healthy women followed across one complete menstrual cycle. The purpose of our main study was to evaluate variation in plasma volume and micronutrient biomarker concentrations in healthy eumenorrheic women across the menstrual cycle. In this paper, we evaluated within-person change in plasma volume across the menstrual cycle among study participants. The study protocol was approved by The Office for Research Protections (ORP) at The Pennsylvania State University (STUDY00008383) and conducted in line with the Declaration of Helsinki. The trial is registered at www.clinicaltrials.gov (NCT03422809). Participants were recruited by using internal emails among Penn State staff and students and recruitment platforms such as StudyFinder and ResearchMatch. The study was also advertised in a student newspaper (The Daily Collegian) and a local newspaper (Centre Daily Times). The potential participants were pre-screened via telephone and those eligible were scheduled to visit the Clinical Research Center (CRC), a service unit of The Pennsylvania State University (where the research occurred), for further screening and enrollment into the study. Data collection occurred between January and November, 2018.

Participants

Forty-seven healthy eumenorrheic women were enrolled into the study. Inclusion criteria for the study were as follows: 1) female, 2) age 18–44 y, 3) regular menstrual cycle (26-35 days), 4) general good health (does not have a known, ongoing health condition/medical issue that requires regular monitoring by a doctor or regular visits to the hospital), 5) BMI from 18.5 to 29.9 kg/m², 6) nonsmoking, 7) nonpregnant, and 8) if ever pregnant, her last pregnancy was more than or equal to 12 months ago. Participants were excluded if any of the following applied: 1) known allergy to shellfish or iodine, 2)
blood pressure on the day of measurements was low or high (SBP < 90 or > 130 mmHg and/or DBP < 60 or > 80 mmHg), 3) current hypertension or previous hypertensive disorder in pregnancy (gestational hypertension or preeclampsia), or 4) taking regular physician-prescribed medication(s) for a health condition, 5) were trying to conceive, 6) using hormonal birth control (within the past 3 months, or used depot medroxyprogesterone acetate (DMPA) in the past 12 months), 7) diagnosed with polycystic ovary syndrome, or 8) breastfeeding.

Sample size calculation

Sample size for plasma volume was estimated based on Bernstein et al. study (n = 21) (159). In that study, plasma volume change ranged from 1% to 5% across the menstrual cycle with a mean estimate in the early follicular phase of 2156 ± 292 mL. We wanted to detect a difference of 10% in plasma volume between any two menstrual phases (early vs. late follicular phase; late follicular phase vs. midluteal phase; and early follicular phase vs. midluteal phase), with a power of 80%, and an alpha-level of 0.05. This resulted in an estimated sample size of 28 subjects. We also anticipated that some women may have an anovulatory cycle during the study. For instance, in the BioCycle Study among women with a mean age of 27.5 years and BMI of 24.1 kg/m², anovulatory cycle incidence varied widely from 3.4% to 18.6% (328, 329). Adjusting for 10% non-ovulatory cycles, and 10% noncompletion rate, the estimated sample size was 34.6 (rounded up to 35). Thus, we aimed to enroll 35 women to reach our desired sample size of 28 women for plasma volume testing. The sample size for the overall study was based on micronutrient biomarker concentrations. We estimated that if a total of 51 women were enrolled, it would be sufficient to for detecting changes in micronutrient biomarker concentrations between any two timepoints in the menstrual cycle.
Clinical visits and study measures

All of the visits were conducted at the CRC. All outcomes of the study were assessed at all three visits, that is, during the early and late follicular phase, and the midluteal phase. These correspond to low estrogen and progesterone concentrations, high estrogen concentration, and high progesterone concentration. Participants were scheduled for visit 1 around cycle day 2. At all three visits, pregnancy test was completed and weight, blood pressure, and hydration (USG) were measured at the CRC. Blood samples were also collected for plasma volume and measurements of micronutrient biomarkers and ovarian hormones concentrations. Pregnancy tests were performed with the use of the QuickVue pregnancy test (an early pregnancy detection test for human chorionic gonadotropin in urine), and blood pressure measured with Omron Blood Pressure Monitor at all visits. We decided a priori that if a participant weight changed by more than 10% between visits, she would be dropped. Participants completed a short questionnaire about their background, health and pregnancy histories and height was measured only at visit 1.

At the end of visit 1, participants were provided with a fertility monitor (Clearblue® Fertility Monitors Swiss Precision Diagnostics GmbH, Procter and Gamble, Cincinnati, OH), and PREGMATE Ovulation test strips. Women were instructed on how to use the fertility monitor to track daily changes in urine hormone concentrations, starting at cycle day 6. Briefly, on rising, women collected their first morning urine sample, and dipped a fresh test strip into the urine before inserting it into the fertility monitor. The monitor displays the results within 5 minutes, and automatically stores the results. For each measurement, the monitor internally estimates the concentration of urinary E3G (the dominant estradiol metabolite) and LH and displays one of three results, “low”, “high” or “peak” fertility, which corresponds to low estrogen and low LH, high estrogen concentration, or high LH concentration (prior to ovulation), respectively.

When the fertility monitor read “high”, the participants began daily testing with PREGMATE Ovulation test strips while continuing to test with the fertility monitor. The ovulation test strips were used
as a backup to ensure that peak LH was detected (if it did occur but was not detected by the fertility monitor). Participants recorded daily test results on a form provided for this purpose, and results were cross-checked by research staff when the monitor was returned at the end of the study. Participants were scheduled for visit 2 immediately after the monitor read high fertility (which indicates an estrogen surge)—mostly within 2-3 days of high fertility, before LH peak. If a participant never reached “high” on the fertility monitor, she was dropped because we anticipated this would be a non-ovulatory cycle and we did not have information to schedule visit 2. The estrogen surge is necessary to trigger an LH surge (199, 378, 379). Visit 3 was scheduled (based on the results of the fertility monitor) to occur 9 days after LH surge—the expected progesterone peak, because, ovulation occurs 0.81 days following LH surge (195), and pregnanediol-3- glucuronide (P3G, the major metabolite of progesterone) peaks 7.65 days after ovulation (307). Instructions for preparation before study visits were the same across time.

Participants arrived after a 12-hour overnight fast and were instructed to refrain from intense physical exercise for the previous 24 hours, and to be well hydrated. Participants also refrained from drinking alcohol or caffeine at least 12 hours before each visit. Participants provided a fresh urine sample for a hydration test measured with a digital hand-held urine specific gravity "pen" refractometer (Atago PEN Urine S.G. Refractometer; ATAGO U.S.A., Inc) which measures to the nearest 0.0001 unit. The remaining urine samples were frozen at -20 °C until analysis for urine osmolality with the use of freezing-point depression osmometer. Body weight (kg) was measured to the nearest 0.1 kg on a digital scale, and height (cm) was measured to the nearest 0.1 cm with the use of a stadiometer; both by trained study staff. Body fat percentage was measured using a segmental bioelectric impedance analyzer (Tanita BC534 Glass InnerScan Body Composition Monitor, Tanita Corporation of America Inc., Arlington Heights, IL).

After these measurements, participants were then instructed to lie on a bed in a supine position with a small heating pad over the inside of the arm selected for IV insertion. Before IV insertion a temporary tourniquet was applied to aid in identifying an antecubital vein. Blood samples were collected into a 6 mL trace element-free vacutainer blood collection tube coated with K2 ethylenediaminetetraacetic
acid (K$_2$ EDTA) for plasma volume and for plasma osmolality assays. Blood samples were also collected into EDTA-free vacutainer blood collection tubes (trace element-free) for micronutrient biomarkers and hormone analysis. Additional samples were collected into EDTA-containing 2 mL vacutainer tubes for whole blood complete blood count (CBC) assays. This was followed by a bolus injection of ICG through the IV line and flushed with saline solution. Starting at 2 minutes after ICG injection, blood samples were collected into 3 mL EDTA-containing vacutainer blood collection tubes every 45 seconds, up to 5 minutes (total of 5 draws at exactly (min:sec) 2:00; 2:45; 3:30; 4:15; and 5:00) for plasma volume determination.

EDTA-treated tubes were gently inverted 10 times immediately after the tube was filled. Tubes were centrifuged (within 10 minutes of collection) at 1500 x g (3200 rpm) for 15 minutes (Drucker Diagnostics, Philipsburg, PA, USA) to separate plasma from blood cells. The plasma samples were aliquoted into cryovials and transported to the laboratory for PV determination. EDTA-free tubes were gently inverted 5 times immediately after collection and allowed to clot (~30-45 min) and centrifuged at the same conditions as above and aliquoted into microcentrifuge tubes. Serum and plasma samples were stored at -80º C within 90 minutes of blood collection.

**Plasma volume measurement**

A detailed description of the method for plasma volume measurement is published elsewhere (331). In brief, standard concentrations of 2.5 mg/L to 15 mg/L were prepared by mixing appropriate volumes of indocyanine green (ICG) solution and participant’s plasma (obtained before ICG injection). A 100 µL of each blank (pre-injection plasma), standard solution and plasma samples were pipetted into a 96-well plate and read on an Epoch plate reader (BioTek Instruments, Inc., Winooski, VT, USA) powered by Gen5™ Software, set to a wavelength of 805 nm. Samples were read in triplicates and the mean calculated. A standard curve was constructed from the standard concentration and absorbance, and used to
estimate ICG concentration in plasma samples (obtained after ICG injection). Sample concentrations were natural log transformed and plotted against the time they were collected. The concentration of ICG dye in plasma was extrapolated to the virtual time of complete mixing, \( t = 0 \). Plasma volume was obtained by dividing the dose of ICG injected by the extrapolated plasma ICG concentrations. The mean within-person coefficient of variation for a single study was 2.2%. Plasma volume measurements were completed within two hours of blood collection.

**Biomarker measurements**

Samples were transported on dry ice to analytical laboratories as a complete cycle batch (three samples) from each participant. Samples were measured consecutively, within a single run, to limit analytical variability. Estradiol and progesterone were measured at the Women's Health and Exercise Laboratory (Penn State, University Park, PA, USA) directed by Dr. Mary Jane De Souza. The samples were run for estradiol and progesterone using ELISA kits produced by Siemens for the Immulite, SAP #10702832 and SAP #10381128, respectively (Siemens Medical Solutions Diagnostics, Norwood, MA, USA) on the Diagnostics Product Corporation Immulite 2000 Analyzer (Siemens Medical Solutions Diagnostics, Deerfield, IL, USA). Inter- and intra-assay coefficients of variation (CV) were, 16.0% and 15.0% for estradiol, and 13.2% and 12.5% for progesterone.

Frozen plasma and urine samples were assayed by freezing point-depression osmometry with the use of the K-7400S Semi-Micro Osmometer (KNAUER, Wissenschaftliche Geräte GmbH, Berlin, Germany) by Dr. Asher Rosinger’s Laboratory (Department of Biobehavioral Health. The coefficient of variation across assays was < 1%.
Statistical analysis

Categorical descriptive variables were presented as frequencies (%). We assessed normality by testing the distribution of continuous variables against a normal distribution using the Shapiro-Wilk test and by visual inspection of kernel density plots. Continuous variables were presented as mean ± SD for normally distributed data or median (interquartile range) for non-normally distributed data. A linear mixed-effects model was used to investigate the bivariate association between menstrual phase and plasma volume. In this model, plasma volume and menstrual phase (time of visit as a categorical variable) were entered into the model as fixed variables. The participant identification (ID) number was entered as a random effect variable to account for repeated measurements within an individual. Similarly, for plasma osmolality, blood pressure, weight, BMI, body–fat (%), estradiol and progesterone, were each entered into separate models with menstrual phase as fixed effect variables, and ID as random effect variable. The change in each variable from visit 1 (early follicular phase measurements) were obtained from the respective models for plasma volume, plasma osmolality, blood pressure, weight, BMI, body–fat (%), estradiol and progesterone. Estimated changes from the early follicular phase to the late follicular phase, the early follicular phase to the midluteal phase were produced by the models. To estimate changes from the late follicular phase to the midluteal phase, we entered the late follicular phase as the reference timepoint.

In a second model, we adjusted for the effect of hydration status, ovarian hormone concentrations and background characteristics on plasma volume by adding plasma osmolality, urine void number (whether or not urine collected for osmolality was the first void of the day), estradiol and progesterone, age, BMI, and race (classified as White, Asian, and others) to the model. The menstrual phase, plasma osmolality, and other variables were defined as fixed factors. The subject identifier was considered the random factor to account for repeated measures on individual subjects. These models compared mean plasma volume across the menstrual cycle (late vs early follicular phase; midluteal phase vs late follicular phase, and midluteal phase vs early follicular phase). Akaike Information Criterion (AIC) was used to
select the covariance structure based on goodness of the fit, and the maximum likelihood was used for estimating each parameter.

We also assessed plasma volume pattern across the cycle by selecting participants that exhibited significant fluctuations across the menstrual cycle. A threshold of $\Delta \pm 8\%$ is considered a significant change (364). In this analysis, plasma volume at each phase of the menstrual cycle were categorized into three groups (reduce plasma volume ($<-8\%$ decrease), no change ($-8$ to $+8$) and increase plasma volume ($> +8\%)$). A Spearman’s correlation was run to examine the correlation between plasma volume and plasma osmolality, blood pressure, and anthropometric variables. Cycle days were standardized to the presumed day of ovulation by adding 2 days to the day of LH surge recorded by the fertility monitor, because it is expected that ovulation occurs within 2 days of LH surge for most women (202, 211). We used fractional polynomial regression (prediction plots) to examine bivariate relationships between plasma volume and plasma osmolality, blood pressure, and anthropometric measures. The same approach was used to assess the relationship between plasma volume, estradiol and progesterone concentrations, on the menstrual cycle day they were measured.

In sensitivity analysis, we restricted to participants aged < 35 years, because plasma volume is thought to be associated with age/parity. We also examined changes in plasma volume across the cycle by restricting data to participants that were presumed to have ovulated (progesterone concentration $>5$ ng/mL at midluteal phase) (380-382). All statistical analyses were conducted with the use of STATA Version 14 software (Stata Corp, College Station, Texas).
Results

Participant characteristics, rates of participation, and attrition

A total of 178 women were screened, out of which 47 women were enrolled into the study, and blood samples were available for 45 participants (Figure 4-1). Blood samples were not collected for two women because the phlebotomist could not successfully establish an IV in these subjects. Six participants were dropped between visits 1 and 2 (one did not use the fertility monitor correctly, one had a monitor malfunction, two did not reach “high” fertility, one was not available to schedule for visit 2, and one withdrew because she was uncomfortable with ICG). Two were dropped between visit 2 and visit 3 (one had bruising on her arm and did not want to continue, one experienced lightheadedness after study visits), and two were dropped at visit 3 because IV was unsuccessful. A total of 45 women had blood draw and plasma volume determination at visit 1. One plasma volume determination was unsuccessful at visit 1. Therefore, plasma volume is reported for 44, 39 and 35 women at visits 1, 2 and 3, respectively. Actual cycle day of visits were 3.3 ± 0.9, 13.1 ± 2.7, and 23.8 ± 2.2 for visits 1, 2, and 3, respectively. The mean ± SD age of participants was 24.2 ± 5.6 years as shown in Table 4-1. Participants were predominantly nulliparous (86.7%), and more than half were undergraduate students (53.3%). Mean menstrual cycle length calculated during the study was 28.0 ± 2.0 days.

Plasma volume changes and the associations with other participant characteristics

As shown in Figure 4-2, there was a small decline in plasma volume across the menstrual cycle. Overall, the highest mean plasma volume was observed during the early follicular phase. Mean plasma ± SD of volume was 2276 ± 478 mL in the early follicular phase, 2232 ± 509 in the late follicular phase,
and 2228 ± 502 mL in the midluteal phase (Table 4-2). Plasma volume estimates ranged from 1211 to 3468 mL with a 95% CI of 2170 to 2361 mL. The highest mean plasma osmolality was also observed during the early follicular phase (Table 4-2). The ratio of plasma volume to plasma osmolality remained constant across the menstrual cycle. Body weight and percent body fat were relatively constant across measurements, by study design (Table 4-2). On average, plasma volume went down by 49 mL (2.2%; \( P = 0.481 \)) between the early and late follicular phase, and by 6 mL (0.3%; \( P = 0.933 \)) between the late follicular phase and the midluteal phase. Plasma volume fell by 55 mL (2.4%; \( P = 0.536 \)) between the early follicular phase and the midluteal phase. Plasma osmolality showed a similar but smaller relative decrease of 3 mosm/kg (1.1%) between the early and late follicular phase (\( P < 0.036 \)). The reduction between the late and midluteal phase was negligible (0.8 mOsm/kg; 0.3%). Between the early follicular phase and the midluteal phase, plasma osmolality dropped by 4 mOsm/kg (1.3%; \( P < 0.038 \)). No change in systolic blood pressure was observed across the cycle, but diastolic blood pressure decreased by 1.6 mmHg (2.3%) between the early follicular phase and the midluteal phase (\( P < 0.036 \)). Body weight showed a reduction of less than 0.1 kg across the menstrual cycle.

Though plasma volume and plasma osmolality both went down across the menstrual cycle, the two have no clear association, as depicted in Figure 4-3. Plasma volume was also not related to blood pressure (\( r < 0.1, P > 0.740 \)). However, plasma volume was strongly associated with anthropometry measures, particularly with BMI, body surface area, and lean body mass. The correlation between plasma volume and anthropometry measures ranged from a low of 0.22 (\( P = 0.018 \)) to a high of 0.55 (\( P < 0.001 \)), for BMI and body surface area, respectively. Plasma volume also correlated positively with age. Change in plasma volume was weakly correlated with changes in plasma osmolality (\( r = 0.05–0.15, \text{ all } P > 0.05 \)), blood pressure (\( r = -0.01–0.25, \text{ all } P > 0.05 \)), or weight (\( r = -0.02–0.2, \text{ all } P > 0.05 \)) (Appendix 4-1). Estradiol and progesterone concentration from this study followed the normal expected pattern in a cycle (Figure 4-4), but did not show a clear association with plasma volume (Figure 4-5).

After adjusting for hydration status, plasma osmolality, estradiol, progesterone, age, and
BMI, a small non-significant reduction of 29 mL or 1.3% in plasma volume was observed between the early and late follicular phase. Plasma volume increased by 77 mL (3.4%) between the late follicular phase and midluteal phase. A 48 mL (2.1%) rise in plasma volume was observed between early follicular phase and midluteal phase (Table 4-3).

Similarly, we did not find any difference in the plasma volume between participants that were hydrated (< 297 mOsm/kg H₂O) vs dehydrated (≥ 297 mOsm/kg H₂O) as measured by plasma osmolality. Overall, less than a fifth (18%) of the women experienced a significant change in plasma volume across the menstrual cycle. We found that 12% (n = 4) of the women showed plasma volume expansion of >8% from the early follicular to midluteal phase, while 2 women showed a contraction of > 8% over the same period.

