The Pennsylvania State University

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MEASURING PLASMA VOLUME IN WOMEN OF REPRODUCTIVE AGE – A COMPARISON STUDY OF HYDROXYETHYL STARCH TO OTHER METHODS OF PLASMA VOLUME MEASUREMENTS

A Thesis in

Nutritional Sciences

by

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ABSTRACT

Background: Plasma volume (PV) is rarely measured, and further research is needed to understand if PV could be an important biomarker and how it may relate to other commonly measured clinical markers of nutritional status and health. Hydroxyethyl starch (HES) has previously been used to measure blood volume in pregnancy, but additional work and validation is needed for this method when measuring PV.

Objectives: To 1) develop and evaluate a method to measure PV using HES; and to 2) examine relationships between PV measured by HES and key biomarkers of health (blood pressure, body mass index (BMI), body surface area (BSA), body fat percentage, and urine specific gravity) in healthy women of reproductive age.

Research Design and Methods: We recruited a convenience sample of 12 healthy women of reproductive age for a cross-sectional comparison study. At enrollment, we collected data on demographics and key health biomarkers (blood pressure, BMI, body fat percentage, and urine specific gravity). For Aim 1, baseline blood samples were collected, followed by an injection of indocyanine green (ICG) and 5 serial blood samples collected over 5 minutes. An injection of HES followed, with 3 serial blood samples collected over 15 minutes. We constructed a dye dilution curve using the post-ICG injection samples to back-extrapolate and estimate PV at the time of injection. HES in plasma was assayed using a handheld glucometer to estimate PV at 5, 10, and 15 minutes post-injection. PV was then estimated using the Kaplan, Hurley, and Nadler equations. The HES PV at 10 minutes post-injection was compared to each of the other methods using the Bland-Altman method. For Aim 2, we used Spearman's rank-order

correlation to assess the correlation between PV measured by HES and each biomarker (blood pressure, BMI, BSA, body fat percentage, urine specific gravity).

Results: A total of 12 participants had complete visit data and were included in the final analysis. Participants were 25.8 ± 7.5 years old with mean BMI of 21.7 kg/m^2. They were predominantly white, non-Hispanic, college-educated, and never married. Almost all participants were nulliparous, and 58% reported using contraceptives. For Aim 1, the mean (SD) PV estimations were 2,046 (392) mL, 2,765 (820) mL, 2,443 (464) mL, 2,407 (301) mL, and 2,373 (406) mL, respectively. The mean differences between methods were -718 mL (HES versus ICG), -397 mL (HES versus Kaplan), -361 mL (HES versus Hurley), and -327 mL (HES versus Nadler). For Aim 2, we found that BSA and body fat percentage had Spearman's rho of close to 0.3, but they were not statistically significant. The other correlations were close to 0.1 and not statistically significant.

Conclusions: In a sample of healthy women of reproductive age, our comparison study showed that PV measured by HES was on average >300 mL different from other PV estimates (ICG and estimation equations). We also found no statistically significant correlations between PV and key biomarkers of health (blood pressure, BMI, BSA, and urine specific gravity). Additional research is needed to investigate PV in healthy individuals and develop sound methods for measurement.

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Chapter 1

Introduction

Blood is a critical body fluid that circulates throughout an individual's body, transporting oxygen and nutrients to target sites, along with waste products from various metabolic processes (1). Total blood volume, or the total amount of whole blood within the circulatory system at any given time, is necessary to maintain normal perfusion throughout the body (1). It is comprised of red and white blood cells (erythrocytes and leukocytes, respectively), platelets, and plasma (1). In general, blood and its components are important because it is responsible for the transport of nutrients and other substances that the body needs to function. Researchers collect and study blood specimens to measure biomarkers of health and nutrition status, which is used to understand the importance and influence of interventions that may be used. However, there are challenges in health and nutrition biomarker measurement, which make it difficult to understand how they may relate to health outcomes or establish a normal range for each.

Plasma Volume

In a healthy individual of normal physiologic status, plasma accounts for approximately 60% of blood, with the remaining 40% consisting of red blood cells and other blood components (1). Hematocrit is a measure of the volume of red blood cells compared to the total blood volume (both red blood cells and plasma); the normal range of hematocrit is 40-54% and 36-48% for males and females, respectively (2). Females tend to have lower plasma volume when compared to males; this difference is also seen when establishing cutoff values for anemia, in which <120 g/L is used for females and <130 g/L is used for males (3,4). An individual's plasma volume

(PV), or the total amount of plasma (the watery base of whole blood) in the whole blood at any given moment, contains approximately 90% water and 10% solids. Serum is the liquid component of blood that remains after it has been allowed to clot, while plasma is the liquid component of blood that remains when clotting is prevented using an anticoagulant. The non-water components include coagulants, plasma proteins, electrolytes, immunoglobulins, and small amounts of enzymes, hormones, and vitamins. In the normal physiologic state, plasma is important for the following functions: coagulation (fibrinogen, thrombin, and factor X aid in blood clotting); immune defense (immunoglobulins and antibodies defend against bacteria, viruses, fungi, and parasites); maintenance of osmotic pressure (albumin and other plasma proteins maintain pressure at around 25 mmHg); thermoregulation; and nutrition (transport of nutrients, including electrolytes, throughout the body as needed) (5). Plasma volume is related to blood pressure, BMI, body surface area, body fat percentage, and hydration markers, and it may be related to micronutrient status. Details on these relationships are below.

Plasma Volume Regulation

Total fluid volume, including PV, is regulated by the renin-angiotensin-aldosterone system (RAAS); blood pressure is regulated by the same system and pathways (6). In the RAAS, a decrease in fluid volume or blood pressure will trigger the kidneys to release renin, which is responsible for converting angiotensinogen (produced by the liver) to angiotensin I. At the same time, the lungs and pulmonary system will release angiotensin-converting enzyme (ACE), used to convert angiotensin I to angiotensin II. If the cascade was triggered by decreased blood pressure, angiotensin II will act directly on blood vessels throughout the circulatory system, causing vasoconstriction and therefore increasing blood pressure. If the cascade was triggered by decreased fluid volume, angiotensin II will act on the adrenal gland to stimulate the release of

aldosterone. Aldosterone will then act on the kidneys to stimulate the reabsorption of sodium chloride and water, therefore increasing fluid volume in the circulatory system. When there is any change in fluid volume, it is detected primarily by stretch receptors in the cardiopulmonary circulation and in the kidney (specifically, in the afferent glomerular arterioles and the early distal tubule) (6–8). For acute or temporary changes in PV, there is short-term regulation; these changes often occur during physical activity or short periods of dehydration. For chronic or prolonged changes in PV, there is long-term regulation; these changes occur under prolonged physiological states, including pregnancy.

Relationships between Plasma Volume and Health Biomarkers

It is widely accepted that changes in PV influence arterial blood pressure. Increased fluid volume in circulation leads to increased central venous pressure, which then increases ventricular stroke volume. This results in higher cardiac output and therefore arterial blood pressure (6). However, recent research on the relationship between blood volume and hypertension conflicts with this. Most studies have found an inverse relationship between blood pressure and plasma or blood volume in individuals with hypertension, and a recent study found no relationship (9).

Anthropometry, including height and weight, and body composition (fat mass and non-fat mass) are important when assessing an individual's health status. Body mass index (BMI), body surface area (BSA), and body fat percentage are calculated anthropometric measurements that are often studied together, and there is data that supports a relationship between each and blood or PV (10–13). It has been shown that as body density and weight status increase, total blood volume and PV increase as well. However, PV and red cell volume per unit of body weight both decrease as weight status or body density increase; individuals with normal or underweight have higher PV and red cell volume per unit of body weight. It is believed that this is likely because fat

mass is underperfused compared to lean or non-fat body mass (10–12). The relationship between BSA and PV is similar to BMI and body fat percentage; as BSA increases (meaning an individual's height and/or weight increase), their total blood and PV also increase (12,13).

There is an obvious relationship between PV and hydration, as plasma is almost entirely comprised of water. Hydration is broadly defined as having the appropriate amount of water in an individual's body to maintain blood volume and hydration-related processes, including temperature regulation, muscle contraction, and cellular metabolism (14). If an individual is adequately hydrated, they are more likely to have a higher PV because there is more water for their body to absorb. If they are dehydrated, they are more likely to have a lower PV because there is less water for their body to absorb; however, the body naturally tries to homeostatically maintain hydration and PV by controlling how much water is reabsorbed by the kidneys (8). This relationship is also observed in endurance training and physical activity; proper hydration during physical activity aids in the maintenance of PV levels (15,16).

Micronutrient deficiencies are of global public health concern, as they can lead to adverse outcomes in vulnerable populations, such as children, pregnant people, and older adults. As micronutrients are transported throughout the body in blood, their status is often assessed by measuring circulating biomarker concentrations in plasma or serum. An individual's PV may influence micronutrient biomarker concentrations because they are transported in the plasma. There are very few studies aimed at examining the specific relationship between micronutrient concentration and plasma volume in non-pregnant women, but it has been shown that the circulating mass of biomarkers, rather than the biomarker concentrations, and PV are often positively correlated (17).

Plasma Volume Expansion in Pregnancy

In a healthy pregnancy, PV expansion is greater and more rapid than that of red blood cell mass, leading to hemodilution and decreased hemoglobin (and other nutrient) concentrations (18). It begins at approximately 6 weeks gestation and rapidly increases until mid-pregnancy, at which point it slowly increases from approximately 32 weeks gestation until delivery (18). Few studies have measured the expansion of PV across pregnancy, likely due to method constraints, but the overall agreement is that PV expands by approximately 45-60%, with most researchers citing approximately 50% (19–23).

Appropriate PV expansion in pregnancy is related to healthy outcomes, such as adequate fetal growth (22,24,25). When PV does not expand enough to accommodate the pregnancy and its new physiologic demands, adverse outcomes may occur, including hypertensive disorders of pregnancy (preeclampsia), fetal growth restriction, low birthweight, and a small-for-gestational age neonate (21,26,27). For example, recurrent preeclampsia has been found in one quarter of pregnant women with low PV (<1,243 mL/m^2), compared to pregnant women with normal PV (>1,373 mL/m^2) (27). However, both low and high PV expansion have been seen in pregnant women with preeclampsia when compared to healthy women, showing the importance of examining PV expansion in pregnancy in women with both normal and adverse pregnancy outcomes (28,29).

Methods to Estimate Plasma Volume

It is important to accurately assess or estimate PV when possible, as it can change as a result of both normal and abnormal conditions. This can lead to hypovolemia (low blood volume) and hypervolemia (high blood volume). For example, intense exercise has been found to increase

plasma volume due to the temporarily increased plasma albumin concentration (30). Dehydration has been shown to decrease plasma volume after periods of heat exposure (31). When PV fluctuates in these conditions, it can impact perfusion and blood pressure regulation. Estimating PV is important for the evaluation of clinical disorders and diseases related to the blood, such as anemia, and blood loss during surgical procedures (32). It is also important to examine PV in research studies to better understand health status in different populations and settings.

Blood volume and PV are easily interchangeable when measuring hematocrit, and some methods utilize equations that were established to calculate blood volume, rather than PV. Additionally, there is great overlap in research of blood volume and PV, with some studies measuring blood volume and then converting those values to PV. In pregnancy and other physiologic states, plasma increases proportionally more than red blood cells, so it is important to specifically measure PV. Some micronutrient biomarkers are measured in plasma, so studies that are examining these should also specifically measure PV.

There are three general categories for the methods of PV measurement: radiolabeled molecules, dye dilution, and estimation equations using health data (Figure 1-1). All laboratory-based methods, including radiolabeled molecules and dye dilution methods, follow the indicator dilution technique, which is based on the concept of diluting a known amount of a test substance via administration (typically injection) into the circulation and then measuring the amount of the substance found in serial blood samples. The estimation equations use health data, such as height and weight, to calculate PV.

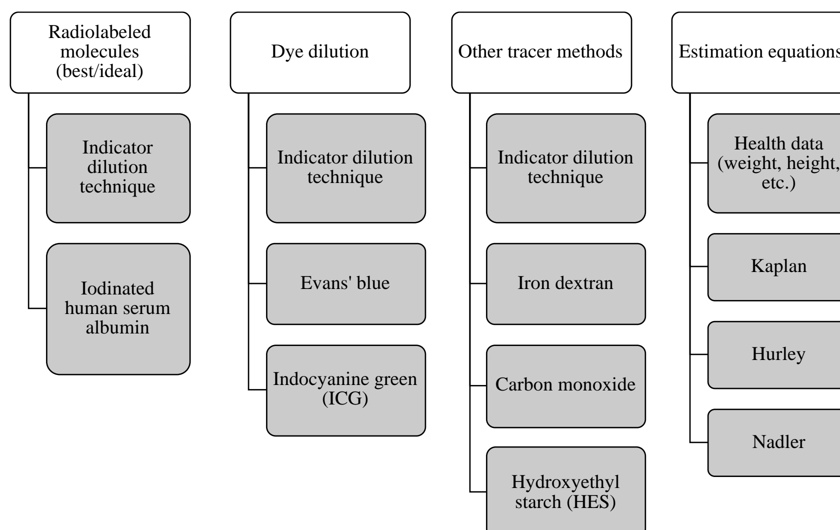

Figure 1-1: Methods to measure PV.

Laboratory-based Methods

The gold-standard laboratory-based method uses radiolabeled iodinated human serum albumin, in which an iodine isotope (either ^{125}I or ^{131}I) are tagged to human serum albumin and a small amount of the radiolabeled albumin is injected into the participant's circulation. The radioactivity of the plasma is measured and compared against a prepared standard solution (33). While this method is very accurate compared to others, its primary disadvantages are the required exposure to radioactive material and expensive and technologically advanced laboratory methods (34).

Carbon monoxide rebreathing, used to measure total blood volume, has been used to estimate plasma volume in research studies despite not measuring it directly. It has recently been adapted and optimized to remedy some of the original disadvantages, which included a long respiration time and many blood samples throughout the study process (35,36). In the optimized method, the individual inhales and rebreathes a bolus dose of carbon monoxide through a

spirometer for 2 minutes. The increase of carboxyhemoglobin content in capillary blood samples after inhalation is assessed, and this data is used to estimate blood volume (which can then be converted to estimate PV) (36,37). The main advantage of this method is the inexpensive tracer substance compared to other methods. As well, the newly optimized portion includes a shorter respiration time of 2 minutes. However, while there is no radioactivity exposure, there is still exposure to carbon monoxide, and this may influence participation in research studies and exclude use in pregnancy. The method also uses expensive equipment that may not be readily available in all settings. Finally, there has been some concern that it reduces oxygen transport capacity, but this may have been remedied through the recent optimization (38).

Iron dextran has previously been used to estimate plasma volume, although there is little literature to document this method. The product is used for iron replacement for treatment of iron deficiency anemia or blood loss (39). It follows the aforementioned indicator dilution technique: a known bolus amount of iron dextran is intravenously injected and the increased iron content in the blood is used to calculate PV (40–42). While this method is simple and can be used in a variety of settings and populations, it is not ideal in nutrition research and studies in which iron and other micronutrients may be measured outcomes.