In sensitivity analysis, when data were restricted to participants aged 18-34 y, the mean plasma volume was 2257 ± 456 mL, 2204 ± 479 mL, and 2185 ± 469 mL at the early follicular, the late follicular and the midluteal phases, respectively. Plasma volume declined by 57 mL (2.5%) from the early to late follicular phase, and 13 mL (0.6%) between the late follicular phase and the midluteal phase. From the early follicular phase to midluteal phase plasma volume rose by 71 mL (3.1%).
Discussion

In this prospective cohort of healthy women of reproductive age, we found that plasma volume showed a very small decrease across the menstrual cycle. The estimated mean plasma volume ranged from 2276 mL in the early follicular phase to 2228 mL in the midluteal phase, a decline of 55 mL (2.4%) [95% CI: -10.1%, 5.3%]. Fewer than a fifth of the women showed a significant change (>8% up or down) in plasma volume between the early follicular phase and the midluteal phase. Plasma osmolality showed a similar pattern with a mean of 300 mOsm/kg in the early follicular phase and 296 mOsm/kg in the midluteal phase, which is a drop of 4 mOsm/kg (1.3%). Plasma volume showed a strong positive correlation with body weight, lean body mass, and body surface area (0.46 \leq r \leq 0.55, P < 0.001).

The mean plasma volume estimates and variations in the current study are comparable to those of Bernstein et al. (159) – we both found that plasma volume estimates at the early follicular phase were the highest. However, the Bernstein et al. study found a small rise between the late follicular phase and the midluteal phase, which was not observed in this study. Plasma volume variability in this study, as shown by the standard deviation, was also larger compared to the Bernstein et al. study which could be due to differences in the population. The participants in this study were mostly students who were not planning for pregnancy while those from Bernstein et al. were women planning to conceive. The other study was largely Caucasian women (90%) compared with our study which was more racially heterogeneous (~50% Caucasians). Spaanderman et al. reported an increase of 13% in plasma volume between the follicular phase and luteal phase. Participant of Spaanderman study were white women, slightly older (31 ± 2 years), and parous. The sample size for that study was relatively small (n = 10) compared to the current study population (220). Another study, where changes in plasma volume were calculated from hemoglobin and hematocrit values, found a 4% reduction between the early and late follicular phase. Similar to the current study, the authors reported a wide within-person variability, indicated by a standard deviation of 9% (160).
In the previous studies that measured plasma volume across the menstrual cycle, the effect of endogenous ovarian hormones on plasma volume change were not reported. In this study, both plasma volume and estradiol and progesterone concentrations were measured. Our result suggests that estrogen and progesterone play some role in the plasma volume changes during the menstrual cycle. For instance, in this study when estradiol and progesterone were controlled for in the analysis, the reduction in plasma volume between the early and late follicular was much smaller < 1%, and plasma volume actually went up by 2.9% between the late follicular phase and midluteal; and by 2.7% from the early follicular phase to the midluteal phase. This is supported by animal studies that showed that prolonged administration of estradiol-17ß in ewes led to classical cardiovascular alteration associated with pregnancy, and increased plasma volume by 14% (25, 26, 30). Other studies have also reported increases in plasma volume following the administration of estrogen and progesterone (32, 33), but in this study plasma volume was somewhat lower during time of expected estrogen and progesterone peaks (the late follicular and the midluteal phases, respectively). In an experiment to examine the influence of exogenous and endogenous estrogen, Fortney et al. observed that, while administration of exogenous estrogen was shown to significantly attenuate plasma volume loss, menstrual cycle fluctuation of endogenous estrogen had only a small transient effect in healthy women (n =19; aged, 21-39 years) (214). Furthermore, estrogen regulates the expression of angiotensinogen genes (27) and influences plasma renin activity (28-30). Both exogenous estrogen administration and endogenous estrogen production influence RAAS, leading to vasodilation (31). Though the exact mechanism driving plasma volume changes during the menstrual cycle is unknown, it at least appears that changes in estradiol and progesterone may be involved.

Taken together, increases in plasma volume following the administration of exogenous estrogen and/or progesterone are inconsistent with those observed in natural cycles changes associated with endogenous hormone concentrations. This suggests that the physiological influence of exogenous and endogenous estrogen and progesterone on plasma volume may be different in women of reproductive age.
To examine hydration as a factor in plasma volume, we also measured osmolality. Our findings of reduced plasma osmolality across the menstrual cycle is consistent with that of Spruce et al., who found a reduction in plasma osmolality between the early follicular phase and the luteal phase (383). Moderate increases/decreases in blood volume have been shown to be associated with a small change in plasma osmolality, with the process being tightly regulated by the antidiuretic hormone arginine vasopressin (384). Neither plasma volume nor changes in volume were associated with plasma osmolality.

Previous studies have reported that change in plasma volume was associated with changes in plasma osmolality during intensive exercise (155), but the present study did not find any association between plasma volume and osmolality, though hydrated participants tended to have a slightly larger plasma volume across the cycle. In this study, women were well-hydrated as indicated by urine osmolality and urine specific gravity, which may partially explain the lack of difference in plasma volume between those participants that were hydrated vs. dehydrated (as measured by plasma osmolality).

Surprisingly though, the correlation between plasma osmolality and urine markers of hydration (urine osmolality and urine specific gravity) were very weak. Though plasma volume and osmolality both tended to decrease across the menstrual cycle, they were not associated with each other.

During pregnancy, there is an increase in systemic vasodilation and vascular capacity as a result of the increase in activity of renin-angiotensin-aldosterone system (RAAS). This helps maintain blood pressure, retain water and salt, and reduce atrial natriuretic peptide concentrations. The loss of water and salt creates an underfilled vascular system (385). In response, plasma volume increased by filling the vascular space so created (30). Previous studies have shown plasma rennin, plasma renin activity, and aldosterone concentrations are higher in the luteal phase compared to the follicular phase (25, 26). If the changes in plasma volume across the menstrual cycle were driven by RAAS, we would at least expect to find higher plasma volume in the luteal phase. This has been observed in one small study (220). However, in our study and others, plasma volume was higher in the early follicular phase (159, 160, 219). Though
the exact reason is unknown, it likely that the higher plasma volume observed in the early follicular phase may be due to left over effects of progesterone from the previous luteal phase.

**Strengths and limitations**

The strengths of this study include a careful design to include women with healthy menstrual cycles and BMI. Participants were carefully tracked before they enrolled into the study to allow visit 1 to be as close to the beginning of the cycle as possible. All visits were conducted at a similar time in the morning. We also used a fertility monitor to help us prospectively track and schedule participants’ visits, timed to specific phases of the menstrual cycle in order to reflect concentrations of key ovarian cycle hormones. In addition to the fertility monitor, participants were provided with LH test kits to help confirm ovulation. We instructed participants to be well hydrated before study visits to eliminate effect of hydration on plasma volume, coupled with the measurement of three quantitative markers of hydration status.

This study also has some limitations. Plasma volume measurement using ICG is somewhat invasive, and that might have prevented some women from participating in the study. Cycle length also varies with age, and considering the wide range of participants, 18-44 years, this included women in the early stage of reproductive cycle. It is unclear if this has some on impact of the results. However, when we restricted data analysis to participants aged 18-35, the estimates for plasma volume were comparable to the whole sample. This suggests that, the inclusion of this wide age range may not have impacted the results. Because plasma volume may be influenced by parity, we performed sensitivity tests restricted to nulliparous women, and found that the results were not affected. Most of the study participants were nulliparous, which may limit the applicability of these findings to parous women. It remains unclear if these findings would apply to overweight/obese and/or women with irregular menstrual cycles.
Conclusion

This study was designed to determine whether healthy eumenorrheic women would have higher plasma volume at a particular timepoint in the menstrual cycle. Our results suggest that plasma volume is stable overall across the menstrual cycle, but is slightly lower in the late follicular phase and the midluteal phase compared to the early follicular phase. It is unclear if these small changes could have clinical implications or not. Larger studies with several repeated follow-ups are needed to investigate changes in plasma volume across the menstrual cycle.
Figure 4-1: Flow chart of participants. IV; intravenous

Screened via phone (n=178)

- Excluded (n=122)
  - Not meeting inclusion criteria (n=102)
  - Other reasons (n=20)
    - Not available for scheduling visits (n=9)
    - Cancelled visit (n=2)
    - Did not consent (n=2)
    - Lost to lack of communication (n=5)
    - Relocated (n=2)

Screened at study visit (n=56)

- Excluded (n=9)
  - Not meeting inclusion criteria (n=8)
    - BMI (n=3)
    - Blood pressure (n=3)
    - Taking medication (n=1)
    - Reported smoking (n=1)
  - Did not fast before visit and not available thereafter (n=1)

Completed consent form (n=47)

- IV was unsuccessful (n=2)
- Blood collected (n=45)

- Excluded (n=9)
  - Not meeting inclusion criteria (n=8)
    - BMI (n=3)
    - Blood pressure (n=3)
    - Taking medication (n=1)
    - Reported smoking (n=1)
  - Did not fast before visit and not available thereafter (n=1)

- Dropout (n=6)
  - Did not use fertility monitor correctly (n=1)
  - Monitor malfunction (n=1)
  - Did not reach high fertility (n=2)
  - Not available for scheduling (n=1)
  - Participant withdrew (n=1)

Attended visit 2 (n=39)

- Dropout (n=2)
  - Had bruising and did not want to continue (n=1)
  - Experienced lightheadedness (n=1)

Attended visit 3 (n=37)

- IV was unsuccessful (n=2)

- Excluded (n=9)
  - Not meeting inclusion criteria (n=8)
    - BMI (n=3)
    - Blood pressure (n=3)
    - Taking medication (n=1)
    - Reported smoking (n=1)
  - Did not fast before visit and not available thereafter (n=1)

- Dropout (n=6)
  - Did not use fertility monitor correctly (n=1)
  - Monitor malfunction (n=1)
  - Did not reach high fertility (n=2)
  - Not available for scheduling (n=1)
  - Participant withdrew (n=1)

Analysis

- Analyzed (had blood data)
  - Visit 1 (n=45)
  - Visit 2 (n=39)
  - Visit 3 (n=35)
Table 4-1: Baseline descriptive characteristics and menstrual cycle information of study participants (n = 45)\(^1\)

<table>
<thead>
<tr>
<th>Variation</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>24.2 ± 5.6</td>
</tr>
<tr>
<td>Height, cm</td>
<td>163.9 ± 7.0</td>
</tr>
<tr>
<td>Race, n (%)</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>22 (48.9)</td>
</tr>
<tr>
<td>Asian</td>
<td>14 (31.1)</td>
</tr>
<tr>
<td>Others (^2)</td>
<td>9 (20.0)</td>
</tr>
<tr>
<td>Ethnicity, n (%)</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>6 (13.3)</td>
</tr>
<tr>
<td>Non-Hispanic</td>
<td>39 (86.7)</td>
</tr>
<tr>
<td>Socio-economic status, n (%) (^3)</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>6 (13.3)</td>
</tr>
<tr>
<td>Middle</td>
<td>33 (73.3)</td>
</tr>
<tr>
<td>High</td>
<td>6 (13.3)</td>
</tr>
<tr>
<td>Educational status, n (%)</td>
<td></td>
</tr>
<tr>
<td>At least bachelor’s degree</td>
<td>18 (40.0)</td>
</tr>
<tr>
<td>Associate degree</td>
<td>3 (6.7)</td>
</tr>
<tr>
<td>Undergraduate students</td>
<td>24 (53.3)</td>
</tr>
<tr>
<td>Never married, n (%)</td>
<td></td>
</tr>
<tr>
<td>Nulliparous, n (%)</td>
<td></td>
</tr>
<tr>
<td>Never married</td>
<td>39 (86.7)</td>
</tr>
<tr>
<td>Nulliparous</td>
<td>39 (86.7)</td>
</tr>
<tr>
<td>Menstrual cycle length, days</td>
<td></td>
</tr>
<tr>
<td>Self-reported</td>
<td>28.9 ± 1.8</td>
</tr>
<tr>
<td>Recorded during study</td>
<td>28.0 ± 2.0</td>
</tr>
</tbody>
</table>

\(^1\) Mean ± SD or n (%) for categorical data  
\(^2\) Black or African American (3), White & Black or African American (1); White & American Indian or Alaska native (1); White and Other (not specified) (1); Others (2); Not specified (1)  
\(^3\) Self-reported by participant
Table 4-2: Mean within-person differences in plasma volume, blood pressure and body weight across a single menstrual cycle (n = 45)  

<table>
<thead>
<tr>
<th>Variables</th>
<th>EFP</th>
<th>LFP vs. ELP</th>
<th>MLP vs. LFP</th>
<th>MLP vs. EFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma volume</td>
<td>2276 ± 476</td>
<td>-49 (-189, 88)</td>
<td>-6 (-149, 137)</td>
<td>-55 (-231, 120)</td>
</tr>
<tr>
<td>Plasma volume by weight, mL/kg</td>
<td>39 ± 6</td>
<td>-0.8 (-3.1, 1.6)</td>
<td>0.2 (-2.3, 2.6)</td>
<td>-0.6 (-3.5, 2.3)</td>
</tr>
<tr>
<td>Plasma volume by lean body mass, mL/kg</td>
<td>52 ± 9</td>
<td>-1.6 (-4.8, 1.6)</td>
<td>0.7 (-2.7, 4.0)</td>
<td>-1.0 (-4.9, 3.0)</td>
</tr>
<tr>
<td>Plasma volume by BSA, mL/m²</td>
<td>1389 ± 237</td>
<td>-28 (-111, 55)</td>
<td>1.1 (-86, 88)</td>
<td>-27 (-130, 75)</td>
</tr>
<tr>
<td>Plasma osmolality</td>
<td>300 ± 7</td>
<td>-3.2 (-6.2, -0.2)</td>
<td>-0.8 (-4.0, 2.4)</td>
<td>-4.0 (-7.3, -0.7)</td>
</tr>
<tr>
<td>Plasma volume/plasma osmolality</td>
<td>7.6 ± 1.6</td>
<td>-0.10 (-0.58, 0.38)</td>
<td>-0.002 (-0.48, 0.53)</td>
<td>-0.08 (-0.69, 0.53)</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>106 ± 10</td>
<td>-2.2 (-5.1, 0.7)</td>
<td>0.9 (-1.0, 2.9)</td>
<td>-1.3 (-4.9, 2.3)</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>69 ± 6</td>
<td>-1.4 (-2.8, 0.01)</td>
<td>-0.2 (-1.7, 1.3)</td>
<td>-1.6 (-3.1, -0.10)</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>58.3 ± 6.9</td>
<td>-0.08 (-0.30, 0.15)</td>
<td>-0.02 (-0.25, 0.22)</td>
<td>-0.09 (-0.32, 0.14)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>21.7 ± 1.9</td>
<td>-0.03 (-0.11, 0.06)</td>
<td>-0.002 (-0.09, 0.08)</td>
<td>-0.03 (-0.11, 0.06)</td>
</tr>
<tr>
<td>Body-fat percentage, %</td>
<td>25 ± 5</td>
<td>-0.78 (-2.03, 0.47)</td>
<td>0.83 (-0.48, 2.15)</td>
<td>-0.06 (-1.25, 1.36)</td>
</tr>
<tr>
<td>Estradiol, pg/mL</td>
<td>30.1 ± 13.2</td>
<td>75.5 (55.6, 95.4)</td>
<td>20.7 (-0.5, 41.8)</td>
<td>96.1 (74.4, 117.8)</td>
</tr>
<tr>
<td>Progesterone, ng/mL</td>
<td>0.8 ± 1.8</td>
<td>0.08 (-1.20, 1.36)</td>
<td>8.26 (6.89, 9.62)</td>
<td>8.34 (7.09, 9.58)</td>
</tr>
</tbody>
</table>

1 All values are model-estimated means difference (95% CIs). All regression estimates were determined with the use of a linear mixed-effects regression with individual-level random intercepts and a fixed effect for the outcome variable and time. Plasma volume and menstrual phase were entered as fixed variables. Subject identification was included as a random effect variable to account for repeated measurements.