Dye dilution methods have been popularly chosen to measure plasma volume in research studies, to avoid exposure to radioactivity and carbon monoxide. The two primary dyes are Evans' blue and indocyanine green (ICG). Evans' blue dye is a chemical tracer dye that reversibly binds to serum albumin (43,44). It was widely used in the past but is no longer approved for use nor available in the United States, and as a result, some researchers have begun to use ICG to measure PV (34). ICG is another tracer dye, similar to Evans' blue dye, that is highly soluble in water and binds to beta-lipoproteins in the blood, particularly to albumin (45). In this method, a known amount of ICG is intravenously injected; after full mixing throughout the circulation, serial blood samples are collected and the amount of dye in each sample is measured

using a plate reader at 805 nm (46). These samples are used to back-extrapolate to calculate the PV at the time of injection (46). This method is advantageous because it does not require any radioactivity exposure and is safe for repeated use due to its very short half-life. However, ICG can be expensive and has a higher participant burden because of frequent post-injection sampling. As well, it requires individual standard curves for each participant (34). ICG is not approved by the Food and Drug Administration for use to measure PV, and there are some noted concerns in pregnancy (mostly due to lack of research), which is a significant disadvantage for those who want to measure plasma volume expansion in pregnancy.

Hydroxyethyl starch (HES) has previously been used to measure blood volume in critically ill patients (38) and pregnant study participants (11). HES is a molecule comprised of glucose and hydroxyethyl groups attached throughout, interconnected by alpha-glycosidic bonds; it was derived as an alternative to similar products containing albumin (47). The molecular structure of hetastarch is large, and the addition of hydroxyethyl groups makes it resistant to enzymatic hydrolysis or degradation in the body. It has been used in clinical settings as a 6% solution in saline, provided at doses of 500 to 1,000 mL; in these settings, it is predominantly used as a volume expander in instances of blood loss or disease (47). To our knowledge, there has been no attempt to use HES to measure or estimate plasma volume. In this method, a known amount of HES is intravenously injected, followed by a post-injection blood sample. The samples are acid-hydrolyzed to release glucose from the molecules. The glucose at baseline and after the injection are assayed and put into an equation, along with the individual's hematocrit at the time of blood draws, to estimate blood volume (11,38). This is then converted to plasma volume using hematocrit. The main advantages for this method are that HES is inexpensive and easy to acquire, compared to other tracer substances, and the laboratory work associated with the method is simple; these advantages together contribute to this method being ideal for use in low- and middle-income countries (LMICs). As well, it is approved and safe for use in pregnancy, making

it one of the few methods that can be effectively and safely used in this vulnerable population (48).

Estimation Equations

There are several equations that are used to estimate blood volume, red cell volume, and PV. These equations use health data that is often readily available, such as age, height, weight; some equations utilize body surface area (BSA), which is calculated using a validated equation and an individual's weight and height (49). These equations are easy to use and non-invasive, but they may not be as accurate as laboratory-based methods that more directly measure PV.

The equation developed by Kaplan was published because clinicians and researchers needed to prescribe plasma exchange therapy but could only do so with reliable predictions of the individual's PV (50). The equation was first developed as a simplified formula used to evaluate the predicted and actual decline in serum levels of 18 participants. However, the original publication does not include any details on the development or validation of the equation, or the study population. This equation uses a constant (0.065), weight (kilograms), and hematocrit (percentage) to calculate PV in liters, which can then be converted to milliliters (50). The Kaplan PV equation has been used frequently in research studies to estimate PV, both alone and in comparison with other methods (51).

The estimation equation developed and published by Hurley aimed to provide insight into the range of red cell volume and PV in normal, healthy individuals, as previous equations had been based on ill participants (3). To develop the equation, Hurley compiled published red cell volume and PV results in healthy men and women determined using various tracer methods and used them to calculate mean volumes. The mean volumes, each ± 2 standard deviations, were

plotted against BSA, and the resulting equations with the highest correlation coefficients were selected. In the equation for estimating PV in healthy women, the health data being used is BSA, calculated from an individual's height (centimeters) and weight (kilograms) (49); the Hurley equation uses a constant (1,278) as well (3). This equation has been used less frequently in research, but it is still helpful and easy to use because it requires basic information to estimate PV.

Equations that were originally developed to estimate blood volume can also be used to estimate PV through a simple conversion using an individual's hematocrit. Nadler *et al.* developed an equation to estimate circulating total blood volume in normal, healthy individuals, so that it could both be used to estimate total blood volume in various disease states and be used in place of the laboratory methods for measuring total blood volume (52). This equation, referred to as the height cubed-body mass formula in the original publication, was developed using regression analysis of blood volume measured by radiolabeled iodinated human serum albumin and the health data of the volunteers. It uses an individual's cubed height (centimeters) and weight (kilograms), along with two constants (0.3561 and 0.03308) to estimate an individual's total blood volume (52). Like other estimation equations, this is easy to calculate using basic and often easily available health data.

There have been many calls for future research in plasma volume, specifically to develop methods for other populations (including pregnant individuals) and to estimate PV in various settings and/or disease states. Studies have been conducted in the past to compare laboratory-based and estimation equation methods, with a few studies using the gold standard method to validate newly developed methods or equations. However, more work is needed in this space to further compare new laboratory methods to previously validated methods.

Validation and Comparison Studies

Validation studies are conducted to compare the accuracy of a new method with the ideal or gold standard method, while comparison studies are conducted to compare the accuracy of one method to that of another method. It is important to recognize that validation and comparison studies are not the same, with the key difference being the inclusion of a gold standard method in validation studies. However, the terms "validation" and "comparison" are often incorrectly used interchangeably in research; some researchers label their work as a validation study when it is in fact a comparison study. Most laboratory-based and estimation equation methods have previously been compared and/or validated several times in research. HES has been compared to the carbon monoxide rebreathing method, as well as estimation equations, but it has not been validated against the radiolabeled gold standard method (11,38).

Study Aims and Hypotheses

This study is part of a vitamin B12 dose escalation trial in Tanzania, in which pregnant (n = 40) and non-pregnant (n = 10) women will be given a vitamin B12 supplement and their B12 status will be assessed over 4 weeks. During the parent trial, PV will be measured to estimate expansion across pregnancy.

The current study is being conducted to develop and pilot a method to measure PV that is safe to use in pregnancy and easier to use in low-resource settings. The new method will be used to measure PV expansion in the parent trial. This study has the following aims:

<u>Aim 1</u>: Develop and evaluate a method to measure plasma volume using HES in healthy women of reproductive age.

Hypothesis for Aim 1: We hypothesize that the HES method will produce similar results to the other tested and validated methods, each within a 10% difference.

Within this aim we will:

a. Test time points and other parameters of an existing HES method to establish an HES method for use in larger studies.

b. Compare PV by HES to PV measured by ICG.

c. Compare PV by HES to PS estimated by equations.

<u>Aim 2</u>: Examine relationships between PV (measured by HES) and key biomarkers of health status (blood pressure, BMI, body surface area, body fat percentage, urine specific gravity) in healthy women of reproductive age.

Hypothesis for Aim 2: We hypothesize that there will be a positive correlation between PV and blood pressure, BMI, and body surface area, and a negative correlation between PV and urine specific gravity.

Chapter 2

Methods

In this cross-sectional comparison study, a convenience sample of eligible participants were recruited via paper fliers and various electronic methods (StudyFinder, ResearchMatch, FIRSt Families) in State College, Pennsylvania, and the surrounding areas within Centre County. Interested individuals completed a phone pre-screening; if deemed provisionally eligible based on the pre-screening, individuals were scheduled to visit the Penn State Clinical Research Center (CRC) for enrollment into the study and to complete an in-person screening.

Sample Size and Eligibility Criteria

In this pilot study, we aimed to recruit a minimum of 10 participants to test the new method for measuring PV. With this sample size, we had 16% power to detect associations (rho = 0.3) between PV measured by HES and key biomarkers in Aim 2. For this study, eligible individuals were non-pregnant females aged 18 to 44 years, in general good health (did not have a known, ongoing health condition or medical issue that required regular monitoring by a doctor or regular visits to the hospital), that had a BMI of 18.5 to 24.9 kg/m^2, were non-smokers, and had at least 12 months since their last pregnancy if applicable. Individuals were ineligible if they had any of the following: 1) known allergy to corn, HES, shellfish, or iodine; 2) low or high blood pressure on the day of measurements (SBP <90 or ≥130 mmHg and/or DBP <60 or ≥80 mmHg); 3) self-reported low or high blood pressure (SBP <90 or ≥130 mmHg and/or DBP <60 or ≥80 mmHg) (53); 4) current hypertension or previous hypertensive disorder in pregnancy (such as gestational hypertension); 5) taking regular medication prescribed by a doctor for a health condition, other than hormonal contraception; 6) polycystic ovarian syndrome (PCOS), or renal,

liver, autoimmune, or bleeding disorders; 7) congestive heart failure; 8) pregnant or currently trying to conceive; 9) currently breastfeeding; and 10) self-reported history of difficult blood draws or IVs. The study protocol was approved by the Institutional Review Board of the Office for Research Protections at the Pennsylvania State University (STUDY00016189) and participants signed informed consent at their study visit.

Study Procedures

Before the in-person screening visit, participants were asked to refrain from alcohol consumption, as well as drink water to ensure adequate hydration (we recommended that participants drink approximately 8 cups or 4 16-ounce bottles of water) for 48 hours prior to the visit and to fast (no food or beverages except water) for 12 hours prior to the visit. We also asked them to void the first morning urine and then consume an additional 1-2 cups of water on the morning of their visit. At the start of the visit, participants provided voluntary informed consent and were enrolled into the study; enrollment at this point was needed to complete the in-person eligibility screening and the remainder of the visit. They then provided a urine sample, which was used to conduct a pregnancy test and assess hydration status later in the visit. Participants were weighed to the nearest 0.1 kg using a digital scale, and their height was measured to the nearest 0.1 cm using a portable stadiometer; body mass index (BMI: kg/m^2) was calculated with these measurements. Both weight and height were measured once. Blood pressure and pulse were each measured twice following the American Heart Association protocol, starting after 5 minutes of undisturbed rest using an electronic blood pressure monitor (OMRON HEM-712C Blood Pressure Measuring Machine), and mean systolic and diastolic blood pressure were used for eligibility (53).

If eligible based on the in-person screening, participants then completed a questionnaire to collect demographic, health history, and pregnancy history data. 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). Hydration status was assessed using the urine sample and a urine specific gravity pen refractometer (Atago 3741 PEN-Urine S.G. Digital Handheld Pen-Style Refractometer, Atago USA, Inc.); hydration status was categorized as hydrated (1.000-1.019), moderately dehydrated (1.020-1.027), or severely dehydrated (1.028-1.035) (54). If not eligible based on the in-person screening, participants were compensated $50 cash, provided with copies of their signed consent forms, and escorted from the CRC.

Participants were then asked to lay on their left side and rest undisturbed in a clinic room for 15 minutes in preparation for establishment of the intravenous (IV) catheter in the left arm. A heating pad was placed on the left arm during the rest period. A trained nurse inserted the IV, collected baseline blood samples into vacutainers (6 mL trace element-free vacutainer for serum, 6 mL trace element-free K2EDTA vacutainer for plasma, and 2 mL K2EDTA vacutainer for whole blood; BD Vacutainer, BD, New Jersey, USA), injected the ICG and HES solutions, and collected post-injection samples into vacutainers after each solution. We chose to inject the ICG solution first followed by the HES solution because HES is used clinically as a volume expander, and we did not want the HES volume to influence the PV estimated by ICG. After the IV was removed, the participants were observed for approximately 15 minutes, during which we measured blood pressure and pulse twice and provided $50 cash as a stipend for participation.

Indocyanine Green (ICG)

After the establishment of the IV catheter and collection of baseline blood samples, the syringe used for the ICG solution injection was weighed using a high-precision scale before the solution was prepared, after the syringe was filled, and then again after the solution was injected; these weights were later used to calculate how much ICG was injected. The solution (IC-Green, AKORN Inc, Lake Forest, IL, USA) was prepared by the nurse manager as 0.25 mg/kg body weight, which was measured at the start of the visit as part of the in-person screening. The nurse then injected the solution as a bolus dose over 5 seconds. Once the injection was complete, the nurse collected 3 mL EDTA vacutainers for plasma, starting at 2 minutes and occurring every 45 seconds, up to 5 minutes (a total of 5 blood draws at exactly (min:sec) 2:00, 2:45, 3:30, 4:15, and 5:00). Further details on the preparation and administration of the ICG solution, along with sample collection, have previously been published by the Micronutrients and Pregnancy Laboratory (46).

Plasma volume was measured using the indicator-dilution principle; baseline plasma (called the “blank” plasma sample) and the 5 post-injection samples were used to estimate PV. The blank plasma sample, prepared standard solutions, and post-injection samples were each loaded into a 96-well plate in triplicate and read at a wavelength of 805 nm on an Epoch plate reader (BioTek Instruments, Inc.) powered by Gen5 Software. We then constructed a standard curve of absorbance against the standard concentrations; this was used to estimate the ICG concentrations of the 5 post-injection plasma samples. We back-extrapolated the ICG concentration to the time of injection (t = 0 seconds) because of its rapid hepatic clearance and calculated PV for each participant as:

$$Plasma\ Volume\ (L) = \frac{D}{C_0}$$

where D was the dose of ICG administered in mg and C_0 was the plasma concentration of ICG in mg/L at t = 0 seconds. Further details on the PV determination have previously been published (46).

Hydroxyethyl Starch (HES)

We developed a method to measure PV using HES by adapting previously published methodology (11,38). Immediately following the post-ICG injection sample collection, the trained nurse injected a 120-170 mL bolus of HES (Hespan, B. Braun Medical Inc.) through the IV catheter over 4 minutes. Ten participants received 120 mL, and 2 participants received 170 mL of HES; we specifically tested different volumes of HES as part of our method development. The timer was reset and the nurse collected 3 mL post-HES injection blood samples into vacutainers. We collected samples at 5, 10, and 15 minutes after the injection as part of our method development, to confirm the timing of collection in the previous study (11). The samples were centrifuged, and plasma was aliquoted into cryovials for plasma volume determination.

Post-HES injection plasma samples were transported to the lab and immediately assayed before being frozen for storage. The whole blood samples were assayed for hematocrit, using a HemoPoint H2 Analyzer (EKF Diagnostics, USA). If a participant was anemic based on hemoglobin assay (HemoCue Hb801, HemoCue AB, Sweden), we measured hematocrit using a microhematocrit centrifuge. We transferred 0.6 mL of each plasma sample to a cryovial; 0.15 mL of hydrochloric acid was added to each sample and vortexed thoroughly. The cryovials were placed in a boiling water bath for 7 minutes and then in a room-temperature water bath for 2 minutes. The samples were then vortexed, and 0.65 mL of tris buffer was added to each, followed by additional vortex mixing and benchtop incubation for 6 minutes. After incubation, the samples were vortexed again and the pH was measured using pH strips; the target pH was 7.0 +/- 0.5, and

additional tris buffer was added to adjust the pH if needed. The samples were then centrifuged at 3600 rpm for 16 minutes, and the supernatant was obtained and assayed for glucose using a handheld glucometer (HemoCue Glucose 201, HemoCue AB, Sweden). The laboratory protocol is included in Appendix A.

The plasma glucose readings at baseline, 5, 10, and 15 minutes post-HES injection and hematocrit from the baseline whole blood sample were entered into the blood volume equation below, where change in glucose is equal to each post-HES injection glucose reading minus the baseline glucose reading (11,38).

$$Blood\ Volume\ (mL) = 3082 * \frac{HES\ volume}{Change\ in\ glucose \Big/ 1 - \left(\frac{Hematocrit\ (\%)}{100}\right)}$$

Plasma volume was then calculated using the following equation to convert blood volume to PV.

$$Plasma\ Volume\ (mL) = Blood\ Volume * \left(1 - \left({Hematocrit\ (\%)}/{100}\right)\right)$$

The original study conducted by Tschaikowsky *et al.* used a solution of 10% HES, but we were only able to purchase a solution of 6% HES in the United States (38). Due to this, we converted the volume of HES injected to the weight in grams of HES injected based on the original product used to develop the equation.

We compared PV estimates across the three collection timepoints (5, 10, and 15 minutes) as part of our method development. For comparison of PV measurements between methods, we used 10 minutes post-HES injection as this time was used by previous studies (11,38). However, we used all HES timepoints to compare measurements within the HES method.

Estimation Equations

There are over a dozen published equations to estimate PV or blood volume. We wanted to select equations that included at least two datapoints collected as part of our study, which drastically narrowed the options since some equations use only one datapoint (e.g., weight). As well, we wanted to select equations that were easy to implement clinically, using data that would be simple to collect or already in an individual's health record. Therefore, we selected the following equations based on the health data used in each; we did not take into account the accuracy or validation for each equation, as this data was not always easily accessible in the original publications.

Kaplan Equation

Kaplan's equation for estimated PV was used to estimate PV for each participant. The equation is (50):

$$PV\ (mL) = [0.065\ \times\ Body\ Weight\ (kg)] \times\ [1 - \frac{Hematocrit\ (\%)}{100}] \times 1000$$

The participant's body weight was measured at the start of the visit, and the hematocrit was measured from the baseline whole blood sample.

Hurley Equation

We used Hurley's equation to estimate Hurley PV for each participant. The equation is (3):

$$PV\ (mL) = 1278 \times Body\ Surface\ Area^{1.289}$$

Body surface area (BSA) was calculated for each participant using the following equation (49):

$$Body\ Surface\ Area = 0.20247 \times (\frac{Height\ (cm)}{100})^{0.725} \times Weight\ (kg)^{0.425}$$

BSA was also used in Aim 2 for comparison with PV estimates.

Nadler Equation

Nadler's equation for total blood volume (TBV) was used to estimate blood volume for each participant, which was then converted to PV. The equation is (52):

$$TBV\ (mL) = [\left(0.3561 \times \left(\frac{Height\ (cm)}{100}\right)^{3}\right) + (0.03308 \times Weight\ (kg))] \times 1000$$

Plasma volume was then estimated by converting total blood volume to PV using the conversion equation above.

Statistical Analysis

Categorial descriptive data were presented as frequencies (%) and continuous variables were presented as mean ± SD. Normality of continuous variables was assessed by testing the distribution of each variable against a normal distribution using the Shapiro-Wilk test and inspection of kernel density plots.

For Aim 1, mean PV values were compared for HES at 10 minutes, ICG at t = 0 seconds, the Kaplan PV equation, the converted Nadler PV equation, and the Hurley PV equation. We compared the difference in PV between HES at 10 minutes and all other PV estimation methods using Bland-Altman analyses (55). This analysis is used to compare a new clinical measurement technique with an established one, to determine if they agree enough for the new method to replace the established one. For Aim 2, we conducted Spearman rank-order correlation between

HES at 10 minutes and systolic blood pressure (SBP), diastolic blood pressure (DBP), body mass index (BMI), body surface area (BSA), body fat percentage, and urine specific gravity (USG). Statistical significance was considered as $p\text{-value} < 0.05$. Statistical analyses were conducted in R (version 4.1.1).

Chapter 3

Results

Study Population

Seventeen participants were enrolled in the study, and a total of 12 participants had complete visit data and were included in the final analysis. Participants were 25.8 ± 7.5 years old with mean BMI of 21.7 kg/m^2. They were predominantly white, non-Hispanic, college-educated, and never married. Over 80% of participants reported middle/average income; no participants reported that their income was low. Almost all participants were nulliparous, and 58% reported using contraceptives (Table 3-1).

Table 3-1: Characteristics of study participants (healthy women of reproductive age) (n=12).

Participant Characteristics	Mean (SD) or n (%)
Age, years	25.8 (7.5)
BMI, kg/m^2	21.7 (1.7)
Body surface area, m^2 [a]	1.64 (0.16)
Systolic blood pressure, mm Hg	110.0 (10.3)
Diastolic blood pressure, mm Hg	72.1 (5.7)
Body fat percentage, %	25.8 (5.6)
Urine specific gravity	1.0161 (0.0075)
Urine specific gravity, n (%) [b]	
Hydrated	8 (67)
Moderately dehydrated	3 (25)
Severely dehydrated	1 (8)
Race/Ethnicity, n (%)	
White, Non-Hispanic	7 (58)
White, Hispanic	1 (8)
Black, Non-Hispanic	1 (8)
Asian, Non-Hispanic	3 (25)
Education, n (%)	
High school	4 (33)
Associate/Bachelor’s degree	4 (33)
Master’s/Doctorate degree	4 (33)
Marital status, n (%)	
Never married	9 (75)
Married	3 (25)
Income level, n (%) [c]	
Middle/Average	10 (83)
High	2 (17)
Contraceptive use, n (%)	7 (58)
Nulliparous, n (%)	11 (92)

[a] BSA=0.20247*(height (cm)/100)$^{0.725}$ *weight (kg)$^{0.425}$
[b] Hydration cutoff values: 1.000-1.019 (Hydrated), 1.020-1.027 (Moderately dehydrated), 1.028-1.035 (Severely dehydrated) (54).
[c] No participants reported that their average income was low.

Aim 1: Method Comparisons

For Aim 1, plasma volume was estimated using HES and ICG, along with the Kaplan, Hurley, and Nadler equations. The mean (SD) PV estimations were 2,046 (392) mL, 2,765 (820) mL, 2,443 (464) mL, 2,407 (301) mL, and 2,373 (406) mL, respectively (Appendix Table 1). Figure 3-1 shows the comparison of PV estimations across all methods used in this study.

Individual participant estimations for each method are included in Appendix Table 1.

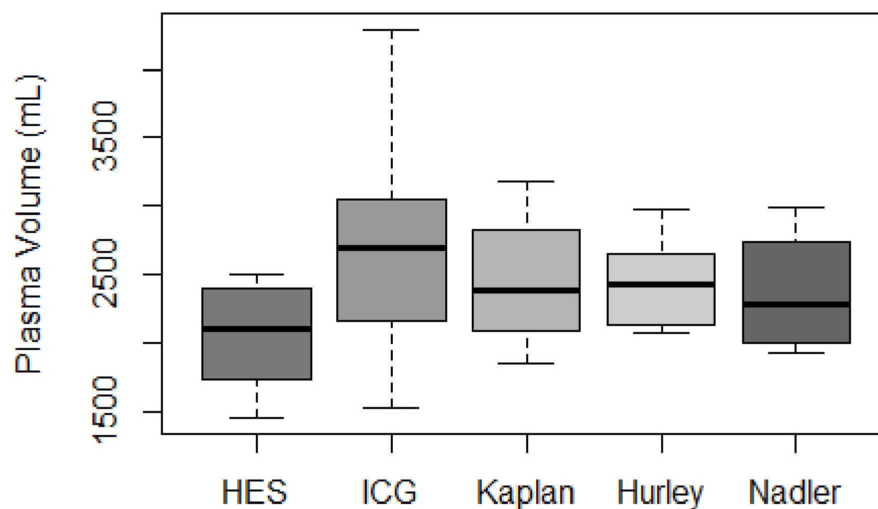

Figure 3-1: Comparison of PV across all methods (n=12).

Aim 1: Method Development

For Aim 1a, we tested key aspects of the protocol as part of method development. We tested the boiling time of the plasma samples (7 minutes versus 10 minutes), the time point selected for comparison to other methods (5 minutes, 10 minutes, or 15 minutes), and the influence of hemolysis on plasma glucose. The results of each experiment and the conclusion of how to proceed are listed below in Table 3-2.

Table 3-2: HES method development experiments.

Experiment	Result	Conclusion
Boiling time – 7 minutes versus 10 minutes[a]	2,384 mL (7 minutes) 1,917 mL (10 minutes)	Proceed with 7 minutes, in line with previously published protocol
Time point for comparison[b]	Mean ± SD mL 5 mins: 3,959 ± 7,706 mL 10 mins: 2,046 ± 392 mL 15 mins: 1,427 ± 2527 mL	Proceed with 10 minutes, in line with previously published protocol
Sample hemolysis[c]	Plasma glucose (mg/dL) No hemolysis: 90 mg/dL Mild hemolysis: 89 mg/dL Moderate hemolysis: 90 mg/dL	Proceed with inclusion of all samples, even those with hemolysis

[a] We tested 4 samples (n = 1; blank pre-injection sample and 3 post-injection samples).
[b] We tested 12 samples for each time point.
[c] We tested 1 clean sample that was then hemolyzed to mild and moderate levels.

Aim 1: HES versus ICG Plasma Volume Estimations

For Aim 1b, we compared the HES PV estimation to the ICG PV estimation using Bland Altman analyses (Figure 3-2 A). Each plot shows the mean difference between the two compared methods and a lower and upper limit of agreement (LOA). If the methods are in perfect agreement, the center mean difference line would be at zero. The mean difference, upper, and lower LOA for the comparison was -718 mL, 748 mL, and -2,185 mL, respectively.

Aim 1: HES versus Estimation Equations

For Aim 1c, we compared the HES PV estimation to the results produced from each estimation equation using Bland Altman analyses (Figure 3-2 B-D). The mean difference, upper, and lower LOA for each comparison are as follows, respectively: HES versus Kaplan (-397 mL, 407 mL, -1,201 mL); HES versus Hurley (-361 mL, 383 mL, -1,106 mL); HES versus Nadler (-

327 mL, 392 mL, -1,046 mL). The mean difference for each comparison with the estimation equations was smaller than HES PV versus ICG PV.

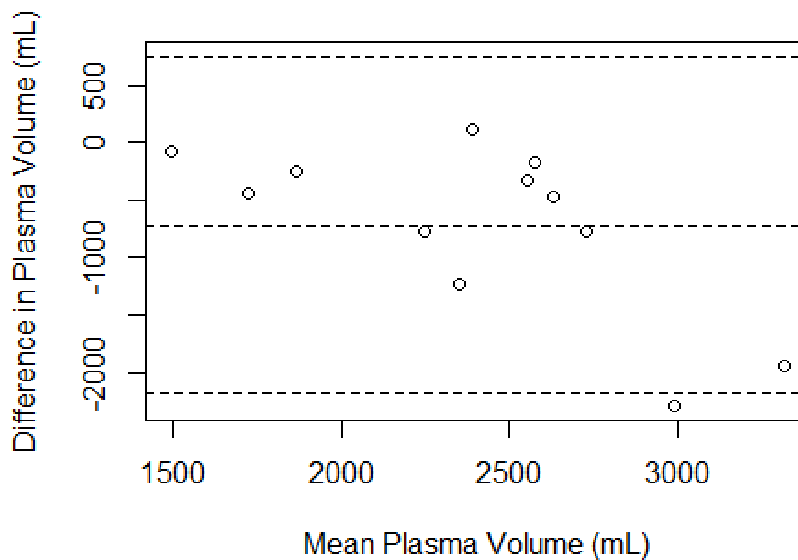

Figure 3-2: Bland Altman analysis plot for PV comparison between HES and ICG (n=12). The open circles represent each participant's difference in PV (mL) between the compared methods. The middle dashed line represents the mean difference in PV (mL) between the compared methods. The top dashed line represents the upper limit of agreement (LOA), placed +2 SD from the mean difference. The bottom dashed line represents the lower limit of agreement (LOA), placed -2 SD from the mean difference.

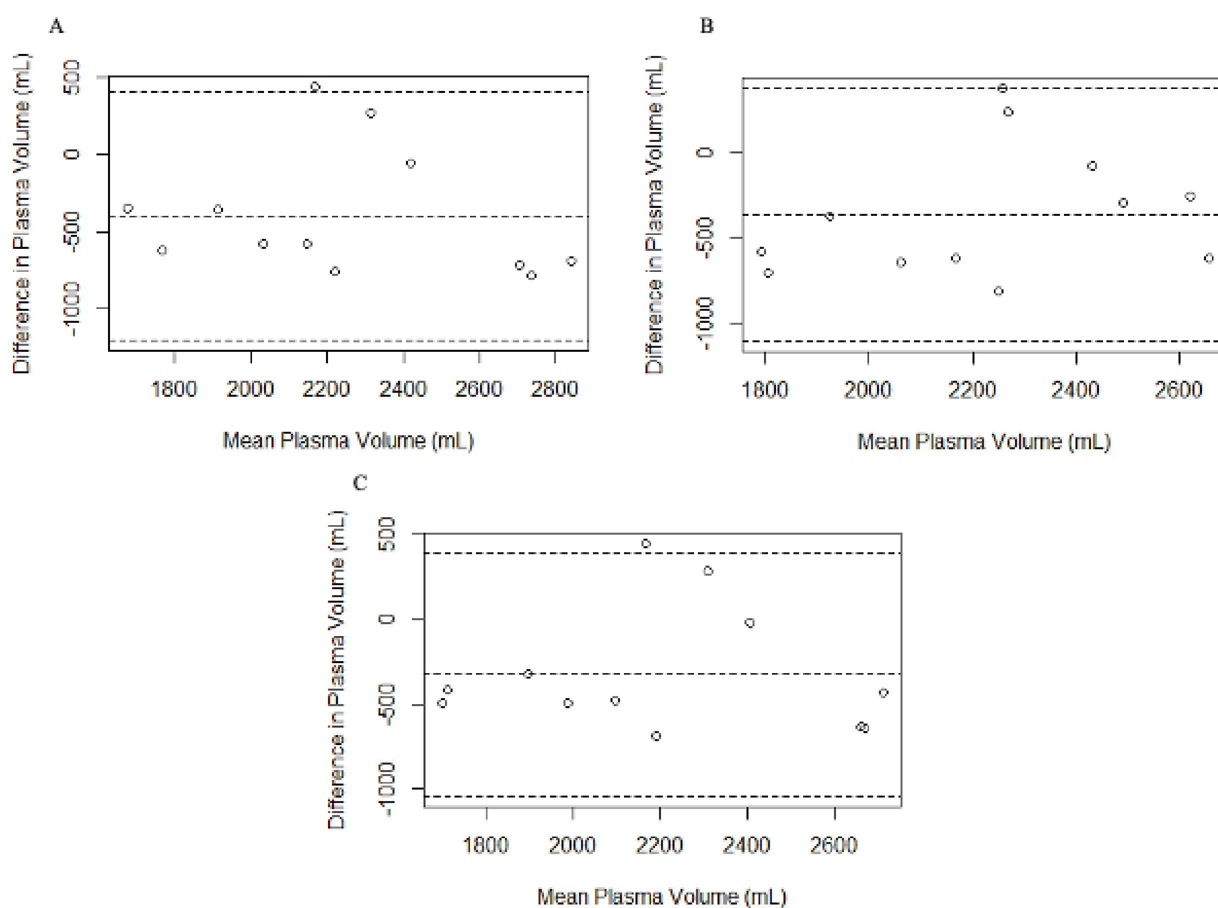

Figure 3-3: Bland Altman analysis plots for PV comparison between HES and A) Kaplan; B) Hurley; and C) Nadler estimation equations (n=12). The open circles represent each participant's difference in PV (mL) between the compared methods. The middle dashed line represents the mean difference in PV (mL) between the compared methods. The top dashed line represents the upper limit of agreement (LOA), placed +2 SD from the mean difference. The bottom dashed line represents the lower limit of agreement (LOA), placed -2 SD from the mean difference.