2 Plasma volume EFP (n=44), LFP (n=39), MLP (n=35)

3 Plasma osmolality, EFP (n=44), LFP (n=37), MLP (n=34)

4 Plasma volume/plasma osmolality ratio, EFP (n=43), LFP (n=37), MLP (n=34)
Table 4.3: Mean adjusted within-person differences in plasma volume across a single menstrual cycle (n = 45) (adjusted)^1

<table>
<thead>
<tr>
<th></th>
<th>LFP vs. ELF</th>
<th>MLP vs. LFP</th>
<th>MLP vs. EFP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β (95% CI)</strong></td>
<td>β (95% CI)</td>
<td>β (95% CI)</td>
<td></td>
</tr>
<tr>
<td><strong>Model 1</strong></td>
<td>-60 (-204, 84)</td>
<td>-0.2 (-150, 150)</td>
<td>-61 (-242, 121)</td>
</tr>
<tr>
<td><strong>Model 2</strong></td>
<td>-29 (-241, 184)</td>
<td>77 (-195, 349)</td>
<td>48 (-283, 380)</td>
</tr>
</tbody>
</table>

EFP, early follicular phase; LFP, late follicular phase prospective; MLP, Midluteal phase
Model 1: β (95% CIs) and P values were estimated using a linear mixed-effects model including maternal age, BMI, race (White, Asian, and others), plasma osmolality, urine void (1st or 2nd) and time as fixed effects, with subject identification as a random effect.
Model 2: model 1 + estrogen and progesterone
Figure 4-2: Fractional polynomial with regression (line) of plasma volume on day of cycle when measurement occurred (left), cycle day relative to presumed date of ovulation (right). Number visits = 118 (95 graph on right). Black dots represent data from study participants; solid black line represents prediction based on all data; short dash gray line represents the 95% CI around the prediction.
Figure 4-3: Fractional polynomial with regression (line) of plasma volume on plasma osmolality (A), systolic blood pressure (B), diastolic blood pressure (C), age (D), weight (E), BMI (F), lean body weight (G), body fat (%) (H), body surface area. Number visits = 118 (114 for plasma osmolality). Black dots represent data from study participants; solid gray line represents prediction based on all data; short dash gray line represents the 95% CI around the prediction.
Figure 4-4: Locally weighted regression (line) of estrogen (left) and progesterone (right) concentrations versus cycle day from ovulation. Number visits = 95. Black dots represent data from study participants; solid black line represents prediction based on all data.
Figure 4-5: Locally weighted regression (line) of plasma volume versus log concentration of estrogen (left) and progesterone (right). Number visits = 118. Black dots represent data from study participants; solid gray line represents prediction based on all data; short dash gray line represents the 95% CI around the prediction.
Appendix 4-1: Associations between within-person changes in plasma volume and changes in plasma osmolality, blood pressure, and body weight across the menstrual cycle (n = 34)

<table>
<thead>
<tr>
<th></th>
<th>∆ Plasma osmolality</th>
<th>∆ Systolic blood pressure</th>
<th>∆ Diastolic blood pressure</th>
<th>∆ Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>β (95% CI)</td>
<td>r</td>
<td>β (95% CI)</td>
</tr>
<tr>
<td>PV, mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LFP vs EFP</td>
<td>0.15</td>
<td>-1.05 (-4.18, 2.09)</td>
<td>0.08</td>
<td>0.22 (-0.85, 1.29)</td>
</tr>
<tr>
<td>MLP vs LFP</td>
<td>0.04</td>
<td>0.53 (-1.79, 2.85)</td>
<td>0.20</td>
<td>0.12 (-0.54, 0.79)</td>
</tr>
<tr>
<td>MLP vs EFP</td>
<td>0.07</td>
<td>0.36 (-2.48, 3.19)</td>
<td>0.18</td>
<td>0.65 (-0.60, 1.91)</td>
</tr>
<tr>
<td>PV by LBM, ml/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LFP vs EFP</td>
<td>-0.14</td>
<td>-0.61 (-2.35, 1.14)</td>
<td>-0.01</td>
<td>0.02 (0.58, 0.61)</td>
</tr>
<tr>
<td>MLP vs LFP</td>
<td>0.08</td>
<td>0.27 (-0.95, 1.50)</td>
<td>0.16</td>
<td>0.07 (-0.28, 0.42)</td>
</tr>
<tr>
<td>MLP vs EFP</td>
<td>0.06</td>
<td>0.14 (-1.39, 1.66)</td>
<td>0.15</td>
<td>0.38 (-0.29, 1.06)</td>
</tr>
<tr>
<td>PV by Weight, ml/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LFP vs EFP</td>
<td>-0.11</td>
<td>-0.42 (-1.69, 0.85)</td>
<td>0.08</td>
<td>0.12 (-0.31, 0.55)</td>
</tr>
<tr>
<td>MLP vs LFP</td>
<td>0.06</td>
<td>0.22 (-0.69, 1.14)</td>
<td>0.16</td>
<td>0.04 (-0.22, 0.30)</td>
</tr>
<tr>
<td>MLP vs EFP</td>
<td>0.07</td>
<td>0.14 (-0.99, 1.27)</td>
<td>0.19</td>
<td>0.33 (-0.17, 0.83)</td>
</tr>
<tr>
<td>PV by BSA, mL/m²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LFP vs EFP</td>
<td>-0.14</td>
<td>-1.04 (-4.18, 2.10)</td>
<td>0.07</td>
<td>0.22 (-0.86, 1.29)</td>
</tr>
<tr>
<td>MLP vs LFP</td>
<td>0.05</td>
<td>0.53 (-1.79, 2.85)</td>
<td>0.20</td>
<td>0.12 (-0.54, 0.79)</td>
</tr>
<tr>
<td>MLP vs EFP</td>
<td>0.07</td>
<td>0.35 (-2.48, 3.18)</td>
<td>0.18</td>
<td>0.65 (-0.60, 1.91)</td>
</tr>
</tbody>
</table>

PV, plasma volume; EFP, early follicular phase; LFP, late follicular phase prospective; MLP, Midluteal phase; LBM, lean body mass; BSA, body surface area.

1 Changes (%) between any two study visits (timepoints).

2 Linear regression estimates: changes in plasma osmolality was used to predict changes in plasma volume at three separate intervals (LFP vs EFP, MLP vs LFP and MLP vs EFP). This was repeated for the changes in blood pressure and weight, on change in plasma volume.
### Appendix 4-2: Characteristic of indocyanine green half-life and clearance characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Early follicular phase</th>
<th>Late follicular phase</th>
<th>Midluteal phase</th>
<th>Normal value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>44 Mean ± SD</td>
<td>39 Mean ± SD</td>
<td>35 Mean ± SD</td>
<td>NA</td>
</tr>
<tr>
<td>Half-life, min</td>
<td>3.2 ± 0.8</td>
<td>3.0 ± 0.8</td>
<td>2.8 ± 0.6</td>
<td>3.5 (280, 341, 386)</td>
</tr>
<tr>
<td>K, min⁻¹</td>
<td>0.23 ± 0.05</td>
<td>0.24 ± 0.06</td>
<td>0.26 ± 0.05</td>
<td>–</td>
</tr>
<tr>
<td>PDR, %/min</td>
<td>23 ± 5</td>
<td>24 ± 6</td>
<td>26 ± 5</td>
<td>&gt; 18 (386)</td>
</tr>
<tr>
<td>ICG clearance, mL/min¹</td>
<td>510 ± 132</td>
<td>530 ± 128</td>
<td>558 ± 124</td>
<td>500-750 (387)</td>
</tr>
<tr>
<td>ICG clearance, mL/min¹ kg⁻¹</td>
<td>9 ± 2</td>
<td>9 ± 2</td>
<td>10 ± 2</td>
<td>6-12 (386)</td>
</tr>
<tr>
<td>ICG¹ mg</td>
<td>14.6 ± 1.8</td>
<td>14.6 ± 1.6</td>
<td>14.6 ± 1.7</td>
<td>NA</td>
</tr>
<tr>
<td>ICG volume, mL</td>
<td>6.1 ± 1.8</td>
<td>5.8 ± 0.7</td>
<td>5.8 ± 0.7</td>
<td>NA</td>
</tr>
<tr>
<td>ICG² mg</td>
<td>14.2 ± 1.9</td>
<td>14.6 ± 1.6</td>
<td>14.6 ± 1.6</td>
<td>NA</td>
</tr>
<tr>
<td>Fraction ICG injected (%)</td>
<td>99.0 ± 3.0</td>
<td>99.9 ± 2.4</td>
<td>100.2 ± 1.7</td>
<td></td>
</tr>
</tbody>
</table>

¹ICG estimated for injection
²ICG Actual amount of ICG injected
K, Disappearance constant; PDR, plasma disappearance rate; ICG, indocyanine green
Chapter 5

Micronutrient status across the menstrual cycle: a prospective cohort study

Abstract

**Background**: Some micronutrient concentrations have been reported to vary across the menstrual cycle. However, data collection is not often timed to the menstrual phase or key hormone concentrations, which could influence how biomarkers are evaluated.

**Objective**: The aim of this study was to evaluate variations in micronutrient biomarker concentrations in a cohort of healthy eumenorrheic women across a complete menstrual cycle.

**Design**: This was prospective cohort study among healthy women of reproductive aged, 18-44 y, living within the State College area. Data collection occurred within a single complete menstrual cycle. Women made three separate visits to the study center; one each during the early follicular phase (EFP), the late follicular phase (LFP), and the midluteal phase (MLP). Visits were timed to key ovarian hormones: low estrogen and progesterone, high estrogen, and high progesterone, respectively. Blood samples were collected at each timepoint to measure micronutrient biomarkers (zinc, copper, magnesium, manganese, iron, ferritin, and retinol) and ovarian hormones (estrogen and progesterone). Linear mixed-effects models were used to evaluate changes in concentrations across the menstrual cycle.

**Results**: At the start of the menstrual cycle (EFP), the mean ± SD concentration for micronutrients was 81.8 ± 16.2 μg/dL, 80.1 ± 12.8 μg/dL, 17.9 ± 1.4 mg/L, 1.51 ± 1.97 μg/L, 106.7 ± 1.7 μg/dL, 26.4 ± 2.3 μg/L, and 39.4 ± 9.3 μg/dL, for zinc, copper, magnesium, manganese, iron, ferritin, and retinol, respectively. Mean concentrations of all micronutrient biomarkers declined between EFP and LFP. Reductions in zinc and magnesium concentrations were relatively large. Zinc concentration declined by 3.4% between EFP and LFP ($P = 0.204$), 3.3% between LFP and MLP ($P = 0.064$), and 6.6% between
EFP and MLP ($P = 0.009$). Across the same interval, magnesium concentration decreased by 2.8% ($P = 0.001$), 1.8% ($P > 0.05$), and 4.6% ($P < 0.001$). The prevalence of micronutrient deficiencies was lower at EFP than at LFP or MLP. For instance, prevalence estimates were 15% (zinc) to 20% (magnesium) higher in the midluteal phase than at the early follicular phase.

**Conclusions:** Our study suggests that serum zinc and magnesium concentrations decline across the menstrual cycle in healthy women of reproductive age. Micronutrient deficiency estimates were dependent on the menstrual phase when measurements were done and were lowest in the early follicular phase. Future studies repeated over several cycles should be conducted to confirm these findings in a large group of women across a wider range of BMI and parity.
Introduction

Cyclic fluctuations in ovarian hormones influence several biological processes in women of reproductive age and can impact research findings (221, 222). Estrogen and progesterone concentrations are associated with both food intake and lipoprotein concentrations across the menstrual cycle (223). Frequent craving for foods, and consumption of food higher in fat, carbohydrate and proteins during the luteal phase compared to follicular phase have been reported (224-231). It is speculated that estrogen may be playing a suppressive role during the periovulatory phase while progesterone stimulates appetite (232, 233). However, few studies have examined changes in micronutrients homeostasis during the menstrual cycle. For instance, in a small study among US women aged 20-34 y for 4-6 menstrual cycles, intake of 11 out of 16 micronutrients did not change between the follicular phase and luteal phase, while energy, protein, carbohydrates and fat intakes were all higher during the luteal phase (228). However, a larger study conducted in US among women aged 18-44 y, showed that except for zinc (lowest during the periovulatory phase compared to the mid-luteal phase), intake of 15 other micronutrients did not vary across 2 cycles (224).

Studies of dietary intake may be helpful, but for many micronutrients (e.g., zinc), serum concentrations do not reflect dietary intake (388-392) and so micronutrient concentrations should be examined directly to assess nutritional status across the menstrual cycle. Limited studies have reported differences in micronutrient concentrations across the menstrual cycle. Among women of reproductive age, studies have reported a decline in zinc concentration between the early and late follicular phase/ovulation (n = 5) (234) while others have shown an increase across the menstrual cycle in healthy women (n=14) (235). In the same study, serum copper concentrations were reported to decrease across the cycle (235). A study among medical students in India, aged 18-25 y (n=25), by Pandya et al. showed that serum magnesium concentration dropped by 1.0 mg/L (6.4%) between menses and the follicular phase (unspecified, mid or late follicular phase), and rose by 1.7 mg/L (11.6%) between the follicular phase and
luteal phase (236). In another study in India (n = 50), Lanje et al. reported that serum magnesium concentration showed a significant decline of 1.7 mg/L (8.0%) between menses (cycle day 2) and the follicular phase (day 8-14), then increased by 2.5 mg/L (11.8%) between the follicular phase and the luteal phase (after day 22) among healthy women, aged 18-35 y, with regular menstrual cycles (237). In both the Pandya et al. and Lanje et al., studies, the decline in serum magnesium concentration between the early (menses) and the follicular/ovulation was between 6 and 12%.

Inconsistent results have been reported for serum iron and ferritin concentrations across the menstrual cycle. While some studies have reported the lowest serum ferritin concentration during menses (239, 240), others found no change across the menstrual cycle in iron-depleted women (111). Previous studies based on data from the second National Health and Nutrition Examination Survey (NHANES II) by Kim et al. reported a significantly lower serum ferritin during menses compared with the luteal phase (239). This was a large study (n = 1712) and the authors statistically controlled for several possible confounders. However, it was a cross-sectional study where different groups of women were compared and intra-individual changes were unknown. In a study among 43 Japanese college students, aged 19-22 y, serum ferritin concentration was higher at the follicular phase among participants that were marginally iron deficient (n = 23) or had iron-deficiency anemia (n = 5), but not in participants with adequate iron status (n = 15) (393).

Taken together, some micronutrient concentrations appear to fluctuate across the menstrual cycle, though the pattern is inconsistent. These inconsistencies may be due to small sample sizes, inaccurate timing of sample collection to cycle phase, and use of cross-sectional data by comparing different groups, not the same women across the menstrual cycle. Moreover, some researchers often assume a 28-day cycle for all women, which is not accurate for many women (201-204). Fluctuations in micronutrient concentration across the menstrual cycle may lead to different estimates of deficiencies in the population, depending on cycle phase when measurements occur.

Currently, there is scarce data on how micronutrient concentrations vary across the menstrual cycle and no data, to our knowledge, on the prevalence of micronutrient deficiencies across the menstrual
cycle. Therefore, the objective of this study was to evaluate the variation of selected micronutrient concentrations across the menstrual cycle, under free-living conditions, in healthy eumenorrheic women. The micronutrient selected were based on their clinical and public health relevance, and those that resources allowed us to measure. The primary outcomes were serum concentrations of zinc, copper, magnesium, manganese, iron, ferritin, and retinol.

**Materials and Methods**

**Subjects**

Women of reproductive age, 18–44 y, were recruited via advertisements posted across the State College, PA area. Inclusion criteria as assessed by the screening questionnaire specified healthy women with a BMI of 18.5 to 24.9; were not allergic to iodine or shell fish; have a regular menstrual cycle, defined as a self-reported average cycle duration of 26 to 35 days for the last three months; not using hormonal birth control; nonpregnant and not planning a pregnancy; if ever pregnant, her last pregnancy was more than or equal to 12 months ago; and normal blood pressure. Exclusion criteria included the following: diagnosed with medical condition (or under regular monitoring by a doctor); smoking; blood pressure higher than normal at any visits; use of antidepressants or chronic use of any medication; currently breastfeeding; used any hormonal birth control hormonal within last the 3 months; used depot medroxyprogesterone acetate (DMPA) in the past 12 months; and diagnosed with polycystic ovary syndrome. Participants were dropped if they could not adhere to the study protocol, use the fertility monitors correctly or reported a weight change of more than 10% between any two visits. The protocol was approved by The Office for Research Protections (ORP) at the Pennsylvania State University (STUDY00008383) and conducted in line with the Declaration of Helsinki. The trial is registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (NCT03422809). All women signed an informed consent form before enrollment.
Study design

This was a prospective cohort study among women of reproductive age with a regular menstrual cycle in the last three months. Women responded to study information posted on several platforms including print advertisements (printed/online newspaper), online message boards (StudyFinder and State College Craigslist) and online recruitment sites (ResearchMatch) available to women in the State College area and surrounding towns within Centre County, PA.

Women were prescreened via telephone, and those eligible were invited to attend in-person screening and enrollment. All study activities occurred at the Penn State's Clinical Research Center (CRC), a service unit in Pennsylvania State University's Clinical and Translational Science Institute, University Park. Each participant completed 3 separate visits which occurred during key phases of their menstrual cycle. Study visits were timed using results from the fertility monitors (Clearblue® Fertility Monitors Swiss Precision Diagnostics GmbH, Procter and Gamble, Cincinnati, OH): one each in the early follicular phase (2–5 d), late follicular phase (~12 d), and midluteal phase (~21 d). These visits correspond to menstruation, time of estrogen peak, and time of progesterone peak, respectively.

Participants were provided the fertility monitors and instructed on how to use them for home-based daily urine tests. The fertility monitor measures the concentration of urinary estrone-3-glucuronide and luteinizing hormone (LH), and displays the results within 5 minutes on the monitor’s LCD. The results showed one of three options, “low”, “high” or “peak” fertility, which corresponds to low estrogen and LH concentration, estrogen surge, and LH surge, respectively. Participants were scheduled for visit 2 during the estrogen surge and visit 3 occurred 9 days after the LH surge. The readings of the fertility monitors were recorded on a form provided to each participant and cross-checked by trained research staff when the monitor was returned at the end of visit 3.

On each occasion, participants arrived at the CRC after a 12-h overnight fast. They were instructed to be well hydrated. At visit 1, participants completed a short questionnaire about their background and health and pregnancy histories. A pregnancy test was conducted at each visit using
QuickVue Pregnancy test kits which detects human chorionic gonadotropin in urine. Blood pressure was measured with an Omron blood pressure monitor (Omron Blood Pressure Monitor, OMRON HEM-712C; Omron Healthcare, Inc, Bannockburn, IL) at each visit to confirm normal blood pressure (330). Participants were instructed to empty their bladder in the bathroom, then to rest on a chair for 5 minutes in a relaxed position, before blood pressure was measured.

Body weight (kg) was measured to the nearest 0.1 kg on a digital scale, and height (cm) was measured to the nearest 0.1 cm with the use of a stadiometer by trained staff. Body composition was measured with the use of a segmental bioelectric impedance analyzer (Tanita BC-534; Tanita Corporation of America, Inc.) to 0.1%. Hydration status was assessed with a digital hand-held urine specific gravity "Pen" refractometer (Atago PEN Urine S.G. Refractometer; ATAGO U.S.A., Inc.) to the nearest 0.0001 resolution unit. This was followed by preparation for blood collection.

Participants were instructed to lie on a bed in a supine position and remained relaxed but awake for 15 min. An IV was inserted, then they rested for another 5 minutes and blood samples were collected into an EDTA-free vacutainer trace element-free tubes (BD, Becton Dickinson, Franklin Lakes, NJ) for micronutrients and hormones analysis. Samples were also collected into EDTA-treated vacutainer trace element-free tubes for plasma osmolality and for a complete blood count (CBC) assays. Blood samples for whole blood assays were not centrifuged. EDTA-treated tubes were centrifuged immediately, while EDTA-free tubes were allowed to clot (30 to 45 minutes) first. Both were centrifuged at 3200 rpm for 15 minutes. Samples were aliquoted into microcentrifuge tubes (serum) or cryovials (plasma) and frozen within 90 min of blood collection at -80°C, until analysis.

**Laboratory assays**

Whole blood was run for CBC using the Beckman Coulter Ac-T Diff 2 hematology analyzer (Beckman Coulter Inc, Brea, CA) with 3-level quality-control material (Beckman Coulter, Inc. Brea, CA) within one hour of blood collection. Serum and plasma samples were transported on dry ice to analytic
laboratories as a complete cycle (per woman), and measured simultaneously, within a single analytic run, to limit analytic variability. Serum ferritin was measured using enzyme-linked immunosorbent assay (ELISA) methods (Ramco Laboratories, Inc., Strafford, Texas) in the laboratory of Dr. Laura Murray-Kolb. We used ELISA methods to measure acute-phase proteins, serum α1-acid glycoprotein (AGP; Kent Laboratories Inc., Bellingham, Washington) and C-reactive protein (CRP; Kent Laboratories Inc., Bellingham, Washington) to account for the influence of inflammation that falsely elevates ferritin (137) and depresses serum zinc and retinol concentrations (135, 136). The intra- and inter-assay coefficient of variations were 5.8% and 5.9% for ferritin, 3.2% and 4.9% for AGP, and 0.1% and 1.3% for CRP.