Aim 2: Relationship between PV and Health Biomarkers

For Aim 2, we measured the correlation between HES PV and SBP, DBP, BMI, BSA, body fat percentage, and urine specific gravity (Table 3-3). Body surface area and body fat percentage had positive correlations of approximately 0.3, but with p-values well above the 0.05 threshold for statistical significance. The other correlations were positive but much smaller (range

0.004-0.16) and not statistically significant. LOWESS plots for each association also show a general lack of relationship between PV and each biomarker (Appendix A).

Table 3-3: Spearman rank-order correlation between HES PV and health markers (n=12).

Health Biomarker	Spearman's rho	p-value
Systolic blood pressure	0.13	0.69
Diastolic blood pressure	0.004	0.99
BMI	0.16	0.62
Body surface area	0.29	0.37
Body fat percentage	0.30	0.34
Urine specific gravity	0.07	0.83

Sensitivity Analysis

Ten participants received 120 mL of HES, and 2 participants received 170 mL of HES for the injection. We conducted a sensitivity analysis by dropping the 2 participants who received 170 mL. For Aim 1, we did not find any difference in mean PV for each method. For Aim 2, the Spearman's rho increased for SBP, DBP, BMI, BSA, and body fat percentage and decreased for decreased for USG. None of the correlations were statistically significant after the sensitivity analysis, but all Spearman's rho were above 0.3, except urine specific gravity. The correlation between HES PV and body surface area was trending towards statistical significance after the sensitivity analysis (p-value = 0.07). LOWESS plots for each correlation have been included in the appendix.

Chapter 4

Discussion and Conclusions

In this comparison study of HES and other methods to measure PV in healthy women of reproductive age, we sought to develop a method to measure PV that was safe to use in pregnancy and easier to use in low-resource settings, compared to current methods. Our HES method measured PV in a range expected for women of reproductive age. We found that, contrary to our hypothesis, HES estimates were lowest and did not closely align with any of the other methods; the equations produced similar results and were closer to HES estimates (~300-400 mL difference) than those of ICG (~700 mL difference). Overall, the estimations across all methods were far apart and likely not comparable to each other across people or studies. We also found no statistically significant correlations between PV by HES and SBP, DBP, BMI, BSA, and urine specific gravity; the correlations for DBP and urine specific gravity were both positive but close to zero, while all other correlations were positive and between 0.13 and 0.3.

In Aim 1, we found that the HES method produced reasonable estimates for PV, but they were farther apart than we expected from the other method estimates. To our knowledge, only 2 studies have been conducted to compare HES to other methods for measuring blood volume. Tschaikowsky *et al*. compared the HES method to carbon monoxide rebreathing and an estimation equation in critically ill hospital patients undergoing surgery (n = 12) (38). In their study, there was a high level of agreement between the laboratory methods, with a mean difference of 1%, indicating that the HES method could be used in place of the carbon monoxide method to estimate blood volume. The mean difference in blood volume between the laboratory methods was 52.3 ± 183 mL; the mean blood volume estimates using the HES and carbon monoxide methods were 5,261.1 ± 616.5 mL and 5,208.8 ± 639.9 mL, respectively. However, there was poor agreement between the HES method and the predicted blood volume by the

estimation equation, showing that the equation should not be used clinically in place of direct blood volume measurement. Our study confirmed the latter findings that the laboratory methods do not closely agree with the estimation equations (38). Vricella *et al*. compared HES to a weight-based estimation equation developed by Feldschuh and Enson to measure blood volume in pregnant women with (n = 30) and without obesity (n = 29) (11,12). Their study found that the HES method produced a consistently higher mean blood volume compared to the estimation equation for the full cohort (7,500 ± 2,600 mL versus 5,000 ± 500 mL, respectively). The mean blood volume estimate using HES was 6,944 ± 2,830 mL for pregnant women with normal weight, and the mean blood volume estimate using the equation was 4,417 ± 436 mL (11). Our study found the opposite, where the HES method produced lower mean plasma volume values compared to our selected estimation equations. This may be explained because the blood volume equation chosen by Vricella *et al*. included sex, height, weight, and deviation from desired weight, while our chosen equations included only weight, height, and hematocrit at the time of the visit (11).

In our study, the mean PV estimates for HES and ICG were 2,046 ± 392 mL 2,765 ± 820 mL, respectively. There has been little other research conducted to examine PV in healthy women of reproductive age. Aguree and Gernand conducted a pilot study to examine the association between PV measured by ICG and micronutrient biomarker concentrations and mass in healthy women of reproductive age (n = 9) (17). The mean PV was 2,067 ± 470 mL in this study, which is close to our mean PV estimation using HES. In another study conducted by Aguree *et al.*, ICG was used to examine PV variation across the menstrual cycle in healthy women of reproductive age (n = 45) (56). The mean PV estimations were 2,276 ± 478 mL (early follicular phase), 2,232 ± 509 mL (late follicular phase), and 2,228 ± 502 mL (midluteal phase); these mean values are higher than our HES estimations but lower than our ICG estimations, but our ICG standard deviation is much higher (820 mL versus ~500 mL) (56). We chose to inject the ICG solution

first followed by the HES solution, so that the HES volume would not influence PV estimated by ICG. Due to this, participants were lying down for a longer period before the HES injection compared to the ICG injection and it is possible that this contributed to the inconsistency of the PV estimates by ICG. Given that we injected a small amount of HES (120-170 mL compared to 500-1,000 mL used clinically), it is likely that we would see similar results if the method order was switched, but we should consider accounting for the HES volume when estimating PV using ICG.

Other studies have been conducted to compare laboratory methods for measuring plasma volume. Poulsen *et al*. compared PV measured by carbon monoxide rebreathing and Evans' blue dye in healthy male participants (n = 10) exposed to sea level and high altitude (57). They found that PV measured by carbon monoxide rebreathing decreased by almost 10% at high altitude compared to sea level, while PV measured by Evans' blue dye did not change. The mean difference between the methods was significantly different from zero in those who experienced hypoxia due to the elevation change; the mean difference at sea level was 0.11, and the mean difference at high altitude was 0.43. The mean PV estimates using Evans' blue and carbon monoxide were 3.49 L and 3.39 L, respectively (57). Menth-Meier *et al*. compared PV measured by ICG and radiolabeled iodinated human serum albumin in both healthy (n = 10) and post-operative (n = 21) participants (58). The mean PV measured by ICG produced results consistent with PV measured by radiolabeled iodinated human serum albumin; the maximum lack of agreement was -9.1 mL/kg body weight (58). Unlike these studies, our study did not find consistent results between our chosen laboratory methods of HES and ICG, although we hypothesized that they would be in better agreement.

We did not find any statistically significant associations between PV measured by HES and key biomarkers of health (SBP, DBP, BMI, BSA, and urine specific gravity). We hypothesized that there would be a positive correlation between PV and blood pressure, BMI, and

BSA, and a negative correlation between PV and urine specific gravity. Our study found a weak positive correlation between PV measured by HES and both systolic and diastolic blood pressure, although not significant. This confirms the directionality of the relationship seen in previous research (6). However, it is difficult to compare our results to previous studies that examine the relationship between PV and blood pressure because they have been conducted primarily in individuals with hypertension or related disorders. Some of these studies have shown an inverse relationship between PV and blood pressure. Spitz *et al*. conducted a study using NHANES data to investigate the relationship between estimated PV and blood pressure, and whether it was dependent on weight status (n = 46,059) (59). They found a significant inverse relationship between PV and blood pressure: for every 1 unit increase in estimated PV, the odds of having hypertension decreased by 10% (59). Other studies have been conducted, but only include males (60).

We found positive correlations, although not statistically significant, between PV measured by HES and BMI, BSA, and body fat percentage; after the sensitivity analysis, the correlation for PV and BSA increased but remained insignificant. These correlations support the relationships found by previous literature. However, these studies are outdated and include populations other than healthy women of reproductive age, including men and pregnant women with normal weight and obesity. In a convenience sample of hospitalized men (n = 49) and women (n = 41), Gibson and Evans, Jr. found that there was an increase in total blood volume as the participants' height, weight, and body surface area increased; their statistical analysis is unclear and they did not provide correlation or regression data (13). Huff and Feller found similar results in their study of healthy men (n = 42) and women (n = 20); as body density increased, the calculated PV increased as well (r = 0.59) (10). Vricella *et al.* conducted a study to measure blood volume in a sample of pregnant women with normal weight and obesity. They found that blood volume was significantly higher in pregnant women with obesity; as BMI increased, unit blood

volume decreased. As well, they found a weak positive correlation when evaluating the relationship between calculated blood volume and percent lean body mass (adjusted $r^2 = 0.1$) (11).

Our study found a weak positive correlation between PV and urine specific gravity; after the sensitivity analysis, the correlation changed to weakly negative but remained insignificant. These results conflict with both our hypothesis and previous literature. It is again difficult to compare our results to those of previous work because the other studies were conducted under prolonged endurance performance or temperature duress, while our study enrolled normal volunteers in a research clinic. In a study of healthy men under prolonged exercise and varying dehydration and heating conditions (n = 32), Kenefick *et al.* found that as hydration status worsened (i.e., the individual became more dehydrated), plasma volume decreased as a response to this (16). We expected to see a similar inverse relationship in our study but were surprised to see a positive correlation instead.

The main strength of our study is the comparison nature; there are few studies that have compared PV methods to examine their agreement and consistency, and there is even less work comparing the HES method to other methods of PV measurement. Our study compared both laboratory-based (HES and ICG) and estimation equation (Kaplan, Hurley, Nadler) methods to measure PV, while other studies have compared fewer methods. As well, we measured PV in a convenience sample of healthy volunteers, and other studies have often focused on critically ill or hospitalized participants. A key limitation of our study is the small sample size; it is possible that this prevented us from seeing any statistical significance in correlations for Aim 2. We also did not include the gold-standard radiolabeled iodinated human serum albumin method for measuring PV. Instead, we compared the HES method to a validated method that we have used in previous research. We could not find any specific reasons for the differences between our PV estimations and others. Throughout our study, we did troubleshooting with the laboratory work, including

taking replicate measurements of glucose and hematocrit for each participant and reanalyzing samples that produced biologically implausible results when possible. We also closely reviewed any parameters that may have had an influence in the PV estimations, such as height (shorter versus taller participants) and body fat percentage. In our study, participants were instructed to rest lying down for 15 minutes before the IV was established. It is possible that the ICG results would have been more consistent and stable if participants had been lying down for a longer period of time before the IV was established, such as 30 minutes.

In a convenience sample of healthy women of reproductive age, our comparison pilot study showed that PV measured by HES did not closely align with other methods to measure PV (ICG and estimation equations). We also found no statistically significant correlations between PV and key biomarkers of health (blood pressure, BMI, BSA, and urine specific gravity). The HES method is easy to implement and use, and it is safe for use in a variety of populations, including pregnancy. However, further research is needed to investigate PV in healthy individuals, so that we can better understand PV under normal conditions and apply that knowledge to individuals with abnormal PV or disease states that may alter PV.

Appendix A

HES Laboratory Protocol

Pre-procedure

Make tris buffer: 1 L of 3.33M tris buffer = 403.4g tris + 1000 mL ddH2O. Stir with magnetic stirrer on stirring plate. May take up to 60 minutes to fully dissolve.

Procedure

1. Fill and turn on water bath to 100°C. Allow to reach boiling.
2. Prepare room temperature water bath by putting tap water into container.
3. Complete quality control check of pH meter.
4. Assay whole blood and pre-HES injection plasma for glucose using HemoCue glucometer.
5. Label all tubes.
6. Transfer 0.6 mL plasma into microcentrifuge tube. Add 0.15 mL HCl and vortex.
7. Place tube in rack in boiling water bath for 7 minutes.
8. Remove tube rack and put into room temperature water bath for 2 minutes. Vortex well.
9. Add 0.65 mL of 3.33M tris buffer. Vortex and incubate sample for 6 minutes on bench.
10. Vortex samples again.
11. Measure pH of each sample using pH strips. Target pH is 7.0 +/- 0.5. If needed, add X mL 3.33M tris buffer to achieve desired pH.
12. Centrifuge sample at 3600 rpm for 16 minutes. Obtain supernatant.
13. Assay supernatant using HemoCue glucometer.
14. Repeat steps 5 through 12 for all pre- and post-injection plasma samples.

Appendix B

Supplemental Plasma Volume Data

Table A-1: PV across all methods for each participant (n=12).

Participant[a]	HES (mL)	ICG (mL)	Kaplan (mL)	Hurley (mL)	Nadler (mL)
Mean (SD)	2046 (392)	2765 (820)	2443 (464)	2407 (301)	2373 (406)
1	1737	1991	2092	2110	2062
2	2344	3116	3127	2640	2978
3	2495	2661	3184	2748	2929
4	2389	2723	1949	2148	1945
5	1741	2965	2323	2379	2238
6	1503	1940	1848	2078	1924
7	2393	2864	2448	2468	2419
8	2346	4290	3062	2967	2991
9	1454	1527	2079	2155	1947
10	1809	4133	2598	2656	2538
11[b]	2431	2328	2177	2066	2170
12[b]	1833	2637	2434	2474	2335

[a] The participant number in this column is not each participant's study identifier code.
[b]These participants received 170 mL of HES; all other participants received 120 mL of HES.

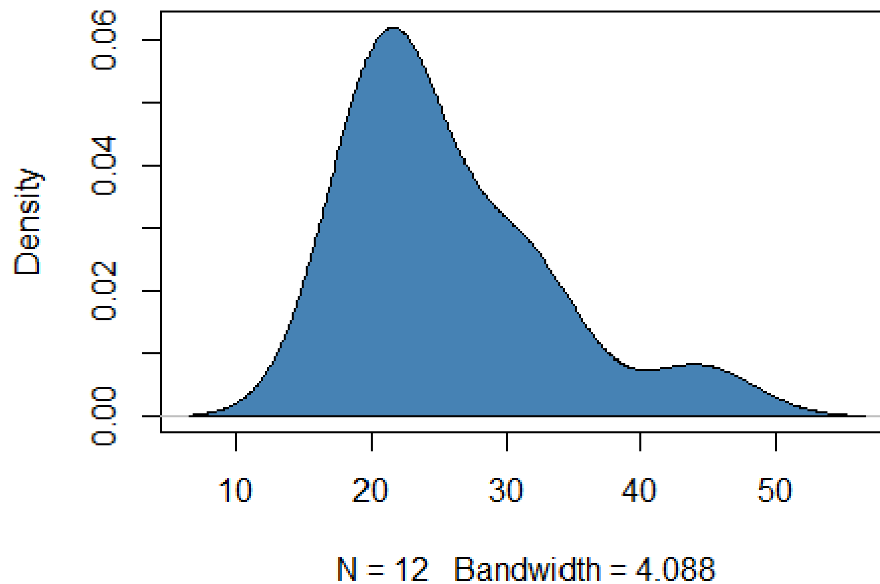

Figure A-1: Kernel density plot for participant age (n=12).

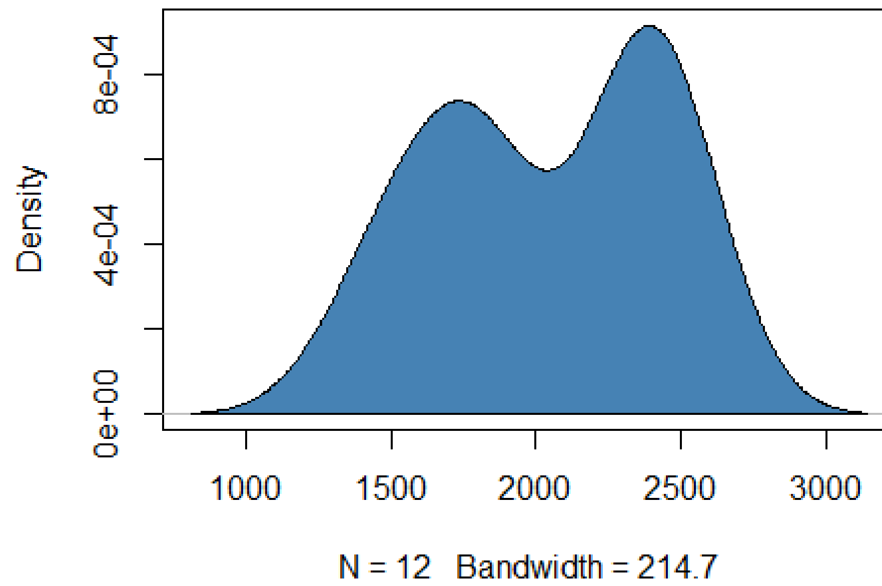

Figure A-2: Kernel density plot for HES PV at 10 minutes (n=12).

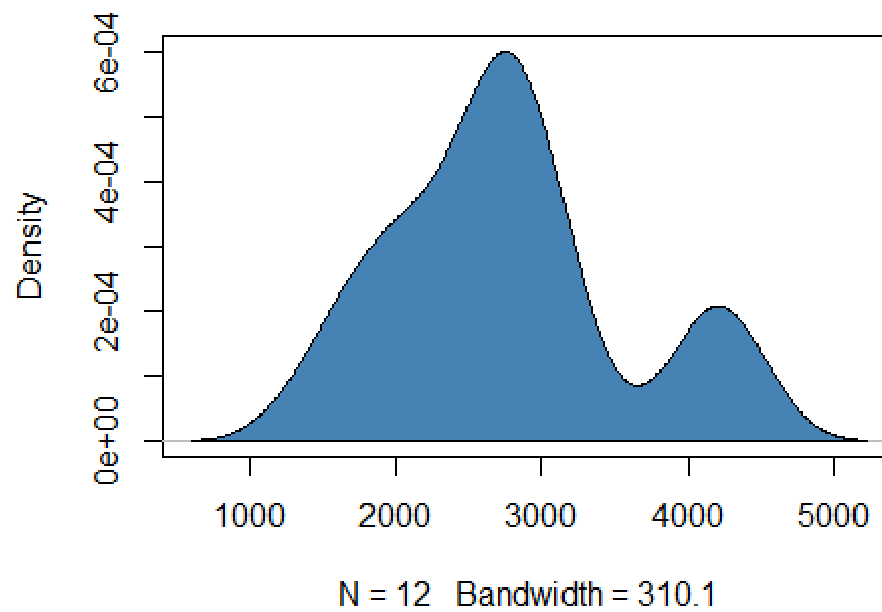

Figure A-3: Kernel density plot for ICG at time of injection (n=12).

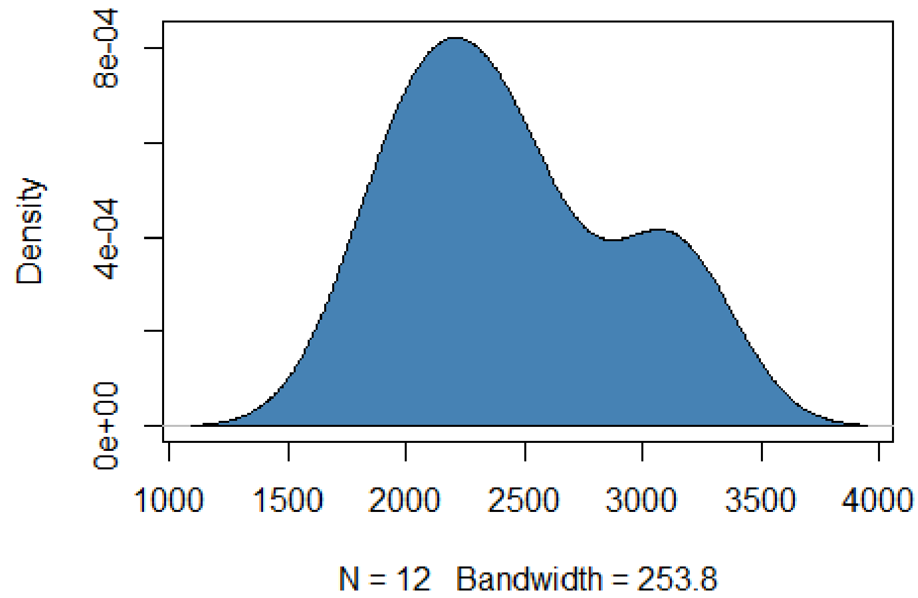

Figure A-4: Kernel density plot for Kaplan EPV (n=12).

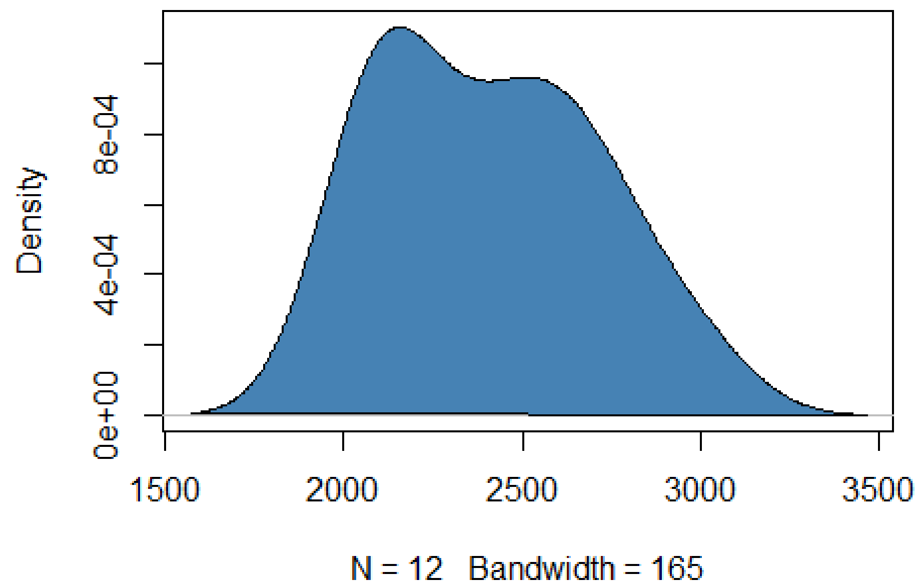

Figure A-5: Kernel density plot for Hurley PV (n=12).

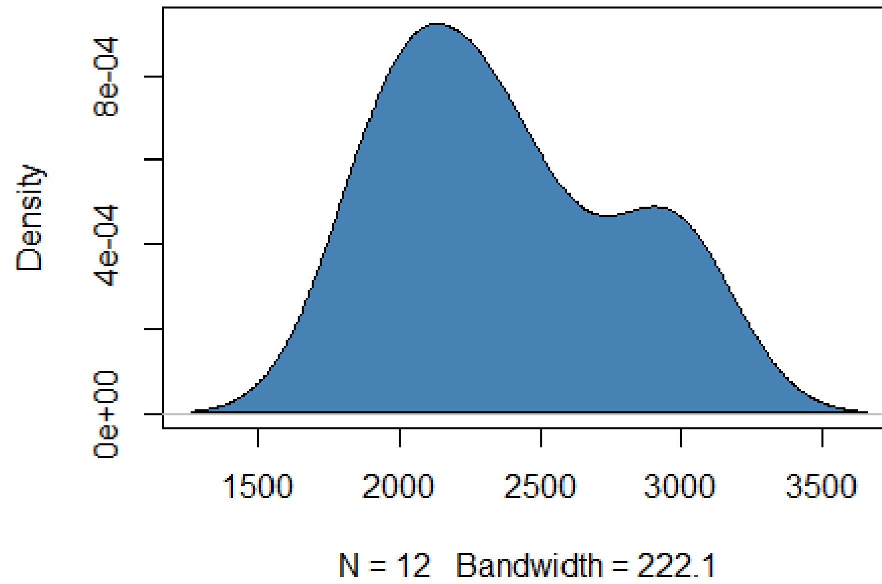

Figure A-6: Kernel density plot for Nadler PV (n=12).

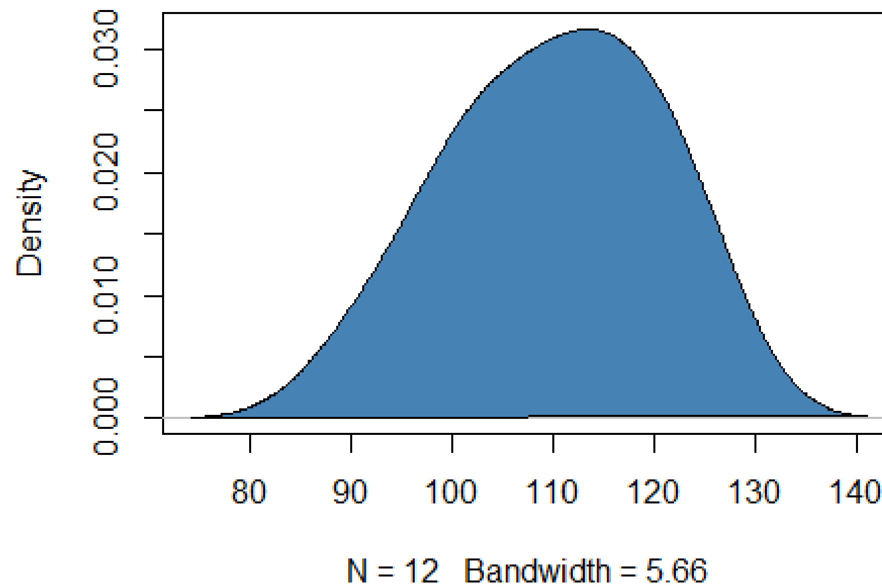

Figure A-7: Kernel density plot for average SBP (n=12).

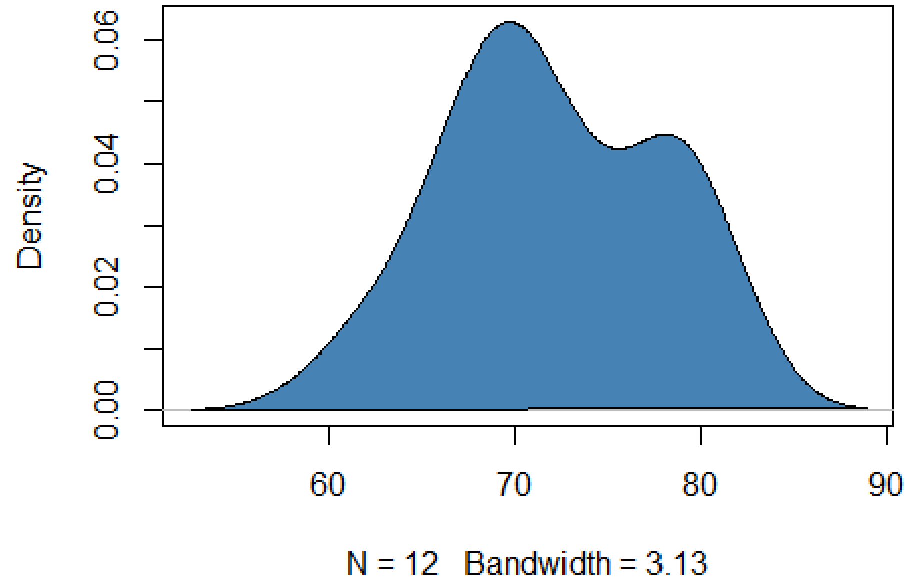

Figure A-8: Kernel density plot for average DBP (n=12).

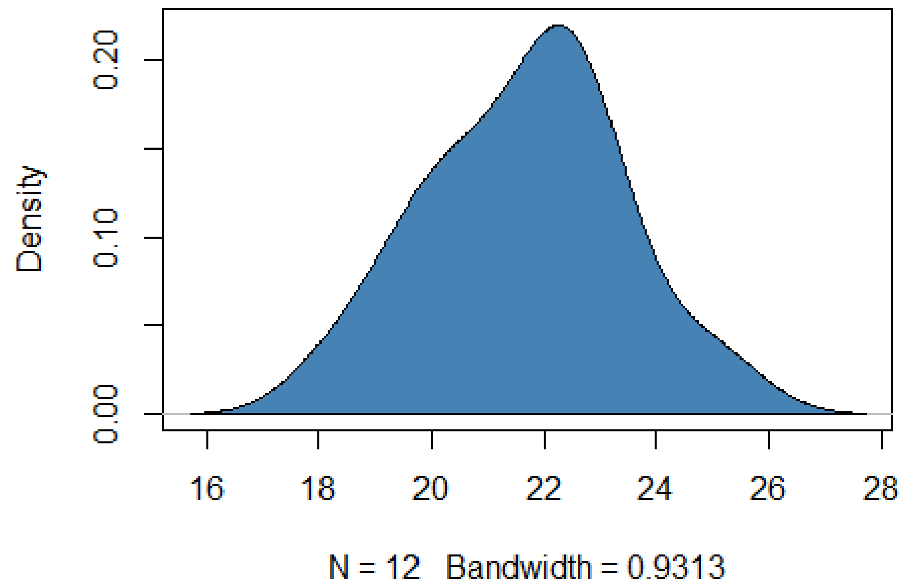

Figure A-9: Kernel density plot for BMI (n=12).