Serum concentration of zinc (Zn), copper (Cu), magnesium (Mg), Manganese (Mn) and iron (Fe) were determined by using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) with Collision Cell Technology (CCT) (ICP-MS, Thermo Fisher Scientific X Series 2) at the Laboratory for Isotopes and Metals in the Environment (LIME) at The Pennsylvania State University. The method was adapted from previous studies (332-334). A 25-fold dilution of the serum samples were prepared by transferring 200 µL of samples into 15 mL trace element-free tubes, and 4.8 mL of 0.1N double distilled NHO3 (HNO3, trace metal grade, EMD Millipore Corporation, Darmstadt, Germany) was then added. Actual dilution factors were determined gravimetrically using a high precision scale. The samples were vortexed at high speed for 15 seconds and stored overnight for at least 24 hours before analysis. Control reference materials (Seronorm™ trace element serum L-1 and L-2 RUO, SERO AS, Billingstad Norway) were prepared and run the same way as the serum samples. Calibration standards were prepared using NIST traceable standard for each element.

External standards traceable to NIST (National Institutes of Standards and Technology, NIST 1640a) were run for every 10 samples to monitor the stability and repeatability of the actual samples. Intra- and inter-assay CVs for Zn, Cu, Mg, Mn, and iron were 2.0% and 2.6%, 3.7% and 4.0%, 3.4% and 7.0%, 5.1 and 4.9%, and 2.3 and 3.1%, respectively. Serum retinol concentration was measured using ultra performance liquid chromatography (ACQUITY UPLC System, Waters Corporation, Milford, MA)
in the laboratory of Dr. A. Catharine Ross. The same NIST standard (SRM® 1950, Gaithersburg, Maryland) was used for quality control.

Menstrual cycle hormones assays were performed by the Women’s Health and Exercise Laboratory (The Pennsylvania State University, University Park, PA) directed by Dr. Mary Jane De Souza. Plasma osmolality was measured by freezing point depressing osmometry with the use of the K-7400S Semi-Micro Osmometer (KNAUER, Wissenschaftliche Geräte GmbH, Berlin, Germany) by Dr. Asher Rosinger’s laboratory (Department of Biobehavioral Health). The coefficient of variation was < 1% across assays. Glassware were acid washed (3% nitric acid and 0.3% HCL) overnight and rinsed with double distilled water, air dried before used. High purity deionized water, 18 MΩ cm⁻¹, obtained from a milli-Q water purification system (Millipore, Bedford, MA) was used in all analytic processes.

The following cutoffs were used to define deficiency; anemia was defined using the Centers for Disease Control and Prevention criteria, hemoglobin < 12 g/dL (394); iron deficiency anemia (IDA) was defined as anemia with low serum ferritin (< 15 µg/L) (248, 394); marginal vitamin A deficiency, serum retinol < 0.70 µmol/L (20 µg/dL) (120, 395); zinc deficiency, serum zinc concentration < 70 µg/dL (10.7 µmol/L) (57, 246); and copper deficiency, serum copper < 10.0 µmol/L (63.5 µg/dL) (246). Magnesium deficiency was defined as serum magnesium concentration less than 17 mg/L (0.70 mmol/L) (396) and manganese deficiency was defined as serum concentration < 0.54 µg/L (397). We defined signs of infection or inflammation as an abnormal CRP (> 5.0 mg/L) or AGP > 1.0 mg/L (398). A combination of CRP and AGP were used to classify inflammation. Thurnham et al. categories are; no inflammation (normal CRP and AGP), incubation (raised CRP and normal AGP), early convalescence (raised CRP and AGP), late convalescence (normal CRP and raised AGP) (147).

Sample size calculation

The sample-size estimation was based on a previous report by Micho et al. where serum copper and zinc concentrations were measured across the menstrual cycle (n = 14) (235). In that study, zinc
concentration increased by more than 8-13%, while copper concentration changed by 9-17% across the menstrual cycle. The mean estimates for zinc (75.6 ± 12.4 µg/dL) and copper (107.1 ± 16.2 µg/dL) in our pilot study (n = 8, the early follicular phases) were comparable to those of Michos et al. study, 85 ± 11 µg/dL and 105 ± 11 µg/dL, respectively. We wanted to be able to detect a 10% change with an effect size of 0.66 (Cu) and 0.61 (Zn) in order to have statistical power of 80% and type I error of 5% in a two-sided test, and thus be able to detect a difference between any two of three time points. We applied a Bonferroni correction for a family-wide error rate to give a type I error rate of 1% (i.e., 0.05/5 = 0.01), to account for simultaneous testing of micronutrient concentrations as response variables. From this data, we estimated a sample size of 31 for copper and 35 for zinc (using the Power Analysis package in R). Thus, a sample size of 35 is sufficient to detect differences in zinc and copper concentrations. Applying a 29% expected dropout rate (loss to follow), yielded a sample size of 45.2, which rounds up to 46 subjects. Further, we adjusted for non-ovulatory cycles based on estimates from the BioCycle Study among women with a mean age of 27.5 years and BMI of 24.1 kg/m² (328, 329). Participants predicted to have anovulatory cycles were dropped based on fertility monitor results. Thus, we accounted for an additional 10% of women that may not ovulate, with estimated sample size of 51. The estimated sample size for other micronutrients was smaller than 35.

Statistical analysis

Data were checked for normality using the Shapiro-Wilk test and visual inspection of kernel density plots. Categorical descriptive variables were presented as frequencies (%). Continuous descriptive variables were presented as mean ± SD. Serum manganese, iron and ferritin were log transformed for regression analysis, and results were back-transformed for easier interpretation. Two extremely high values of serum iron (greater than Mean+3SD) were removed from analysis. To account for the effect of inflammation which has the potential to increase or decrease micronutrient concentrations, serum zinc, retinol, and ferritin concentrations were corrected for inflammation based on AGP and CRP using
external correction factors. Stages of inflammation: no inflammation (CRP < 5 mg/L and AGP < 1 g/L); incubation (CRP > 5 mg/L and AGP < 1 g/L); early convalescence (CRP > 5 mg/L and AGP > 1 g/L); late convalescence (CRP ≤ 5 mg/L and AGP > 1 g/L) (147). The correction factors with reference to no inflammation were; 0.92,1.05, and 1.09 for zinc (399); 0.83,0.48, and 0.65 for ferritin (399); and 1.14,1.31, and 1.12 for retinol (147). Total micronutrient biomarker circulating mass was calculated as the product of its concentration and total circulating plasma volume.

A linear mixed-effects model was used to investigate the bivariate association between menstrual phase and each micronutrient biomarkers (model 1). In this model, the micronutrient biomarker and menstrual phase (time of visit as a categorical variable) were entered into the model as fixed variables. The participant identification (ID) was included as a random effect variable to account for repeated measurements. Separate models were run for each biomarker. From these models, we obtained the changes in biomarker concentrations at visits 2 and 3 from visit 1 (early follicular phase measurements). Differences in biomarker concentrations between the late follicular phase and the midluteal phase were obtained by repeating each analysis, and with visit 2 (the late follicular phase) as the reference timepoint.

In a second model, we adjusted for the effect of background characteristics on micronutrient concentrations by adding participant’s, age, BMI and race (classified as White, Asian, and others) to model 1. These models compared mean micronutrient biomarker concentrations across the menstrual cycle (late follicular phase vs early follicular phase; midluteal phase vs late follicular phase and midluteal phase vs early follicular phase). Akaike information criterion (AIC) was used to select the covariance structure based on goodness of the fit, and the maximum likelihood was used as the parameters for the models. Wilcoxon signed-rank test was used to test the difference in differences in micronutrient concentrations between any two phases of the menstrual cycle.

Spearman correlations were run to assess the correlation between micronutrient biomarkers across all visits. In sensitivity analysis, we restricted data to participants without inflammation to examine changes in micronutrient biomarker concentrations across the menstrual cycle. Statistical analyses were conducted with the use of STATA Version 14.2 (Stata Corp, College Station, Texas).
Results

A total of 178 women were screened for the study, and of this 47 met the inclusion criteria and were enrolled. Blood data were available for 45 participants at enrollment. Six participants were dropped between the early and late follicular phase. Two more were dropped between the late follicular phase and midluteal phase visits. Additionally, at the midluteal phase visit, blood samples could not be collected from two participants because we could not successfully establish an IV line. Thus, results are presented for 45 in the early follicular phase, 39 in the late follicular phase and 35 in the midluteal phase. Participants were mostly college students, never married, and nulliparous. Overall, micronutrient concentrations were lowest during the late follicular phase compared with the early follicular phase, but only serum zinc, and magnesium concentrations showed large decreases across the menstrual cycle. All micronutrient biomarkers declined from the early to late follicular phase and the midluteal phase (except manganese (Table 5-1)).

Zinc concentration dropped by 2.8 µg/dL (3.4%) between the early and late follicular phase; 2.6 µg/dL (3.3%) between the late follicular phase and the midluteal; and 5.4 µg/dL (6.6%) from the early follicular phase to midluteal phase. Magnesium concentration fell by 0.51 mg/L (2.9%) between the early and late follicular phase; 0.31 mg/L (1.8%) between the late follicular phase and midluteal phase; and 0.82 mg/L (4.6%) between the early follicular phase and midluteal phase. Among all women serum copper, manganese, iron, ferritin, and retinol concentration remained fairly stable across the menstrual cycle (Table 5-1). Micronutrient distributions showed the same pattern across the menstrual cycle; serum zinc ranged from 48.9 to 141.7 µg/dL, copper ranged from 51.4 to 116.6 µg/dL, magnesium ranged from 13.4 to 20.9 mg/L, manganese ranged from 0.49 to 9.367 µg/L, iron ranged from 20.7 to 245.8 µg/dL, ferritin ranged from 3.7 to 169.5 µg/L, and retinol ranged from 24.6 to 63.8 µg/dL.

When we adjusted for participant age, BMI, and race, the pattern of micronutrient biomarker concentrations across the menstrual cycle was similar to the unadjusted results (Table 5-2). The point
estimates for change in micronutrient biomarker was less than 1% different between the adjusted and unadjusted models.

The prevalence of micronutrient deficiencies reflects the pattern of their concentration changes across the menstrual cycle. Generally, a lower prevalence was observed at the early follicular phase compared with either the late follicular phase or the midluteal phase. Zinc deficiency rose from 22% to 26% at the late follicular phase and further to 37% in the midluteal phase. The prevalence of magnesium deficiency rose across the menstrual cycle, and was much higher at the midluteal phase compared to the early follicular phase. Deficiency rose from 29% in the early follicular phase to 39% in the late follicular phase, and then to 49% in the midluteal phase (Figure 5-1). No participant was vitamin A deficient. More than a third of the participants were anemic at any time in the cycle, 35% (early follicular phase), 44% (late follicular phase), and 34% (midluteal phase). About 13% had iron deficiency anemia (IDA) in the early follicular phase; 18% had IDA in the late follicular phase, and 17% had IDA in the midluteal phase.

Given that most micronutrient biomarker concentrations decline between the early and late follicular phase (with higher deficiencies), it was surprising that the correlations between micronutrients varied widely. Serum zinc correlated positively with copper, magnesium, manganese, iron, ferritin, retinol and hemoglobin concentration (0.10 < r < 0.28); copper correlated with iron (r = 0.21) and retinol (r = 0.22; P < 0.05 for both); iron with retinol (r = 0.23) and hemoglobin (r = 0.31; P < 0.05 for both); and ferritin with hemoglobin (r = 0.22, P < 0.05). Magnesium showed a weak negative correlation with copper (r = -0.16, P > 0.05) and retinol (r = -0.18, P > 0.05). Retinol and ferritin showed a similar weak negative correlation (r = -0.17, P > 0.05). The correlations between other biomarkers were weak (Table 5-3).

Just like micronutrient concentration, estimated micronutrient biomarker mass tended to be lower at the late follicular phase than the early follicular phase. However, only ferritin mass showed a significant drop from the early to late follicular phase, and no difference was observed between late follicular phase and midluteal phase (Table 5-4). Inflammation as measured by AGP and CRP was higher at the early follicular phase 24.4% (n = 11), compared with 18.0% (n = 7), and 11.4% (n = 4) at the late follicular phase.
follicular phase and midluteal phase, respectively. Increasing estradiol concentration was associated with a slight decline in serum zinc and magnesium concentration, but no other biomarkers (Figure 5-2). Furthermore, the rise in progesterone was associated with a decline in serum magnesium concentration (Figure 5-3). Figure 5-4 also show that serum zinc and magnesium concentrations decline across the menstrual cycles.

In sensitivity analysis, when participants with inflammation were dropped, results were similar for all biomarkers except zinc and ferritin. The reduction in zinc concentration was much higher: there was a decline of 3.4 µg/dL (4.2%) between the early and late follicular phase ($P > 0.05$), 4.0 µg/dL (5.1%) between the late follicular phase and the midluteal phase ($P < 0.05$), and 7.4 µg/dL (9.1%) between the early follicular phase and midluteal phase ($P < 0.001$). Serum ferritin declined by 0.083 µg/L (3.0%, $P < 0.05$) from the early to late follicular phase. When we adjusted for age, BMI and race and hydration status among participants without inflammation, changes in micronutrient biomarker concentrations were comparable to the unadjusted results.
Discussion

Fluctuations in ovarian hormones are thought to play an important role in regulating intake of macronutrients but it is unclear if these variation effect micronutrient biomarker concentrations. Data on how micronutrient biomarker concentrations vary across the menstrual cycle are scarce. The few existing studies did not appropriately time blood collection in order to take into considerations key hormone changes based on objective hormone markers. Our aim was to evaluate micronutrient biomarker concentrations and changes across the menstrual cycle in a cohort of healthy women of reproductive age. We found that while all micronutrient biomarker concentrations were lowest during the late follicular phase, the reduction in zinc and magnesium concentrations were relatively large.

An analysis of serum zinc concentration from a representative data from the US (NHANES III) by Hennigar et al. showed that mean serum zinc concentration of women was 81 μg/dL (391). Though this was a cross-sectional data and results were not stratified by the menstrual phase, it compares favorably with the results from the current study, 76 μg/dL (midluteal phase) to 82 μg/dL (early follicular phase). Our results support the findings of Deuster et al. who found that plasma zinc concentrations were higher during the menses and follicular phase than at ovulation (234). However, this is in contrast with the increase in plasma zinc concentration noted by Michos et al., who reported that the highest concentrations were observed at the time of ovulation and the lowest at menstruation (235). Though the sample size was small in the Deuster et al. study, its strength lies on the fact that participants were measured across three menstrual cycles allowing for examining individual change across time.

In two studies from India (236, 237), serum magnesium concentration was reported to decline between the early (menses) and the follicular/ovulation by 6-12%, comparatively larger than observed in the current study. In other studies, and the current study, magnesium concentration was higher during the early follicular phase than the late follicular phase/ovulation. However, while other studies showed an increase between the late follicular phase/ovulation and luteal phase, we observed a decrease in concentration across the same time. The differences could be due to timing of measurement. For instance,
while measurements occurred during the midluteal phase in our study, Lanje et al. reported luteal phase measurements that occurred after cycle day 22. Consistent with our study, however, a reduction in serum magnesium concentration at ovulation and the luteal phase have been reported by Deuster et al. (222). Furthermore, Deuster et al., reported that plasma magnesium concentration was highest during menses and lowest during the late follicular phase and ovulation (234). However, Rosenstein et al. reported no changes in serum magnesium concentration across the menstrual cycle (238).

Data on serum manganese across the menstrual cycle are lacking but mean estimates of 0.58 µg/L to 1.14 µg/L (85, 400-404) have been reported in reproductive age women. One study reported an even wider range of 1.8 to 16.5 µg/L (405). Our estimates of 0.49 to 9.67 µg/L are comparable to these estimates.

Mean ferritin concentration increased by 4.2% between menses and follicular phase as reported by Kim et al. (239), and 11.6% in the luteal phase. These estimates are comparably larger than the change estimates obtained from our study, 3.4% between both timepoints. We did not find any difference in serum iron concentration across the menstrual cycle, contrary to previous reports (240). These changes are smaller than the day-to-day changes observed for serum ferritin, 8.2% to 18.8% (108-112). Our retinol estimates did not vary across the cycle and the mean estimates are comparable to a previous study (n = 9) in women who were studied across the menstrual cycle, aged 38.5 ± 4.7 years and BMI 25.9 ± 5.1, 39.0 µg/dL (406). In that study retinol concentrations were not reported across the menstrual cycle for comparison. However, in one study serum retinol concentration was lowest during menstruation, and rose to peak values at around cycle day 14(n = 6) (242). An increase in retinol concentration during the follicular phase has been also reported in another study (407). The findings from these studies contradict the results from the current study. Retinol concentration remained stable across the menstrual cycle among our study participants, consistent with previous studies that reported that retinol concentration remains fairly constant across the menstrual cycle, with negligible day-to-day variations (125).
The estimated circulating zinc mass from this study is within the expected range in healthy women. The estimates from the current study are expected to represent ~ 0.1% of total body zinc mass of 1.5 to 2.5 g (408, 409) – because circulating zinc mass constitutes about 0.1% of total body zinc (410). Thus, our estimated serum zinc mass is remarkably close to the expected value in the adult female. Mean estimated circulating copper mass of 1.8 mg is lower than the 6 mg reported for whole blood. But this is not unexpected as whole blood copper levels are higher than plasma or serum concentrations (408).

Though total body manganese is often quoted as 12-20 mg (88), data on circulating mass of magnesium is scarce. Serum magnesium constitutes about 0.3% of the total body content of ~24 g (411). The mean estimate from this study is ~0.2% of the expected value of ~24 g. These estimates are reasonable given the high prevalence of magnesium deficiency in our population. Previous studies have shown that among women aged 18-60 y, 25–27% of Polish and French, and 40% of UK women, magnesium intakes were below the EAR (412). In the United States, about 45% of Americans ≥ 2 y do not meet EAR for magnesium (70). An estimated drop of 80-90% magnesium content in fruit and vegetable over the last 100 y has been linked to the high prevalence of magnesium deficiency in US and UK (76).

Overall, micronutrient deficiencies were found to be dependent on the cycle phases when measurements were conducted. Higher prevalence was reported during the late follicular phase and the midluteal phase compared to the early follicular phase. For instance, a difference of up to 20% was observed for magnesium when measurements in the midluteal phase were compared to the early follicular phase.

Clinical and research implications

Our results suggest that menstrual cycles should be taken into consideration when evaluating serum zinc and magnesium concentrations in women of reproductive age to improve the interpretation of findings. In population settings where talking of menses is a taboo topic, it is likely that in a study involving women of reproductive age, most study participants would be available for data collection after
the early follicular phase. Therefore, without considering the cycle phase, the overall deficiencies could be overly influenced by the cycle phase (large number of participants in later phase of cycle) s– probably driven by physiological changes, which may not require any intervention.