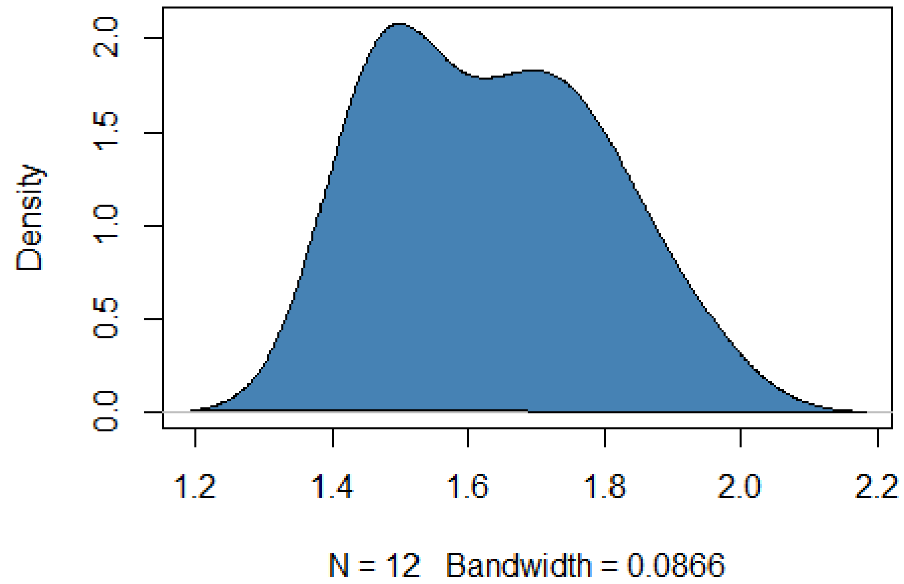

Figure A-10: Kernel density plot for BSA (n=12).

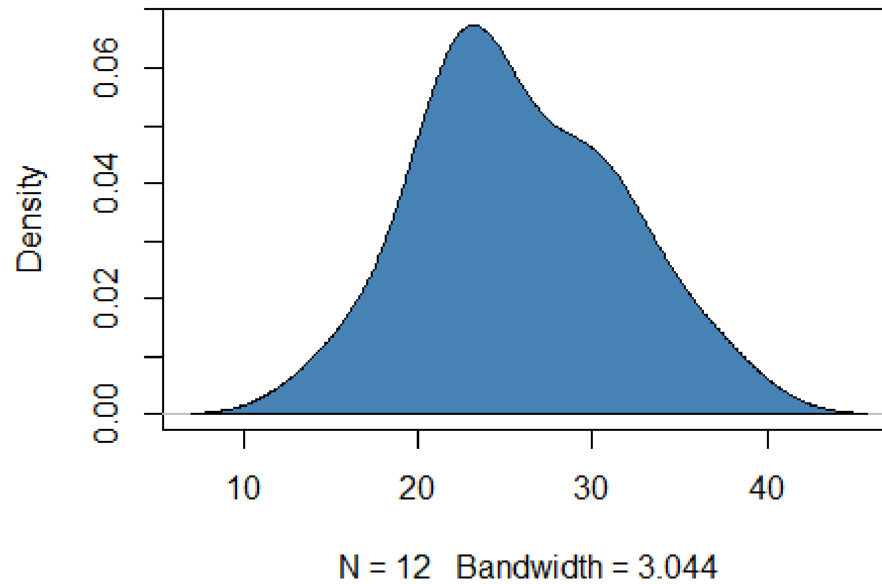

Figure A-11: Kernel density plot for average body fat percentage (n=12).

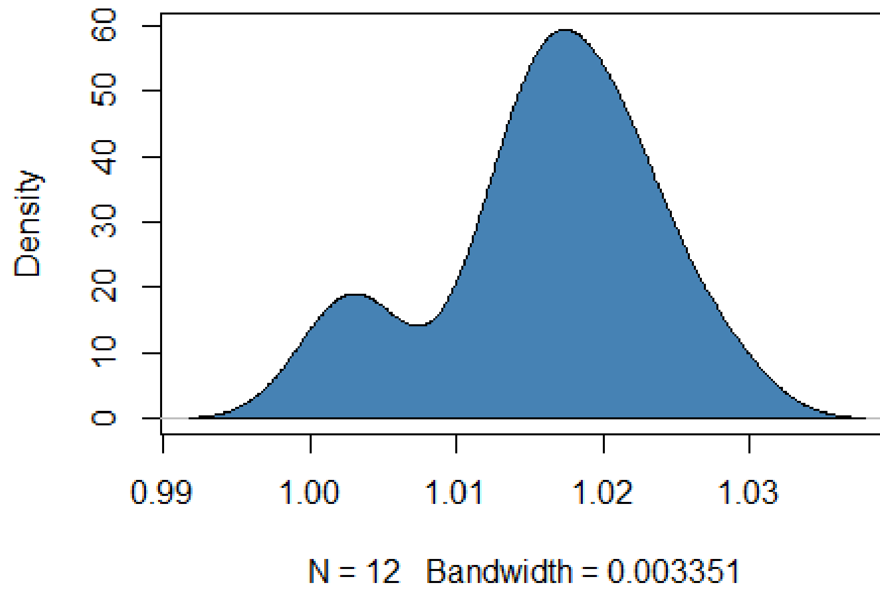

Figure A-12: Kernel density plot for average urine specific gravity (n=12).

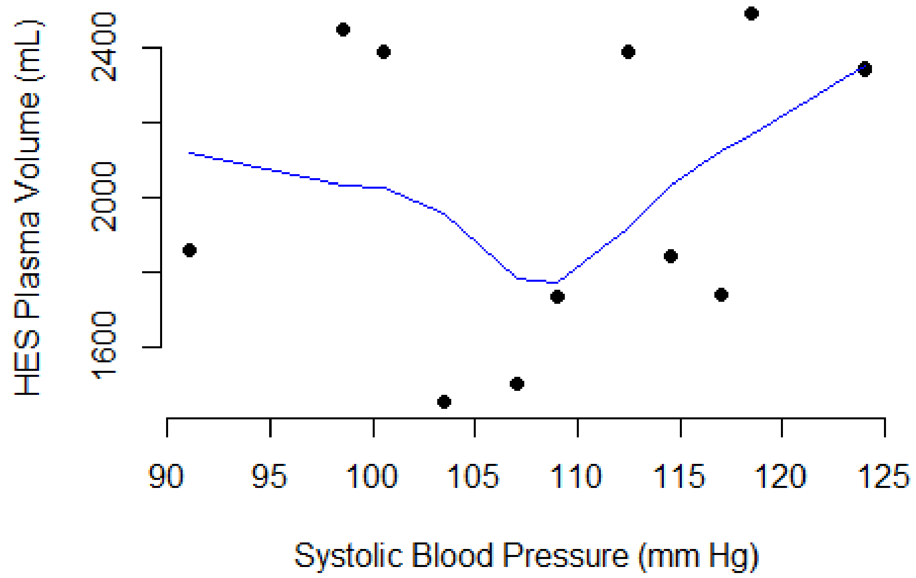

Figure A-13: LOWESS plot for SBP and HES PV (n=12).

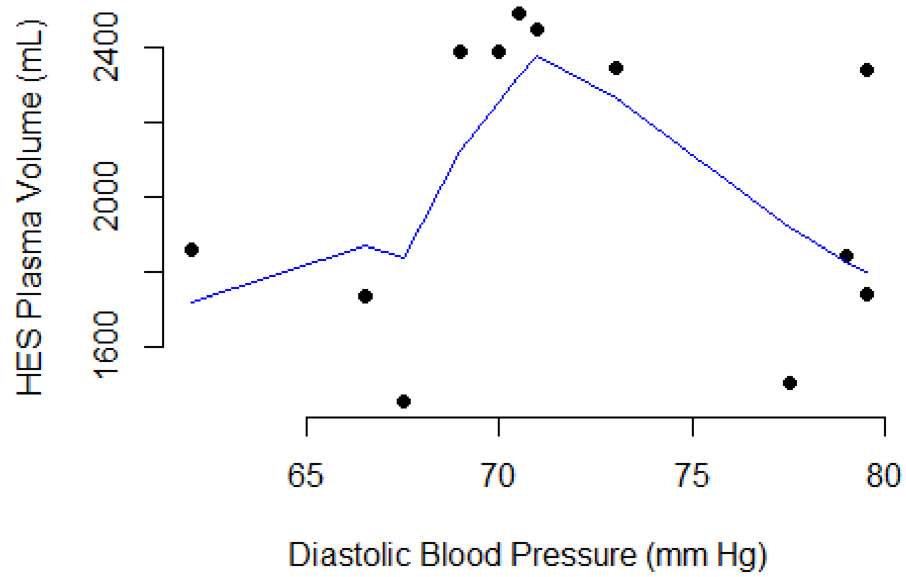

Figure A-14: LOWESS plot for DBP and HES PV (n=12).

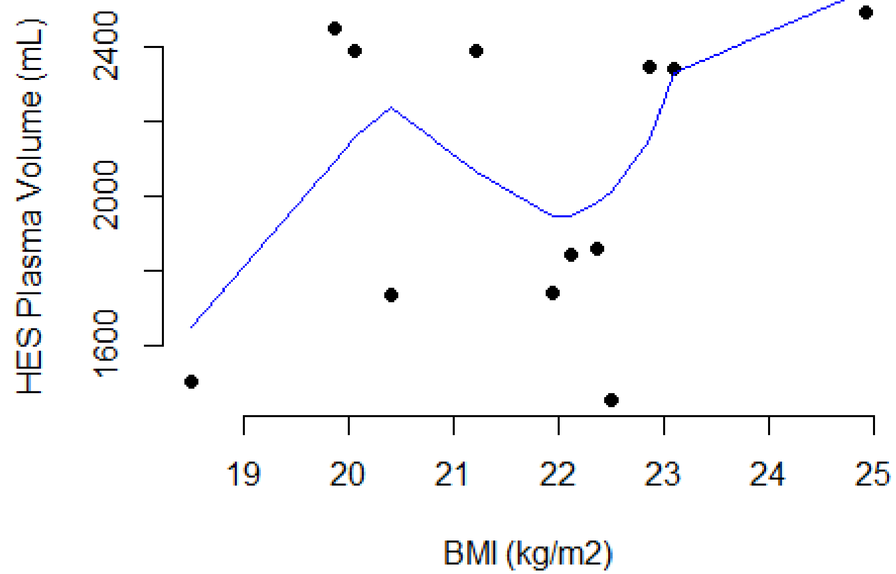

Figure A-15: LOWESS plot for BMI and HES PV (n=12).

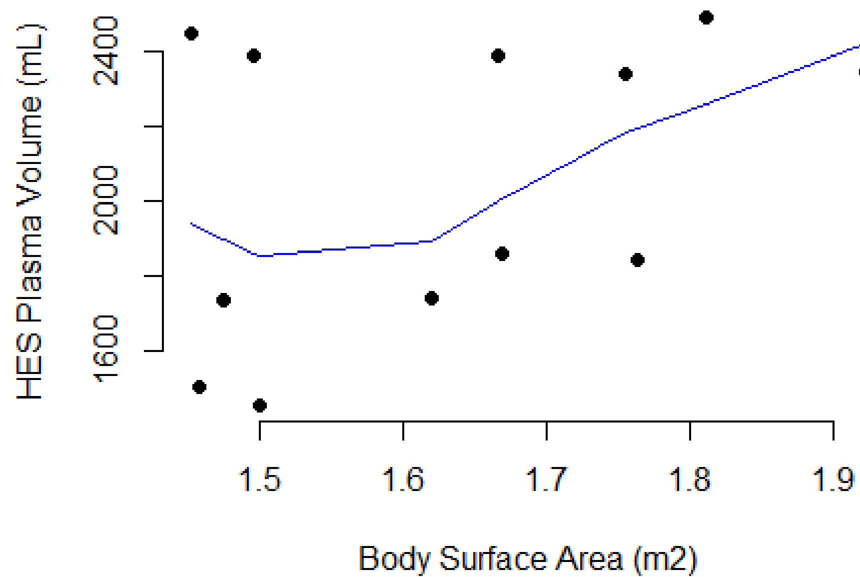

Figure A-16: LOWESS plot for BSA and HES PV (n=12).

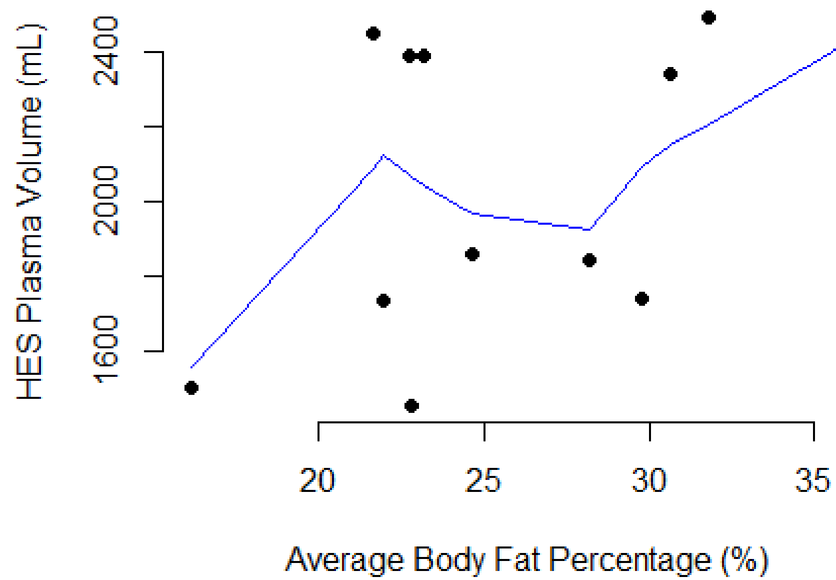

Figure A-17: LOWESS plot for body fat percentage and HES PV (n=12).

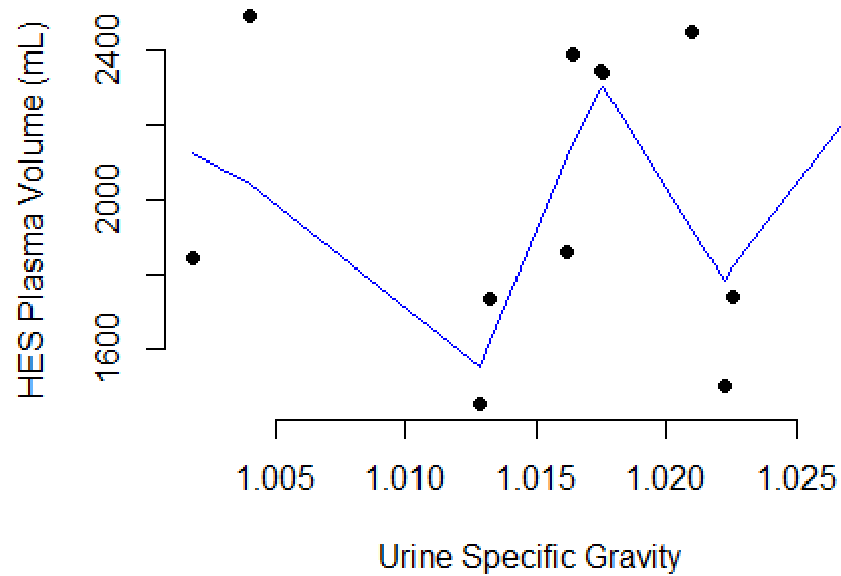

Figure A-18: LOWESS plot for urine specific gravity and HES PV (n=12).