Furthermore, though our study was not able detect statistically significant difference between menstrual phases for most of the biomarkers, we did see that differences between deficiency prevalence were quite large. This suggests that in population settings where micronutrient deficiencies are high, there is the potential to classify the public health significance of the deficiency into different categories based on the time measurements are conducted. Therefore, while we wait for these findings to be confirmed, research design should take into consideration the menstrual phase in evaluating micronutrient status, particularly for serum zinc and magnesium.

In conclusion, serum zinc and magnesium concentrations decline across the menstrual cycle in healthy women of reproductive age. The prevalence of micronutrient deficiency may be dependent on the cycle phase when measurements are conducted. Although more studies will be needed to confirm these findings, we suggest that the menstrual cycle variability should be considered, or standardized, when evaluating micronutrient concentrations or deficiencies (particularly zinc and magnesium) in women of reproductive age. Future studies should also examine the relationship between ovarian hormones and micronutrient concentrations.
Table 5-1: Mean differences in micronutrient biomarker concentrations across a single menstrual cycle in women of reproductive age (n= 45)  

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>EFP</th>
<th>LFP vs. EFP</th>
<th>MLP vs. LFP</th>
<th>MLP vs. EFP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>β (95 % CI)</td>
<td>β (95 % CI)</td>
<td>β (95 % CI)</td>
</tr>
<tr>
<td>Adjusted zinc, µg/dL</td>
<td>81.8 ± 16.2</td>
<td>-2.8 (-7.1, 1.5)</td>
<td>-2.6 (-5.4, 0.2)</td>
<td>-5.4 (-9.6, -1.3) ***</td>
</tr>
<tr>
<td>Zinc ², µg/dL</td>
<td>81.1 ± 16.5</td>
<td>-3.4 (-8.1, 1.2)</td>
<td>-4.0 (-7.3, -0.6)</td>
<td>-7.4 (-12.3, -2.6) ***</td>
</tr>
<tr>
<td>Copper, µg/dL</td>
<td>80.1 ± 12.8</td>
<td>-2.2 (-4.6, 0.2)</td>
<td>1.9 (-0.6, 4.4)</td>
<td>-0.3 (-2.8, 2.2)</td>
</tr>
<tr>
<td>Magnesium, mg/L</td>
<td>17.9 ± 1.4</td>
<td>-0.51 (-0.81, -0.22) **</td>
<td>-0.31 (-0.72, 0.11)</td>
<td>-0.82 (-1.16, -0.48) ***</td>
</tr>
<tr>
<td>Manganese ³, µg/L</td>
<td>1.51 ± 1.97</td>
<td>-0.91 (-0.77, 1.07)</td>
<td>1.18 (-0.99, 1.40)</td>
<td>1.08 (-0.87, 3.4)</td>
</tr>
<tr>
<td>Iron ³, µg/dL</td>
<td>106.7 ± 1.7</td>
<td>-0.98 (-0.81, 1.19)</td>
<td>-0.99 (-0.85, 1.17)</td>
<td>-0.98 (-0.80, 1.20)</td>
</tr>
<tr>
<td>Adjusted ferritin ², µg/L</td>
<td>26.4 ± 2.3</td>
<td>-0.87 (-0.74, 1.03)</td>
<td>-1.00 (-0.85, 1.17)</td>
<td>-0.87 (-0.73, 1.03)</td>
</tr>
<tr>
<td>Ferritin ², µg/L</td>
<td>28.2 ± 2.3</td>
<td>-0.83 (-0.71, -0.96) *</td>
<td>1.04 (-0.89, 1.21)</td>
<td>0.86 (-0.73, 1.00)</td>
</tr>
<tr>
<td>Adjusted retinol, µg/dL</td>
<td>39.4 ± 9.3</td>
<td>-0.04 (-1.96, 1.88)</td>
<td>-0.37 (-2.56, 1.82)</td>
<td>-0.41 (-3.03, 2.21)</td>
</tr>
<tr>
<td>Retinol ², µg/dL</td>
<td>37.7 ± 7.7</td>
<td>-0.15 (-2.42, 2.13)</td>
<td>-0.21 (-2.19, 1.78)</td>
<td>-0.35 (-3.05, 2.35)</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>12.1 ± 0.7</td>
<td>-0.11 (-0.23, 0.01)</td>
<td>0.08 (-0.05, 0.21)</td>
<td>-0.03 (-0.17, 0.12)</td>
</tr>
</tbody>
</table>

EFP, early follicular phase; LFP, late follicular phase prospective; MLP, Midluteal phase
1Within-group differences were calculated as recent visit minus previous visit (LFP-EFP; MLP-EFP and MLP-LFP) using a linear mixed-effects model including time as a fixed effect and subject identification as a random effect.
2 Estimates for participants with no inflammation (all such values)
3 Variables were log transformed for analysis, the results are back-transformed for proper interpretation.
**Table 5-2:** Adjusted mean differences in nutritional biomarkers concentrations across a single menstrual cycle in women of reproductive age (n = 45)\(^1\)

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>LFP vs. EFP</th>
<th>MLP vs. LFP</th>
<th>MLP vs. EFP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\beta) (95% CI)</td>
<td>(\beta) (95% CI)</td>
<td>(\beta) (95% CI)</td>
</tr>
<tr>
<td>Adjusted zinc, (\mu g/dL)</td>
<td>-2.4 (-6.8, 1.9)</td>
<td>-2.7 (-5.5, 0.1)</td>
<td>-5.1 (-9.3, -1.0) *</td>
</tr>
<tr>
<td>Zinc (^2), (\mu g/dL)</td>
<td>-3.5 (-8.2, 1.3)</td>
<td>-3.9 (-7.2, -0.6) *</td>
<td>-7.4 (-12.3, -2.5) **</td>
</tr>
<tr>
<td>Copper, (\mu g/dL)</td>
<td>-2.2 (-4.6, 0.2)</td>
<td>1.9 (-0.6, 4.4)</td>
<td>-0.3 (-2.8, 2.2)</td>
</tr>
<tr>
<td>Magnesium, mg/L</td>
<td>-0.50 (-0.80, -0.21) **</td>
<td>-0.31 (-0.72, 0.11)</td>
<td>-0.81 (-1.15, -0.47) ***</td>
</tr>
<tr>
<td>Manganese (3), (\mu g/L)</td>
<td>-0.91 (-0.77, 1.06)</td>
<td>1.18 (-0.99, 1.40)</td>
<td>1.07 (-0.86, 1.32)</td>
</tr>
<tr>
<td>Iron (^3), (\mu g/dL)</td>
<td>-0.98 (-0.81, 1.19)</td>
<td>-0.99 (-0.85, 1.16)</td>
<td>-0.97 (-0.80, 1.19)</td>
</tr>
<tr>
<td>Adjusted ferritin (^3), (\mu g/L)</td>
<td>-0.87 (-0.75, 1.02)</td>
<td>-1.00 (-0.85, 1.17)</td>
<td>-0.87 (-0.74, 1.02)</td>
</tr>
<tr>
<td>Ferritin (2^3), (\mu g/L)</td>
<td>-0.87 (-0.96, -0.72)</td>
<td>-1.04 (-0.89, 1.21)</td>
<td>-0.86 (-0.74, 1.01)</td>
</tr>
<tr>
<td>Adjusted retinol, (\mu g/dL)</td>
<td>-0.0002 (-1.89, 1.89)</td>
<td>-0.31 (-2.50, 1.89)</td>
<td>-0.31 (-2.91, 2.30)</td>
</tr>
<tr>
<td>Retinol (^2), (\mu g/dL)</td>
<td>-0.11 (-2.39, 2.17)</td>
<td>-0.15 (-2.07, 1.78)</td>
<td>-0.25 (-2.93, 2.42)</td>
</tr>
<tr>
<td>Hemoglobin, g/dl</td>
<td>-0.11 (-0.23, 0.01)</td>
<td>0.08 (-0.05, 0.21)</td>
<td>-0.03 (-0.17, 0.12)</td>
</tr>
</tbody>
</table>

EFP, early follicular phase; LFP, late follicular phase prospective; MLP, Midluteal phase

1 Within-group differences were calculated as recent visit minus previous visit (LFP-EFP; MLP-EFP and MLP-LFP) using a linear mixed-effects model including time as a fixed effect and subject identification as a random effect.

*\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\); \(P\)-value refers to maximum likelihood estimator.

2 Estimates for participants with no inflammation (all such values)

3 Natural logarithm-transformed to improve normality (results presented in logarithmic terms, not back-transformed).
Figure 5-1: Prevalence of micronutrients deficiencies and anemia across the menstrual cycle. EFP, early follicular phase; IDA, iron deficiency anemia (hemoglobin<12g/dL and ferritin<15µg/L); LFP, late follicular phase prospective; MLP, Midluteal phase. There was no vitamin A deficiency (i.e., retinol (<20µg/dL)) at any visit.
Table 5-3: Correlations between micronutrient biomarker concentrations and hemoglobin (n = 45) 1

<table>
<thead>
<tr>
<th></th>
<th>Zinc</th>
<th>Copper</th>
<th>Magnesium</th>
<th>Manganese</th>
<th>Iron</th>
<th>Ferritin</th>
<th>Retinol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.10</td>
<td>-0.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Manganese</td>
<td>0.28***</td>
<td>0.10</td>
<td>-0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>0.17</td>
<td>-0.21*</td>
<td>0.17</td>
<td>-0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferritin</td>
<td>0.13</td>
<td>0.04</td>
<td>-0.01</td>
<td>0.07</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retinol</td>
<td>0.28***</td>
<td>0.22**</td>
<td>-0.18</td>
<td>0.09</td>
<td>-0.21*</td>
<td>-0.17</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>0.26***</td>
<td>0.01</td>
<td>-0.03</td>
<td>-0.02</td>
<td>0.31**</td>
<td>0.22*</td>
<td>-0.02</td>
</tr>
</tbody>
</table>

1All values are Spearman’s rank correlations. *P < 0.05, **P < 0.01, ***P < 0.001

Table 5-4: Mean serum circulating mass of micronutrient biomarkers and inflammatory marker at early follicular phase, late follicular phase, and midluteal phase in menstrual cycle of reproductive age women (n =45) 1.

<table>
<thead>
<tr>
<th>Biomarker mass</th>
<th>EFP</th>
<th>LFP</th>
<th>MLP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>N</td>
<td>44</td>
<td>39</td>
<td>35</td>
</tr>
<tr>
<td>Zinc, mg</td>
<td>1.9 ± 0.6a</td>
<td>1.8 ± 0.5ab</td>
<td>1.7 ± 0.4bc</td>
</tr>
<tr>
<td>Copper, mg</td>
<td>1.8 ± 0.4</td>
<td>1.7 ± 0.5</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td>Magnesium, mg</td>
<td>41 ± 9</td>
<td>39 ± 9</td>
<td>38 ± 10</td>
</tr>
<tr>
<td>Manganese, µg</td>
<td>2.8 [2.0, 5.5]</td>
<td>3.1 [2.0, 4.3]</td>
<td>3.3 [2.1, 5.6]</td>
</tr>
<tr>
<td>Iron, mg</td>
<td>2.4 [1.7, 3.9]</td>
<td>2.3 [1.7, 3.3]</td>
<td>2.1 [1.3, 3.7]</td>
</tr>
<tr>
<td>Ferritin, µg</td>
<td>54.0 [29.8, 134.3]</td>
<td>49.8 [23.5, 99.8]</td>
<td>44.7 [28.0, 89.7]</td>
</tr>
<tr>
<td>Retinol, mg</td>
<td>0.9 ± 0.3</td>
<td>0.9 ± 0.3</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>Hemoglobin, g</td>
<td>419 ± 97</td>
<td>409 ± 104</td>
<td>409 ± 101</td>
</tr>
</tbody>
</table>

1Within-group differences were calculated as recent visit minus previous visit (LFP-EFP; MLP-EFP and MLP-LFP); Means (95 % CIs) and P values were estimated using a linear mixed-effects model including nutritional biomarkers and time as fixed effects, with subject identification as a random effect. Biomarker mass was obtained by multiplying plasma volume by biomarker concentration.

*P < 0.05, **P < 0.01; P-value refers to maximum likelihood estimator.

a,b,c Mean values with different superscripts are statistically significantly different from each other (P<0.05).

2 Samples size were (EFP = 39; LFP = 33; MLP = 30)

3 Median; interquartile range (all such values).
Figure 5-2: Fractional polynomial regression (prediction line) of estradiol concentration (log) on biomarker concentration; log of progesterone concentration on zinc (A), copper (B), magnesium (C), manganese (D), log of iron (E), log of ferritin (F), retinol (G), and hemoglobin (H). Solid black lines represent the prediction line. Short-dash gray lines represent the 95% prediction interval for the prediction line. (118 data points, n=45).
Figure 5-3: Fractional polynomial regression (prediction line) of progesterone concentration (log) on biomarker concentration: log progesterone concentration on zinc (A), copper (B), magnesium (C), log of manganese (D), log iron (E), log ferritin (F), retinol (G), and hemoglobin (H). Solid black lines represent the prediction line. Short-dash gray lines represent the 95% prediction interval for the prediction line (118 data points, n=45).
Figure 5-4: Fractional polynomial regression (prediction line) of micronutrient biomarker concentration on day of cycle when measurement occurred; zinc (A), copper (B), magnesium (C), manganese (D), log of iron (E), log of ferritin (F), retinol (G), and hemoglobin (H). Solid black lines represent the prediction line. Short-dash gray lines represent the 95% prediction interval for the prediction line. (118 data points, n=45).
Chapter 6

The relationship between plasma volume and micronutrient biomarker concentrations and mass in women of reproductive age

Abstract

**Background**: Plasma volume changes have been linked to changes in biomarker concentrations due to hemodilution, but most of our current understanding is based on studies during pregnancy. Little is known about the relationship between plasma volume and micronutrient biomarkers in nonpregnant women.

**Objectives**: Our aim was to examine the relationship between plasma volume and micronutrient biomarkers in healthy nonpregnant women.

**Methods**: We conducted a longitudinal study with 3 visits across a single menstrual cycle (n=45). At each visit, participants provided fasting blood samples to measure estrogen, progesterone, micronutrient biomarker concentrations (zinc, copper, magnesium, manganese, iron, retinol) and plasma volume. A bolus dose of ICG (0.25 mg/kg body weight) was injected followed by a series of blood draws from 2 minutes to 5 minutes post-ICG injection. Plasma volume was measured using blood samples collected before and after the ICG injection. Total circulating micronutrient biomarker mass was calculated from plasma volume and biomarker concentration. We used Spearman's correlation to assess the relationship between plasma volume and each micronutrient biomarker concentration and mass. Fractional polynomial regression was used to examine bivariate relationships between plasma volume and micronutrient biomarkers.

**Results**: The correlations between plasma volume and micronutrient biomarker concentrations ranged from -0.05 to 0.10 ($P > 0.05$); none showed a meaningful relationship. Plasma volume and biomarker mass were strongly positively correlated, ranging from 0.26 to 0.95 ($P < 0.05$). For mass, five out of the eight biomarkers showed a correlation coefficient > 0.70 ($P < 0.001$) with plasma volume.
**Conclusion:** In this prospective cohort study, we found that plasma volume correlated strongly with biomarker mass of zinc, copper, magnesium, retinol, and hemoglobin; plasma volume did not correlate with concentrations.

**Keywords:** fluid retention, intravascular volume, hemodilution, blood volume, menstrual cycle, ovarian cycle, micronutrient, nutritional biomarker, indocyanine green.
Introduction

Plasma volume, total of the liquid component of blood, is the medium for transporting micronutrient biomarkers and other metabolites in the body. Small changes in plasma volume occur with increasing temperatures and intensive exercise (149, 150, 152, 156), but a comparatively large change is observed during pregnancy—an average rise of 42% to 50% has been reported (168, 254, 255). Changes in plasma volume have been associated with changes in micronutrient biomarkers, such as zinc and folate. The rise in plasma volume during pregnancy, which starts after gestational week six (159, 163), peaking in the late pregnancy (259) is associated with a decline in the concentration of hemoglobin (259, 260), zinc (257) and folate (258) but an increase in the concentration of copper (257), compared to the nonpregnant state.

While the changes in concentrations of zinc and copper are in an opposite direction with the change in plasma volume during pregnancy, the mass of both micronutrients increase with that of plasma volume (257). This would suggest that micronutrient concentrations and plasma volume are independent, such that biomarker mass increases in responses to plasma volume but that concentrations are maintained based on the specific nature of the biomarker. Poor plasma volume expansion has been associated with adverse pregnancy outcomes, such as pre-eclampsia and fetal growth-restriction (335-338). Similarly, micronutrient deficiencies are associated with poor fetal growth, low birthweight, and SGA (36).

Compared to pregnancy, observed changes in plasma volume in nonpregnant women are small. Intravascular fluid retention is highest in the early follicular phase of the cycle (413), but little is known about the influence of hemodilution on micronutrient biomarkers during the menstrual cycle. Furthermore, studies have reported associations between ovarian hormones and micronutrient biomarker concentrations during the menstrual cycle (222, 243, 414, 415), and fluctuations in plasma volume across the cycle (159, 160, 220). However, little is known about the relationship between plasma volume and micronutrient status in nonpregnant women. Only two studies have evaluated the impact of plasma volume on biomarker concentrations (lipoproteins) across the menstrual cycle (160, 219). Yet in those
studies, plasma volume calculated from changes in hemoglobin and hematocrit values, and not measured
with direct methods.

To our knowledge, no previous study has concurrently measured plasma volume and
micronutrient biomarker concentrations across the menstrual cycle to examine their relationship, though
this area of research has been encouraged (221, 416). The aim of the present study, therefore, was to
examine the relationship between plasma volume and micronutrient biomarkers in a cohort of healthy
women of reproductive age.
Subjects and Methods

Study design and participants

This was longitudinal prospective cohort study in healthy women of reproductive age. Nonpregnant and non-breastfeeding women with regular menstrual cycles, 18-44 years of age, and with normal BMI and blood pressure were eligible. Women were not using any hormonal contraceptive for the last three months preceding the study. We screened out participants with known allergy to shellfish or iodine or low/high blood pressure (SBP < 90 or > 130 mmHg and/or DBP < 60 or > 80 mmHg). A convenience sample was recruited from the State College area by advertisement.

Participants were primarily screened via telephone and those eligible were further screened at the study site – the Clinical Research Center (CRC), which is a service unit in The Pennsylvania State University's Clinical and Translational Science Institute, University Park. Participants arrived at the study site after an overnight fast (12 hours). Protocols and measurements across all visits were similar, except that during visit 1 participants completed a more detailed screening form as well as a socio-demographic, health history, and pregnancy history questionnaire and height measurement. To complete the study, participants made 3 separate visits across the menstrual cycle, timed to key menstrual cycle hormones, thus visit 1 (around cycle day ~2), visit 2 (cycle day ~12) and visit 3 (cycle day ~21), adjusted for menstrual cycle length. Forty-seven healthy women of reproductive age volunteers were enrolled and 45 had micronutrients measured in blood samples for inclusion in the analysis.