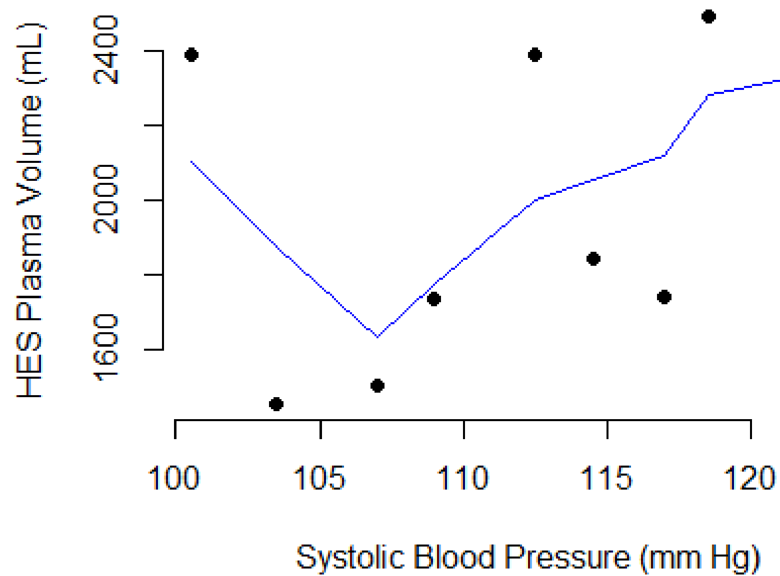

Figure A-19: LOWESS plot for SBP and HES PV (n=10).

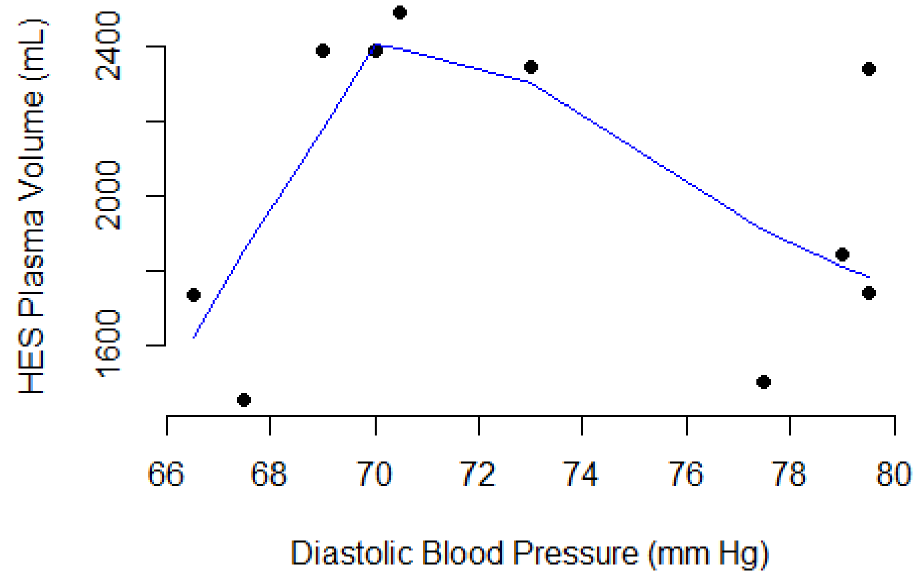

Figure A-20: LOWESS plot for DBP and HES PV (n=10).

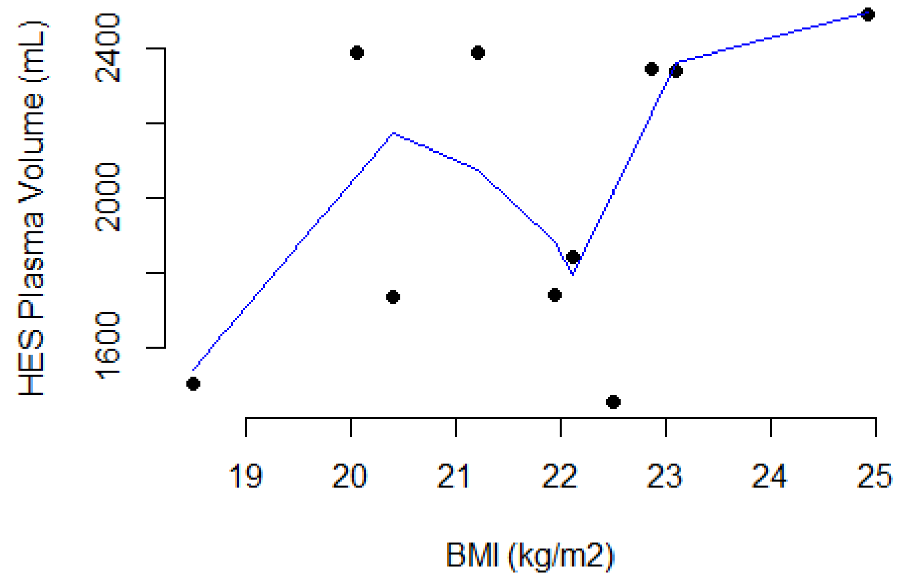

Figure A-21: LOWESS plot for BMI and HES PV (n=10).

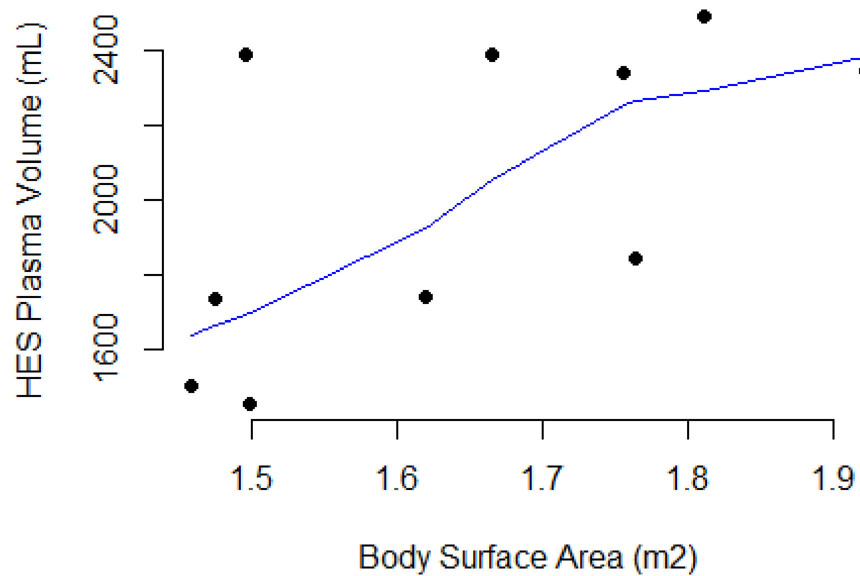

Figure A-22: LOWESS plot for BSA and HES PV (n=10).

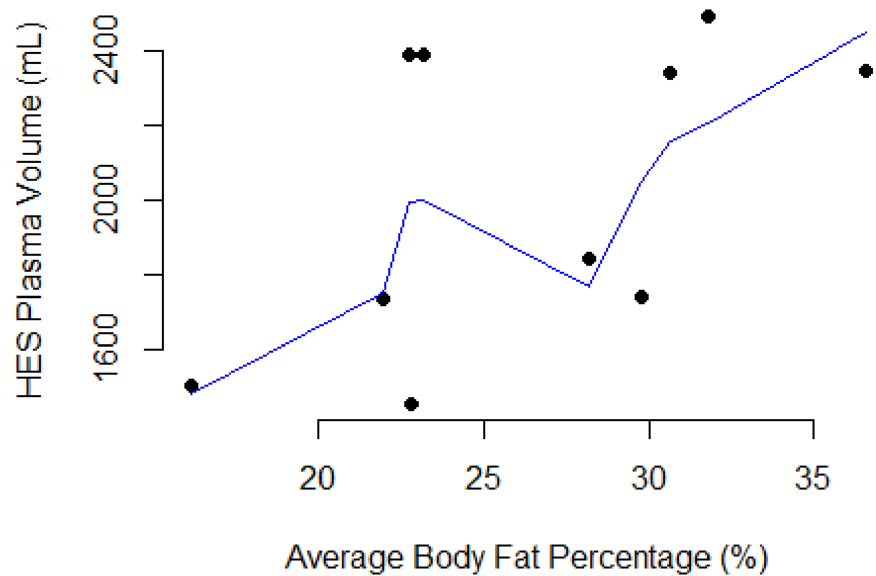

Figure A-23: LOWESS plot for body fat percentage and HES PV (n=10).

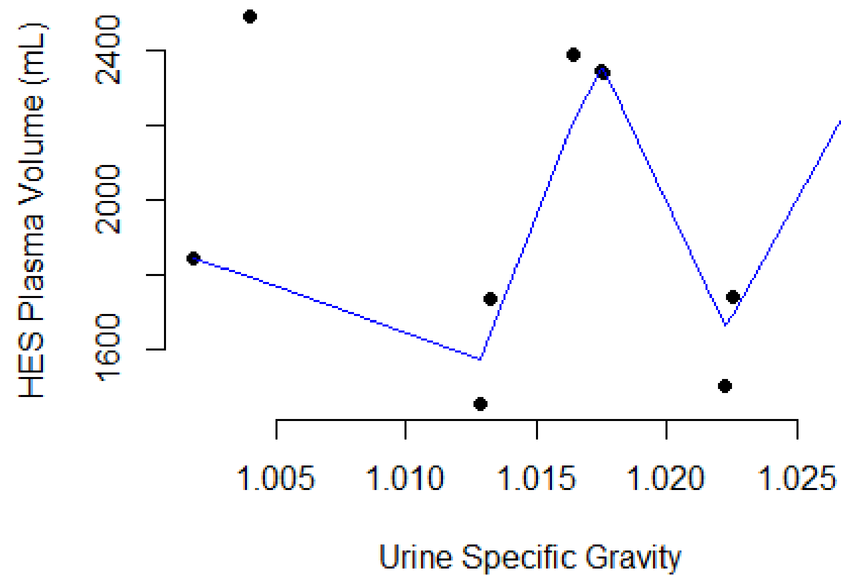

Figure A-24: LOWESS plot for urine specific gravity and HES PV (n=10).

Appendix C

R Code

Load dataset into R

```
library(readxl)
PV_Pilot_Phase_1_copy <- read_excel("C:/Users/yahoo/OneDrive - The Penn
sylvania State University/Masters Degree Thesis/Data Analysis/PV Pilot
Phase 1_copy.xlsx")

## New names:
## * form1a_q19a -> form1a_q19a...37
## * form1a_q19a -> form1a_q19a...38

View(PV_Pilot_Phase_1_copy)
phase1 <- PV_Pilot_Phase_1_copy
head(phase1)
```

Drop participants without full data

```
phase1 <- phase1[-c(5,6,7,12,15),]
head(phase1)
```

Create new variables

```
#BMI
phase1$bmi = phase1$form1a_q20wt/((phase1$form1a_q20ht/100)^2)

#Average body composition
phase1$avgbodycomp <- (phase1$form3_q1a+phase1$form3_q1b)/2

#Body surface area
phase1$bsa = 0.20247 * (phase1$form1a_q20ht/100) ^ 0.725 * phase1$form1
a_q20wt ^ 0.425

#Average urine specific gravity
phase1$avgusg <- (phase1$form3_q2_1+phase1$form3_q2_2)/2

#ICG plasma volume values from L to mL
phase1$icg_pv1ml = phase1$icg_pv1 * 1000
phase1$icg_pv2ml = phase1$icg_pv2 * 1000
```

Plasma volume equations (Kaplan, Hurley, Nadler)

```
#Kaplan estimated plasma volume
phase1$kaplan = (0.065 * phase1$form1a_q20wt * (1 - (phase1$avghct/100)
)) * 1000
```

```
#Hurley estimated plasma volume equation
phase1$hurley = 1278 * (phase1$bsa ^ 1.289)

#Nadler estimated total blood volume
phase1$nadlertbv = ((0.3561 * (phase1$form1a_q20ht / 100) ^ 3) + (0.033
08 * phase1$form1a_q20wt) + 0.1833) * 1000

#Nadler estimated tbv converted to plasma volume
phase1$nadlerpv = phase1$nadlertbv * (1 - (phase1$avghct / 100))
```

Demographic data

```
#Age
mean(phase1$form1a_age)

sd(phase1$form1a_age)

median(phase1$form1a_age)

#BMI
mean(phase1$bmi)

median(phase1$bmi)

sd(phase1$bmi)

#Body surface area
mean(phase1$bsa)

median(phase1$bsa)

sd(phase1$bsa)

#Systolic blood pressure
mean(phase1$form1a_q18avgsbp)

median(phase1$form1a_q18avgsbp)

sd(phase1$form1a_q18avgsbp)

#Diastolic blood pressure
mean(phase1$form1a_q18avgdbp)

median(phase1$form1a_q18avgdbp)

sd(phase1$form1a_q18avgdbp)

#Average body composition
mean(phase1$avgbodycomp)

median(phase1$avgbodycomp)
```

```
sd(phase1$avgbodycomp)

#Average urine specific gravity
mean(phase1$avgusg)

median(phase1$avgusg)

sd(phase1$avgusg)

#Tabulate race and ethnicity
table(phase1$form2_q4)

table(phase1$form2_q3)

#Tabulate marital status
table(phase1$form2_q2)

#Tabulate education level
table(phase1$form2_q6)

#Tabulate income
table(phase1$form2_q11)

#Tabulate use of contraceptives and type
table(phase1$form2_q15)

table(phase1$form2_q16)

#Tabulate number of pregnancies
table(phase1$form2_q17)

#Tabulate number of pregnancies resulting in a delivery past 20 weeks g
estation
table(phase1$form2_q18)

#Tabulate USG categories
phase1$usg_cat <- as.factor(ifelse(phase1$avgusg < 1.0190, 'Hydrated',
                            ifelse(phase1$avgusg < 1.0270, 'Moderately
Dehydrated',
                            ifelse(phase1$avgusg < 1.0360, 'Severely De
hydrated'))))
table(phase1$usg_cat)
```

Summary plasma volume data

```
#ICG plasma volume at time = 0
mean(phase1$icg_pv1ml)

sd(phase1$icg_pv1ml)

median(phase1$icg_pv1ml)
```

```
#ICG plasma volume at time = 60 seconds
mean(phase1$icg_pv2ml)

sd(phase1$icg_pv2ml)

median(phase1$icg_pv2ml)

#HES plasma volume at 5 minutes
mean(phase1$hes_pv1)

sd(phase1$hes_pv1)

median(phase1$hes_pv1)

#HES plasma volume at 10 minutes
mean(phase1$hes_pv2)

sd(phase1$hes_pv2)

median(phase1$hes_pv2)

#HES plasma volume at 15 minutes
mean(phase1$hes_pv3)

sd(phase1$hes_pv3)

median(phase1$hes_pv3)

#Kaplan equation
mean(phase1$kaplan)

sd(phase1$kaplan)

median(phase1$kaplan)

#Hurley equation
mean(phase1$hurley)

sd(phase1$hurley)

median(phase1$hurley)

#Nadler equation
mean(phase1$nadlerpv)

sd(phase1$nadlerpv)

median(phase1$nadlerpv)
```