Pregnancy tests were performed with the use of the QuickVue pregnancy test (an early pregnancy detection test for human chorionic gonadotropin in urine), and blood pressure was measured by the American Heart Association procedure, including 5 minutes of seated rest beforehand (330). Height was measured and recorded to the nearest 0.1 cm (Scale–Tronix, Inc., White Plains, NY) and weight to the nearest 0.1 kg (wall-mounted Seca stadiometer) by trained research staff. Percentage body fat mass (%) was measured using Tanita InnerScan Body Composition Monitor (Tanita Corporation of America Inc.,
Arlington Heights, IL). Following screening and measurements of weight, height and body fat percentage, women rested in supine position on a hospital-style room in preparation for an intravenous (IV) catheter insertion and blood collection.

**Blood collection and plasma volume determination**

The phlebotomist inserted an IV in the antecubital vein in the left or right arm, after the participant rested for 15 minutes. Blood samples were collected into 3 trace element-free vacutainer tubes: a 6 mL EDTA-free tube and 6 mL and 2 mL EDTA-treated tubes, for acquiring serum, plasma, and whole blood, respectively. Detailed methods for our plasma volume measurement has been published elsewhere (331). In brief, a bolus dose of indocyanine green (ICG, IC-GREEN®, AKORN Inc, Lake Forest, IL) (0.25 mg/kg body weight) was injected through the IV line and immediately flushed with saline solution. A timer was set and used to guide blood collection, starting at 2 minutes and ending at 5 minutes after injection. Blood samples were collected at an interval of ~45 seconds into a 3 mL EDTA-containing trace element-free tubes (5 tubes total). A 10 mL syringe used for the dye injection was weighed on a high precision scale to determine the actual weight of the dye injected. To obtain serum, the 6 mL EDTA-free tube was allowed to clot then centrifuged at 3200 rpm for 15 minutes; the supernatant was aliquoted into microcentrifuge tubes and stored at −80°C, until analysis. Plasma samples (6 mL and 3 mL EDTA tubes were centrifuged immediately at the same settings as serum samples and the supernatant aliquoted into the cryovials. Samples for CBC were not centrifuged but sent to the lab for analysis within 1 hour of blood collection.
**Measurement of micronutrient biomarkers**

Serum zinc, copper, magnesium, manganese, and iron were analyzed by means of inductively coupled plasma mass spectrometry (ICP-MS) with collision cell technology using a Thermo Fisher Scientific X Series 2 (Thermo Fisher Scientific, Lanham, MD) at the Laboratory for Isotopes and Metals in the Environment (LIME) at The Pennsylvania State University. Seronorm serum samples were used as quality control check (seronorm L-1 and L-2; Seronorm™ Trace Elements Serum; SERO AS, Billingstad, Norway). Serum retinol concentration was measured in the laboratory of Dr. Catherine Ross using ultra performance liquid chromatography (ACQUITY UPLC System, Waters Corporation, Milford, MA). NIST (National Institute of Standards and Technology, SRM® 1950, Gaithersburg, Maryland) samples were run as for quality control. Ferritin assays (Ramco Laboratories, Inc., Strafford, Texas) were performed in Dr. Murray-Kolb’s laboratory using enzyme linked immunosorbent assay (ELISA) methods. Similarly, α1-acid glycoprotein (AGP) and C-reactive protein (CRP) were assayed by ELISA methods using commercial kits from Kent Laboratories (Kent Laboratories Inc., Bellingham, Washington).

Manganese concentrations were below the instrument detection limit for 16 samples. No other micronutrient biomarker concentrations were below or above the limits of detection.

**Statistical analysis**

All data points across the menstrual cycle were included in analysis without restriction to cycle phase. We calculated mean ± SD for data with normal distribution otherwise, median [interquartile range] were presented. Ferritin and iron data were log transformed for normality and used in analysis. Results were back-transformed for proper interpretation. Serum zinc, ferritin and retinol were corrected for inflammation using measured CRP and AGP values, and external correction factors. Two values of serum iron were considered extremely high and were removed from all analysis. Stage of inflammation: no inflammation (CRP < 5 mg/L and AGP < 1 g/L); incubation (CRP > 5 mg/L and AGP < 1 g/L); early
convalescence (CRP > 5 mg/L and AGP > 1 g/L); late convalescence (CRP ≤ 5 mg/L and AGP > 1 g/L) (147). The correction factors with reference to no inflammation were: 0.92, 1.05, and 1.09 for zinc (399); 0.83, 0.48, and 0.65 for ferritin (399); and 1.14, 1.31, and 1.12 for retinol (147). Biomarker mass was obtained by multiplying plasma volume and biomarker concentration. Body surface area was estimated with the Dubois equation (363). Relationships between plasma volume and biomarker concentrations and mass were examined using Spearman’s correlation. Fractional polynomial regression was performed to graphically examine the relationship between plasma volume and biomarker concentrations and mass. In sensitivity analyses, extreme values were removed for serum iron and women with inflammation were dropped. Values ± 3SD from the mean were considered extreme. Data were analyzed using Stata version 14.3 (Stata-Corp., College Station, TX).
Results

Characteristics of the study samples

Out of the total 45 women who had micronutrient and plasma volume data for this study, a total of 39 (86.7%) women were not married; 48.9% self-identified as white, 31.1% as Asian, 6.7% as black, and 4.4% as two races (2.2% did not identify a race). Characteristics of the study participants are presented in Table 6-1. Most women were in their 20s. The mean ± SD of the menstrual cycle length was 28.0 ± 1.9 days.

Micronutrient biomarker concentration and mass

Table 6-2 presents a summary of micronutrient biomarker concentrations and mass. Data for serum zinc, copper, magnesium and retinol as well as whole blood hemoglobin were normally distributed and values ranged from 49–142 µg/dL, 51–117 µg/dL, 13–21 mg/L, 25–64 µg/dL and 9–14 g/dL, respectively. Manganese, iron, and ferritin were not normally distributed. The ranges of concentrations for manganese, iron, and ferritin were 0.49–9.7 µg/L, 21–285 µg/dL, and 4–170 µg/L. Circulating mass for zinc, copper, magnesium, and retinol had ranges of 1.0–4.0 mg, 1.0–3.8 mg, 23–63 mg, and 0.4–2.0 mg, respectively. Total circulating hemoglobin mass ranged from 197–706 g.

None of the participants were deficient in vitamin A (as measured by low retinol concentration). The deficiencies of zinc, copper, magnesium, manganese and iron were 28%, 22%, 38%, 16% and 13%, respectively. About 38% were anemic and 16% had iron-deficiency anemia.
Correlation of plasma volume with micronutrient biomarker concentrations and mass

The correlation between plasma volume and micronutrient concentrations were weak, ranging from -0.05 to 0.10 (Table 6-3). Except for copper all other correlations were positive.

Correlations were positive and stronger between biomarker mass and plasma volume, ranging from 0.26 to 0.95 with low p values (most < 0.001). The correlations were greater than 0.70 for five out of the eight biomarkers. The correlation coefficients for biomarker concentrations and mass with plasma volume were similar overall when examined separately at each of 3 timepoints in the menstrual cycle (Appendix 6-1).

Figure 6-1 shows the relationship between plasma volume and biomarker concentration across measurements. Plasma volume is evenly distributed across the ranges of each biomarker concentration with no clear pattern. On the other hand, plasma volume showed a strong positive correlation with zinc, copper, magnesium, retinol and hemoglobin mass (Figure 6-2). Both serum iron and ferritin showed a weak positive trend with plasma volume. Changes in plasma volume between any two timepoints were not associated with changes in biomarker concentration across the same time. Changes in plasma volume were strongly associated with changes in biomarker mass (Appendix 6-2).

In sensitivity analysis when extreme manganese values were removed, the concentration and plasma volume were moderately negatively correlated, \( r(94) = -0.24, p = 0.023 \). Manganese mass was not associated with plasma volume across the cycle. Removing extreme serum iron and ferritin values did not change their correlations with plasma volume. However, circulating iron mass and plasma volume were moderately negatively correlated, \( r(114) = -0.37, P < 0.001 \), after removing the two extreme values. The correlations between plasma volume and biomarker concentration and mass did change when women with inflammation were dropped.
In this study among healthy women of reproductive age, plasma volume was not correlated with micronutrient biomarker concentrations. On the other hand, plasma volume and biomarker mass were strongly positively correlated for zinc, copper, magnesium, retinol, and hemoglobin. Weak to moderate correlations were observed between manganese, iron, and ferritin mass with plasma volume. Participants were healthy women and plasma volume and micronutrient concentrations were comparable to other studies in women of reproductive age.

In studies of pregnancy, plasma or serum zinc concentrations decline as gestation progresses (251, 257, 417, 418). As the plasma volume expands, the mean plasma zinc concentrations decline steadily from 8 and 22 weeks of gestation and thereafter plateau (251). Some studies report that plasma zinc concentration starts to decrease from as early as six weeks gestation (419), which is about the same time that plasma volume starts to increase (159, 164). A review by Donangelo and King (53) shows that plasma or serum zinc concentration declines 15%–35% by late pregnancy compared to pre-pregnancy or early pregnancy concentrations and that this decline in plasma zinc levels is related to the plasma volume expansion. Similarly, concentrations of folate (258) and hemoglobin (259, 260) fall during pregnancy partly related to rise in the plasma volume. Both plasma zinc concentration and plasma volume return to non-pregnancy state by 6-8 weeks postpartum (252, 253, 420). Plasma volume has been shown to be related strongly with circulating mass of zinc and copper (257).

We speculate that differences in plasma volume observed during the menstrual cycle are not large enough to offset zinc homeostasis– the plasma volume increased by only 3% compared with about 50% increased during pregnancy, which is associated with a decrease of 15-35% in serum zinc concentration (168, 254). There is evidence from our work that plasma volume may have a bigger influence on circulating micronutrient mass than on their concentration across the menstrual cycle. Physiologically, this suggests that as plasma volume increases, the body adapts by releasing more solutes into the plasma.
thereby increasing the total circulating mass of the biomarker to maintain normal function without changing the concentration available to the cells. These mechanisms appear to work differently for various biomarkers. By looking the strength of the correlation between biomarker mass and plasma volume, it seems that larger changes in plasma volume maybe associated with significant changes in hemoglobin and magnesium mass. Though the literature is not clear on magnesium, a significant reduction in hemoglobin concentration is observed in normal physiological pregnancy. Copper, zinc and retinol also follow the same pattern as magnesium and hemoglobin but to a lower extent. It appears that manganese, serum iron and ferritin respond to plasma volume differently. Suggesting that though their status may be influenced by increases in plasma volume, changes are relatively small compared to that of zinc, copper, magnesium and retinol. The physiological mechanism regulating these biomarkers probably responds to plasma volume changes differently.

Increases in zinc mass with plasma volume has been reported by other authors (252). For copper, it appears that increases in intravascular volume are associated with increases in circulating copper as has been reported in pregnant women, and this appears to increase in the presence of both estrogen and progesterone (midluteal). Manganese and iron follow the same pattern as zinc, as confirmed by their correlation coefficients, suggesting that they may share the same mechanism of physiological regulations. As more studies are conducted in this area, a clear pattern of how plasma volume influences these micronutrient biomarker concentrations will be established. For instance, measurement of blood volume has been reported to reduce the number of patients diagnosed with anemia and has the potential to help physician decide whether fluid infusion is necessary (421). Because biomarker concentrations are usually maintained within a tight range, the mass is changing with plasma volume to ensure that concentration is kept within a relatively narrow range.
Strengths and limitations

Several micronutrient biomarkers were measured in this study, which is not commonly done. All biomarkers and plasma volume were based on repeated measures further strengthening results. Finally, to our knowledge, this the first time that plasma volume and micronutrient biomarkers were concurrently measured in nonpregnant women over time.

However, plasma volume measurement was done using an invasive method that involved repeated blood draws and injection of a tracer, which may be difficult for other investigators to replicate. Also, the time window within which plasma volume should be measured following blood collection is narrow, within 6 hours of preparing the ICG solution, which means that application of the method requires adequate preparation as the plasma samples cannot be frozen, and analyzed latter as done for micronutrients. Participants in this study were healthy women with regular menstrual cycle, finding may differ for overweight and or obese women.

Conclusions and recommendations for future research

Plasma volume was not correlated with micronutrient biomarker concentrations in healthy women of reproductive age, but circulating mass of micronutrient biomarkers were strongly positively correlated with plasma volume. Future studies will be needed to evaluate changes in plasma volume are related to circulating biomarker mass in population where micronutrient deficiencies are high.
Table 6-1: Characteristics and health measurements of women of reproductive age (n = 45)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>24.3 ± 5.6</td>
</tr>
<tr>
<td>Height, cm</td>
<td>164.2 ± 6.9</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>58.5 ± 6.7</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>21.7 ± 1.9</td>
</tr>
<tr>
<td>Fat mass, kg</td>
<td>24.9 ± 5.4</td>
</tr>
<tr>
<td>Fat mass, %</td>
<td>14.8 ± 4.5</td>
</tr>
<tr>
<td>Fat-free mass, kg</td>
<td>43.6 ± 3.8</td>
</tr>
<tr>
<td>Fat-free mass, %</td>
<td>75.1 ± 5.4</td>
</tr>
<tr>
<td>Body surface area, m²</td>
<td>1.63 ± 0.12</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>68 ± 6</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>105 ± 8</td>
</tr>
<tr>
<td>Plasma volume</td>
<td>2247 ± 491</td>
</tr>
<tr>
<td>mL/kg body weight</td>
<td>39 ± 7</td>
</tr>
<tr>
<td>mL/kg lean mass</td>
<td>51 ± 10</td>
</tr>
<tr>
<td>mL/m² body surface area</td>
<td>1373 ± 262</td>
</tr>
<tr>
<td>Hydration</td>
<td></td>
</tr>
<tr>
<td>Urine specific gravity</td>
<td>1.0167 ± 0.0081</td>
</tr>
<tr>
<td>Urine osmolality, mOsm/kg of H₂O</td>
<td>603 ± 280</td>
</tr>
<tr>
<td>Plasma osmolality, mOsm/kg of H₂O</td>
<td>298 ± 8</td>
</tr>
</tbody>
</table>

1Body fat percentage was measured by bioelectrical impedance analysis using a TANITA body scan scale.
2Body surface area was estimated by using height and weight equation of Dubois (363).
Table 6-2: Micronutrient biomarker and hemoglobin concentrations and mass of women of reproductive age (n = 45)\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>Biomarker concentration</th>
<th>Biomarker mass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Unit</td>
</tr>
<tr>
<td>Zinc</td>
<td>79.2 ± 13.3</td>
<td>µg/dL</td>
</tr>
<tr>
<td>Copper</td>
<td>79.4 ± 13.2</td>
<td>µg/dL</td>
</tr>
<tr>
<td>Magnesium</td>
<td>17.4 ± 1.4</td>
<td>mg/L</td>
</tr>
<tr>
<td>Manganese</td>
<td>1.4 [0.9, 2.4]</td>
<td>ng/mL</td>
</tr>
<tr>
<td>Iron</td>
<td>100.1 [77.9, 156.5]</td>
<td>µg/dL</td>
</tr>
<tr>
<td>Ferritin</td>
<td>21.1 [12.9, 47.3]</td>
<td>ng/mL</td>
</tr>
<tr>
<td>Retinol</td>
<td>39.0 ± 7.9</td>
<td>µg/dL</td>
</tr>
<tr>
<td>Hemoglobin(^2)</td>
<td>12.1 ± 0.8</td>
<td>g/dL</td>
</tr>
</tbody>
</table>

\(^1\)Estimates are based on all samples across 118 data points for each biomarker, except for manganese which had 16 values below the limit of detection and iron, which had 2 extreme outliers (423 µg/dL and 2705 µg/dL were removed).

\(^2\)Measured in whole blood, all others were measured in serum.

Table 6-3: Spearman’s correlations of plasma volume with micronutrient biomarker and hemoglobin concentrations and mass of women of reproductive age (n = 45)\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>Biomarker concentration</th>
<th>Biomarker mass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>P</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.01</td>
<td>0.899</td>
</tr>
<tr>
<td>Copper</td>
<td>-0.02</td>
<td>0.858</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.08</td>
<td>0.412</td>
</tr>
<tr>
<td>Manganese</td>
<td>0.04</td>
<td>0.719</td>
</tr>
<tr>
<td>Iron</td>
<td>-0.05</td>
<td>0.579</td>
</tr>
<tr>
<td>Ferritin</td>
<td>0.10</td>
<td>0.270</td>
</tr>
<tr>
<td>Retinol</td>
<td>0.10</td>
<td>0.278</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>0.01</td>
<td>0.921</td>
</tr>
</tbody>
</table>

\(^1\)Estimates are based on all samples across 118 data points for each biomarker, except for manganese which had 16 values below the limit of detection and iron, which had 2 extreme outliers (423 µg/dL and 2705 µg/dL were removed).
Figure 6-1: Fractional polynomial regression (prediction line) of plasma volume on biomarker concentrations: plasma volume on zinc (A), copper (B), magnesium (C), manganese (D), iron (E), ferritin (F), retinol (G), and hemoglobin (H). Solid black lines represent the prediction line. Short-dash gray lines represent the 95% prediction interval for the prediction line. (118 data points for 44 women).
Figure 6-2: Fractional polynomial regression (prediction line) of plasma volume on biomarker mass: plasma volume on zinc (A), copper (B), magnesium (C), manganese (D), iron (E), ferritin (F), retinol (G), and hemoglobin on plasma volume (H). Solid black lines represent the prediction line. Short-dash gray lines represent the 95% prediction interval for the prediction line. (118 data points).
Appendix 6-1: Spearman’s correlations of plasma volume and micronutrient biomarkers and hemoglobin concentrations and mass of women of reproductive age across the menstrual cycle (n=45)

<table>
<thead>
<tr>
<th>Biomarker concentration</th>
<th>Biomarker mass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EFP</td>
</tr>
<tr>
<td>N</td>
<td>44</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.15 (0.35)</td>
</tr>
<tr>
<td>Copper</td>
<td>-0.13 (0.39)</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.06 (0.70)</td>
</tr>
<tr>
<td>Manganese ²</td>
<td>0.08 (0.63)</td>
</tr>
<tr>
<td>Iron</td>
<td>-0.04 (0.82)</td>
</tr>
<tr>
<td>Ferritin</td>
<td>0.07 (0.66)</td>
</tr>
<tr>
<td>Retinol</td>
<td>0.13 (0.40)</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>-0.002 (0.99)</td>
</tr>
</tbody>
</table>

PV, plasma volume; EFP, early follicular phase; LFP, late follicular phase prospective; MLP, Midluteal phase. For manganese

² Values are correlation coefficient (r) (P-value)

Samples size were (EFP = 39; LFP =33; MLP = 30)

Appendix 6-2: Spearman’s correlation between change (%) in plasma volume with change (%) in biomarker concentration and mass (n=45)

<table>
<thead>
<tr>
<th>Biomarker concentration</th>
<th>Biomarker mass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V1-V2</td>
</tr>
<tr>
<td>Zinc, %</td>
<td>-0.05 (0.769)</td>
</tr>
<tr>
<td>Copper, %</td>
<td>-0.05 (0.757)</td>
</tr>
<tr>
<td>Magnesium, %</td>
<td>-0.20 (0.236)</td>
</tr>
<tr>
<td>Manganese, %</td>
<td>-0.10 (0.584)</td>
</tr>
<tr>
<td>Iron, %</td>
<td>-0.07 (0.665)</td>
</tr>
<tr>
<td>Ferritin, %</td>
<td>-0.20 (0.227)</td>
</tr>
<tr>
<td>Retinol, %</td>
<td>-0.17 (0.312)</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>0.05 (0.925)</td>
</tr>
</tbody>
</table>

V1, early follicular phase; V2, late follicular phase; V3, midluteal phase
Chapter 7

Research Summary, Discussion and Future Perspectives

The purpose of this dissertation was to evaluate how the menstrual cycle impacts plasma volume and micronutrient concentrations. We were particularly interested in evaluating the influence of the menstrual cycle phase on plasma volume and nutritional status markers of vitamins and minerals. Specifically, we examined zinc, copper, magnesium, manganese, iron, and vitamin A. Additionally, we evaluated the relationship between plasma volume and micronutrient biomarkers in women of reproductive age, to determine if plasma volume might be an important factor in interpreting biomarkers.