Kernel density plots

```
#Age
#Title: Kernel Density Plot for Participant Age
```

```
age_kd <- density(phase1$form1a_age)
plot(age_kd)
polygon(age_kd, col = 'steelblue', border = 'black')

#HES at 10 minutes
#Title: Kernel Density Plot for HES PV
hespv_kd <- density(phase1$hes_pv2)
plot(hespv_kd)
polygon(hespv_kd, col = 'steelblue', border = 'black')

#ICG at 0 seconds
#Title: Kernel Density Plot for ICG PV
icgpv_kd <- density(phase1$icg_pv1ml)
plot(icgpv_kd)
polygon(icgpv_kd, col = 'steelblue', border = 'black')

#Kaplan EPV equation
#Title: Kernel Density Plot for Kaplan EPV
kaplanepv_kd <- density(phase1$kaplan)
plot(kaplanepv_kd)
polygon(kaplanepv_kd, col = 'steelblue', border = 'black')

#Hurley HPV equation
#Title: Kernel Density Plot for Hurley HPV
hurleyhpv_kd <- density(phase1$hurley)
plot(hurleyhpv_kd)
polygon(hurleyhpv_kd, col = 'steelblue', border = 'black')

#Nadler converted PV equation
#Title: Kernel Density Plot for Nadler PV
nadlerpv_kd <- density(phase1$nadlerpv)
plot(nadlerpv_kd)
polygon(nadlerpv_kd, col = 'steelblue', border = 'black')

#Average systolic blood pressure
#Title: Kernel Density Plot for Systolic Blood Pressure
sbp_kd <- density(phase1$form1a_q18avgsbp)
plot(sbp_kd)
polygon(sbp_kd, col = 'steelblue', border = 'black')

#Average diastolic blood pressure
#Title: Kernel Density Plot for Diastolic Blood Pressure
dbp_kd <- density(phase1$form1a_q18avgdbp)
plot(dbp_kd)
polygon(dbp_kd, col = 'steelblue', border = 'black')

#Calculated BMI
#Title: Kernel Density Plot for BMI
bmi_kd <- density(phase1$bmi)
```

```
plot(bmi_kd)
polygon(bmi_kd, col = 'steelblue', border = 'black')

#Calculated body surface area (BSA)
#Title: Kernel Density Plot for Body Surface Area
bsa_kd <- density(phase1$bsa)
plot(bsa_kd)
polygon(bsa_kd, col = 'steelblue', border = 'black')

#Average body fat percentage
#Title: Kernel Density Plot for Body Fat Percentage
bodyfat_kd <- density(phase1$avgbodycomp)
plot(bodyfat_kd)
polygon(bodyfat_kd, col = 'steelblue', border = 'black')

#Average urine specific gravity (USG)
#Title: Kernel Density Plot for Urine Specific Gravity
usg_kd <- density(phase1$avgusg)
plot(usg_kd)
polygon(usg_kd, col = 'steelblue', border = 'black')
```

Visualize data using scatter plots

```
#Average SBP vs HES
#Title: Systolic Blood Pressure vs HES PV
plot(phase1$form1a_q18avgsbp, phase1$hes_pv2, xlab = "Systolic Blood Pr
essure (mm Hg)", ylab = "HES Plasma Volume (mL)", pch = 19, frame = F)
lines(lowess(phase1$form1a_q18avgsbp, phase1$hes_pv2), col = 'blue')

#Average DBP vs HES
#Title: Diastolic Blood Pressure vs HES PV
plot(phase1$form1a_q18avgdbp, phase1$hes_pv2, xlab = "Diastolic Blood P
ressure (mm Hg)", ylab = "HES Plasma Volume (mL)", pch = 19, frame = F)
lines(lowess(phase1$form1a_q18avgdbp, phase1$hes_pv2), col = 'blue')

#BMI vs HES
#Title: BMI vs HES PV
plot(phase1$bmi, phase1$hes_pv2, xlab = "BMI (kg/m2)", ylab = "HES Plas
ma Volume (mL)", pch = 19, frame = F)
lines(lowess(phase1$bmi, phase1$hes_pv2), col = 'blue')

#BSA vs HES
#Title: Body Surface Area vs HES PV
plot(phase1$bsa, phase1$hes_pv2, xlab = "Body Surface Area (m2)", ylab
= "HES Plasma Volume (mL)", pch = 19, frame = F)
lines(lowess(phase1$bsa, phase1$hes_pv2), col = 'blue')

#Body fat % vs HES
#Title: Body Fat Percentage vs HES PV
plot(phase1$avgbodycomp, phase1$hes_pv2, xlab = "Average Body Fat Perce
```

```
ntage (%)", ylab = "HES Plasma Volume (mL)", pch = 19, frame = F)
lines(lowess(phase1$avgbodycomp, phase1$hes_pv2), col = 'blue')

#USG vs HES
#Title: Urine Specific Gravity vs HE PV
plot(phase1$avgusg, phase1$hes_pv2, xlab = "Urine Specific Gravity", yl
ab = "HES Plasma Volume (mL)", pch = 19, frame = F)
lines(lowess(phase1$avgusg, phase1$hes_pv2), col = 'blue')
```

Test to see if the data follow a normal distribution

```
#HES at 10 minutes
shapiro.test(phase1$hes_pv2)

#average systolic blood pressure
shapiro.test(phase1$form1a_q18avgsbp)

#average diastolic blood pressure
shapiro.test(phase1$form1a_q18avgdbp)

#BMI
shapiro.test(phase1$bmi)

#BSA
shapiro.test(phase1$bsa)

#Average body fat percentage
shapiro.test(phase1$avgbodycomp)

#Average USG
```

Correlation between HES plasma volume at 10 minutes and health markers of interest (SBP, DBP, BMI, BSA, body fat %, USG)

```
#Average SBP and HES PV
cor.test(phase1$form1a_q18avgsbp, phase1$hes_pv2, method = "spearman")

#Average DBP and HES PV
cor.test(phase1$form1a_q18avgdbp, phase1$hes_pv2, method = "spearman")

#BMI and HES PV
cor.test(phase1$bmi, phase1$hes_pv2, method = "spearman")

#BSA and HES PV
cor.test(phase1$bsa, phase1$hes_pv2, method = "spearman")

#Average body fat % and HES PV
cor.test(phase1$avgbodycomp, phase1$hes_pv2, method = "spearman")
```

```
#Average USG and HES PV
cor.test(phase1$avgusg, phase1$hes_pv2, method = "spearman")
```

Bland Altman Plots for HES compared to all other methods (ICG and equations)

```
#load bland altman package
library(BlandAltmanLeh)
library(blandr)

#Bland Altman output for HES and ICG
blandr.statistics(phase1$hes_pv2, phase1$icg_pv1ml, sig.level = 0.95)

#Bland Altman output for HES and Kaplan EPV
blandr.statistics(phase1$hes_pv2, phase1$kaplan, sig.level = 0.95)

#Bland Altman output for HES and Hurley
blandr.statistics(phase1$hes_pv2, phase1$hurley, sig.level = 0.95)

#Bland Altman output for HES and Nadler
blandr.statistics(phase1$hes_pv2, phase1$nadlerpv, sig.level = 0.95)

#Bland Altman plot for HES and ICG
bland.altman.plot(phase1$hes_pv2, phase1$icg_pv1ml, xlab="Mean Plasma V
olume (mL)", ylab="Difference in Plasma Volume (mL)")

#Bland Altman plot for HES and Kaplan EPV
bland.altman.plot(phase1$hes_pv2, phase1$kaplan, xlab="Mean Plasma Volu
me (mL)", ylab="Difference in Plasma Volume (mL)")

#Bland Altman plot for HES and Hurley
bland.altman.plot(phase1$hes_pv2, phase1$hurley, xlab="Mean Plasma Volu
me (mL)", ylab="Difference in Plasma Volume (mL)")

#Bland Altman plot for HES and Nadler
bland.altman.plot(phase1$hes_pv2, phase1$nadlerpv, xlab="Mean Plasma Vo
lume (mL)", ylab="Difference in Plasma Volume (mL)")
```

Boxplot to compare PV across all methods

```
boxplot(phase1$hes_pv2, phase1$icg_pv1ml, phase1$kaplan, phase1$hurley,
phase1$nadlerpv, names = c("HES", "ICG", "Kaplan", "Hurley", "Nadler"),
ylab = "Plasma Volume (mL)", col = c("gray48", "gray60", "gray70", "gra
y80", "gray40"))
```

Sensitivity analysis - removing 2 participants that received 170 mL HES

```
#Creating new dataset without the 2 participants
phase1sa <- phase1[-c(11,12),]
head(phase1sa)
```

Summary PV data (n=10)

```r
#ICG plasma volume at time = 0
mean(phase1sa$icg_pv1ml)

sd(phase1sa$icg_pv1ml)

median(phase1sa$icg_pv1ml)

#ICG plasma volume at time = 60 seconds
mean(phase1sa$icg_pv2ml)

sd(phase1sa$icg_pv2ml)

median(phase1sa$icg_pv2ml)

#HES plasma volume at 5 minutes
mean(phase1sa$hes_pv1)

sd(phase1sa$hes_pv1)

median(phase1sa$hes_pv1)

#HES plasma volume at 10 minutes
mean(phase1sa$hes_pv2)

sd(phase1sa$hes_pv2)

median(phase1sa$hes_pv2)

#HES plasma volume at 15 minutes
mean(phase1sa$hes_pv3)

sd(phase1sa$hes_pv3)

median(phase1sa$hes_pv3)

#Kaplan equation
mean(phase1sa$kaplan)

sd(phase1sa$kaplan)

median(phase1sa$kaplan)

#Hurley equation
mean(phase1sa$hurley)

sd(phase1sa$hurley)

median(phase1sa$hurley)

#Nadler equation
mean(phase1sa$nadlerpv)

sd(phase1sa$nadlerpv)
```

```
median(phase1sa$nadlerpv)
```

Visualize data using scatter plots (n=10)

```
#Average SBP vs HES
#Title: Systolic Blood Pressure vs HES PV
plot(phase1sa$form1a_q18avgsbp, phase1sa$hes_pv2, xlab = "Systolic Bloo
d Pressure (mm Hg)", ylab = "HES Plasma Volume (mL)", pch = 19, frame =
F)
lines(lowess(phase1sa$form1a_q18avgsbp, phase1sa$hes_pv2), col = 'blue'
)

#Average DBP vs HES
#Title: Diastolic Blood Pressure vs HES PV
plot(phase1sa$form1a_q18avgdbp, phase1sa$hes_pv2, xlab = "Diastolic Blo
od Pressure (mm Hg)", ylab = "HES Plasma Volume (mL)", pch = 19, frame
= F)
lines(lowess(phase1sa$form1a_q18avgdbp, phase1sa$hes_pv2), col = 'blue'
)

#BMI vs HES
#Title: BMI vs HES PV
plot(phase1sa$bmi, phase1sa$hes_pv2, xlab = "BMI (kg/m2)", ylab = "HES
Plasma Volume (mL)", pch = 19, frame = F)
lines(lowess(phase1sa$bmi, phase1sa$hes_pv2), col = 'blue')

#BSA vs HES
#Title: Body Surface Area vs HES PV
plot(phase1sa$bsa, phase1sa$hes_pv2, xlab = "Body Surface Area (m2)", y
lab = "HES Plasma Volume (mL)", pch = 19, frame = F)
lines(lowess(phase1sa$bsa, phase1sa$hes_pv2), col = 'blue')

#Body fat % vs HES
#Title: Body Fat Percentage vs HES PV
plot(phase1sa$avgbodycomp, phase1sa$hes_pv2, xlab = "Average Body Fat P
ercentage (%)", ylab = "HES Plasma Volume (mL)", pch = 19, frame = F)
lines(lowess(phase1sa$avgbodycomp, phase1sa$hes_pv2), col = 'blue')

#USG vs HES
#Title: Urine Specific Gravity vs HE PV
plot(phase1sa$avgusg, phase1sa$hes_pv2, xlab = "Urine Specific Gravity"
, ylab = "HES Plasma Volume (mL)", pch = 19, frame = F)
lines(lowess(phase1sa$avgusg, phase1sa$hes_pv2), col = 'blue')
```

Test to see if the data follow a normal distribution (n=10)

```
#HES at 10 minutes
shapiro.test(phase1sa$hes_pv2)
```

```
#average systolic blood pressure
shapiro.test(phase1sa$form1a_q18avgsbp)

#average diastolic blood pressure
shapiro.test(phase1sa$form1a_q18avgdbp)

#BMI
shapiro.test(phase1sa$bmi)

#BSA
shapiro.test(phase1sa$bsa)

#Average body fat percentage
shapiro.test(phase1sa$avgbodycomp)

#Average USG
shapiro.test(phase1sa$avgusg)
```

Correlation between HES plasma volume at 10 minutes and health markers of interest (BP, BMI, BSA, body fat %, USG) (n=10)

```
#Average SBP and HES PV
cor.test(phase1sa$form1a_q18avgsbp, phase1sa$hes_pv2, method = "spearma
n")

#Average DBP and HES PV
cor.test(phase1sa$form1a_q18avgdbp, phase1sa$hes_pv2, method = "spearma
n")

#BMI and HES PV
cor.test(phase1sa$bmi, phase1sa$hes_pv2, method = "spearman")

#BSA and HES PV
cor.test(phase1sa$bsa, phase1sa$hes_pv2, method = "spearman")

#Average body fat % and HES PV
cor.test(phase1sa$avgbodycomp, phase1sa$hes_pv2, method = "spearman")

#Average USG and HES PV
cor.test(phase1sa$avgusg, phase1sa$hes_pv2, method = "spearman")
```

Bland Altman Plots for HES compared to all other methods (ICG and equations) (n=10)

```
#load bland altman package
library(BlandAltmanLeh)
library(blandr)

#Bland Altman output for HES and ICG
blandr.statistics(phase1sa$hes_pv2, phase1sa$icg_pv1ml, sig.level = 0.9
5)
```

```
#Bland Altman output for HES and Kaplan EPV
blandr.statistics(phase1sa$hes_pv2, phase1sa$kaplan, sig.level = 0.95)

#Bland Altman output for HES and Hurley
blandr.statistics(phase1sa$hes_pv2, phase1sa$hurley, sig.level = 0.95)

#Bland Altman output for HES and Nadler
blandr.statistics(phase1sa$hes_pv2, phase1sa$nadlerpv, sig.level = 0.95
)

#Bland Altman plot for HES and ICG
bland.altman.plot(phase1sa$hes_pv2, phase1sa$icg_pv1ml, xlab="Mean Plas
ma Volume (mL)", ylab="Difference in Plasma Volume (mL)")

#Bland Altman plot for HES and Kaplan EPV
bland.altman.plot(phase1sa$hes_pv2, phase1sa$kaplan, xlab="Mean Plasma
Volume (mL)", ylab="Difference in Plasma Volume (mL)")

#Bland Altman plot for HES and Hurley
bland.altman.plot(phase1sa$hes_pv2, phase1sa$hurley, xlab="Mean Plasma
Volume (mL)", ylab="Difference in Plasma Volume (mL)")

#Bland Altman plot for HES and Nadler
bland.altman.plot(phase1sa$hes_pv2, phase1sa$nadlerpv, xlab="Mean Plasm
a Volume (mL)", ylab="Difference in Plasma Volume (mL)")
```

Boxplot to compare PV across all methods

```
boxplot(phase1sa$hes_pv2, phase1sa$icg_pv1ml, phase1sa$kaplan, phase1sa
$hurley, phase1sa$nadlerpv, names = c("HES", "ICG", "Kaplan", "Hurley",
"Nadler"), ylab = "Plasma Volume (mL)", col = c("gray48", "gray60", "gr
ay70", "gray80", "gray40"))
```

Calculate power for the study

```
pwr.r.test(n = 12, r = 0.3, sig.level = 0.05)
```

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