The first objective was to estimate plasma volume change across the menstrual cycle in women of reproductive age. Plasma volume was measured at three time points: early and late in the follicular phase and at the midpoint of the luteal phase. Measurements were timed to the period of low estrogen and progesterone, high estrogen, and high progesterone, respectively. We found that plasma volume was highest at the early follicular phase and lowest at the midluteal phase. Plasma volume decreased by 2.2% between the early and late follicular phase and 2.4% by the midluteal phase. Thus, changes across the menstrual cycle were small.

The second objective was to examine changes in micronutrient biomarkers (for zinc, copper, magnesium, manganese, iron, and retinol) and hemoglobin. The concentrations of all micronutrients were lowest in the late follicular phase. Serum zinc and magnesium showed a large decrease from the early to late follicular phase and from the early follicular phase to the midluteal phase. All micronutrient biomarker concentrations showed some reduction between the early and late follicular phase. As a consequence of these variations, the prevalence of micronutrient deficiencies was higher at the late follicular phase than at the early follicular phase. In general, concentrations for all micronutrient biomarkers were higher at the early follicular phase than at the late follicular phase but the direction of
change between the late follicular phase and midluteal phase was different across micronutrients. Micronutrient deficiencies were also higher in the midluteal phase than the early follicular phase.

These changes in micronutrient concentration and deficiency prevalence estimates are not usually considered when evaluating micronutrient status in women of reproductive age. The implication of ignoring cyclic variations in micronutrient concentrations and deficiencies across the menstrual cycle is that different estimates of deficiencies could be observed depending on the cycle phase when measurements are conducted. For instance, if measurements occurred in the late follicular phase, the estimates of zinc deficiency could be higher. However, some micronutrient deficiencies did not change across the cycle. This suggests that the menstrual cycle phase when measurements occurred may contribute to differences in prevalence estimates across time in a population, but perhaps only for some micronutrients.

Our third objective was to evaluate the relationship between plasma volume and micronutrient concentrations in healthy women of reproductive age. While we hypothesized that plasma volume would have a small impact on micronutrients circulating in the plasma, we found that plasma volume was not correlated with micronutrient concentrations, and this was true across a range of biomarkers. On the other hand, plasma volume was strongly positively related to micronutrient biomarker mass. These findings support the physiologic concept that under normal conditions, plasma volume is tightly regulated in healthy individuals (158, 422).

Though more attention is often focused on micronutrient status during pregnancy, nutritional status during adolescence is important for optimal growth and development, and to establish body nutrient reserve in preparation for pregnancy and beyond (423, 424). Therefore, research on micronutrient status in women of reproductive age is important, including measuring micronutrient status, and accurately classifying deficiency. Reducing deficiencies in women is a major focus of public health nutrition globally. From the results in our small but rigorous study, measuring micronutrient concentrations in
different phases of the menstrual cycle may affect how we estimate the prevalence of their deficiencies in a population.

Micronutrient status preconception is important as women prepare for pregnancy, and an understanding of how micronutrients vary across the menstrual cycle can help clinicians and researchers in deciding when it is appropriate to assess the nutritional status of a woman. For instance, just before and until immediately after ovulation, lack of zinc in the diet causes severe problems with egg quality (in animal models) and pregnancy success (54, 425). Accurately measuring micronutrients and plasma volume in the nonpregnant state will also provide useful information for estimating how much biomarkers change into pregnancy. Effort should be made in understanding how micronutrient biomarkers vary within the cycle and whether this affects the deficiency estimates in the population.

This study has the advantage that we prospectively tracked healthy women with regular menstrual cycles across the menstrual cycle, with each visit timed to specific key hormones using a simple urine-based technology that relies on personal fertility testing. This allowed us to accurately define menstrual cycle phases; to predict when relevant hormones were likely to be high; and to schedule study visits accordingly. In addition, we measured serum estradiol and progesterone concentration across three time points to confirm results from the fertility monitor, and to evaluate ovulatory cycles.

To our knowledge, no previous study has simultaneously measured plasma volume and several micronutrient biomarker concentrations across the menstrual cycle for the same group of women. Furthermore, no study has measured plasma volume and micronutrients across the menstrual cycle. By measuring several micronutrient biomarkers across the menstrual cycle in addition to plasma volume and ovarian hormones, it allowed us to describe changes across menstrual phases. Additionally, because of the longitudinal nature of the study, we were able use mixed-effect modelling to estimate inter- and intra-individual changes in plasma volume and micronutrient biomarkers across the menstrual cycle.

Furthermore, we expect that this study is the first to prospectively provide data on the relationship between plasma volume and micronutrient biomarkers across the menstrual cycle. The study
demonstrates the importance of cyclic variation on serum zinc and magnesium concentrations but also to a lesser extent other micronutrient biomarkers, and their deficiencies, in women of reproductive age. We provided preliminary data that could serve as a guide for future studies evaluating micronutrient status and deficiencies in women of reproductive age.

Overall, we found that plasma volume changes slightly across the menstrual cycle among healthy women of reproductive age, while changes in micronutrient concentrations are considerably large for a few nutrients. Plasma volume and micronutrient concentrations are not related in healthy nonpregnant women. This implies that in normal healthy nonpregnant women of reproductive age, it is not expected that plasma volume will be needed to aid in the interpretation of micronutrient status from blood-based biomarkers. However, the menstrual cycle phase may be needed to interpret micronutrient status in nonpregnant women. Future investigations of zinc and magnesium concentrations in nonpregnant women of reproductive age should take into consideration the menstrual cycle phase when measurements occurred.

Further studies are needed to create a body of evidence on micronutrient concentrations and variations across the menstrual cycle to help evaluate how micronutrient biomarker concentrations vary across the menstrual cycle. Studies should be powered to examine changes in deficiencies over several cycles to estimate the population prevalence by cycle phase across time, parity, age, and BMI, population settings (e.g., low-income countries). Studies should aim to provide estimates of the normal range for each phase of the menstrual cycle to allow for easy comparison, such that irrespective of the time measurements occurred, sound conclusions can be made about micronutrient status.
Recruitment material for ResearchMatch

We welcome you to participate in a study that examines the relationship between the menstrual cycle (often called a period), blood volume, and vitamins and minerals, like iron. Both the amount of plasma in the blood and levels of vitamins and minerals can be important for a woman’s health and the health of a pregnancy she might have in the future. If you choose to participate, this study will take place over one complete menstrual cycle and involves three visits to the Penn State Clinical Research Center (CRC) in State College, Pennsylvania to collect study data and specimens. Each visit takes approximately 1.5 hours. Free and convenient research parking is provided on site. You would receive a fertility monitor at the first visit to track your menstrual cycle at home and these at-home testing results will be used to schedule your remaining visits. The monitor would be returned but the results of the tests are yours to keep for your own knowledge.

You might be eligible for this study if you are:
- A female between 18 and 44 years old
- Not allergic to iodine or shellfish
- Have a regular menstrual cycle
- Are not using hormonal birth control (e.g., Mirena, intrauterine devices or IUDs, Depo-Provera – “the shot”, “the pill”)
- Not currently pregnant
- Not trying to become pregnant
- Generally healthy with normal blood pressure and body mass index (BMI – check yours at https://bit.ly/2y5ZFGk)

There may be possible benefits if you take part in this study:
- You will receive information on your BMI, blood pressure, and hydration status
- You will contribute to the scientific understanding of blood volume and nutrients in women of reproductive age

If you choose to participate, you will receive $90 compensation for participating in this study.

The Institutional Review Board (IRB) of The Pennsylvania State University has approved this study.
## Screening form 1a

### Form 1a: Screening

**Cyclic Variation in Micronutrient Concentrations and Plasma Volume in Reproductive-Age Women**

**To be administered at visit 1 only**

Time of form completion: ___ : ___ am/pm (hh:mm)

Date: __ __ / __ __ / __ __ __ ___

Staff name: ______________________

### SECTION A: Screening criteria

<table>
<thead>
<tr>
<th>Question</th>
<th>Participant’s response</th>
<th>Criteria</th>
<th>Criteria met?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Are you a female?</td>
<td>□ Yes □ No</td>
<td>Yes</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>2. What is your date of birth?</td>
<td>_______________</td>
<td>18-44 years</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>(Is subject aged between 18 to 44 years?)</td>
<td>□ Yes □ No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Are you allergic to iodine or iodine containing compounds?</td>
<td>□ Yes □ No</td>
<td>No</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>4. Are you allergic to shellfish?</td>
<td>□ Yes □ No</td>
<td>No</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>5. Have you had a regular menstrual cycle that lasts 26-35 days per cycle over the last 3 months?</td>
<td>□ Yes □ No</td>
<td>Yes</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>6. 6A) Are you currently using hormonal birth control of any type?</td>
<td>□ Yes □ No</td>
<td>A) No</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>6B) Have you used any hormonal birth control in the past 3 months?</td>
<td>□ Yes □ No</td>
<td>B) No</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>6C) Have you used depot medroxyprogesterone acetate (DMPA) in the past 12 months?</td>
<td>□ Yes □ No</td>
<td>C) No</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>7. Are you currently pregnant?</td>
<td>□ Yes □ No</td>
<td>No</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>8. Are you currently trying to get pregnant?</td>
<td>□ Yes □ No</td>
<td>No</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>9. Have you ever been pregnant?</td>
<td>□ Yes □ No</td>
<td>Yes/No</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>(If yes, ask 9a-c. If no, skip to 10)</td>
<td>_______________</td>
<td>A) ≥ 12 mo.</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>9A) When did your last pregnancy end? (≥ 12 months or &lt; 12 months)</td>
<td>□ Yes □ No</td>
<td>B) No</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>9B) Are you currently breastfeeding?</td>
<td>□ Yes □ No</td>
<td>C) No</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>9C) Have you been diagnosed by a doctor with any form of hypertension during pregnancy (gestational hypertension or preeclampsia)?</td>
<td>□ Yes □ No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. Do you currently have hypertension, as diagnosed by a doctor?</td>
<td>□ Yes □ No</td>
<td>No</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>11. Do you have any health conditions or medical issues that require regular monitoring by a doctor or regular visits to a clinic or hospital?</td>
<td>□ Yes □ No</td>
<td>No</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>12. Are you taking any regular medication prescribed by a doctor for a health condition?</td>
<td>□ Yes □ No</td>
<td>No</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>13. Have you ever been diagnosed with polycystic ovary syndrome (PCOS)?</td>
<td>□ Yes □ No</td>
<td>No</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>14. Do you currently smoke?</td>
<td>□ Yes □ No</td>
<td>No</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>15. <strong>Seated Blood Pressure Measurement</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First blood pressure measurement (mmHg)</td>
<td>__ / __ __ (SBP/DBP)</td>
<td>SBP ≥ 90 and &lt; 130 mmHg</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>*Wait 1 minute after first reading before taking the second reading</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second blood pressure measurement (mmHg)</td>
<td>__ / __ __</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average of the two blood pressure measurements (mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Is the average SBP ≥90 and <130 mmHg and DBP ≥60 and <80 mmHg? (If no, subject must be rescheduled for screening and a blood pressure measurement.)

<table>
<thead>
<tr>
<th>SBP/DBP</th>
<th>PULSE:</th>
<th>DBP ≥60 and &lt;80 mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

16. Pregnancy Test Result (Staff Initials: __________)

<table>
<thead>
<tr>
<th>Is this the subject’s first voided urine sample for today?</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Note: If yes, you will need a second urine sample to conduct the hydration test. If no, conduct hydration test on the same urine sample for this subject.)</td>
</tr>
<tr>
<td>□ Yes     □ No</td>
</tr>
</tbody>
</table>

17. Height (cm) *

<table>
<thead>
<tr>
<th>Weight (kg) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>*without shoes/coat</td>
</tr>
<tr>
<td>BMI</td>
</tr>
<tr>
<td>Is BMI 18.5-24.9 kg/m²?</td>
</tr>
<tr>
<td>□ Yes □ No</td>
</tr>
</tbody>
</table>

18. Eligibility of participant (determined by study staff)

<table>
<thead>
<tr>
<th>Is this participant eligible?</th>
</tr>
</thead>
<tbody>
<tr>
<td>If yes, inform woman she is eligible, continue with questions about protocol before taking consent.</td>
</tr>
<tr>
<td>If no, inform the woman she is not eligible to participate.</td>
</tr>
<tr>
<td>□ Yes □ No</td>
</tr>
</tbody>
</table>

19. Have you consumed caffeine in the last 12 hours? (If yes, reschedule subject.)

| □ Yes □ No | No |

20. Did you eat or drink anything (except water) in the last 12 hours? (If yes, reschedule subject.)

| □ Yes □ No | No |

21. Have you consumed alcohol (e.g. beer, wine, or liquor) in the past 12 hours? (If yes, reschedule subject.)

| □ Yes □ No | No |

22. When was the first day of your last menstrual cycle? (If not day 2, 3, 4, or 5 of the LMP, reschedule the subject.)

<table>
<thead>
<tr>
<th>(DD/MM/YYYY)</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ Yes □ No</td>
</tr>
</tbody>
</table>

23. Did the eligible woman provide consent for the study? (If yes, proceed with study measurements. If no, participation ends.)

| □ Yes □ No | N/A |

Enrolled Subject

Assigned ID number _____ _____ ___
Form 1b: Screening visits for 2 and 3  
ID number: ___

**Screening form 1b**

**Form 1b: Screening for visits 2 and 3**  
Cyclic Variation in Micronutrients Concentrations and Plasma Volume in Reproductive-Age Women

Time of form completion: ___ ___ : ___ ___ am/pm (hh:mm)
Date: ___/___/___ ___
Staff name: ___________________

To be administered at visits 2 and 3

**SECTION A: Screening criteria**

<table>
<thead>
<tr>
<th>Question</th>
<th>Participant’s Response</th>
<th>Criteria</th>
<th>Meet the criteria?</th>
</tr>
</thead>
</table>
| 1. **Seated Blood Pressure Measurement** (The woman should rest, sitting on the chair, for at least 5 minutes before blood pressure measurements.)  
First blood pressure measurement (mmHg)  
*Wait 1 minute after first reading before taking the second reading*  
Second blood pressure measurement (mmHg)  
Average of the two blood pressure measurements (mmHg)  
Is the average SBP ≥90 and <130 mmHg and DBP ≥60 and <80 mmHg? (If no, subject must be rescheduled for screening and a blood pressure measurement.) | ___ ___ / ___ ___  
PULSE: ___ ___  
___ ___ / ___ ___  
PULSE: ___ ___  
___ ___ / ___ ___  
PULSE: ___ ___ | SBP ≥90 and <130 mmHg  
DBP ≥60 and <80 mmHg | □ Yes □ No |
| 2. **Pregnancy Test Result** (Staff Initials: _________)  
Is this the subject’s first voided urine sample for today?  
(Note: If yes, you will need a second urine sample to conduct the hydration test. If no, conduct hydration test on the same urine sample for this subject.) | □ Pos. □ Neg.  
□ Yes □ No | Negative | □ Yes □ No |
| 3. **Weight from last visit (kg.)** | N/A | N/A |
| 4. **Weight (kg.)**  
*without shoes/coat* | N/A | N/A |
| 5. What is the change in weight (%) since last visit? | <10% | □ Yes □ No |
| **Eligibility of continuous participation** | □ Yes □ No | N/A |
| 6. Is this participant eligible? (If yes, inform woman she is eligible, continue with questions about protocol before completing Form 3. If no, | N/A | |
inform the woman she is not eligible to continue participating in the study.

Was protocol followed to take measurements today?

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>7. Have you consumed caffeine in the last 12 hours? (If yes, reschedule subject.)</td>
<td>□ Yes □ No</td>
<td>No</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>8. Did you eat or drink anything (except water) in the last 12 hours? (If yes, reschedule subject.)</td>
<td>□ Yes □ No</td>
<td>No</td>
<td>□ Yes □ No</td>
</tr>
<tr>
<td>9. Have you consumed alcohol (e.g. beer, wine, or liquor) in the past 12 hours? (If yes, reschedule subject.)</td>
<td>□ Yes □ No</td>
<td>No</td>
<td>□ Yes □ No</td>
</tr>
</tbody>
</table>
Appendix B

Study consent form

CONSENT FOR RESEARCH
The Pennsylvania State University

Title of Project: Cyclic Variation in Micronutrient Concentrations and Plasma Volume in Reproductive-Age Women

Principal Investigator: Dr. Alison Gernand

Address: The Pennsylvania State University, 110 Chandlee Laboratory, University Park, PA

Telephone Numbers: 814-867-4752 (Dr. Gernand); 814-865-7103 (PSU Clinical Research Center)

Subject’s Printed Name: _____________________________

We are asking you to be in a research study. This form gives you information about the research.

Whether or not you take part is up to you. You can choose not to take part. You can agree to take part and later change your mind. Your decision will not be held against you.

Please ask questions about anything that is unclear to you and take your time to make your choice.

1. Why is this research study being done?

We are asking you to be in this study because you are a woman in good health with a regular menstrual cycle.

This research is being done to measure the amount of plasma (the water component) in the blood of women of reproductive age. We will use a dye called indocyanine green (ICG) to measure plasma. This dye is approved by the Food and Drug Administration (FDA) and is used for many types of medical procedures.
The study is also being done to measure levels of vitamins and minerals across the menstrual cycle. Both the amount of plasma and levels of vitamins and minerals can be important for a woman’s health and the health of a pregnancy she might have in the future. At least 35 women will take part in this study.

2. What will happen in this research study?

If you agree to participate in this study, you will be asked to complete a set of measurements in three visits at the Clinical Research Center. Each visit should take less than 1 and a half hours to complete. We will ask you to fast overnight before each visit, and refrain from alcohol for 12 hours before each visit. This is needed so that measurements we take in your blood are not changed by what you have recently been eating or drinking.

At the first visit, we will ask you some questions about yourself, including information about your age, education, health, and previous pregnancies (if any). You are free to skip any questions that you would prefer not to answer. The questions should take less than 10 minutes. Then, we will conduct a series of measurements that will be the same for all 3 visits. We will measure your body composition using a body composition analyser. This is a small machine that passes a weak current through your body to measure body fat percentage. It takes a few minutes to perform and you do not feel anything during the measurement. We will also test hydration using a urine sample.

Next, a nurse will insert a small IV into your arm. This IV will be used to draw blood and to inject the ICG dye. The nurse will draw a few small tubes of blood (less than 1 ounce). Then the dye will be injected. We will inject a very small amount of dye, about 15 mg (0.25 mg/kg body weight). Over the next five minutes following the ICG injection, the nurse will draw about half an ounce of blood from you into very small tubes. The whole process of blood draws should take less than 15 minutes.

Your blood will be processed and some measurements will be done right after each visit (e.g. hemoglobin). The rest of the blood will be stored for measurements in a lab that will be able to determine the volume of plasma and levels of vitamins and minerals. The blood samples we get from you will be destroyed within 10 years from completion of the study.

As part of the study, we will be assessing fluctuations in your fertility for one specific cycle. Because cycles fluctuate within women over time, this information does not represent your overall level of fertility. At the end of the first visit, you will be provided a home-based fertility monitor and individual test strips to track your cycle. We will instruct you on how to them. In brief, you will conduct simple urine tests for about two weeks using the monitor and individual test strips, and you will report the results to us so that we can schedule visits 2 and 3. We will provide you with a daily check sheet to make it easier for you to track your daily tests. You will return the monitor on your 3rd visit.

At the beginning of visits 2 and 3, we will measure your blood pressure and weight and do a pregnancy test, before doing the rest of the measurements described above. After the pregnancy and hydration tests are completed, we will store about 3 oz of your urine sample to be used for measuring other biomarkers associated with hydration. At the end of the third visit your study
participation at the CRC is complete, but we will keep in touch with you to record the first day of your next menstrual cycle.

**What are your responsibilities if you take part in this research?**
If you take part in this research, your major responsibilities will include:

- Fast and refrain from alcohol before each visit. Drink plenty of water.
- Complete 2 daily urine tests (for about two weeks) and indicate the fertility test results on a tracking sheet.
- Take good care of the fertility monitor and return it at the end of your participation.

**3. What are the risks and possible discomforts from being in this research study?**

ICG dye is considered to be safe for repeated use. It is quickly cleared out of your blood by the liver and your stool may appear green.

ICG contains sodium iodide, so someone with an unknown allergy to iodine could have an allergic reaction to the injection. If this occurred, the nurse or other medical staff would provide medical attention and monitor your symptoms.

Blood draws may cause mild pain, swelling or bleeding. You may feel a small pinch or slight discomfort when the IV is inserted. If the initial IV placement is unsuccessful, it may need to be repeated, with your permission. There may be some bruising (blood under the surface of the skin), which can be minimized by pressing on the site after the IV is removed. There is also a slight chance of infection, dizziness, fainting. These risks will be minimized and most likely eliminated by having trained medical staff draw the blood in a clinical setting using sterile supplies. If dizziness or fainting occurs, the symptoms will be alleviated by having you lie flat with your feet raised.

There is a risk of loss of confidentiality if your information or your identity is obtained by someone other than the investigators, but precautions will be taken to prevent this from happening. The confidentiality of your electronic data created by you or by the researchers will be maintained to the degree permitted by the technology used. Absolute confidentiality cannot be guaranteed.

Fasting for 12 hours overnight should not pose a risk to healthy adults, as long as you drink plenty of water during the fasting time.

**4. What are the possible benefits from being in this research study?**

4a. What are the possible benefits to you?

- You may benefit from learning about your body composition, weight, and blood pressure.

4b. What are the possible benefits to others?
Information from this study will help to plan future studies on the health of women. This information may help us find important factors in women’s health before a pregnancy.

5. **What other options are available instead of being in this research study?**

You may decide not to participate.

6. **How long will you take part in this research study?**

If you agree to take part, it will take you approximately 5 to 6 hours of your time across 1 month (one menstrual cycle) to complete the study. You will be asked to return to the research site 2 times after your first visit. Each visit will last approximately 1 and a half hours. You will conduct brief daily urine tests using the fertility monitor starting from day 6 of your cycle until the monitor asks you to stop testing (about two weeks).

7. **How will your privacy and confidentiality be protected if you decide to take part in this research study?**

Efforts will be made to limit the use and sharing of your personal research information to people who have a need to review this information. You will be assigned a unique study code number.

- A list that matches your name with your code number will be kept in a locked file and the master code list will be kept in Dr. Gernand’s office at The Pennsylvania State University.
- Your research records will be labelled with your code number and will be kept in a locked office of the Principal investigator: Dr. Gernand.
- Your research samples will be labelled with your code number and will be stored in a freezer in the Clinical Research Center.

If we send specimens to a different lab outside of Penn State University, you will be identified by a code number such that the outside lab cannot identify you on the labelled specimen.

In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.

We will do our best to keep your participation in this research study confidential to the extent permitted by law. However, it is possible that other people may find out about your participation in this research study. For example, the following people/groups may check and copy records about this research.

- The Office for Human Research Protections in the U. S. Department of Health and Human Services
- The Food and Drug Administration of the United States Department of Health and Human Services
- The Institutional Review Board (a committee that reviews and approves research studies) and
• The Office for Research Protections.
• The Clinical Research Center (CRC) at The Pennsylvania State University

Some of these records could contain information that personally identifies you. Reasonable efforts will be made to keep the personal information in your research record private. However, absolute confidentiality cannot be guaranteed.

8. What happens if you are injured as a result of taking part in this research study?

In the unlikely event you become injured as a result of your participation in this study, medical care is available.

If you experience a side effect and emergency medical treatment is required, seek treatment immediately at any medical facility. If you experience a side effect and you believe that emergency treatment is not necessary, you should contact the principal investigator and the Clinical Research Center listed on the first page of this consent form as soon as possible.

You should also let any health care provider who treats you know that you are in a research study.

PSU compensation for injury

▪ There are no plans for PSU to provide financial compensation or free medical treatment for research-related injury.
▪ If an injury occurs, medical treatment is available at the usual charge.
▪ Costs will be charged to your insurance carrier or to you.
▪ Some insurance companies may not cover costs associated with research injuries.
▪ If these costs are not covered by your insurance, they will be your responsibility.

By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

9. Will you be paid or receive credit to take part in this research study?

You will receive $90 cash as stipend for completing this study ($30 for each completed visit). If you come to a visit and you are no longer eligible to participate based on screening, you will receive $5 cash.

10. What are your rights if you take part in this research study?
Taking part in this research study is voluntary.

- You do not have to be in this research.
- If you choose to be in this research, you have the right to stop at any time.
- If you decide not to be in this research or if you decide to stop at a later date, there will be no penalty or loss of benefits to which you are entitled.

If you stop being in the research, already collected data may not be removed from the study database. If you agree, this data will be handled in the same way as research data. If you withdraw completely from the research study, no further information will be collected and your participation will end.

The person in charge of the research study can remove you from the research study without your approval. Possible reasons for removal include:

- You do not reach “high fertility” according to the home fertility monitor
- You test positive for pregnancy
- You have high or low blood pressure (at the beginning of a visit)
- You gain or lose 10% of your body weight between 2 visits

11. If you have questions or concerns about this research study, whom should you call?

Please call the head of the research study (principal investigator), Dr. Alison Gernand at 814-867-4752 or the Clinical Research Center at 814-865-7103 if you:

- Have questions, complaints or concerns about the research.
- Believe you may have been harmed by being in the research study.

You may also contact the Office for Research Protections at (814) 865-1775, ORProtections@psu.edu if you:

- Have questions regarding your rights as a person in a research study.
- Have concerns or general questions about the research.
- You may also call this number if you cannot reach the research team or wish to talk to someone else about any concerns related to the research.
INFORMED CONSENT TO TAKE PART IN RESEARCH

Signature of Person Obtaining Informed Consent

Your signature below means that you have explained the research to the subject or subject representative and have answered any questions he/she has about the research.

______________________________
Signature of person who explained this research       Date       Printed Name
(Only approved investigators for this research may explain the research and obtain informed consent.)

Signature of Person Giving Informed Consent

Before making the decision about being in this research you should have:
  • Discussed this research study with an investigator,
  • Read the information in this form, and
  • Had the opportunity to ask any questions you may have.

Your signature below means that you have received this information, have asked the questions you currently have about the research and those questions have been answered. You will receive a copy of the signed and dated form to keep for future reference.

Signature of Subject

By signing this consent form, you indicate that you voluntarily choose to be in this research and agree to allow your information to be used and shared as described above.

______________________________       Date       Printed Name
Consent: Optional Part of the Study

In addition to the main part of the research study, there is another part of the research.

You can be in the main part of the research without agreeing to be in this optional part.

Optional Storage of Blood and Urine Samples for Future Research

As part of this study, we are obtaining blood and urine from you. If you agree, the research team would like to store leftover samples of your blood and urine that is collected so that it can be studied in the future after this study is over. These future studies may provide additional information that will be helpful in understanding plasma volume and nutritional biomarkers, but it is unlikely that these studies will have a direct benefit to you. Neither your doctor nor you will receive results of these future research tests, nor will the results be put in your health record. If you have any questions, you should contact Dr. Alison Gernand at 814-867-4752.

Your leftover samples will be labelled with a code number and stored in Dr. Alison Gernand’s laboratory and locked. If you consent to the storage of leftover samples of your blood and urine for future research, the period for the use of the samples is unknown. If you agree to allow your blood and urine to be kept for future research, you will be free to change your mind at any time. You should contact Dr. Alison Gernand at 814-867-4752 and let her know you wish to withdraw your permission for your samples to be used for future research. Should you choose not to allow for future testing of your blood and urine, they will be destroyed 10 years from study completion.

You should initial below to indicate your preferences regarding the optional storage of your leftover blood for future research studies.

a. Your samples may be stored and used for future research studies to learn about plasma volume and nutritional biomarkers and related health conditions.
   
   ______ Yes    ______ No

b. Your samples may be shared with other investigators/groups without any identifying information.
   
   ______ Yes    ______ No
**Signature of Person Obtaining Informed Consent**

Your signature below means that you have explained the optional part(s) to the research to the subject or subject representative and have answered any questions she has about the research.

________________________________________________________________________
Signature of person who explained this research Date Printed Name
(Only approved investigators for this research may explain the research and obtain informed consent.)

**Signature of Subject**

By signing below, you indicate that you have read the information written above and have indicated your choices for the optional part(s) of the research study.

________________________________________________________________________
Signature of Subject Date Printed
Appendix C

Questionnaire

Background, and health & pregnancy histories

Form 2: Questionnaire (background, and health & pregnancy histories)
Cyclic Variation in Micronutrients Concentrations and Plasma Volume in Reproductive-Age Women

Time of form completion: ___ ___ : ___ ___ am/pm (hh:mm)
Date: ___ ___ / ___ ___ / ___ ___ ___ ___
Staff name: ___________________

First visit (administered at visit 1 only)
SECTION A: Identification
1. ID Number: ___ ___ ___

SECTION B: Background characteristics
2. Are you now
   - Married
   - Widowed
   - Divorced
   - Separated
   - Never married
   - Living with partner
   - Refused
   - Don’t Know

3. Do you consider yourself to be Hispanic, Latino, or of Spanish origin?
   - Yes
   - No
   - Refused
   - Don’t Know
4. Which race(s) do you consider yourself to be?
   - White
   - Asian
   - Black or African American
   - American Indian or Alaska Native
   - Native Hawaiian or Pacific Islander
   - Other _________________________
   - Refused
   - Don’t Know

5. Have you ever been to school?
   - Yes
   - No
   - Refused
   - Don’t Know

6. What is the highest grade or level of school you have completed or the highest degree you have received?
   - High school
   - Associate’s degree
   - Bachelor’s degree
   - Master’s degree
   - Doctorate degree
   - Refused
   - Don’t Know
   - Others (specify) _________________________

7. Are you now
   - Going to school
   - Between grades
   - Neither
   - Refused
   - Don’t know

8. Which of the following were you doing last week?
   - Working at a job or business part time
   - Working at a job or business full time
   - Have a job or business but I was not at work
   - Looking for work
   - Not working at a job or business
   - Refused
   - Don’t know
9. What kind of business or industry is this? (fill in) ____________________________
   - Refused
   - Don’t Know
   - Not Applicable

10. What kind of work were you doing? ____________________________ (Enter Name of Occupation)
    - Refused
    - Don’t Know

11. In general, do you consider yourself or your household to be:
    - Low income
    - Average/middle income
    - High income

12. How many times have you been pregnant? ________________
    - Refused
    - Don’t Know

13. How many pregnancies resulted in delivery of a baby after 20 weeks gestation? (fill in) ________________
    - Refused
    - Don’t Know
    - Not Applicable (N/A)

14. Has a doctor, nurse, or health professional ever told you that you had any of the following during a pregnancy?

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>Don't know</th>
<th>Refused</th>
<th>N/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational hypertension</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preeclampsia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eclampsia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational Diabetes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other diseases (specify)______________</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Blood collection form

Form 3: Blood collection and body comp.

**ID Number: ____ ____ ____**

Form 3: Blood collection and body composition

Cyclic Variation in Micronutrient Concentrations and Plasma Volume in Reproductive-Age Women

Time of form completion: ____ : ____ am/pm (hh:mm)

Date: ____ / ____ / ____

Staff name: ___________________

To be administered at all three visits

<table>
<thead>
<tr>
<th></th>
<th>Result 1</th>
<th>Result 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Body Composition Measurement (% Fat)</td>
<td>____ <em><strong>.</strong></em></td>
<td>____ <em><strong>.</strong></em></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Result 1</th>
<th>Result 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Urine Specific Gravity (USG)?</td>
<td>____ . ____ ___</td>
<td>____ . ____ ___</td>
</tr>
</tbody>
</table>

2a) Using first or second urine sample? □ 1st □ 2nd

<table>
<thead>
<tr>
<th>Urine collection</th>
<th>Time (hh:mm)</th>
<th>Collected? (y/n)</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-3 oz (60-90 mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3. Amount of ICG to inject: 0.25 mg x _____ kg of participant = ________ mg

4. Volume of ICG solution to inject: _____ mg ÷ 2.5mg/ml = ______ ml

5. Weight of ICG injected: ____________ mg

6. Blood volumes (ml) measurements pre-and post-ICG injection

<table>
<thead>
<tr>
<th>Pre-injection</th>
<th>Time (hh:mm)</th>
<th>Collected? (y/n)</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 ml Serum tube</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 ml EDTA plasma tube</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 ml EDTA tube</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Post-injection ICG infusion (3 ml each)</th>
<th>Time (mm:sec)</th>
<th>Collected? (y/n)</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1 (start@ 2min)</td>
<td>2:00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V2</td>
<td>2:45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V3</td>
<td>3:30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V4</td>
<td>4:15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V5</td>
<td>5:00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| ICG Injection time (hh:mm): ________________|

<table>
<thead>
<tr>
<th></th>
<th>Result 1</th>
<th>Result 2</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>7. Blood pressure (mmHg)</td>
<td>____ ___./ ____ ___ (SBP/DBP)</td>
<td>____ ___./ ____ ___ (SBP/DBP)</td>
<td>____ ___./ ____ ___ (SBP/DBP)</td>
</tr>
<tr>
<td>Pulse (BPM)</td>
<td>____ ___</td>
<td>____ ___</td>
<td>____ ___</td>
</tr>
</tbody>
</table>
Daily Home Testing - Menstrual Cycle and Blood Volume Study
Testing Window: ________________ ID Number ________ Monitor Number ________

Check box as appropriate each morning after urine tests, comment where necessary

<table>
<thead>
<tr>
<th>Cycle Day</th>
<th>Calendar Date</th>
<th>Fertility monitor</th>
<th>LH test strip</th>
<th>Comment(s):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>High</td>
<td>Peak</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
References


120. Pee Sd, Dary O. Biochemical Indicators of Vitamin A Deficiency: Serum Retinol and Serum Retinol Binding Protein. J Nutr 2002;132(suppl):2895S–901S.


doi:10.1017/S0007114508006818 Indicators. doi: 10.1017/S0007114508006818.


142. Namaste SM, Aaron GJ, Varadhan R, Peerson JM, Suchdev PS, Group. BW. Methodologic approach for the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia


196. Stricker R, Eberhart R, Chevailler MC, Quinn FA, Bischof P, Stricker R. Establishment of detailed reference values for luteinizing hormone, follicle stimulating hormone, estradiol,
progesterone during different phases of the menstrual cycle on the Abbott ARCHITECT analyzer. 


178


182


183


VITA

SIXTUS AGUREE

EDUCATION

The Pennsylvania State University
PhD, Nutritional Sciences (Minor in Statistics)  University Park, PA
August 2019

University of Ghana
MPhil., Human Nutrition  Legon-Accra, Ghana
July 2010

B.Sc. with Honors, Nutrition and Food Science  May 2007

SELECTED GRANTS AND HONORS

John A. Milner Graduate Student Endowment, The Pennsylvania State University  2019
HHD Professional Development Endowment, Penn State University  2018
Best Poster award, The Huck Institutes of the Life Sciences, The Pennsylvania State  2018
Bergen Summer Research School (BSRS) Scholarship, University of Bergen, Norway  2015
Graham Endowed Fellowship at The Graduate School, The Pennsylvania State University  2014-15
Excellence in Graduate Recruitment (FEGR) award, The Pennsylvania State University  2014

PEER-REVIEWED PUBLICATIONS


ABSTRACTS AND CONFERENCE PRESENTATIONS


- Aguree S. Gernand AD. Plasma volume, hydration and complete blood count across the menstrual cycle: preliminary results. Poster presentation, Life Sciences Symposium, May 18, 2018, The Pennsylvania State University, University Park, PA

- Aguree S. Gernand AD. Nutritional biomarkers correlate with plasma volume in regular cycling women. Poster presentation, fourth annual Women's Health Research Day, November 6, 2017, Nittany Lion Inn, The Pennsylvania State University, University Park, PA